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Strategies for estimating human exposure to mycotoxins via food

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REVIEW ARTICLE

Abstract

In this review, five strategies to estimate mycotoxin exposure of a (sub-)population via food, including data collection, are discussed with the aim to identify the added values and limitations of each strategy for risk assessment of these chemicals. The well-established point estimate, observed individual mean, probabilistic and duplicate diet strategies are addressed, as well as the emerging human biomonitoring strategy. All five exposure assessment strategies allow the estimation of chronic (long-term) exposure to mycotoxins, and, with the exception of the observed individual mean strategy, also acute (short-term) exposure. Methods for data collection, i.e. food consumption surveys, food monitoring studies and total diet studies are discussed. In food monitoring studies, the driving force is often enforcement of legal limits, and, consequently, data are often generated with relatively high limits of quantification and targeted at products suspected to contain mycotoxin levels above these legal limits. Total diet studies provide a solid base for chronic exposure assessments since they provide mycotoxin levels in food based on well-defined samples and including the effect of food preparation. Duplicate diet studies and human biomonitoring studies reveal the actual exposure but often involve a restricted group of human volunteers and a limited time period. Human biomonitoring studies may also include exposure to mycotoxins from other sources than food, and exposure to modified mycotoxins that may not be detected with current analytical methods. Low limits of quantification are required for analytical methods applied for data collection to avoid large uncertainties in the exposure due to high numbers of left censored data, i.e. with levels below the limit of quantification.

Keywords: exposure assessment, food consumption, mycotoxins, human biomonitoring, total diet study, duplicate diet study, food monitoring

1. Introduction

Food safety authorities aim at protecting the health of consumers by taking measures to avoid exposure of the population to harmful levels of these compounds. The measures should ensure that exposure of the population is below health-based guidance values, such as an acute reference dose or tolerable daily or weekly intake. The measures are preferably based on science-based risk evaluations from competent food safety authorities. Risk assessment studies, composed of hazard identification, characterisation and exposure assessment, are an integral part of these evaluations (Brera *et al.*, 2014; FAO/WHO, 2012; Marin *et al.*, 2013; Van Egmond *et al.*, 2007).

Food safety authorities of EU Member States are obliged to monitor the occurrence of harmful compounds in food by implementing risk-based monitoring programs (EC, 2004). Results from long-term monitoring of the occurrence of undesirable substances in food can also provide input for a trend analysis on levels and exposure in order to evaluate the effectiveness of implemented measures, to discover unknown sources of mycotoxins, and to give insight in changing trends in contamination of crops.

Food can be contaminated with a mixture of mycotoxins, natural toxins produced by fungal species in all parts of the world. Fungal invasion and mycotoxins production can take place both in the field and/or during storage of the crops (Han *et al.*, 2014). The actual contamination

levels are determined by complex interaction among fungal species, plant cultivars and environmental factors (Battilani *et al.*, 2012; Van der Fels-Klerx, 2014). The occurrence of fungi on crops varies between geographic regions, climatic conditions and pre- and post-harvest management (De Rijk *et al.*, 2015). The types and levels of mycotoxins in food can therefore vary considerably. Industrial processes and household preparation processes, such as cleaning or peeling, may furthermore alter the profile and the levels of mycotoxins in consumed food (Cano-Sancho *et al.*, 2013; Meca *et al.*, 2013; Tittlemier *et al.*, 2014; Vidal *et al.*, 2014).

International trade of food and feed adds variation to the exposure to mycotoxins and introduces extra challenges to assess the exposure (Cressey, 2009; Kendra and Dyer, 2007). Additionally, dietary exposure varies between geographic regions due to variability in the diets consumed and specific individual consumption patterns (Boon *et al.*, 2009; Brera *et al.*, 2014; EFSA, 2014b; FAO/WHO, 2012).

