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Report of the Scientific Committee of the Spanish Agency for Food Safety and Nutrition (AESAN) on the prospection of chemical hazards of interest in food safety in Spain

Section of Food Safety and Nutrition
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Abstract

Along the food chain different chemical hazards may be present, incorporated or produced that could pose a risk to the consumer.

The Scientific Committee has reviewed the chemical hazards of most concern for food safety in Spain that are not specifically regulated, identifying them and drawing attention to those foods or conditions which, a priori, may involve a greater risk to consumers, with the purpose of eventually carrying out prospective studies.

The following chemical hazards and matrices have been considered in the report: Cylinderpermopsin (cyanobacteria toxin) in drinking water (not bottled), Chloropropanols and Glycidol in baby food, Furan and derivatives in processed foods in general, and particularly in baby food, Hydrocarbons of mineral oils, Mycotoxins produced by fungi of the *Claviceps* genus in cereals and derived foods, *Alternaria* toxins in fruits, vegetables, cereals and tomatoes, *Fusarium* mycotoxins (Enniatins, Nivalenol) in cereals and Pyrrolizidine alkaloids in baby food, food supplements, honey, pollen, tea, infusions and cereals.

The identification, characterisation, and exposure assessment of each are described, and recommendations and future considerations are also included.

Furthermore, the identification of new hazards which may have a significant exposure, or the risk assessment derived from a new or substantially increased exposure or susceptibility to a known hazard is important in order to not only eventually control these emerging hazards, but to also promote research and improve the knowledge of both consumers and the scientific community.

Key words

Cylinderpermopsin, Chloropropanol, Furan, Hydrocarbons of mineral oils, *Claviceps*, *Alternaria* and *Fusarium* Mycotoxins, Pyrrolizidine alkaloids.

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1. Introduction

Throughout the food chain, different chemical or biological hazards that may pose a risk to consumers may be present, incorporated or caused.

The official control programmes attempt to guarantee that controls of the hazards of interest in food safety are implemented in accordance with the hazard but they only affect parameters with maximum limits established in certain foods.

However, there are other hazards of interest in food safety for which there is no specific regulation, or it exists but only in certain foods, which may be subject to prospecting programmes in order to obtain data that, in addition to protecting consumers from specific exposure to a hazard, allow risk assessment.

Moreover, the identification of new hazards for which a significant exposure may occur, or the assessment of the risk arising from a new or significantly increased exposure or susceptibility to a known hazard is important, not only for the purpose of potential control of these emerging hazards, but also for consumers and the scientific community to promote research and improve their knowledge.

For this reason, the Section of Food Safety and Nutrition of the Scientific Committee of the Spanish Agency for Food Safety and Nutrition (AESAN) has been requested to carry out a review of the hazards of greatest interest in food safety in Spain that do not have a specific regulation, identifying them and indicating those foods or conditions that, a priori, could involve a greater risk to consumers, in order to potentially carry out prospective studies.

2. Chemical hazards

The following chemical hazards and matrices have been considered:

- Cylindrospermopsin (cyanobacterial toxin) in drinking water (not bottled).
- Chloropropanols and glycidol in baby food.
- Furan and derivatives in processed foods in general and baby food in particular.
- Hydrocarbons of mineral oils in all the matrices.
- Mycotoxins produced by fungi of the *Claviceps* genus in cereals and derived foods.
- *Alternaria* toxins in fruits, vegetables, cereals and tomatoes.
- *Fusarium* mycotoxins (Enniatins, Nivalenol) in cereals.
- Alkaloids of pyrrolizidine in children's foods, food supplements, honey, pollen, tea, infusions and cereals.

2.1 Cylindrospermopsin

2.1.1 Identification and hazard characterisation

Cylindrospermopsin (CYN) is a toxin produced by different species of cyanobacteria, including *Cylindrospermopsis raciborskii*, *Aphanizomenon* (currently *Chrisosporum*) *ovalisporum, Anabaena lapponica, Aphanizomenon flos-aquae* or *Raphidiopsis curvata* (Buratti et al., 2017). It is a tricyclic alkaloid derived from guanidine linked to a hydroxymethyluracil group (Ohtani et al., 1992), with a molecular weight of 415 Daltons and a high solubility in water (Figure 1). Structural variants have also been identified, such as 7-epi-CYN and 7-deoxy-CYN (Norris et al., 1999) (Banker et al., 2000).

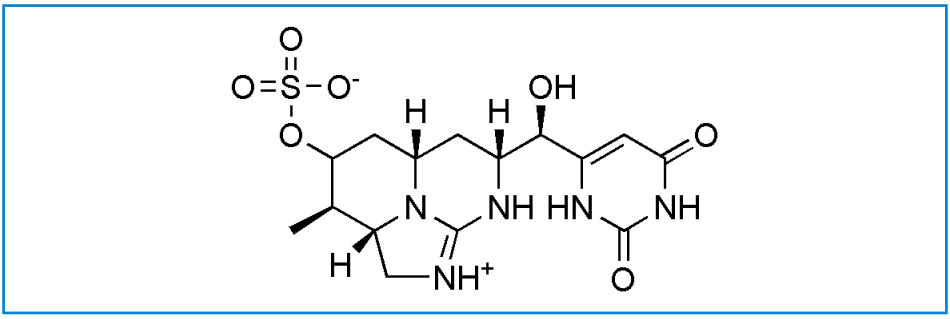


Figure 1. Chemical structure of CYN

With respect to its toxicity, the liver and kidney are the main target organs of its acute toxicity, however, it also has effects on other organs (Terao et al., 1994) (Falconer et al., 1999) (Seawright et al., 1999).

It has different mechanisms of toxic action. Thus, CYN is a potent inhibitor of protein synthesis, which leads to cytotoxicity (Terao et al., 1994) (Froscio et al., 2003). In addition, it also inhibits the synthesis of glutathione (Runnegar et al., 1995), induces the production of reactive oxygen species, oxidative stress and cell death by apoptosis (Buratti et al., 2017). Its metabolism by cytochrome P450 seems to play an important role in its toxicity (Norris et al., 2002) and it is considered a pro-genotoxic substance (Zegura et al., 2011), not yet classified by the International Agency for Research on Cancer (IARC).

The main human toxicity accident associated with CYN took place on Palm Island (Queensland, Australia) in 1979 where more than 100 children from Aboriginal families had to be hospitalised with symptoms of hepato-enteritis (Byth, 1980). The accident occurred after copper sulphate was applied to eliminate the blooming of *C. raciborskii* in the island's only drinking water reservoir. One of the reasons put forward to explain the scarcity of toxic episodes due to blooms of cyanobacteria in humans is the difficulty in establishing a causal relationship when the symptoms are subclinical (Buratti et al., 2017).

In the scientific literature there are several studies on the toxicity of CYN mainly *in vitro* (Pichardo et al., 2017) but also *in vivo*, in experimental models of mammals (for example, Terao et al. (1994), de Almeida et al. (2013)) and fish (for example, Gutiérrez-Praena et al. (2012), Guzmán-Guillén et al. (2015)).

The mean lethal dose (LD_{50}) of pure CYN in mice intraperitoneally depends on the observation time being 2.1 mg/kg b.w. after 24 hours, and 0.2 mg/kg b.w. after 120-144 hours (Ohtani et al., 1992). Orally, the LD_{50} was 4.4-6.9 mg CYN equivalents/kg b.w. after 2-6 days (Seawright et al., 1999). Humpage and Falconer (2003) exposed mice to an extract of cyanobacteria containing CYN both through drinking water for 10 weeks and by stomach tube for 11 weeks and established a non observed adverse effect level (NOAEL) of 30 µg/kg b.w./day, from which they derived a tolerable daily intake (TDI) of 0.03 µg/kg b.w./day and a guideline value in water of 1 µg/l. However, at present there are no legislated limits of CYN in water in Spain.

2.1.2 Exposure evaluation

Cyanobacteria that produce CYN have been detected in all continents. In Europe, among others, *Chrysosporum ovalisporum* in Spain, *Anabaena laponica* in Finland, *Aphanizomenon flos-aque* in Germany, *A. gracile* in Germany and Poland, *Anabaena planctonica* in Portugal and France, etc. The variety of producers indicates that this production is not species-specific and that the list of producer species may remain incomplete (Kokocinski et al., 2017). *Aphanizomenon gracile* and *A. flos-aquae* are the most important CYN-producing species in Europe (Cires and Ballot, 2016).

With regard to CYN levels, the highest datum recorded in the environment is 173 µg/l in an arid lake in Saudi Arabia (Mohamed and Al-Shehri, 2013). Rzymski and Poniedziałek (2014) include a table with maximum levels of CYN in surface waters in different countries, for example 12.1 µg/l in Germany, 126 µg/l in Italy, 9.4 µg/l in Spain (Quesada et al., 2006), etc., higher than the value proposed by Humpage and Falconer (2003) of 1 µg/l. However, the presence of CYN in low concentrations in drinking water has been generally documented (Buratti et al., 2017). Given its presence in water, CYN can also be present in foods such as fish, plants and food supplements, although data on this are scarce (Buratti et al., 2017).

Human exposure to CYN can take place mainly dermally through swimming and recreational activities in contaminated water, and orally by ingesting contaminated food and water or swallowing water during aquatic activities.

There are different analytical techniques that allow the detection and/or quantification of CYN in different matrices (water, food), such as the ELISA (Enzyme-Linked ImmunoSorbent Assay), liquid chromatography with ultraviolet detector (LC-UV) or liquid chromatography coupled to mass spectrometry (LC-MS/MS), with the latter being considered that of choice. Published protocols are available for its identification and quantification (Guzmán-Guillén et al., 2012) (Triantis et al., 2017), and there are commercial standards, but not certified reference materials.

2.1.3 Future considerations

A higher incidence of cyanobacteria blooms (producing or not producing cyanotoxins) is expected both in number and in distribution for different reasons, such as its genotypic plasticity, climate change and eutrophication of waters. In fact, CYN was identified in surface waters for the first time in 2000 in Germany, in 2004 in Spain and Italy, in 2006 in France, etc. (Rzymski and Poniedziałek, 2014) indicative of the emerging nature of this type of hazard.

Currently, CYN is not a parameter to be controlled according to Royal Decree 140/2003, which establishes sanitary criteria for the quality of water for human consumption (BOE, 2003), which does include another cyanotoxin, microcystin with a value of 1 µg/l (the same proposed by Humpage and Falconer (2003) for CYN). It is only mandatory to determine it when there is suspicion of eutrophication in the catchment water, at the exit of the drinking water treatment station or upper-level reservoir.

Recently, Testai et al. (2016) published an external scientific report for the European Food Safety Authority (EFSA) in relation to the analysis of the presence, exposure and toxicity of cyanobacterial toxins in food. With respect to CYN, it is indicated that more toxicological data are neces-

sary, mainly as regards their genotoxicity, from which health guideline values can be derived. The scientific literature also establishes the need to systematically monitorize the presence of CYN in reservoirs.

2.2 Chloropropanols and Glycidol

Chloropropanols are a group of chemical contaminants derived from glycerol that are formed during the processing and preparation of certain foods and ingredients.

Structurally, chloropropanols are formed by a chain of three carbon atoms, chlorine atoms and alcohol groups. Chloropropanols that are usually found in food are distinguished by the number of chlorine atoms, hydroxyl groups and their position in the molecule: 3-monochloropropane-1,2-diol (3-MCPD), 2-monochloropropane-1,3-diol (2-MCPD), 1,3-dichloropropanol (1,3-DCP), 2,3-dichloropropanol (2,3-DCP), and 3-chloropropan-1-ol. 3-MCPD is the most common contaminant in the group of chloropropanols in foods, followed by 2-MCPD while compound 2,3-DCP is generally found in foods at concentrations much lower than 1,3-DCP and 3-chloropropan-1-ol.

Different formation routes in food are known:

a. Acid hydrolysis of vegetable proteins (acid-HPV)

3-MCPD was detected for the first time in vegetable protein hydrolysed by acid hydrolysis (acid-HVP) which is an ingredient widely used as a flavour enhancer in processed food products (Velíšek et al., 1978). In this process the proteins (corn, wheat, casein, yeast and rice proteins) undergo a hydrolysis process with hydrochloric acid (at a temperature between 70 and 135 ºC), which also reacts with triglycerides, phospholipids and glycerol present in the raw material, leading to the formation of chloropropanols. They are also found in soy sauce and related condiments; whose manufacturing process includes the processing of soybeans with hydrochloric acid. In this mechanism, the formation of an epoxide (glycidol) is proposed as an intermediate reaction product.

b. Thermal processing of foods

Subsequently, chloropropanols have also been detected in smaller amounts in other foods that do not undergo acid hydrolysis during manufacturing, such as cereal-based and bakery products, processed meats, smoked fish, beer and coffee (Crews et al., 2002). It has been shown that 3-MCPD can form when foods containing lipids and sodium chloride are subjected to high temperatures, such as bread and pastry products (JECFA, 2006). Furthermore, cooking/grilling (high temperature treatment) can lead to some 3-MCPD formation in the food. Hamlet et al. (2004a) propose the formation of 3-MCPD when glycerol reacts with sodium chloride in the presence of other acids, such as citric and acetic acid at high temperatures. It has also been suggested that the use of sucralose in baked goods can lead to the formation of chloropropanols through pyrolysis of the synthetic sweetener sucralose, a polychlorinated compound, in the presence of glycerol (Rahn and Yaylayan, 2010). In thermally processed foods, the formation and stability of 3-MCPD depends on the pH and temperature to which the food is exposed; in fact, it has been proven that additives such as sodium bicarbonate can inhibit their formation or accelerate their degradation (IFST, 2011).

c. Migration of materials in contact with food

There is another documented route of food contamination through migration of 3-MCPD from moisture-resistant polyamide-epichlorohydrin resins present in paper and cellulose covers for food use (Pace and Hartman, 2010). However, the development and use of resins with lower levels of 3-MCPD suggests that exposure to 3-MCPD from this source will continue to decrease.

d. Hydrolysis of 3-MCPD fatty acid esters

On the other hand, several studies describe a new source of 3-MCPD from the esters of 3-MCPD (3-MCPDE) present in various food products, since 3-MCPD can be released from them *in vivo* by hydrolysis catalysed by the enzyme lipase. These studies suggest that exposures to 3-MCPD released from its esters are significantly higher than those observed in 3-MCPD in free form. The hypothesis of an equivalent bioavailability of 3-MCPD in free form and in ester form is currently accepted (EFSA, 2013).