The present paper will describe and discuss five strategies applied in exposure assessments of mycotoxins. First, four data collection studies are described, followed by the description of the use of these data in four well established exposure assessment strategies. Secondly, a new strategy for exposure assessment, human biomonitoring, is described. Benefits and limits of all five exposure assessment strategies are discussed in the final chapter.

2. Data collection studies for exposure assessment

Application of the four well-established exposure assessment strategies described in Section 3, for the assessment of the dietary exposure to mycotoxins, require data on food consumption as well as data on levels of mycotoxins in food. Table 1 presents a schematic overview of the food consumption and data collection studies for these well-established exposure assessment strategies.

Table 1. Characteristics of: (1) study design; (2) sample number and analysis; (3) ethical issues; (4) costs; and (5) ability to use provided samples for other contaminants.

Data collection study	Characteristics
Food consumption surveys (FCSs)	1. Complex study design. 2. Large number of subjects required, including subgroups in the population. 3. Volunteers involved, requiring ethical approval and management of privacy data. 4. High costs for collection of data and processing of data. 5. Data can be used for exposure assessment to food chemicals and intake assessment to nutrients, both acute and chronic (if covering more than one day per subject).
Food monitoring studies	1. Simple study design. 2. Large number of analytical samples, often from routine monitoring (official control), often analysed with multi-methods with relatively high limits of quantification or reporting limits; no dilution of samples. 3. No volunteers involved. 4. High costs for sample collection, relatively low analysis costs per sample/mycotoxin. Primary aim in general is compliance checks, data from this can be used for exposure assessment studies without additional costs. 5. Samples can be used for analysis of other food chemicals and to estimate acute and chronic exposure to these compounds.
Total diet studies	1. Initial study design complex but can be re-used in follow-up studies. 2. Samples are pooled per food category, resulting in dilution of higher contaminated samples; sensitive analysis required to achieve quantifiable results. 3. No volunteers involved; possibly ethical approval required when investigating diets of ethical groups in the population. 4. High costs for the initial design of the study; high costs for sample collection, preparation and analysis. 5. Samples can be used for other food chemicals but only when the study design is suitable for this topic (so called total diet-like studies); only allows estimation of chronic exposure to contaminants.
Duplicate diet studies	1. Simple study design. 2. Limited number of samples because all foods are pooled into one sample and small sample size; sensitive analysis required to achieve quantifiable results. 3. A representative group of reliable volunteers must be identified; ethical approval is required and privacy must be managed; sample collection puts a high burden on the volunteers. 4. High costs for sample preparation; high costs for studies; high costs for processing of food diaries. 5. Samples may be used for analysis of chemicals in food; allows estimation of chronic (if covering more than one day per subject) and acute exposure to contaminants.

Food consumption surveys

The amount of a single food ingredient consumed influences the potential dietary exposure of humans to mycotoxins (Brera *et al.*, 2014; Cressey and Reeve, 2013; EFSA, 2014b; O'Mahony and Vilone, 2013). Data on food consumption of a population are collected via questionnaires from volunteers and, ideally, at the individual level, at a high level of detail, and covering at least two days. At a national level, such databases are widely used to assess the dietary exposure to mycotoxins by the population (Boon *et al.*, 2009; Sprong *et al.*, 2016b). At the international level, various databases are available for exposure assessment. The situation in the EU is assessed by the European Food Safety Authority (EFSA), while the Food and Agriculture Organisation (FAO) of the United Nations evaluates the global food situation, including countries with insufficient food supplies (EFSA, 2015a; FAO/WHO, 2015).

In 2011, EFSA introduced the Comprehensive European Food Consumption Database, hereafter called EFSA Comprehensive Database (EFSA, 2011d). The various EU Member States provide(d) EFSA with data from their most recent national dietary surveys, at the level of the individual consumer. In addition, the food classification system 'FoodEx1' was developed by EFSA to codify all foods and beverages present in the database. FoodEx1 is a hierarchical system based on 20 main food categories that are further divided into subgroups up to a maximum of 4 levels. In 2015, the EFSA Comprehensive Database was updated with additional, up-to-date, food consumption data (EFSA, 2015b). These data were coded with an upgraded version of EFSA's food classification and description system, FoodEx2, which enables more precise reporting of consumption patterns. The FoodEx1 database, up to now used by EFSA, includes food consumption data concerning infants, toddlers, children, adolescents, adults, elderly and very elderly from 32 different dietary surveys carried out in 22 different Member States. Summary statistics are calculated and are available at the EFSA website (EFSA, 2015a). These statistics include the total number of individuals per survey and age class and, for all four levels of FoodEx1, per age class and survey, the total number of consumers, and the mean, the median and the standard deviation of the consumption, as well as low and high percentiles. Food consumption statistics are reported both in grams per day and in grams per kg body weight per day, for both chronic and acute consumption, and for both the total population and consumers only (i.e. those that reported consumption of the specific food product on the one or more days included in the survey). These summary statistics can be used as a quick screening tool to assess chronic and acute exposure to hazardous substances. EFSA uses the detailed underlying consumption data at the individual level to perform more refined exposure assessments, both acute and chronic.

To assess global food intake, the WHO has managed since 1976 a Food Contamination Monitoring and Assessment Programme, within the Global Environment Monitoring System, commonly known as GEMS/Food (WHO, 2015). WHO implements the programme in cooperation with a network of Collaborating Centres and recognised national institutions located all around the world. As part of its dietary exposure assessment mandate, GEMS/Food has developed supra-national model diets that are currently used for estimating dietary intake of various chemicals according to internationally accepted methodologies. Guidance documents have been developed for International Estimated Short-term Intake (IESTI) (FAO/WHO, 2014), International Estimated Daily Intake (IEDI) and a harmonised Total Diet Study approach (see next section) (EFSA/FAO/WHO, 2011b). A monograph on the principles and methods for the risk assessment of chemicals in food has been published (FAO/WHO, 2009).

WHO and FAO have recently developed a new database for Individual Food Consumption Data. This Chronic Individual Food Consumption database – Summary statistics (CIFOCOss), is hosted by WHO and currently contains summary statistics of 37 surveys from 26 countries (only surveys with a survey duration of two days or more) (WHO, 2015). It has been developed to be used by FAO/WHO scientific committees in particular for dietary exposure assessment purposes. It provides summary statistics at three levels of food categorisation and can therefore only be used for a rough indication of the dietary exposure at national level. CIFOCOss is continuously being expanded with data from additional food consumption surveys (WHO, 2015).

Data collection studies

Occurrence data on mycotoxins collected for exposure assessment purposes always require low limits of detection (LOQs) to prevent left-censored data or non-detects ('below LOQ'). The assumption that the levels of mycotoxins in the samples with left censored data are as high as the LOQ, the upper bound principle, only allows worst case estimates (EFSA, 2010). This approach suffices if the margin between assumed exposure levels and levels causing the toxic effects is sufficiently large, but this is often not the case for mycotoxins. EFSA identified this problem with the genotoxic carcinogen sterigmatocystin (EFSA, 2013b) for which the upper bound approach led to a low margin of exposure, as a result from the fact that virtually all available data were left censored due to high LOQs. EFSA calculated a required LOQ of 1.5 µg/kg: based on consumption of the relevant food products, the level causing adverse effects in laboratory animals and the minimal required margin of exposure for genotoxic carcinogens. A dedicated survey was subsequently performed at an LOQ of 0.5 µg/kg and sterigmatocystin was detected in 10% of the 1,259 samples,

in most cases below 1 µg/kg (Mol *et al.*, 2015). Besides the issue of low LOQs, another issue with certain mycotoxins is the high heterogeneity of the contamination which may result in a high uncertainty of the analysis results (higher than the measurement uncertainty of the analysis as such), but this clearly depends on the type of food product.