Like 3-MCPD, its esters are produced in foods processed at high temperatures, which have low water content, high levels of sodium chloride and are stored for long periods (FAO, 2007). Hamlet et al. (2004b) found this type of esters in processed cereals and showed that they could be generated as stable intermediates or as formation products from mono and diacylglycerols. Also, several studies have revealed the presence of significant levels of 3-MCPD esters in refined edible oils and fats and products made with them (BfR, 2007) (FSA, 2009) (EFSA, 2016). These are formed by reaction of the free fatty acids present in oils and fats with 2- and 3-MCPD. Depending on the fatty acid, 14 different monoesters and 49 diesters of 3-MCPD can be formed.

Moreover, glycidol is an organic compound formed by an epoxide ring and an alcohol group (2,3-Epoxy-1-propanol) considered genotoxic and carcinogenic. It was detected for the first time in palm oil and in smaller quantities in other refined oils (Weibhaar and Pertz, 2010). During the oil refining process, mainly during the deodorisation phase, it reacts with the diglycerides generating glycidyl fatty acid esters (GE), which are hydrolysed in the gastrointestinal tract in their glycidol precursor.

Studies to elucidate the mechanism of formation of these esterified toxic compounds show that in thermally processed foods with low water activity and containing fat, 3-MCPD and its esters are formed from glycerol or/and acylglycerols (triacylglycerols and diacylglycerols) and chloride ions, while GE are formed mainly from diacylglycerols or monoacylglycerols regardless of the presence of chlorinated compounds. The formation of 3-MCPDE takes place at temperatures of 160-200 ºC and the formation process does not accelerate at higher temperatures, while the formation of GE starts at > 200 ºC, increasing exponentially at an increasing temperature when diacylglycerols exceed 3-4 % of total lipids.

Most unrefined oils do not contain detectable levels of 3-MCPDE or GE; however, they have different capacities to form 3-MCPDE and GE during the deodorisation of the refining process. Factors that contribute to this variation include climate, terrain, and growing conditions of the plants, their genotype, collection techniques, and processing conditions, all of which affect precursor levels

of 3-MCPDE and GE (acylglycerols and chlorine-containing compounds). In general, levels of 3-MCPDE and GE in foods made with refined oils correspond to the concentrations of 3-MCPDE and GE in the oils themselves. At present, the presence of these compounds in infant formulae is of particular concern.

2.2.1 Toxicological characterisation

The presence of chloropropanols in food is of concern, due to their toxicological properties. At the present, there is not enough toxicological information on chloroesters and GE in foods to be able to evaluate their importance in health. However, the toxicokinetic data indicate that 3-MCPDE and GE decompose in their non-esterified forms, and therefore, the toxicological evaluations carried out are based on 3-MCPD and glycidol, compounds for which toxicological data are available (JECFA, 2016).

3-MCPD has been classified as a potential carcinogen (Group 2B) by the International Agency for Research on Cancer (IARC, 2012), causing infertility and decreased activity of the immune system in laboratory rats, which causes testicular cancer and kidney cancer. The evaluation carried out in 2001 by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) brought together short- and long-term toxicity studies concluding that the kidney and testes are the target organs and demonstrating its *in vitro* mutagenic activity in the absence of clinical or epidemiological studies in humans (JECFA, 2001). By contrast, 2-MCPD has not been evaluated by the IARC and its toxicological potential (effects on striated muscles, the heart, kidney and liver) has not been confirmed to date.

In relation to 1,3-DCP, in 1993 JECFA concluded that it is carcinogenic and that its content in foods should be reduced as much as possible. It is currently recognised that it induces hepatotoxicity, nephrotoxicity, neurotoxicity, teratogenesis and mutagenicity (Lu et al., 2014), it also shows structure-activity similarities with other known carcinogens identified by the IARC, and has been classified as possibly carcinogenic to humans (group 2B) (IARC, 2012). Currently, the interest of its study is due to its toxic potential as a carcinogen through a genotoxic mechanism, and to its activity as an endocrine disruptor in humans and animals. 2,3-DCP in addition to testicular, hepatic and renal toxicity, has immunotoxic effects on T cells both *in vivo* and *in vitro* (Lu et al., 2013).

In 2001, JECFA provisionally assigned a maximum tolerable daily intake value per kg of body weight (PMTDI) of 2 µg of 3-MCPD based on the lowest observed effect level (LOEL) and a safety factor of 5 for extrapolation from the LOEL up to the no observed effect level (NOEL) (JECFA, 2001), this value was ratified in 2006 (JECFA, 2006). Subsequently, JECFA determined a provisional maximum tolerable daily intake (PMTDI) of 4 µg/kg b.w. for 3-MCPD and 3-MCPDE (individually or in combination, expressed as equivalents of 3-MCPD) based on renal tubular hyperplasia in male rats (JECFA, 2016).

In 2001, the Scientific Committee on Food (SCF) adopted a tolerable daily intake (TDI) of 2 µg/kg b.w. for 3-MCPD (SCF, 2001). In 2016, EFSA's Scientific Panel on Contaminants in the Food Chain (CONTAM) concluded that it was not possible to maintain the previously established TDI for 3-MCPD and determined a tolerable daily intake (TDI) of 0.8 µg/kg b.w./day for the sum of 3-MCPD and its

fatty acid esters based on scientific evidence linking this substance with nephrotoxicity in animal testing (EFSA, 2016). In view of the divergence of opinion with JECFA, EFSA has revised this value and based on the new "Benchmark dose" approach ($BMDL_{10}$ of 0.20 mg/kg b.w./day for renal effects) it has established a TDI of 2 µg/kg b.w./day, closer to the JECFA position, while recommending additional studies to confirm the toxicity of 3-MCPD (EFSA, 2018).

Moreover, glycidol has been classified as a likely carcinogen (Group 2A) by the IARC (2000). It is currently considered to be proven that glycidol, the precursor of GE, is genotoxic and carcinogenic, and, as such, a level of safety for the intake of glycidyl fatty acid esters (expressed as glycidol) cannot be established. Instead JECFA applies the margin of exposure approach (MOE) calculated from the lowest confidence limit in the $BMDL_{10}$ reference dose of 2.4 mg/kg b.w./day for mesotheliomas in male rats while EFSA has used T_{25} of 10.2 mg/kg b.w./day for neoplastic effects in rats as a toxicological reference point, and it is considered that an exposure margin of less than 10 000 and 25 000, respectively, is a health concern (EFSA, 2016) (JECFA, 2016).

2.2.2 Exposure assessment and risk to the infant population

Several scientific reports published in recent years highlight the presence of chloropropanols and chloroesters in food. The opinion published by the German Institute for Risk Assessment (BfR) on 3-MCPD esters concluded that high levels of 3-MCPD esters existed in refined vegetable fats and oils, frying oils, hazelnut cream and infant formula (BfR, 2007). The report of the Food Standards Agency (FSA) noted that the highest contents of 3-MCPD were found in biscuits and pasta (FSA, 2009). The International Life Sciences Institute (ILSI) published data linking the presence of 3-MCPD esters to foods derived from cereals, coffee, fish, meat, potatoes, nuts and refined oils (ILSI, 2011). The scientific opinion published by EFSA (2016) indicated that the highest average values of GE, as well as 3-MCPD and 2-MCPD and their esters were found in palm oils and palm fats and they were 5 to 10 times higher than the average values found in most other refined edible oils.

The most recent studies have concluded that exposure of the population to 3-MCPDE and GE is mainly due to consumption of refined vegetable oils and food products containing them (potato products and fine bakery products), including infant formulae.

In the European Union (EU), the evaluation of analytical data on the presence of 3-MCPD/GE in foods contributed by various countries showed that edible oils made the greatest contribution to the daily intake of these contaminants and that margarines, and bakery and pastry products were the main sources of exposure to these chemical contaminants for children from 3 years of age and for the young population. Likewise, an average daily intake of 2.4 mg/kg b.w. and from 1.8 to 2.1 mg/kg b.w. for 3-MCPD and glycidol, respectively (EFSA, 2013, 2016) was estimated for children fed exclusively with infant formulae. In accordance with the latest evaluation of exposure estimated by EFSA (2018), the average exposure to 3-MCPD and its esters is not exceeded in the adult population while it is above its estimate of a safe dose in younger groups with higher consumption, including adolescents (up to 18 years of age), and in particular for infants who consume only infant formulae. For this population group, the daily intake of 3-MCPD can be three times higher than the TDI, which is, therefore, a potential health concern for these population groups.

JECFA (2016) concluded that the estimated nutritional exposure to 3-MCPD for the general population, even for large consumers, did not exceed its safe dose. However, the mean dietary exposure to 3-MCPD of the groups fed infant formula was 2.5 times greater than the PMTDI.

With regard to glycidol, both EFSA (2016) and JECFA (2016) have found scientific evidence that it is genotoxic and carcinogenic, and assuming a complete conversion of the esters into glycidol after ingestion, both organisms have proposed a high margin of exposure (MOE) to these compounds in order to not affect the health of the consumer. JECFA (2016) estimated that lower limits of MOE ranges determined for infants, children and adults (less than 10 000) can pose a health problem, and EFSA (2016) concluded that GE are a potential concern for the health of young age groups with average exposure, and for all age groups with high exposure, and that the exposure of infants that consume only infant formulae is particularly concerning due to the presence of vegetable oils in these formulas (MOE of 5 400 and 2 100 for average exposure and high exposure, respectively).

Infant formulae as substitutes for breast milk are made from vegetable oils to achieve a qualitative and quantitative profile of fatty acids similar to breast milk. However, the information available on the presence of chloropropanols and chloroesters in these products that form the basis of the diet of infants is still very limited.

In general, the results published to date provide the following conclusions: although free 3-MCPD is not detected, the content of MCPDE and GE found in marketed infant formulae involves a potential risk to the health of infants; a significant variation has been observed in the levels of 3-MCPDE and GE in infant formulae, which may be due to the types of oils used in these preparations; the highest content determined for glycidyl esters of palmitic and oleic acid is consistent with the composition of infant milk in these fatty acids from the added refined vegetable oils; 2-MCPD esters are in a concentration equivalent to half the esters of 3-MCPD; a tendency to reduce concentration over time is observed, which suggests the possibility of implementing mitigation measures in the production process (Zelinková et al., 2009) (Becalski et al., 2015) (Wöhrlin et al., 2015) (Jędrkiewicz et al., 2016) (Leigh and MacMahon, 2017) (Pavesi et al., 2017).

2.2.3 Risk management strategies

According to the provisions of Recommendation 2014/661/EU, the industry has been adapting its processes to control the presence of 2- and 3-MCPD, 2- and 3-MCPD fatty acid esters and fatty acid glycidyl esters in foods (EU, 2014). Thus, various organisations such as the European Federation of the Oil Industry (FEDIOL, 2015) and the German Federation of Food Law and Food Science (BLL, 2016) have developed guidelines to reduce 3-MCPDE and GE in refined oils and in foods that contain them.

In 2008, the *Codex Alimentarius* established a Code of Practice (CAC/RCP 64-2008) on 3-MCPD in acid-hydrolysed vegetable proteins and recently, the *Codex* Committee on Contaminants in Foods (CCCF, 2018) presented a draft Code of Practice to reduce 3-monochloropropane-1,2-diol esters (3-MCPDE) and glycidyl esters (GE) in refined oils, especially in infant formulae, based on three management strategies: good agricultural practices, good manufacturing practices, and selection and uses of refined oils in food products made from these oils, including infant formula. In relation to the latter, it is recommended to select refined vegetable oils with lower levels of 3-MCPDE and GE

(either by their lower natural content or by the application of mitigation measures) and reduce the amount of refined vegetable oils in the finished products, although this could affect the organoleptic or nutritional qualities of the finished products.

In the EU, maximum permitted levels of 20 µg/kg were established for hydrolysed vegetable protein and soy sauce (Regulation (EC) No. 1881/2006 (EU, 2006)). This Regulation was recently modified by Regulation (EU) No. 2018/290 (EU, 2018) which adds the limits of glycidyl esters of 1 000 µg/kg for any vegetable oil and fat available on the market, either for direct consumption, or for use as an ingredient in food, and of 500 µg/kg in the case of vegetable oils and fats intended for the production of baby food and processed cereal-based foods for infants and young children. Furthermore, considering the risk of exposure of infants fed only with infant formula, the maximum content of glycidyl esters of fatty acids expressed as glycidol at 50 µg/kg and 6 µg/kg in infant formula, follow-on formula and food for special medical purposes, in powder and liquid respectively, is limited. However, the Commission considers that the presence of GE in these foods needs to be reduced even more once a reliable methodology is available to determine stricter content.

Moreover, for epichlorohydrin, the EU has established a maximum limit of 1 mg/kg for specific migration in materials and plastics intended to come into contact with food (EU, 2011).

Regulation (EC) No. 333/2007 (EU, 2007) establishes sampling requirements for official control over the content of 3-MCPD in foods, although no specific method has been established at community level for the determination of 3-MCPD in food products and, as such, laboratories can choose any validated method of analysis (if possible, the validation will include certified reference material), provided that the selected method meets the specific performance criteria.

To determine the MCPD and glycidol linked in the form of esters, the Commission (EU, 2014) recommends using the standard methods of the American Oil Chemists' Society (AOCS). These methods based on gas chromatography coupled to mass spectrometry (GC-MS) have been validated by a collaborative study in relation to vegetable oils and fats. It is recommended that the limit of quantification does not exceed 100 µg/kg for the analysis of MCPD and glycidol linked in the form of fatty acid esters in edible oils and fats. For other foods that contain more than 10 % fat, it is preferable that the limit of quantification is not higher when it is related to the fat content of the food, that is, the limit of quantification for the analysis of fatty acid esters of MCPD and glycidol in foods containing 20 % fat should not exceed 20 µg/kg in relation to the total weight. For foods containing less than 10 % fat, the limit of quantification should not exceed 10 µg/kg in relation to the total weight. Likewise, the laboratories must have quality control procedures to prevent, during the analysis, glycidyl esters transform into MCPD esters and vice versa. Furthermore, it is necessary to unequivocally specify the measurand and report separately on the free 2- and 3-MCPD present in the analysed matrix that come from fatty acid esters of 2- and 3-MCPD, since both are measured as 3-MCPD. The following measurands should be reported separately: 2-MCPD, 3-MCPD, 2-MCPD esters, 3-MCPD esters, and glycidyl esters.

The analysis of the various 3-MCPD mono- and diesters and glycidyl esters is very complex and direct and indirect analytical methods are available for their determination in foods.

Indirect methods require alkaline or acid hydrolysis of the fatty acid esters of glycidol or MCPDE before measuring the total amount of 3-MCPD and glycidol without differentiating between the ester

types by GC-MS. They have the advantage of providing very low detection limits but require control of the analysis conditions due to the ability of 3-MCPD and glycidol to interconvert easily. The AOCS (2013a, b, c) has established three validated inter-laboratory indirect methods for determining 3-MCPDE and GE in edible oils and fats (Methods Cd 29a-13, Cd 29b-13, and Cd 29c-13) and the Joint Research Center (JRC) recently published the validation study of a MCPDE and GE analysis method in various food matrices (bakery products, smoked fish, smoked meat, margarine, cereal-based products) (JRC, 2017).

The direct determination of 3-MCPD esters and glycidyl esters is based on isolation of the analytes generally by solid phase extraction and individual identification by HPLC-MS. These methods provide a lower degree of uncertainty in the quantification of analytes but require a range of reference standards that are not yet available for all types of esters. Currently, a direct method is available to quantify seven glycidyl esters (C12-C18), using two consecutive solid phase extraction operations followed by LC-MS based on a recent version of AOCS (American Oil Chemists' Society) (Eurofins). Haines et al. (2011) developed a direct method for the determination of esters of 3-MCPD and glycidol in edible oils and fats based on liquid chromatography coupled to time-of-flight mass spectrometry (LC-TOFMS).

For foods containing refined oils, especially infant formulae, few methods have been validated, both direct and indirect. The validation of a method for the determination of specific 3-MCPD esters and glycidyl esters in infant formulae based on a liquid-liquid extraction of fat, purification by extraction in solid phase and subsequent quantification by LC-MS/MS was recently published (Leigh and MacMahon, 2016).

2.2.4 Future steps

The toxicological information available on 2-MCPD is too limited to establish a safe level of intake.

More data are needed on the presence of fatty acid esters of MCPD and glycidyl fatty acid esters in infant formula and baby food to have a more accurate exposure evaluation.

Despite the progress made, EFSA insists on the recommendation of a significant reduction of 3-MCPD/GE in baby food products, and JECFA has been recommending that everything possible should be done to reduce 3-MCPDE and 3-MCPD in infant formula and that measures to reduce GE and glycidol in fats and oils should continue, especially those used in infant formula.

2.3 Furan and its derivatives

2.3.1 Identification and hazard characterisation

Furan (C_4H_4O; CAS No. 110-00-9) is an aromatic heterocyclic organic compound with one oxygen atom, it is lipophilic and highly volatile with a boiling temperature of 32 ºC. It is a transparent and colourless liquid that is obtained industrially through the catalytic decarbonylation of furfural and is used as an intermediary in the production of chemical substances for industry and agriculture (production of lacquers, as solvent for resins, insecticides, stabilisers and pharmaceutical products).

In addition to its industrial application, furan and its methylfuran derivatives (2-methylfuran, 3-methylfuran and 2,5-dimethylfuran) are part of a group of organic compounds that are naturally

formed in foods processed at high temperatures or by exposure to ionising or ultraviolet radiation. These compounds have been associated for long with aromas of food (FAO/WHO, 2011).

In 2004, the Food and Drug Administration (FDA) reported its presence in foods in cans and glass containers (baby food, infant formulae, canned vegetables, baked beans, soups, sauces, stews, and canned meats and fish) and subsequently in a variety of foods subjected to thermal processes such as coffee, beer, fruit and vegetable juices, soy sauce, nutritional drinks and cereal-based foods, such as biscuits, crackers, breakfast cereals and bread. In general, furan content is higher in packaged foods (FDA, 2004a, 2006, 2007, 2008).

According to the most recent data published by EFSA in 2017, the highest concentrations of furan were found in whole roasted coffee beans, followed by roasted ground coffee, unspecified coffee solids and coffee substitutes and, to a lesser extent, instant coffee in powder. Medium concentrations were also detected in compound foods based on cereals and vegetables and ready-to-eat meals for infants and young children, soy sauce, bread and rolls, raw pasta, breakfast cereals, fine bakery products and liquors. Likewise, the presence of 2-methylfuran, 3-methylfuran and 2,5-dimethylfuran was reported in various foods with 2-methylfuran/furan ratios of 4 (coffee), 1.4 (cereals for children), 1.1 (breakfast cereals) and 0.23 (ready-to-eat meals for infants and young children) (EFSA, 2017). However, according to the Food Standards Agency (FSA) the trend observed on the presence of this process contaminant in high-risk foods, including food for children, does not currently involve an increase in the concern for human health (FSA, 2017).

Furan is produced in foods subjected to heat and different mechanisms of formation have been documented from various precursors naturally present in foods, including the thermal degradation of reducing sugars with or without amino acids, the thermal degradation of amino acids, the thermal oxidation of ascorbic acid and polyunsaturated fatty acids and carotenoids (Crews and Castle, 2007).

Several studies concur that the degradation of ascorbic acid from 120 ºC is the most important route of furan formation in foods, although some research indicates that foods rich in carbohydrates are more prone to furan formation, probably due to the Maillard reaction and that the retention of furans in foods is related to the lipid fraction, especially polyunsaturated fatty acids, which is why carbohydrate-rich foods subjected to frying processes are particularly susceptible. Experiments in model systems have confirmed the formation of furan by pyrolysis of sugars, mainly erythrose followed by ribose, sucrose, glucose and fructose. Likewise, amino acids such as serine and cysteine can generate furan by pyrolysis in the absence of carbohydrates, and polyunsaturated fatty acids such as linoleic and linolenic were considered furan precursors during the thermal treatment at 120 ºC for 25 minutes (Fan, 2015). In general, the furan level tends to be higher in foods with complex mixtures of carbohydrates, fat and protein (FAO/WHO, 2011).

In addition to the abovementioned precursors (sugars, amino acids, unsaturated fatty acids and carotenoids), the role of certain metals such as copper as possible catalysts for the formation of furan in food has been investigated (Lawely et al., 2012). Likewise, the influence of other factors such as pH and the redox potential has been evaluated, although the results are not conclusive. In acidic conditions, sugars lose efficiency as furan precursors and, nevertheless, these conditions

favour the conversion of ascorbic acid into furan (EFSA, 2017). During the storage of food at 25 ºC for 3 days, the formation of furan was 3.5 times greater at pH 9 than at pH 3 or 6 (Fan, 2015). There is also evidence that the use of antioxidants reduces the formation of furan (EFSA, 2017), with liposoluble compounds (BHT and tocopherol) being more effective, with the exception of caffeic acid (Zheng et al., 2015), although the mitigating effect of furan formation exercised by antioxidants decreases with the treatment time (Shen et al., 2017).

Moreover, it has been demonstrated that furan can form in foods not subjected to thermal processes, from carbohydrates and ascorbic acid subjected to ionising radiation (Fan, 2005a, b) (Fan and Geveke, 2007) (Fan and Sokorai, 2008). Furthermore, treatment with UV-C radiation (11.5 J/cm^2) caused the formation of furan from linoleic and linolenic fatty acids, while gamma irradiation up to 20 kGy did not induce the formation of significant amounts of furan from myristic, palmitic, stearic, oleic, linoleic and linolenic acids (Fan, 2015), and even at doses lower than 10 kGy, there was a significant reduction in the furan content formed in ready-to-eat meat-based foods (Fan and Sommers, 2006).

Apart from the characteristics of food, the final concentration of furan at the time of consumption depends on the conditions of its transformation and preparation. Several studies relate the formation of furan to temperatures higher than 120 ºC, and it can even be formed during the reheating of processed foods in closed containers. In this regard, foods for children based on vegetables, mixtures of meat and vegetables or fruits that are commonly made with heat application at high temperatures in sealed containers, are particularly susceptible to furan formation. In this type of food, the highest levels of furan were more related to vegetable products than to fruit, with sterilised instead of pasteurised products and with vitamin C content (natural or added) and a higher pH (Arisseto and Toledo, 2008). In the bread toasting process, the level of furan increased with toasting time and with the degree of browning (FAO/WHO, 2011). Another study revealed that fried fish bars had a higher furan concentration when olive oil (30 µg/g) was used instead of sunflower oil (20 µg/g) and that when baked they generated a much lower amount (10 µg/g), showing that the amount of furan is lower as temperature and frying time decrease (Pérez Palacios et al., 2013).

Moreover, several studies have shown a loss of furan by evaporation in the preparation by the consumer of ready-to-eat foods. This loss is attributed to the instability and volatility of furan in food after preparing or opening commercial products and is related to the temperature of the product and the time of exposure to the atmosphere.

For coffee, the different methods of preparation determined the loss of furan at different degrees, so in boiled/Turkish coffee, the loss was 3 to 4 times greater than in coffee prepared with filter and espressos. It has also been documented that furan levels decreased in coffee beverages when allowed to stand at room temperature for up to 20 minutes without a lid and that furan levels in coffee prepared in automatic machines were higher than in home machines due to higher retention of furan in the closed system of automatic machines (FAO/WHO, 2011).

Likewise, the influence of reheating commercially processed foods on furan concentration has been studied and, although the information is still very limited, it has been indicated that the practice of reheating commercially processed foods for children by means of a hot water bath without a lid

can reduce its exposure by 15-30 % (EFSA, 2017). Likewise, it has been documented that stirring canned and bottled baby food samples increased the release of furan, compared to leaving food unstirred and, on the other hand, the oil added to baby foods heated before their consumption produced a higher retention of furan than those without added oil (FAO/WHO, 2011). A loss of furan was also observed after reheating fried fish sticks in the microwave (Pérez Palacios et al., 2013). On the other hand, other researchers found no significant reductions in furan content by heat application or by stirring prepared foods or heating bottles with commercial foods for children in a special heater for baby food (FAO/WHO, 2011).

As reported by the Food and Environmental Research Agency (FERA), the loss by evaporation of furan from foods is another potential source of inhalation exposure, due to the presence of furan in domestic air, especially after the frying of potatoes in an open container, the preparation of coffee and the baking of some foods (FERA, 2009).

2.3.1.1 Toxicological characterisation

According to the information obtained from *in vitro* and *in vivo* studies in animals, furan can easily pass through biological membranes and after oral administration to mice and rats it is rapidly absorbed at the gastrointestinal tract. Furan has a short half-life, is metabolised by cytochrome P450 2E1 (CYP2E1) to the reactive metabolite, cis-but-2-ene-1,4-dialdehyde (BDA) and is eliminated in urine and faeces as metabolites, and it is exhaled into the air as unmodified furan and carbon dioxide formed as a result of the opening of the rings. Furan is cytotoxic and affects mainly the liver (EFSA, 2004) (JECFA, 2011).

Various studies carried out in rats have shown its carcinogenic potential and for this reason it has been classified in group 2B (potential carcinogen in humans) by the International Agency for Research on Cancer (IARC, 1995). Although the mechanism of carcinogenicity is not clearly defined, there is scientific evidence of *in vitro* genotoxicity of the BDA metabolite formed *in vivo* from furan, and based on the weight of the evidence, furan is considered a genotoxic carcinogen for risk assessment by the European Food Safety Authority (EFSA, 2004, 2017).

The greatest concern for human health corresponds to its effect due to chronic exposure through diet (Lawely et al., 2012). The various studies conducted with experiment animals concluded that liver damage (cholangiofibrosis) and liver cancer (adenoma and carcinoma) were the most critical health effects related to furan. In high doses (<30 µg/kg b.w./day) it can also affect the kidneys and lungs.

The United States National Academy of Sciences (NAS) calculated a $BMDL_{10}$ of 0.09 mg/kg b.w./day for the critical effect of furan-induced cholangiocarcinoma (NAS, 2000). In 2010, JECFA examined the induction of hepatocellular adenomas and carcinomas in female mice as a relevant endpoint and calculated a $BMDL_{10}$ of 1.3 mg/kg b.w./day, corresponding to 0.96 mg/kg b.w./day when adjusting from a programme dosage of 5 days a week at an average daily dose (FAO/WHO, 2011). In 2012, the Norwegian Scientific Committee for Food and Environment (VKM) calculated a $BMDL_{10}$ of 0.02 mg/kg b.w./day for the risk of cholangiocarcinoma by applying a correction factor of 7 to the dose obtained from a 9-month study (VKM, 2012). In 2017, the CONTAM Panel examined the

toxicological information and considered as reference a $BMDL_{10}$ of 0.064 mg/kg b.w./day and 1.31 mg/kg b.w./day for non-neoplastic effects (cholangiofibrosis) and neoplastic effects (adenoma and hepatocellular carcinoma), respectively (EFSA, 2017).

Until now, the toxicological reference points for methylfurans have not been identified, although based on the available information, an additive effect of hepatotoxicity associated with furan, 2-methylfuran and 3-methylfuran is assumed, although the *in vivo* hepatotoxic potential of 2,5-dimethylfuran is still unknown (EFSA, 2017).