Food monitoring study

Food monitoring studies investigate the prevalence and concentration of contaminants, including mycotoxins, in single food ingredients or foods (Lopez *et al.*, 2016b; Schwartzbord and Brown, 2015; Straumfors *et al.*, 2015). This increases the chance of detecting the mycotoxins under investigation.

Samples in monitoring studies are typically collected randomly at various points in the supply chain, ideally assuring that a food product can be traced to the producer and repeatedly sampled over a longer time period. The aim of sampling within national (long-term) monitoring programs often is to check for compliance or law enforcement in risk-based designed studies (EC, 2004). This allows higher LOQs, often relatively close to the maximum legal limits (Sulyok *et al.*, 2010). This may compromise the use of the data in exposure assessments, since samples taken in targeted studies may reflect higher occurrence and/or levels of mycotoxins than to which the consumer is exposed. The aim of the sampling should be clearly indicated to prevent potential bias. In addition, samples can also be collected in surveys (De Nijs *et al.*, 2013; Lopez *et al.*, 2016b; Sanders *et al.*, 2014), certainly in case of non-regulated mycotoxins. Results of such surveys are frequently published in literature, contrary to data from long-term monitoring programmes (Muller and Korn, 2013).

The relevance of occurrence data from food monitoring studies for exposure assessments is determined by the information accompanying the samples. Classification and description are important to derive exposure estimations for the general population as well as for sensitive groups in the population, such as infants or people with specific diets as in the case of celiac disease (EFSA, 2013c). Knowledge on geographic origin is of interest for trend analysis and for adjustments to risk-based monitoring programs. Sample size, sampling strategy and sample preparation must be documented since these may influence the exposure results (De Rijk *et al.*, 2015; Garcia-Cela *et al.*, 2013; Pichler, 2013; Spanjer *et al.*, 2006).

In addition to confirmatory methods, also relatively simple screening methods like immunoassays may be used in food monitoring studies. Screening methods are by definition unsuitable to identify the target compound but can be used to exclude their presence above a certain cut-off value. Data obtained with such screening methods require careful evaluation before use in exposure assessments.

Food monitoring studies analyse mycotoxin levels often in raw food ingredients. Occurrence data from these sources require a processing factor when used in exposure assessments, to translate the levels to contamination levels in the ready-to-eat foods. Mycotoxins can be lost after peeling (e.g. vegetables), cleaning and sorting (e.g. peanuts (Torres *et al.*, 2014)) and, in case of cereals, after a certain degree of debranning, i.e. (partly) removing the outer layer of the kernel which is often more contaminated with mycotoxins. Common processing, such as fermentation, heating and household preparation may have a further effect. In their exposure assessment on deoxynivalenol (DON), Boon *et al.* (2009) applied a processing factor to occurrence data in raw wheat and rice samples to estimate the levels in prepared pasta and rice.

Total diet study

In total diet studies, food samples representing the whole diet and prepared as consumed are analysed for food chemicals. Samples are collected, prepared and pooled into composite samples per food category (EFSA/FAO/WHO, 2011b; Marin *et al.*, 2013). Due to the pooling of samples, results are not appropriate for compliance or law enforcement, although they may trigger follow-up studies. Samples collected within a total diet study should cover at least one of the two main aspects of representativeness: seasonality and geographical variation (EFSA/FAO/WHO, 2011a). The geographical coverage is important because of potential regional differences in dietary patterns and in the levels of mycotoxins in foods. Even if no differentiation is made in the exposure assessment between regions, the sampling has to cover potential geographical differences. For small countries like the Netherlands, this might be less relevant (Sprong *et al.*, 2016b), but for larger countries with different climate zones of very different ethnic groups or large variations in consumption patterns, this must be carefully addressed (Australia New Zealand Food Standards, 2011). For locally produced foods, it is important to ensure that discount shops, supermarkets and farmers' markets are sampled proportionally to their market share. Seasonality should be addressed for foods in which the mycotoxin levels may vary due to climatic conditions (i.e. cereals) or seasonal supply variations.