2.3.2 Evaluation of exposure through the diet and characterisation of the risk to human health

In 2004, the FDA of the United States warned of the presence of furans in foods as an emerging food safety problem and EFSA published its first provisional evaluation on the presence of furans in foods warning that there was a relatively small difference between human exposure and the dose that produces carcinogenic effects in laboratory animals (EFSA, 2004).

Subsequently, on the basis of the data on the presence of furan in the foods subjected to heat treatment provided by the Member States, EFSA published different monitoring reports in 2009, 2010, 2011 and 2017. An analysis of them confirmed that coffee is the foodstuff that contributes the most to the intake of furans for adults followed by beer and instant soups. Cereals and cereal products are those that contribute most in children and adolescents. For young children, they were fruit juices, milk-based products and cereal-based products, and baby foods, which became the main contributors for this age group (EFSA, 2017).

The results of published studies of furan exposure through diet concur that the highest estimates are associated with the infant population group and although the margin of exposure for the majority of consumers indicates a low concern for health, for the population with extreme consumption habits, exposure is up to three times greater than what is considered to be of low concern for public health.

In the European Union, average dietary exposures calculated for infants ranged from 0.14 to 0.99 µg/kg b.w./day while for adults the average intake was 0.78 µg/kg b.w./day. With regard to exposure corresponding to 95th percentiles, the highest estimates were also observed for infants (0.27 to 1.8 µg/kg b.w./day). Furthermore, EFSA estimated that the calculated exposure could be higher due to the presence of 2- and 3-methylfuran in foods, since in many foods, levels of 2-methylfuran are even higher than those of furan (EFSA, 2017).

With all available data, the World Health Organization (WHO), as well as EFSA and other institutions and health authorities, have reported that estimated exposure to furans and methylfurans through food could cause potential long-term liver damage, and they have considered the level of exposure estimated for the infant population through the consumption of canned or ready-to-eat foods to be of particular concern. However, and based on existing uncertainties, they agree that there is a greater probability of overestimation than underestimation of risk, and that it is necessary to obtain new data on methods of analysis, presence, formation, exposure and toxicity, particularly of methylfurans in order to have a more realistic risk assessment (FDA, 2004b) (FERA, 2009) (FSA, 2012) (JECFA, 2011) (VKM, 2012) (ANSES, 2016) (Health Canada, 2016) (EFSA, 2017).

2.3.3 Risk mitigation and management strategies

To date in the European sphere, no legal limits have been established for the concentration of furan in food, nor has the official methodology for the determination of furan in foods been proposed.

In 2007, the European Commission published a recommendation regarding the monitoring of the presence of furan in foods (EU, 2007a) whereby it urged EU Member States to perform a follow-up on the presence of furan in food products subjected to heat treatment in 2007 and 2008. Subsequently, EFSA recommended that furan controls be carried out on heat-treated products for which few data are available and that, whenever possible, the same sample should be analysed as purchased and after preparing it for consumption, with indication of the mode of preparation followed (time, temperature and handling information) (EFSA, 2011).

From an analytical point of view, the European Commission recommended following the sampling procedures set out in Part B of the Annex to Regulation (EC) No. 333/2007 to ensure that samples were representative of the batch being sampled and that there is careful preparation of the samples prior to analysis to ensure that their furan content is not altered (EU, 2007b).

In 2004, the FDA developed the analytical methodology for the quantitative determination of furan in foods based on the gas chromatography coupled to mass spectrometry technique with head-space autosampler (HS-GC/MS) (FDA, 2004c). Subsequently, numerous researchers have proposed the solid phase microextraction (SPME) technique coupled to GC/MS as an alternative technique, although the improvement in terms of sensitivity has not been demonstrated.

Recently, the results of validation of a selective analytical method for the analysis of furan and derivatives (2-methylfuran, 2-ethylfuran, 2-butylfuran, 2-pentylfuran, 2-acetylfuran, furfural and furfuryl alcohol) in baby food based on head-space solid phase microextraction coupled to GC/MS (HS-SPME-GC/MS) were published. The good results obtained in terms of accuracy (RSD <5.02-5.55 %), recovery (98.42-99.8 %), linearity (two orders of magnitude) and sensitivity (detection and quantification limit of 0.018-0.035 ng/g and 0.060-0.117 ng/g, respectively) support its application for obtaining functional data in the risk assessment process (Condurso et al., 2018).

Moreover, considering the numerous uncertainties that still exist in relation to the kinetics of furan formation and stability in foods, the health authorities have not proposed recommended practices to minimise its presence in them. However, the possibilities of mitigation in food have been evaluated experimentally, although the information is limited and specific and cannot be extrapolated to other foods since furan formation is clearly dependent on each type of food matrix.

Below, the recommended practices in domestic food preparation extracted from the scientific literature are listed (Anese and Suman, 2013) (Pérez-Palacios et al., 2013) (Mesías and Morales, 2014) (Palmers et al., 2015) (Becalski et al., 2016) (Juaniz et al., 2016) (Rannou et al., 2016) (Cepeda-Vazquez et al., 2018):

- Follow the recommended preparation that is reported on the food label.
- Heat or stir packaged foods without a lid, to allow partial volatilisation and furan dispersion.
- Cook food in open containers to allow evaporation of the furan formed.
- Cook in the oven or microwave, since these methods generate less furan than frying.
- Adjust the frying conditions by decreasing the temperature and time to 160 ºC for 4 minutes.

- Wait an adequate time (10 minutes) from the preparation of the food until its consumption and stir it regularly.
- Prepare coffee in systems that allow low levels, such as drip filtration or in machines that pass coffee directly from beans to the cup.
- Stir coffee for 5 minutes before consumption or store in a flask for 8 hours.
- Moderate the time and degree of toasting of the bread.

2.4 Mineral oil hydrocarbons

2.4.1 Identification and characterisation of the hazard

Mineral oil hydrocarbons (MOH) are chemical compounds normally obtained by distillation of crude oil, although they can also be produced synthetically from cardboard, natural gas and biomass. MOH are used in a large number of applications: in the food industry as additives or in materials that are in contact with food, in industrial machinery as lubricants or motor oils, in phytosanitary products, in feed, printing ink, pharmaceutical products and cosmetics. They can also be generated naturally in marine organisms, bacteria, fungi, plants, insects and through some operations of food processing such as heat treatments and refined oils, among others. Therefore, MOH are part of our daily lives and may be present in foods either because of their intended use as food additives or technological adjuvants, or by different routes of contamination.

The name MOH is imprecise and includes very heterogeneous and complex mixtures of hydrocarbons with different numbers of carbons and a linear, branched or cyclic structure. Depending on their general structure, MOH can be classified as:

- Paraffins: linear and branched alkanes.
- Naphthenes: cycloalkanes with alkyl substituents.
- Aromatics: alkyl-substituted polycyclic aromatic hydrocarbons (PAH).

All of them can contain small amounts of nitrogen and sulphur compounds (EFSA, 2012) (AECOSAN, 2017).

In 2012, the European Food Safety Authority (EFSA) issued a scientific opinion on MOH in contact with food and defined them as hydrocarbons containing between 10 and 50 carbon atoms, where crude mineral oils are the predominant ones and grouped them into two categories:

- Mineral oil saturated hydrocarbons (MOSH), which basically consist of paraffins and naphthenes. They can accumulate in some tissues of the body, causing damage to the liver, lymph nodes and the spleen.
- Mineral oil aromatic hydrocarbons (MOAH), which consist of PAH replaced with alkyl. They can act as genotoxic carcinogens and damage DNA.

Foods may contain mineral oils resulting from their processing, packaging, food additives, technological adjuvants or environmental contaminants. In this regard, the experts of the Scientific Panel on Contaminants in the Food Chain of EFSA (CONTAM) identified in 2012 the following sources of contamination in food and feed:

- Materials in contact with foods: recycled paper and cardboard, off-set printing inks on paper and cardboard for packaging, additives in the manufacture of plastics, waxed paper and cardboard, treated jute or sisal bags, lubricants in the manufacture of metallic packaging, waxes applied directly on food and adhesives.
- Contaminants: a) environmental origin: lubricating oil for engines without a catalytic converter, unburnt fuel, tyre debris and road asphalt; b) machinery used in the harvesting and processing of food: diesel, pump lubricant, dispensers and other machines, cleaning agents and solvents.
- Food additives, adjuvants and other uses: non-stick (bakery and confectionery), surface treatment (rice), binding agents for minor additives, defoamers, anti-dust agents (cereals), feed and phytosanitary co-formulants.

MOSH, especially the fraction comprising carbon chains of 16-35 atoms, can accumulate in the human body, especially in the lymph nodes, the spleen and the liver, but, according to EFSA, this has not been associated with adverse consequences for health. MOAH with 3 or more monoalkylated or non-alkylated aromatic rings can be mutagenic and carcinogenic, which is why they are considered more concerning than the MOSH fraction. In general, toxicologists focus on polycyclic aromatic hydrocarbons (PAH), especially on 3-7-ring PAH, some of which are carcinogenic, while highly-alkylated 1-2-ring systems are not genotoxic and are not considered concerning in terms of carcinogenicity. In conclusion, the presence of MOAH itself is not indicative of its carcinogenic potential (FEICA, 2017).

EFSA (2012) has not been able to establish an acceptable daily intake value (ADI), since it considers that the ADI values taken as a reference previously are not adequate (EFSA, 2009). Given the deficiencies in the databases, EFSA has decided to use the margin of exposure (MOE) approach. As there are no dose-response data for MOAH with regard to carcinogenicity, EFSA has not been able to establish a reference point on which the exposure margin can be based, but considers exposure to MOAH through diet to be a potential concern.

With regard to MOSH, EFSA (2012) has considered as a critical effect the formation of microgranulomas in the livers of Fischer 344 rats in 90-day studies due to the presence of MOSH with a number of carbon atoms between 16 and 35 coming from various products with MOSH for food use, in particular white oils as release agents for bread and grain spraying. 45 mg/kg b.w./day was considered as a reference, taken based on the NOAEL of the studies reviewed and the MOEs have been established based on the different possible scenarios. In conclusion, EFSA also sees a potential concern associated with the current levels of these MOH in Europe.

The EFSA opinion (2012) provides a basis for reviewing the low and medium viscosity MOH ADIs for food use. These MOH were evaluated by the previous Scientific Committee on Food (SCF) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 2002 (FAO/WHO, 2002) and are currently under review. According to EFSA, the accumulation of MOSH in the lymph nodes of the intestines of laboratory animals is less relevant to human health than was thought when their ADI was established. EFSA considers the revision of highly viscous MOH ADI to be a low priority.

2.4.2 Exposure assessment

Given their presence as complex mixtures, it is impossible to separate individual compounds to quantify them, with the added problem of limited availability of certified analytical standards. The concentration of saturated and aromatic fractions can be measured separately by gas chromatography. Currently, the analysis of their presence in foods consists of an extraction followed by pre-separation by liquid chromatography coupled to another of gas with flame ionisation detector (AECOSAN, 2017), and on it is based the only European standardised method for their determination in some foods that is used to determine concentrations above 10 mg/kg but whose value depends on the food matrix and especially the fat content, with the values being less reliable the greater the content in it (DIN EN, 2017). MOH specifications usually refer to physical-chemical properties such as viscosity, related to their intended use. Products with the same characteristics may have a different chemical composition depending on the origin of the oil and its processing. Technical grade MOH typically contain 15-35 % PAH, and food grade MOH undergo additional distillation to minimise the PAH content (EFSA, 2012).

MOH presence data are only available in a limited number of food groups, focusing on MOSH content, without differentiating between paraffins and naphthenes, with a carbon number between 12 and 40. No information is available on MOAH and, as such, only an estimate of its composition can be performed. In general, MOSH levels are low in almost all food groups, except in "breads and buns" and "cereals for human consumption", mainly rice with average values of 261 mg/kg and 132 mg/kg, respectively, which, on the contrary, have very low levels of MOAH. The rest of the groups show significantly lower average values: confectionery products other than chocolate (46 mg/kg), vegetable oils (41-45 mg/kg), canned fish products (40 mg/kg), oil seeds (38 mg/kg), animal fats (22-24 mg/kg), fish meat (21 mg/kg), nuts (20-21 mg/kg) and desserts and ice cream (14 mg/kg) (EFSA, 2012). The presence of both substances in dry foods can be attributed, in part, to the use of recycled paper.

The working group also reviewed the migration of MOH in foods packaged with recycled paper and cardboard, finding that when functional barriers (bags or coverings that impede migration) are not used, there is a significant transfer to food and, as such, a migration and permeability analysis should be performed over time and it should also be considered that migration from the container is influenced by temperature and only MOH of up to 25 carbons migrate at room temperature (FoodDrink Europe, 2018).

EFSA (2012) estimates, considering the average values found in the different food groups, that the average chronic exposure of the European population is in the range of 0.03-0.30 mg/kg b.w./day, and is higher in young consumers, especially in those aged between 3 and 10 years old, than in adults and the elderly. Migration from recycled paper packaging could contribute significantly to total exposure, but there is little information about it.

The exposure of consumers to MOAH from contamination accounts for 20 % of exposure to MOSH saturates, while the contribution of MOH for food use is minimal and exposure to MOAH does not increase due to this use.

The potential concern associated with MOH consumption, both MOAH and MOSH, can be important in consumers loyal to a brand or who usually buy the same product in the same store, because they are exposed to high levels of MOH on a regular basis.

Given the complexity of the mixtures that constitute MOH, additional studies must be undertaken, both related to analytical techniques and to human exposure and its real effects on health in order to establish recommendations and regulations. In this regard, FoodDrink Europe (2018) has proposed a series of tools, considering the three possible routes of contamination, which aim to help reduce the risk of contamination by MOH by implementing measures based on the route of entry and the potential contaminant.

2.4.3 Recommendations and future considerations

MOH can be present in food both due to environmental contamination, as well as by being generated or incorporated during processing, in addition to migration from packaging materials, particularly paper and cardboard. The potential effects of the different compounds constituted by MOH on human health vary considerably depending on their chemical structure. Therefore, certified standards and reference materials of MOH components should be available immediately to improve analytical methods and monitoring systems to better evaluate the risks posed by MOH. Similarly, in the future, MOAH and MOSH should be distinguished from each other, and, depending on the chemical structures and carbon number of the chain, further data on the action of multi-branched and cyclic MOSH are necessary. With regard to the food groups where they are found, those that contribute most to exposure and those that use white oils should be controlled. It is necessary to identify sources of contamination throughout all stages of the food production process in order to design adequate control systems.

Food contamination with MOH due to the use of recycled cardboard as packaging material must be effectively prevented by including materials that serve as a functional barrier in the container. Likewise, it is necessary to perform additional toxicological studies on the various hazards posed by the different fractions of MOH focused on the range of molecular weights and structural subclasses rather than on physicochemical properties, such as viscosity. It should be investigated whether oral exposure to MOSH is associated with systemic autoimmune diseases or with impaired immune function, as well as studying the transfer to humans of the results of studies on MOH in animals. Lastly, EFSA, in its 2012 scientific opinion, suggested the revision of the group of temporary ADIs for low and medium viscosity oils.

In 2017, the European Commission published Recommendation (EU) 2017/84, on the monitoring of MOH in food and in materials and articles intended to come into contact with food (EU, 2017). In this recommendation, it is urged to perform monitoring throughout 2017 and 2018 of the presence of MOH in the following foods, in which the latest data can be submitted before 28 February 2019: animal fats, bread and fine bakery products, breakfast cereals and confectionery (including chocolate and cocoa), fish meat and fish products (canned fish), cereals for human consumption, ice cream and desserts, oil seeds, pasta, cereal products, pulses, sausages, nuts and vegetable oils, as well as the materials in contact with the foods used for these products. However, for a homogeneous application of the Recommendation and to obtain reliable results, the specific guidelines of the EU reference laboratory should be followed, but these guidelines do not yet exist and Member States are urged to collaborate in their preparation.

2.5 *Claviceps* mycotoxins

2.5.1 Identification and characterisation of the hazard

Ergot is the term used to designate the solidified mycelium of the fungus *Claviceps purpurea, africa*n, *fusiformis, sorghi* and related species that can affect pastures and cereals of all kinds. The main types of cereals affected are rye, triticale (*Claviceps purpurea*), sorghum (*Claviceps africana sorghi, sorghicola*) and pearl millet (*Claviceps fusiformis*). In addition, it can affect wheat and barley in springs with long periods of humidity and cold.

Ergot (sclerotium) is a kind of dark-coloured and sometimes white "horn", which is formed instead of the grain in the ears of the infected grain through the inflorescence of the plant. If a good selection of grains is not made before grinding, it mixes with flours. The importance of good agricultural and processing practices was highlighted by the *Codex Alimentarius* Commission, which published a code of practice in 2003 to prevent and reduce mycotoxin contamination of cereals and it was revised in 2016 by CAC/RCP 51-2003 (Codex Alimentarius, 2016).

Sclerotia contain toxic alkaloids. There are 40 known alkaloids of ergot, with ergometrine, ergotamine, ergosine, ergocristine, ergocryptine and ergocornin, and their epimers, being the predominant ones. In the ergot of sorghum, dihydroergosine and related alkaloids are also important (Blaney et al., 2010). The profiles of distribution and concentration of alkaloids vary depending on the strain of *Claviceps*, the host, weather conditions, since moisture facilitates its proliferation, and the geographic area. Therefore, the content of alkaloids in a sclerotium is variable but can reach up to 0.5 % (Codex Alimentarius, 2016).

Poisoning by contamination of flour by ergot is currently known as ergotism and formerly as Saint Anthony's Fire or Holy Fire and it has led to serious collective intoxications: it was very present in the Middle Ages and nowadays, although it no longer creates major epidemics in humans, sporadic local epidemics have continued to occur in more recent years and they are common in domestic animals.

There are two symptomatic forms of ergotism: gangrenous and convulsive. In the gangrenous form, a tingling effect is perceived in the peripheral tissues that eventually leads to the loss of extremities, while in its convulsive form, the tingling is followed by hallucinations, delirium and epileptic seizures (Codex Alimentarius, 2016). After ingestion of small amounts of ergot alkaloids, acute symptoms such as vomiting, spasms, headaches, cardiovascular problems and central nervous system dysfunction, as well as contractions of the uterus that lead to bleeding and miscarriages occur. Consumption of high concentrations gives rise to acute toxic effects, such as circulatory disorders due to vasoconstriction of the cardiac muscle, but also in the kidneys and extremities, accompanied by hallucinations, spasms, diminished sensations, paralysis, and even death due to cardiac or respiratory arrest. Chronic intake of moderate amounts of these alkaloids can affect reproduction (cause miscarriages, low birth weight and deficient breastfeeding). When chronic ingestion is high, it produces symptoms that correspond to acute ingestion of high quantities. Furthermore, in certain consumer groups (young children and pregnant women) there may be undesirable effects on their health when they consume baked goods and flours that contain ergot alkaloids (Mariné, 2012).

In 2000, the European Commission established a limit of 0.05 % of sclerotia as a quality requirement for cereals under intervention and, based on toxicological data, Switzerland and Germany have considered limits of total alkaloids of ergot in rye for human consumption of 100 µg/kg and 400-500 µg/kg, respectively (Mariné, 2012).

The contamination of ergot alkaloids is a major problem in feed as cattle, sheep and poultry are sensitive to ergot toxins. The European Food Safety Authority (EFSA, 2005) concluded that a relationship between the number of sclerotia and ergot alkaloids could not be established, since the concentration of alkaloids in sclerotia is very variable (0.01-0.5 %), but assuming an average of 0.2 %, a level of 0.05 % of sclerotium achieves a total content of 1 000 µg/kg of alkaloids. In the United States and Canada, the maximum permissible level of sclerotia in grain is 300 mg/kg. With regard to feed, Canada and Uruguay have established limits ranging from 450 to 9 000 µg/kg, depending on the animal (Mariné, 2012). Subsequently, EFSA (2017) conducted a study in food and feed on exposure to the 12 major alkaloids of ergot, ergometrine, ergosin, ergocornin, ergotamine, ergocristine, ergocryptin, the α and β isomers, and their corresponding inine-S epimers. A statistically significant linear relationship was found between the content of sclerotia and levels of alkaloids quantified in different cereal grains (barley, oats, rye, triticale and wheat). However, the absence of sclerotia does not exclude the presence of alkaloids in samples where sclerotia were not identified due to having contents below the limits of quantification, which would be false negatives.

Ergot dust is very fixed and sticks easily to the surface of the grains, a fact that must be taken into account in the cleaning tasks in which the bodies of ergot and the powder of the cereal consignment must be removed as much as possible. The cleaning procedures of the grain must be adapted to achieve maximum efficiency and a second cleaning process implemented for the previously cleaned grain (Codex Alimentarius, 2016).

The acute toxicity of rye ergot is relatively low. Fatal doses of sclerotia powder are estimated at 10-15 grammes. Considering that a person consumes 300-400 g of bread a day, this would have to contain about 3 % of ergot, which would be noticeable to the naked eye in the flour, as it would have violet, brownish or blue spots. It is more difficult to detect and evaluate the consequences of repeated consumption of low or very low doses. With respect to oral pharmacological doses of alkaloids, the indications for ergotamine are 6 mg/day or 10 mg/week. It must be considered that the great historical intoxications were due to major infestations, that the quality of the grain was not controlled and that intake was repeated. Moreover, if there is intake of solid sclerotia, only a part of its components is absorbed and, furthermore, the preparation of cereals and their derivatives, such as baking, inactivates, according to some, the activity of the alkaloids by up to 50 %. In addition, it is known that ergot alkaloids are not carcinogenic and some even have the opposite effect, and, as such, their use as cytostatic agents has been investigated, although it is not clear if exposure to these alkaloids in the diet has the capacity to mitigate carcinogenic effects (De Ruyck et al., 2015).

2.5.2 Exposure assessment and potential hazards

EFSA (2012) evaluated the data available on the presence and potential effects of ergot alkaloids in food and feed in the European Union and, considering that a daily intake of 0.6 µg/kg b.w./day and 1

µg/kg b.w./day for the acute reference dose of the group of total alkaloids of rye ergot is tolerable (the toxicity of the main alkaloids is considered quite similar), it concluded that the existing data do not involve a risk for any human population subgroup. Subsequently, it evaluated a much higher number of samples, especially processed foods, obtaining similar results (EFSA, 2017). In both evaluations, it was mentioned that early childhood is a stage with an increased risk of ingestion with an estimated acute exposure of 0.02 µg/kg b.w./day in babies and 0.32 µg/kg b.w./day in older children. There is no greater risk in vegetarians. The foods in which the presence of alkaloids was detected are rye and its derivatives, although it is not ruled out that there may be other sources of contamination that have not been studied so far. One of the recently evaluated products was barley and its derivative, beer, where it was observed how the initial concentrations of alkaloids in barley were reduced throughout the process to very low levels in the final product (<10 µg/l) and, as such, beer cannot be considered a source of ergot alkaloids in the diet (Bauer et al., 2016).

With regard to livestock, EFSA (2012, 2017) indicated that under normal conditions the risk of toxicosis is low, with pigs for fattening being those with the highest level of exposure, but that there is a greater possibility of humans ingesting significant doses of alkaloids.

In the assessments of the Institute for Risk Assessment (BfR), the potential risk to consumers of large portions of contaminated cereal-based products with levels above 64 µg ergot alkaloids per kg of product was revealed and it is considered that the content of ergot alkaloids remains constant during processing (Fajardo et al., 2012). This indicates that the level of 64 µg/kg is reasonable if the initial amount of ergot alkaloids present in cereals or flour is low, between 100 and 250 µg/kg depending on the recipe of the product.

The European Commission established a maximum level of 0.5 g of ergot sclerotia in one kg of unprocessed cereals, marketed for a first phase of cereal processing, with the exception of corn and rice (EU, 2015). The maximum level could be extended in the future, when more data have been collected on the content of ergot alkaloids in processed cereals, where ergot sclerotia are not visible.

In the analytical determination of ergot alkaloids, the existence of several reference compounds of the alkaloids and their high instability is worth mentioning. This fact, together with the preparation of the sample in the absence of light to avoid the formation of compounds derived from its action, must be rigorously taken into account during the different stages of its analysis. There are several analytical techniques that can be used, from spectroscopic methods, the oldest, to high performance liquid chromatography (HPLC) coupled to mass detector, as well as immunology and gas chromatography, and there is an HPLC method with an internationally validated fluorescence detector (EFSA, 2012 and 2017).

2.5.3 Recommendations and future considerations

The real absence of a hazard must be properly demonstrated for it not to be considered in food legislation. In the case of ergot alkaloids, it does not seem that there is a major problem if the recommendations for cultivation and storage are followed, but it is necessary to study it and follow it up because it may be the case that certain agricultural practices involve some risk if the proper precautions are not taken. It seems quite evident that, although the risk, in practice, is low, it is ne-

cessary to include the monitoring and control of the presence of ergot alkaloids in food and feed, in the same way that is carried out with other mycotoxins.

According to the European Commission, it is necessary to obtain more data on the presence of ergot alkaloids in feed and foods, especially processed ones, and to use analytical methods with sufficient sensitivity to detect and quantify them and therefore be able to list the amount of sclerotia in the plant with the concentration of alkaloids. It is possible to consider another sampling plan and a different method for assessing the level of contamination and take into account that ergot dust can also contaminate the cereal without it being visible.

Ergot bodies and their fine powder attaching to the surface of the grains and in the furrows have to be avoided and removed from the processing chain. The prevention of contamination with ergot alkaloids is not fully covered by the general provisions of the Code of Practice for the prevention and reduction of contamination of cereals by mycotoxins (CAC/RCP 51-2003), which requires a specific annex to address points not included in the general provisions.

2.6 *Alternaria* toxins

2.6.1 Identification and characterisation of the hazard

The genus *Alternaria* was originally defined in 1816 and, since then, numerous species of *Alternaria* sp. have been described. The genus produces more than 70 mycotoxins, the most important being alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT), tenazonic acid (TeA), tentoxin (TEN), toxins of *Alternaria alternata* f. sp. *Lycopersici* (AAL toxins) and altertoxins (ATX) I, II, III (ATX-I, -II, -III). The main mycotoxin-producing species include: *A. alternata, A. arborescens, A. brassicae, A. brassiciola, A. citri, A. cucumerina, A. dauci, A. gaisen, A. jaoinica, A. kikuchiana, A. longipes, A. mali, A. pori, A. racina, A. radicina, A. solani, A. tenuissina* (Table 1).