The preparation of the food list is of paramount importance in total diet studies. The food list should cover around 90% of the food intake, should be as close as possible to the actual whole diet and should include beverages and drinking water (EFSA/FAO/WHO, 2011b). Representative food items should be identified and selected from food consumption data. Food processing and at-home food preparation, like addition of water, salt and use of cooking utensils, should

be selected for each type of food and should be as close as possible to the habits of the population. Finally, the degree of pooling into the composite samples should be determined according to the total diet study purposes. Pooling of the processed samples is an essential step and consists of creating a unique food sample (composite sample) for analysis. This is achieved by combining various individually prepared food items either of the same type (individual food approach; e.g. one apple sample made of different varieties of apples) or by mixing several different foods from the same food group (mixed food approach; e.g. one fruit sample made of different types of fruits like apple, pear or banana).

In a total diet-like study, the food items for which contamination levels of the relevant (group of) substances are expected are sampled separately (EFSA/FAO/WHO, 2011a). So in case of patulin, apple products, being the main source of this mycotoxin (Beretta *et al.*, 2000), should be sampled and combined into a separate composite sample, not containing other fruit products (Sprong *et al.*, 2016b). The same applies to aflatoxin M_1 which is only present in dairy products, while e.g. the occurrence of fumonisin B_1 seems more limited to maize derived products and potentially wheat (Cendoya *et al.*, 2014).

Total diet-like studies specifically designed for mycotoxin exposure were carried out in France in 2013 (Sirot *et al.*, 2013) and in the Netherlands in 2014 (Lopez *et al.*, 2016a; Sprong *et al.*, 2016a,b). In the French study, 1,319 food items were collected, prepared and pooled, resulting in 577 composite samples for mycotoxin analysis. In the study in the Netherlands, 1,617 food items were pooled into 213 sub-samples, which were further pooled proportionally according to the consumption habits of the population in the Netherlands, eventually resulting in 88 composite samples for analysis (Sprong *et al.*, 2016b).

Foods contributing most to the exposure can be identified by combining the analysed levels in the total diet(-like) study with food consumption data (Cano-Sancho *et al.*, 2011; Leblanc *et al.*, 2005; Sirot *et al.*, 2013; Sprong *et al.*, 2016a; Vin *et al.*, 2014). EFSA distinguishes two types of total diet study approaches: a total diet study for screening, consisting of a limited number of composite food samples representative for common food categories, and a total diet study for refined exposure assessment containing a large number of samples representative for smaller, more refined, food categories (EFSA/FAO/WHO, 2011a). If the screening indicates high exposures, further evaluation should be performed to identify its source.

Storage of the samples before and after preparation of composites must be assessed, but for mycotoxins this seems less of an issue since they are stable in samples stored in the freezer and during freeze drying. Due to pooling of samples, and thus dilution of the compounds of interest, a low LOQ must be aimed at. Although multi-methods can be applied to the more generic samples, the specific composite samples need dedicated analysis with lower LOQs. For instance, Lopez *et al.* (2016a) applied a dedicated method for trichothecenes using GC-MS/MS after derivatisation, which resulted in ten times lower LOQs than those obtained with multi-methods (Lopez *et al.*, 2016a; Sirot *et al.*, 2013).

No volunteers are involved in total diet(-like) studies and hence no ethical issues are involved in the collection of the samples. However, approval from the competent ethics commission might be required when the study aims at diets of specific ethical groups in the population.

Costs lay in the design of the study, the preparation of the samples and the analysis of the samples. Costs of analysis can be high due to the low LOQs required and can be 50% of the total costs of the study (EFSA/FAO/WHO, 2011b). However, once the total diet study is performed, the design can be used to repeat the study for follow-up studies, thereby reducing their costs considerably. Samples stored may allow trend analysis of known and emerging mycotoxins for risk management information. However, samples collected specifically for certain mycotoxins, may be less useful for other classes of mycotoxins or other contaminants, since these may occur in other specific products that were not sampled separately and/or may be volatile and therefore lost during freeze-drying.