Table 1. Species producing *Alternaria* mycotoxins and foods they contaminate

Mycotoxin	Producing species	Foods involved
Tenazonic acid (TeA)	*A. alternata, A. brassicae, A. brassiciola, A. citri, A. jaoinica, A. kikuchiana, A. mali, A. pori, A. racina, A. tenuissina*	Olives, citrus fruits, apples and juice, pepper, sunflower seeds, sorghum, tomato, wheat, spices, orange, lemon, red beetroot, alcoholic drinks, vegetables and derivatives, baby foods
Altertoxin I-II-II (ATX)	*A. alternata, A. arborescens, A. brassicae, A. gaisen, A. longipes, A. mali, A. radicina, A. tenuissima*	Apple and juice, sorghum
Alternariol (AOH)	*A. alternata, A. arborescens, A. brassicicola, A. citri, A. cucumerina, A. dauci, A. gaisen, A. tenuissima*	Oats, pepper, tomato, apple and juice, spices, sunflower seeds, orange, lemon, wheat, legumes, alcoholic drinks, vegetables and derivatives

Table 1. Species producing *Alternaria* mycotoxins and foods they contaminate

Mycotoxin	Producing species	Foods involved
Alternariol monomethyl ether (AME)	*A. alternata, A. arborescens, A. brassicae, A. brassicicola, A. citri, A. cucumerina, A. dauci, A. gaisen, A. kikuchiana, A. longipes, A. mali, A. porri, A. solani, A. tenuissima*	Olives, barley, rye, citrus fruits, apple and juice, melon, vegetables and derivatives, pepper, alcoholic drinks, sunflower seeds, sorghum, tomato, legumes, wheat, pepper, spices, orange, lemon, baby food
Altenuene (ALT)	*A. alternata, A. arborescens, A. citri, A. gaisen, A. porri, A. tenuissina*	Cereal grains and derivatives, oilseeds, seed oils, vegetables and derivatives
Tentoxin (TEN)	*A. alternata, A. mali, A. porri, A. tenuissima*	Cereal grains and derivatives, seed oils, vegetables and derivatives
AAL-toxin	*A. alternata*	Cereal grains and derivatives

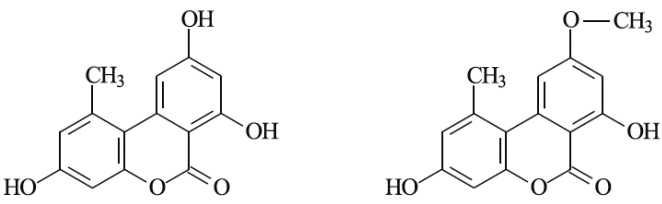
Source: (Soriano, 2007) (Barkai-Golan, 2008) (Ostry, 2008) (Barros et al., 2011) (Pavón et al., 2012).

The optimum growth temperatures for the genus *Alternaria* vary between 22 and 30 ºC, although it can grow and produce mycotoxins between 0 and 6.5 ºC in colder regions and regions with low water activity. The genus *Alternaria* deteriorates food during transportation and storage, even in refrigerated foods below the set temperature. For this reason, *Alternaria* mycotoxins are commonly found in a wide variety of fresh and processed plant products (Table 1).

The presence of *Alternaria* mycotoxins in food is likely under optimum growth conditions (Soriano, 2007) (Barkai-Golan, 2008) (Ostry, 2008) (Barros et al., 2011) (Pavón et al., 2012). Direct human consumption of foods that are visibly infected with fungi is unlikely in humans. The foods most likely contaminated with *Alternaria* mycotoxins are fruits and vegetables (Table 1). However, it is common to find them in processed foods such as tomato sauces, preserves, jams, wine or fruit juices (Fernández-Cruz et al., 2010). The presence of *Alternaria* mycotoxins in cereals is very common due to the storage of grains under favourable conditions for the growth of the fungus (Logrieco et al., 2003). Also, the presence of AME and TeA was detected in infant formulae containing cereals in their composition (Scoot et al., 2012). In oilseeds such as rapeseed, sunflower, sesame and flaxseed the presence of AOH and AME has been determined (Visconti et al., 1986) (Ostry et al., 2004) (Ostry, 2008), as well as in legumes such as lentils and soybeans (Barkai-Golan, 2008) (Barros et al., 2011).

In vivo and *in vitro* studies have shown that AME is poorly absorbed in the gastrointestinal tract; however, the absorbed proportion is metabolised and persists in tissues (Pollock et al., 1982) (Pfeiffer et al., 2007). AOH and the AME produce hydroxylated metabolites, mainly catechols, through cytochrome P450. The importance of catechols lies in their ability to form reactive intermediates such as quinones and semiquinones that are capable of producing reactive oxygen species (ROS)

and binding to DNA, resulting in DNA adducts (Solhaug et al., 2012). AOH and AME (Figure 1) have 3 and 2 phenolic hydroxyl groups respectively, which react with uridine diphosphate glucuronic acid (UDPGA), through uridine glucuronosyltransferase enzymes (UGTs), intestinal and hepatic microsomes forming glucuronide conjugates such as AOH-3-O-glucuronide, AOH-9-O-glucuronide and AME-3-O-glucuronide (Pfeiffer et al., 2009). Both *Alternaria* mycotoxins are easily glucuronidated in hepatic and extrahepatic tissues. These mycotoxins can also form sulphated conjugates by sulphotransferases and biomethylation of O-methylated compounds (Pfeiffer et al., 2007) (Burkhardt et al., 2009, 2011). Therefore, although AOH is not easily absorbed into the gastrointestinal tract, once biotransformed in the liver and excreted via the bile into the duodenum, it is rapidly absorbed from the intestinal lumen and reaches portal blood in the form of aglycone, glucuronide and sulphate (Burkhardt et al., 2011).



Alternariol (AOH) Alternariol monomethyl ether (AME)

Figure 1. Chemical structure of alternariol and alternariol monomethyl ether

The metabolites of *Alternaria* show different biological activities such as antimicrobial, phytotoxic and cytotoxic properties. For example, the porritoxin from the endophytic species of *Alternaria porri* has been studied as a cancer chemopreventive agent (Horiuchi et al., 2006). Depudecin is a metabolite of the species *A. brassicicola*, an inhibitor of histone deacetylase (HDAC), which has antitumour potential (Kwon et al., 2003). On the other hand, TeA and TEN have been studied as potential herbicides (Lou et al., 2013).

There are few studies of experimental acute and chronic toxicity in animal species. As observed in table 2, laboratory animals or embryos are exposed to crude extracts of *Alternaria* fungi or mycotoxins such as AOH, AME, AOH+AME mixtures, ALT, ATX-I, ATX-II and TeA by different routes of administration, oral, intravenous, intraperitoneal and subcutaneous (Table 2). Of all the acute toxicity studies carried out, only TeA showed an LD_{50} value in 1-day-old chickens and in mice, ranging from 37.5 to 225 mg/kg b.w./day. In table 2, some chronic toxicity effects obtained after exposure of experimental animals to different doses of *Alternaria* mycotoxins are also observed.

In vitro studies show that some *Alternaria* mycotoxins cause genotoxicity in bacteria and mammalian cells (Tiessen et al., 2013), clastogenic effects and the induction of DNA breakage in different mammalian cells (Lehmann et al., 2006) (Wollenhaupt et al., 2008) (Fehr et al., 2009).

It has been observed that in immunodeficient people (transplant or Cushing patients) they cause opportunistic infections, cutaneous alternariosis (papulonodular, pustular, or ulcerous-scabby

localised reddish-brown plaques) (Schracher et al., 2001) (Vieira et al., 2006). They are related to rhinosinusitis, oculomycosis, and onychomycosis (Pastor and Guarro, 2008). As in the case of other fungi in some patients, exposure to spores or mycotoxins in the context of the dampness and mould hypersensitivity syndrome can cause inflammatory, rheumatological or neurological spectrum symptoms (Valtonen, 2017).

On the other hand, the genus *Alternaria* causes reactions of rhinoconjunctivitis and asthma mediated by IgE (de Vouge et al., 1998) (Päivi et al., 2006). The species *A. alternata* is considered a potent allergen, since it is associated with severe bronchial asthma and is one of the fungi that produces most sensitisation in allergic patients. However, allergic reactions due to food intake and attributed to fungal contamination are extremely rare (Bobolea et al., 2009).

With the data currently available, an association cannot be established between *Alternaria* mycotoxins and a high incidence of oesophageal cancer in humans due to multiple limiting factors such as the presence in the same samples of high concentrations of other carcinogenic compounds, data bias, etc., not considered in these studies (Liu et al., 1991, 1992) (Yekeler et al., 2001) (EFSA 2011).

Table 2. Toxic effects of *Alternaria* mycotoxins through *in vivo* assays

Species	Mycotoxin	Route and dose	Type of assay	Effects	Reference
Mouse	AOH, AME	*i.p.* 100, 200, 400 mg/kg b.w. AOH+AME (100+100) mg/kg b.w.	Acute toxicity	Gastric spasms	Pero et al. (1973)
	AOH	*s.c.* 100 mg/kg b.w. day Administration on day 9-12 of gestation and on day 13-16 of gestation	Reproduction and development	Increased deaths. Partial or total reabsorption of the foetus	
	AOH	200 and 1 000 mg/kg b.w. (radiolabelled AOH)	Toxicokinetic	Low systemic absorption, 90 % of the total dose is excreted in faeces and up to 9 % in urine. Significant levels in blood (0.5 µM)	Schuchardt et al. (2014)
		3 x 2 000 mg/kg (0, 24 and 45 hours), after 48 hours the mice are slaughtered	Genotoxicity. Micronucleus and comet assays	No genotoxic effect was observed in the bone marrow or the liver tissue	
	AME	Food: 50 mg/kg b.w. 10 months	Carcinogenicity	Dysplasia of the oesophageal mucosa	Yekeler et al. (2001)

Table 2. Toxic effects of *Alternaria* mycotoxins through *in vivo* assays

Species	Mycotoxin	Route and dose	Type of assay	Effects	Reference
Mouse	ALT	*i.p.* 50 mg/kg b.w. (females)	Acute toxicity	Deaths: 1/3	Pero et al. (1973)
	ATX-I	*i.p.* 100 and 200 mg/kg b.w. (females)		Deaths: 0/8 (100 mg/kg b.w.); 8/8 (200 mg/kg b.w.)	
	ATX-II	*i.p.* 100 and 200 mg/kg b.w. (females)		Deaths: 0/2 (100 mg/kg b.w.) 2/2 (100 mg/kg b.w.)	
	TeA	*Sodium salt i.v.*		DL_{50}= 162 (males); DL_{50}= 115 (females)	Smith et al. (1968)
		Sodium salt orally		DL_{50}= 186 mg/kg b.w. (males); DL_{50}= 81 mg/kg b.w. (females)	
		i.v.		DL_{50}= 125 mg/kg b.w. (males)	Woodey and Chu (1992)
		i.p.		DL_{50}= 150 mg/kg b.w. (males)	
		s.c.		DL_{50}= 145 mg/kg b.w. (males)	
		Oral		DL_{50}= 225 mg/kg b.w. (males)	
Rat	AME, AOH	AOH: 21 days with 39 mg/kg and day AME: 21 days with 24 mg/kg and day	Chronic toxicity	There is no evidence of toxicity	Sauer et al. (1978)
	TeA	Sodium salt *i.v.*	Acute toxicity	DL_{50}= 146 mg/kg b.w. (males); DL_{50}= 157 mg/kg b.w. (females)	Smith et al. (1968)
		Sodium salt orally		DL_{50}= 180 mg/kg b.w. (males); DL_{50}= 168 mg/kg b.w. (females)	
Hamster	AME	*i.p.* 200 mg/kg b.w. on day 8 of gestation	Reproduction and development	Toxic doses for the mother. Increase in reabsorptions and decreased foetus weight	Pollock et al. (1982)
1-day-old chicken	TeA	Orally	Acute toxicity	DL_{50}= 37.5 mg/kg b.w.	Giambrone et al. (1978)
Chick embryo	AME, AOH	*i.p.* 1 000 (AOH), 500 (AME) µg per egg		No death	Griffin and Chu (1983)
	TeA	10 µg/ml of TeA		DL_{50}= 0.548 mg/egg	

Abbreviations: AOH: alternariol; AME: alternariol monomethyl ether; TeA: tenazonic acid; *i.p.*: intraperitoneal; *sc.*: subcutaneous; b.w.: body weight.

2.6.2 Exposure assessment and risk characterisation

The highest concentrations of *Alternaria* mycotoxins found in foods according to EFSA are detailed below (EFSA, 2016). For the processing of data in which mycotoxin is not detected, the guidelines (as recommended by EFSA, 2010) are followed that suggest the lower limit (LB) approach where the values below the limit of quantification (LOQ) and the limit of detection (LOD) are replaced by zero and for the upper limit (UB) the values below the LOD are replaced by the LOD and those below the LOQ are replaced by the LOQ value. Thus, the highest concentrations of AOH were found in chestnuts (LB= 43.9 µg/kg, UB= 44.5 µg/kg), followed by oats (LB= 35.3 µg/kg, UB= 39.7 µg/kg), buckwheat (LB= 27.9 µg/kg, UB= 33.1 µg/kg) and sunflower seeds (LB= 22.4 µg/kg, UB= 29.1 µg/kg). In the case of AME, the highest concentrations were found in chestnuts (LB= 16.8 µg/kg, UB= 17.5 µg/kg), followed by sesame seeds (LB= 11.3 µg/kg, UB= 11.8 µg/kg), buckwheat (LB= 10.1 µg/kg, UB= 11.0 µg/kg) and oats (LB= 6.4 µg/kg, UB= 7.1 µg/kg). With respect to TEN, sunflower seeds had the highest concentrations (LB= 79.0 µg/kg, UB= 82.0 µg/kg). For TeA, all the analysed foods contained concentrations much higher than the rest of the *Alternaria* mycotoxins analysed. The highest concentrations were obtained in paprika powder (LB= 8 801.0 µg/kg, UB= 8 802.0 µg/kg) and blackberries (LB= UB= 5 742.0 µg/kg). The rest of the foods contained TeA concentrations that were much higher than the food concentrations of AOH and AME. For example, chestnuts contained TeA levels of 793.0 µg/kg (LB) and 794 µg/kg (UB) and sunflower seeds 563.0 µg/kg (LB) and 570 µg/kg (UB).

There is currently no legislation on *Alternaria* toxins in food or feed in Europe or in other regions of the world. EFSA considers the following mycotoxins AOH, AME, TeA, iso-TaA, ATX, TEN, ALT and AAL toxins (due to their higher presence in food and feed) to carry out a risk assessment in food and feed (EFSA 2011, 2016). Due to the limited toxicity data available on *Alternaria* mycotoxins, EFSA's CONTAM Panel uses the Threshold of Toxicological Concern (TTC) concept to evaluate the relative level of concern of these mycotoxins for human health. The Panel concluded that for *Alternaria* genotoxic toxins (AOH and AME), the average chronic dietary exposures estimated in the upper limit (UB) and the dietary exposures of the 95th percentile exceeded the TTC value (2.5 ng/kg b.w./day). This indicates the need for additional specific toxicity data for the compound as they pose a health risk. For non-genotoxic *Alternaria* toxins by the bacterial mutagenicity test (TeA and TEN with a TTC value of 1 500 ng/kg b.w./day), exposure estimates are probably not a human health problem, considering the concentrations found in the foods evaluated (EFSA, 2016).