Duplicate diet study

In duplicate diet studies, an exact copy of all the foods and drinks as consumed by one person in a certain time period, e.g. 24 hours, is collected by volunteers with an accompanying food diary. In duplicate diet studies carried out in the Netherland this typically yields about 300 g of freeze dried samples per person. These studies are used to measure the actual exposure of consumers to compounds of interest, including the effects of food processing and preparation (Jekel and Van Egmond, 2014; Vaessen and Schothorst, 1999). Since samples are a mix of the foods as consumed, the contamination can seldom be traced to a specific food ingredient or food which makes the method unsuitable for enforcement of legislation, but allows comparing exposure data with health-based guidance values.

Several alternatives of the duplicate diet study are known, such as the cyclic sub-portion duplicate diet, the sub-population duplicate diet, targeted food duplicate diet and the total population diet (Tomerlin *et al.*, 2002). In these alternatives only a portion of the diet is collected or foods are collected based on standardised or average diets. Duplicate diets collected per eating event (Melnyk *et al.*, 2012) allows the identification of a specific group of foods

responsible for the main exposure. For example aflatoxin M_1 and fumonisins may occur more frequent in breakfast samples since dairy products (milk, yoghurt) and maize based cornflakes are, in many regions of the EU, consumed at breakfast. DON can be present in all meals containing cereal products, which are often consumed during the whole day in European type diets.

The study design is quite straightforward, but the sample collection and preparation are complex. As a result, duplicate diet studies are always limited in the number of participants and sampling days, which hampers the extrapolation of the results to larger populations.

The analysis of duplicate diets requires low LOQs due to dilution of the compounds in the sample (Jekel and Van Egmond, 2014). After homogenisation of the entire sample, it is typically freeze dried and stored in the freezer. Mycotoxins are stable under these conditions, which allows the collection of historic information (trends) when analysing emerging mycotoxins in the future. An example is the exposure assessment for T-2/HT-2 toxins in a study in the Netherlands (Jekel and Van Egmond, 2014).

The involvement of volunteers means that ethical issues must be addressed before the start of the collection. The actual collection of the duplicate diet typically involves the use of a cooled container which can be a burden to volunteers and may influence the diet consumed in the period of collection. Costs for collection of duplicate diets are high and only a limited number of samples can be collected. The latter reduces the costs for analysis but these can still be high due to the very low LOQs required.

3. Exposure assessment strategies

Exposure assessment is defined by the Food and Agriculture Organisation of the United Nations (FAO) and World Health Organisation (WHO) as the qualitative and/or quantitative evaluation of the likely intake of chemical agents via food as well as exposure from other sources if relevant (FAO/WHO, 2008). For estimating the dietary exposure of humans to mycotoxins, information on prevalence and levels in foods are combined with consumption information (Brera *et al.*, 2014; Cressey and Reeve, 2013; EFSA, 2014b). A schematic overview of the well-established exposure assessment strategies for among others mycotoxins is given in Figure 1 and the characteristics are described in Table 2. Each strategy yield unique information and has its own merits and challenges. Depending on the approach, these strategies may be used to estimate either acute exposure, i.e. during a period of 24 hours or less, or chronic exposure, i.e. over a longer period of time: from a couple of months up to several years.

Point estimate

In point estimate assessments, a single mycotoxin concentration value is combined with a single input parameter for consumption. The result is one single exposure estimate with a high degree of uncertainty. The input for concentration often comes from a food monitoring study or a total diet study. A mean or high consumption level from a food consumption survey is often used for input for consumption. If exposure via more than one food product is expected, the calculated exposures per food item can be combined to assess the total exposure.