EFSA has determined the daily exposure levels of *Alternaria* mycotoxins for different population groups (Table 3) (EFSA, 2011). In the estimation of chronic exposure to this mycotoxin, only foods of vegetable origin are considered, since the presence of *Alternaria* mycotoxins in foods of animal origin has not been demonstrated (EFSA, 2011, 2016). The foods included in this study were: grains and grain-based products, vegetables and vegetable-based products (mainly tomatoes), fruits and fruit-based products including fruit and vegetable juices, beer, wine, seed oil and vegetable oils (mainly sunflower oil and sunflower seeds). Chronic exposure through diet is only calculated for two age groups, children and adults.

Table 3 shows the average dietary exposure (average consumption in the population) and high dietary exposure (food consumption in the 95th percentile in the population) to *Alternaria* mycotoxins according to EFSA reports (2011, 2016). Exposure was calculated separately for each dietary survey using consumption data at the individual level. Individual food consumption data were combined with average values of mycotoxin presence in food to provide exposure estimates. Exposure estimates were calculated for both scenarios, LB (lower limit) and UB (upper limit). It should be highlighted that the report indicates that there are many samples where *Alternaria* mycotoxins are not detected, since they are below the limit of detection or the limit of quantification. As shown in table 3, the comparison demonstrates that higher food intake per kg of body weight in children means greater dietary exposure compared to adults (factor 2 to 3).

Table 3. Estimation of chronic exposure to alternariol (AOH), alternariol monomethyl ether (AME), tenazonic acid (TeA) and tentoxin (TEN) through intake in adults and children

Mycotoxin	Average exposure through diet (ng/kg b.w./day)		95th percentile (ng/kg)	
	Children (LB-UB)*	Adults (LB-UB)*	Children (LB-UB)*	Adults (LB-UB)*
AOH	3.8-71.6	1.9-39.0	11.4-270.5	5.9-82.0
AME	3.4-38.8	0.8-4.7	10.3-97.3	3.1-15.0
TeA	100.0-1 614.0	36.0-141.0	209.0-1 216.0	89.0-362.0
TEN	1.6-33.4	0.01-7.0	4.9-54.4	0.0-13.0

*Children (12-36 months), adults (18-65 years); LB (lower bound); UB (upper bound). **Source:** (EFSA, 2016).

Due to the presence of these mycotoxins mainly in vegetables, vegetarians may be more exposed to these toxins because of the higher intake of vegetable-based foods. Not many consumption data are available, but considering dietary surveys with vegetarian subjects, as shown in table 4, EFSA's report shows that chronic exposure to the four mycotoxins of *Alternaria* (AOH, AME, TeA and TEN) through ingestion is higher in vegetarians than in the general population (EFSA, 2016). However, due to the sample size, this conclusion must be interpreted with caution.

Table 4. Comparison of exposure to alternariol (AOH), alternariol monomethyl ether (AME), tenazonic acid (TeA) and tentoxin (TEN) in adult vegetarians and the total adult population of a selected dietary survey (National Nutrition Survey II, Germany)

Mycotoxin	Average exposure through the diet (ng/kg b.w./day)		Percentile 95 % (ng/kg)	
	Vegetarians (LB-UB)*	Total population (LB-UB)*	Vegetarians (LB-UB)*	Total population (LB-UB)*
AOH	3.5-14.4	2.1-8.9	11.3-45.5	7.8-31.8
AME	1.8-10.3	1.1-7.4	8.1-29.8	4.2-19.7
TeA	127.0-227.0	87.0-186.0	442.0-592.0	263.0-403.0
TEN	1.6-5.2	0.8-3.3	4.5-12.4	3.3-10.7

*Vegetarians (n= 237), total population (n= 10 419); LB (lower bound); UB (upper bound). **Source:** (EFSA, 2016).

2.6.3 Mycotoxin mitigation strategies

Strategies before and after harvesting are used to try to eliminate the mycotoxins in food and feed and prevent their adverse effects on the health of humans and animals. Avoiding the formation of mycotoxins in the field would be the best method of prevention, but it is often not enough and other methods should be used. Prevention strategies during cultivation and storage are aimed at the selection of crop varieties that can withstand the attack of fungi: avoiding favourable temperatures for the development of fungi; avoiding flowering coinciding with the release of spores; using appropriate cultivation techniques to reduce the risk of fungal contamination; avoiding the attack of insects; use of biocontrol techniques; controlling moisture content and oxygen concentration during storage; etc. However, these measures are often not followed correctly or do not produce cultures free of mycotoxins. In these cases, a stage of decontamination or degradation of mycotoxins is necessary, transforming them into less toxic metabolites.

Among the methods to reduce or eliminate the presence of mycotoxins in foods are chemical reduction through the use of chemical substances during food processing, the use of techniques during food processing and reduction of the bioavailability of mycotoxins once absorbed.

Chemical reduction uses substances that have been shown to be effective in transforming mycotoxins into less toxic metabolites or inactivating them completely. These compounds include isothiocyanates (ITC), natural antimicrobial reactive compounds that inhibit the growth of the fungus (Azaiez et al., 2013) (Nazareth et al., 2016).

Although mycotoxins are stable compounds, some food treatments such as cleaning, grinding, yeast fermentation (beer, baking), cooking, baking, frying, roasting, canning, peeling, etc. can affect the chemical structure of some of them and reduce their toxicity (Bretz et al., 2006) (Meca et al., 2012a, 2013a, b).

And lastly, the reduction of the bioavailability of mycotoxins in the gastrointestinal tract has been proven by the use of probiotic strains (*Lactobacillus animalis, Lb. Casei, Lb. Casei, Lb. Plantarum, Lb.*

Rhuminis, Lb. Casei, Bifidobacterium breve, Bf. Adolescents, Bf. Bifidum, Corynebacterium vitaeruminis, Streptococcus faecalis, Eubacterium crispatus and Saccharomyces cerevisiae). Probiotics are used because the lactic acid produced by bacterias is considered to be a detoxifying agent in the gastrointestinal tract. Greater effectiveness has been obtained with strains of *Lactobacillus rhamnosus* (Meca et al., 2012b). Other systems for reducing bioavailability are the use of cellulose and inulin prebiotics, dietary fibres such as galactomannan, glucomannan, citrus fibre, bamboo fibre, carrot fibre, cake fibre, beta-glucan, xylan, and cellulose and protein ingredients such as whey, beta-lactoglobulin and calcium caseinate (Luz et al., 2017).

2.6.4 Conclusions and future considerations

Alternaria mycotoxins (AOH, AME, TeA and TEN) were found in certain grains and grain-based products, tomatoes and tomato products, sunflower seeds and sunflower oil, fruits, juices and fruit products, in beer and wine. TeA was the *Alternaria* mycotoxin with the highest concentrations found in tomato-based products, nuts, oilseeds, grains and fruits.

The greatest exposure through the diet was estimated in vegetarians and children, mainly due to greater exposure to "cereal-based foods for infants and young children".

More information is required on toxicokinetics, including the metabolism of *Alternaria* mycotoxins with greater toxicological significance, as well as chronic toxicity data, which are scarce in most *Alternaria* mycotoxins.

It is necessary to generate more analytical data on *Alternaria* toxins in relevant food products (for example, fruits and fruit products, tomatoes and tomato-based products, cereal-based foods for babies and young children, among others) and develop more sensitive analytical methods to reduce the uncertainty associated with exposure to different *Alternaria* toxins.

2.7 *Fusarium* mycotoxins (Enniatins, Nivalenol)

2.7.1 Hazard identification and characterisation

Emerging *Fusarium* mycotoxins such as enniatins (Ens) and nivalenol (NIV) are gaining interest because they are not yet regulated and/or because of their concomitant appearance with other mycotoxins (EFSA, 2014b, 2017) (Moretti et al., 2018). Ens can be present in significant amounts in grains such as wheat infected with *F. avenaceum*. NIV is found in wheat, corn, barley, oats and rice infected with *F. crookwellence* or *F. poae* mainly, under certain conditions of humidity and temperature (EFSA, 2013, 2014b). Ens, among the most prevalent of these toxins, are usually found alongside beauvericin, deoxynivalenol, moniliformin and fumonisins (Meca et al., 2010) (Svingen et al., 2017).

2.7.1.1 Chemical structure

Ens are a broad group of structurally related cyclic hex depsipeptides consisting of three alternatively linked residues of D22-hydroxycarboxylic acid and N-methylamino acids. To date, 29 natural analogues have been identified, but only 4 of them, Enniatin A, A1, B and B1 have been detected frequently in foods (EFSA, 2014b). Due to their apolar properties Ens can be incorporated into cell membranes and create selective cation channels (Svingen et al., 2017). The most representative in human exposure is EN-B (Maranghi et al., 2018).

NIV is a type B trichothecene, tetracyclic sesquiterpene with a keto group in the 8th position. Fusarenone-X (FUS-X, 4-acetynivalenol) is a precursor of NIV and the only biotransformation products identified so far are the phase I metabolite de-epoxy-nivalenol (DE-NIV) and the phase II metabolite nivalenol-3-glucoside (NIV3Glc). However, it is expected that other conjugated metabolites will be formed in plants and fungi (EFSA, 2017).

2.7.1.2 Toxicokinetics (ADME)

The *in vitro* data indicate that Ens are absorbed and rapidly metabolised to uncharacterised metabolites (Meca et al., 2011) (Prosperini et al., 2013). *In vivo* studies suggest that EN-B is rapidly eliminated from the blood by hepatobiliary excretion (Rodríguez-Carrasco et al., 2016). EN-B metabolites have been found both in the liver and colon, which potentially contributes to their distribution and the production of toxic effects (Rodríguez-Carrasco et al., 2016) (Maranghi et al., 2018). In addition, some EN-B phase I metabolites have been identified in liver and colon, which suggests the possible contribution of hepatic and intestinal metabolism in the metabolism of the first step of EN-B (Rodríguez-Carrasco et al., 2016).

Information on NIV absorption is limited, but it is rapid and appears to be distributed and eliminated without accumulation (Poapolathep et al., 2003) (EFSA, 2013, 2017). NIV conjugates can break in the gastrointestinal tract, releasing NIV (EFSA, 2014c).

Often, mycotoxin biotransformation products can contribute to global toxicity (EFSA, 2017).

2.7.1.3 Mechanism of action

Ens have a wide range of biological activities: they are ionophores (Meca et al., 2011), enzyme inhibitors (Ivanova et al., 2011), and oxidants (Prosperini et al., 2013). They are cytotoxic and induce apoptosis, apparently in relation to their ionophoric properties (Meca et al., 2011) (EFSA, 2014b) (Fraeyman et al., 2017).

NIV induces *in vitro* apoptosis in cells of the immune system: lymphocytes, dendritic cells and macrophages (EFSA, 2013). In *in vivo* studies, NIV targets the immune system, increasing the apoptosis of lymphocytes in the thymus, Peyer's patches or spleen (Sugita-Konishiet al., 2008). NIV induces both immunotoxicity and hematotoxicity. Reproductive and developmental toxicity has also been observed, but it is not likely to be a critical effect of NIV (EFSA, 2013).

2.7.1.4 Genotoxicity and carcinogenicity

EN-B showed a genotoxic effect in bone marrow and liver cells after acute oral administration in male mice. No DNA damage, gene mutations or chromosomal damage was observed after repeated exposure (Maranghi et al., 2018). No Ens carcinogenicity studies or reports of human toxicosis by Ens have been identified (EFSA, 2014b).

NIV is not likely to be genotoxic (Le Hégarat et al., 2014), and its carcinogenicity is unknown based on the studies available (EFSA, 2013). IARC includes it in group 3, not classifiable as to its carcinogenicity to humans.

2.7.1.5 Guideline levels in health

EFSA concluded that there was not enough data to establish a tolerable daily intake (TDI) or an acute reference intake for the sum of Ens (EFSA, 2014b). For NIV, however, it established a TDI of 1.2 µg/kg b.w. (EFSA, 2017).

2.7.2 Exposure assessment

2.7.2.1 Analytical detection methods.

The quantification of Ens is performed by LC-MS(/MS) often with a multi-analyte approach; UHPLC combined with MS or by immunohistochemical methods (Rodríguez-Carrasco et al., 2016). However, none has been validated inter-laboratory and there are no commercially available reference materials or analytical standards (EFSA, 2014b).

For NIV analytical methods (mainly LC-MS/MS) are available, but their high polarity affects recovery rates. Thus, detection is more difficult compared to other trichothecenes, which explains why other NIV phase II metabolites have not yet been identified. ELISA kits are capable of detecting NIV selectively and surface plasmon resonance immunoassays have been developed, but are still in the research phase and are not suitable for routine application. However, none of the chromatographic or immunological methods have been validated in interlaboratory studies (EFSA, 2013) and standards and reference materials of modified forms of NIV are not commercially available (EFSA, 2017).

2.7.2.2 Presence in foods

EFSA's CONTAM Panel (2014b) reported the following maximum mean concentrations for Ens in unprocessed grains: barley (703 µg/kg), rye (650 µg/kg), and wheat (446 µg/kg), with a limit of quantification (LOQ) of 0.3-10.8 µg/kg. Grain and grain-based products are those that contribute most to the exposure, especially bread and pastries (EFSA, 2014b).

NIV is present along with lower amounts of other trichothecenes in grains, mainly in oats, corn, barley and wheat (Juan et al., 2016) (Rodríguez-Carrasco et al., 2016). Grains and grain-based foods are the main contributors to NIV exposure. In particular bread, pastries, ground grain-based products, pasta, fine bakery products and breakfast cereals (EFSA, 2013).

Both Ens and NIV, like most mycotoxins and their modified forms, concentrate in the outer layers of the grains. The cleaning, classification and milling redistribute them, causing a concentration in the bran and fibre, with a reduction of the fractions used for human consumption. Therefore, products enriched with bran and fibre are more prone to contamination (EFSA, 2014a). They are stable during processing for commercialisation, including drying and silage procedures (EFSA, 2013) (Rodríguez-Carrasco et al., 2016).

2.7.2.3 Exposure in diet

Health concerns due to dietary exposure to Ens focuses on young children (1-3 years) and children, since several reports have indicated them to be the age groups at greatest risk. The highest mean value of chronic dietary exposure to Ens in young children was 0.42-1.82 µg/kg b.w./day, with a 95th percentile of 0.91-3.28 µg/kg b.w./day (EFSA, 2014b).

For NIV, the highest chronic exposure has been estimated for young children (1-3 years), ranging between 4.3-202 ng/kg b.w./day for moderate consumers and 12-484 ng/kg b.w./day for high consumers (EFSA, 2013).

2.7.3 Hazard characterisation

EFSA's CONTAM Panel (2014b) concluded that acute exposure to Ens is not indicative of a human health concern. There could be a concern with regard to chronic exposure, but no definitive conclusions can be drawn since relevant *in vivo* toxicity data are necessary to enable risk assessment in humans.

Exposure to NIV and its modified forms is not a concern since the highest exposure (95th percentile) for high consumers (12-484 ng/kg b.w./day) was less than 20 % of the TDI established for NIV (1.2 µg/kg b.w./day) (EFSA, 2013).

2.7.4 Maximum levels in legislation

Ens and NIV are not included in the Annex to Commission Regulation (EC) No. 1881/2006, which establishes the maximum content of certain contaminants in food products (EU, 2006), and are not regulated under Directive 2002/32/EC on undesirable substances in animal health (EU, 2002).

2.7.5 Future considerations

In conclusion, it is likely that the evaluation of the exposure carried out so far has been underestimated due to the lack of validated methods of screening, detection and quantification of emerging toxins such as Ens, NIV, their modified forms and their mixtures. In addition, there is a clear need to further assess their toxicological potential. More efforts should be made to develop and establish ongoing monitoring programmes. Additional data on the co-presence, prevalence and combined effects of *Fusarium* mycotoxins are necessary. The analytical methods for Ens, NIV and their modified forms should be evaluated in inter-laboratory validation studies as well as the development of protocols and reference materials. Furthermore, more *in vivo* research is needed that is focused on clarifying the metabolic pathways, the toxicokinetics and the toxicity and genotoxicity of both Ens, NIV, their derivatives and metabolites.

2.8 Pyrrolizidine alkaloids

2.8.1 Hazard identification and characterisation

Pyrrolizidine alkaloids (PA) are a large group of secondary metabolites highly toxic to humans and animals (WHO, 2016) (EFSA, 2017). PA are produced by more than 6 000 species of plants of the families *Boraginaceae* (all genuses), *Compositae (Asteraceae)* and *Leguminosae (Fabaceae)* (genus *Crotalaria*) (WHO, 2016).

2.8.1.1 Chemical structure

PA are heterocyclic compounds, most of them derived from four bases of necine (platinecin, retrotecin, heliotridine or otonecin) (EFSA, 2007) (Codex, 2018). Most natural PA are esterified necines

or alkaloid N-oxides (with the exception of otonecin alkaloids), while non-esterified PA occur less commonly in plants (Codex, 2018). However, new PA continue to be identified both in new plant species and in others already studied (WHO, 2016).

Hepatoxic PA have an unsaturated necine base, while in non-hepatotoxic PA this necine base is saturated. The former have greater toxicity because they can undergo metabolic activation and form reactive pyrroles that can react with proteins and form DNA adducts (EFSA, 2011).

2.8.1.2 Toxicokinetics

1,2 unsaturated PA are rapidly absorbed and distributed throughout the body. PA-N-oxides are reduced to their free bases in the digestive tract (Hessel et al., 2014) (WHO, 2016). After ingestion, PA undergo hepatic metabolism. 1,2 unsaturated PA are metabolised in the liver in three ways: (1) breakage of the ester bonds; (2) N-oxygenation of the necine base in PA with retronecin and heliotridine, resulting in N-oxides that are more rapidly excreted in urine; and (3) oxidation through cytochrome P450 (CYP450) which forms 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5 [[H]] - reactive pyrrolizidine esters (DHP). The DHP esters conjugate with glutathione and other nucleophilic substances *in vivo* and are hydrolysed to DHP diols. The reactive esters form DHP adducts with nucleophilic groups in many tissues by alkylation (WHO, 2016) (Yang et al., 2017) (Zhu et al., 2017).

2.8.1.3 Mechanisms of action

PA are not chemically reactive substances, so their toxicity is due to their metabolic activation. The crucial step is the formation of reactive pyrrole derivatives (DHP), while biotransformation to N-oxides is the most common pathway for detoxification (EFSA, 2007). Pyrrolic metabolites bind to nucleophilic groups of proteins and cellular DNA resulting in adducts and cross-links (Dusemund et al., 2018). The degree of bioactivation of toxic pyrroles depends on the degree of esterification and the nature of the ester groups. Moreover, the individual sensitivity to PA comes from the degree of expression of the enzymes involved in their biotransformation (EFSA, 2007).

2.8.1.4 Organ-specific toxicity

PA have a common toxic profile, with the liver being the main target organ (WHO, 2016) (EFSA, 2017) (Codex, 2018). The main signs of toxicity in all animal species include various degrees of progressive liver damage (centrilobular hepatocellular necrosis) (Edgar et al., 2014), and hepatic veno-occlusive disease (Kakar et al., 2010). Other effects observed include bile duct proliferation, hepatic megalocytosis and fibrosis (NTP, 2003) (Merz and Schrenk, 2016) (Codex, 2018). However, effects on other organs have also been reported: lungs (pulmonary hypertension), cardiovascular system (right ventricular hypertrophy) and degenerative damage in the kidneys (Codex, 2018). The most major effect in humans is hepatic veno-occlusive disease (Copple et al., 2003).

2.8.1.5 Genotoxicity and carcinogenicity

The genotoxicity of PAs and of preparations containing them has been extensively studied both *in vitro* and *in vivo* (Merz and Schrenk, 2016) (WHO, 2016) (EFSA, 2017). 1,2-unsaturated PA that have

been tested form adducts with DNA and are mutagenic. Carcinogenicity is the most critical marker after prolonged exposure to certain PA (WHO, 2016). Riddelliine causes hemangiosarcomas in the liver of rats and mice and alveolar and bronchiolar neoplasms in female mice (NTP, 2003). Lasiocarpine causes hepatocellular and angiosarcoma tumours in the liver of both male and female rats and hematopoietic tumours in females. The International Agency for Research on Cancer (IARC) has classified three PA, lasiocarpine, monocrotaline and riddelliine in group 2B "possible human carcinogens", while other PA evaluated could not be classified (Group 3) due to limited information (Codex, 2018) (EFSA, 2017).

2.8.1.6 Toxicological reference values and health guideline values

The WHO (2016) concluded that the mechanism of genotoxic action of PA did not allow health guideline values to be established. EFSA's CONTAM Panel (2011) could not establish an acute reference dose, but identified that a level of 2 mg/kg b.w./day would be associated with acute effects, based on the limited information obtained from intoxications in humans. EFSA's CONTAM Panel (2017) selected the lower limit of the confidence interval of the reference dose that produces an additional 10 % increase in the incidence of hepatic hemangiosarcoma in female rats exposed to riddelliine ($BMDL_{10}$= 237 µg/kg b.w./day) as a reference point for the evaluation of chronic risk.

2.8.2 Exposure assessment

2.8.2.1 Analytical methods for detection

There are various screening methods for PA: thin layer chromatography, electrophoresis, nuclear magnetic resonance and immunological methods. The quantitative analysis of PA is carried out by LC-MS/MS or GC-MS (EFSA, 2011, 2017) (Crews, 2013) (WHO, 2016). However, when HPLC-MS/MS is used, adequate chromatographic separation is not always achieved and PA cannot be distinguished by MS due to their similar molecular weight (Crews, 2013) (Mulder et al., 2015). Therefore, accurate quantification of individual PA is not always possible.

The main issues with respect to the analysis of PA include: high variations in the concentration of PA in food samples; natural variation of PA profiles in plants; the stability of PA during the storage and quantification of individual PA or total neccine. There are no high-quality standards, internal standards or certified reference materials, and, in addition, there are currently no harmonised methods or specific action criteria for PA (WHO, 2016).

2.8.2.2 Presence in foods

Tea and herbal infusions are the main products that contribute to total exposure to PA, however, honey also does so significantly (Kempf et al., 2011) (EFSA, 2017) (Dusemund et al., 2018). According to the EFSA CONTAM Panel (2017) the main PA present in tea and herbal infusions are: lycopsamine, intermedine, intermedine N-oxide, senecionine, senecionine-N-oxide, seneciphylline, seneciphylline-N-oxide and retrorsine-N-oxide. The highest average concentrations of PA were found in rooibos (4.1 µg/l) and mint (3.5 µg/l). And in black tea (1.6 µg/l) they were twice as high as in green tea (0.8 µg/l) (EFSA, 2016).

Their presence has also been identified in fresh pollen from PA producing plants, which explains their content in honey and in pollen-based products (Edgar et al., 2002) (Boppré et al., 2008) (Kempf et al., 2011). In this regard, the CONTAM Panel determined equimidine (44 %) and lycopene (37 %) in commercial honeys (EFSA, 2017).

High levels of PA have also been found in food supplements obtained from producing plants (Mulder et al., 2015) (EFSA, 2016). In plant extracts and pollen-based supplements, the main PA were lycopsamine, intermedine and their N-oxides. Senkirkine was, nevertheless, the main PA in coltsfoot (*Tussilago*) (EFSA, 2017). Average concentrations of PA of 235-253 µg/kg have been found in some extracts of plants consumed as infusions such as borage (*Borago officinalis*) and comfrey (*Symphytum officinale*) (up to 29 694 µg/kg dry product). Hemp-agrimony (*Eupatorium cannabinum*) sold as capsules/tablets for direct intake contained 2 410 275 µg/kg, the highest levels of PA (EFSA, 2016).

In children, the foods that contribute most to PA exposure are teas and herbal infusions (EFSA, 2017).

2.8.3 Risk characterisation

The CONTAM Panel estimated the acute and chronic dietary exposure to PA in the European population (EFSA, 2017). The foods that contribute the most are tea and herbal infusions. It concluded that acute exposure to PA through the consumption of teas, herbal infusions and honey is of low risk. However, the highest estimates of chronic exposure to PA from teas and herbal infusions (154-214 ng/kg b.w./day in children) and honey (0.7-31 ng/kg b.w./day for young high consumers) showed a possible health concern for frequent and high consumers, especially in toddlers (1-3 years) and children.

The consumption of food supplements from PA producing plants can also result in exposure levels that cause acute toxicity. Chronic exposure to PA via pollen-based supplements (0.7-12 ng/kg b.w./day) was also considered to result in risks. However, acute exposure (2.8-44 ng/kg b.w./day) was not (EFSA, 2017).

Thus, the CONTAM Panel (EFSA, 2017) established a set of 17 PA that should be monitored in foods: intermedine/lycopsamine, intermedine-N-oxide/lycopsamine-N-oxide, senecionine/senecivernin, senecionine-N-oxide/senecivernin-N-oxide, seneciphylline, seneciphylline-N-oxide, retrorsine, retrorsine-N-oxide, equimidine, equimidine-N-oxide, lasiocarpine, lasiocarpine-N-oxide and senkirkine.

2.8.4 Risk management

German regulations restrict PA content in herbal products with proven health benefits at 1 µg/day for oral administration, and its use is limited to 6 weeks/year (Edgar et al., 2002). This level is reduced to 0.1 µg/day of oral dose when the product is consumed for a longer time. Its use in pregnant and breastfeeding women is specifically prohibited, as well as in those products that have not demonstrated beneficial effects for health. Similar restrictions regarding PA exposure in herbal products have been imposed in the Netherlands, Austria and Switzerland (Edgar et al., 2002). However, at the

European level, no maximum permissible concentrations of PA have yet been established in any food.

2.8.5 Future considerations

It is necessary to obtain additional toxicological data in relation to the PA most commonly found in food: toxicokinetics, metabolic activation and carcinogenic potency of individual PA (EFSA, 2016, 2017) (Codex, 2018). Additional information on the presence and levels of PA in cereals, baby food, herbal supplements (other than plant extracts) and pollen is also required. But for this, more sensitive analytical methods must be developed and specialised protocols developed for the analysis of the most important PA in foods. Furthermore, additional research is required on the plants responsible for the presence of PA in tea, herbal infusions, pollen or honey and appropriate measures must be developed to control the infestation by them. Other PA not included among the 17 that require EFSA monitoring should also be taken into account. Lastly, and due to the possible risk detected in certain population groups resulting from consumption of PA present in certain foods, consideration should be given to establishing risk management measures such as setting maximum permissible concentrations.

Conclusions of the Scientific Committee

A review has been carried out of some chemical hazards for which there is no specific regulation and which may pose an emerging risk to health. The list of hazards addressed in this report is not intended to be exhaustive, since it does not cover all potential new chemical hazards and its aim is to be used as a starting point for potential prospective studies, which is why special attention is paid to indicating the foods that may be of special importance in relation to the hazards considered.

At the same time, gaps have been identified in the study of such hazards, which can be used to promote research activities aimed at obtaining new relevant data for a correct assessment.

Specific information has been included on the description of the identification and characterisation of each of hazard reviewed, the exposure assessment and a series of recommendations for risk management and future considerations on the possibilities of control in the food chain, which can serve to improve knowledge about them among consumers and other sectors involved.

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Cylinderpermopsin

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