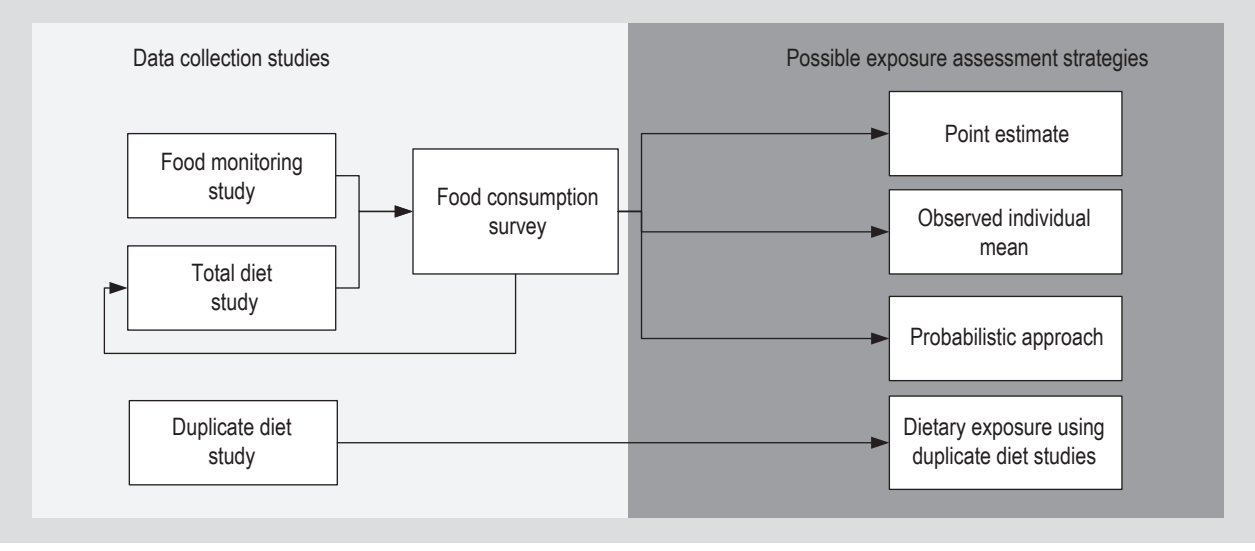

Figure 1. Overview of the methods for data collection and how these serve as input for different strategies to assess the dietary exposure. For total diet studies, the food list included should cover about 90% of the food intake and therefore knowledge of food consumption is a prerequisite for these studies (feed-back loop).

Table 2. Main characteristics of four well-established exposure assessments methodologies.

Exposure assessment strategy	Collection of data on mycotoxin contamination[1]	Volunteers involved in sampling	Takes consumer's food processing into account	Typical samples analysed	Analysis method (scope)[2]	Analysis method (LOQ)[3]	Assessing chronic and/or acute exposure	Characteristics of exposure assessment strategies
Point estimate	FMS	No	No[4]	Individual food ingredients/products	+	–/++	Chronic and acute	Input: single concentration and consumption level per food
	TDS (chronic) combined with data from FCS		Yes	Composite processed food products	–/+	++/–		Output: one chronic or acute intake level (mean, high) Easy to perform
Observed individual mean	FMS	No	No[4]	Individual food ingredients/products	+	–/++	Chronic	Input: distribution of consumption and mean concentration levels
	TDS combined with data from FCS		Yes	Composite processed food products	–/+	++/–		Output: distribution of mean intake levels over all recording days per individual Computer modelling
Probabilistic approach	FMS	No	No[4]	Individual food ingredients/products	+	–/++	Chronic and acute	Input: as Observed Individual Mean
	TDS (chronic) combined with data from FCS		Yes	Composite processed food products	–/+	++/–		Output: distribution of chronic intake, adjusted for within-individual variation; distribution of acute intake per person Computer modelling
Duplicate diet	DDS	Yes	Yes	Composite processed food products	–	++	Chronic and acute	Input: duplicate portions of food Output: measured intake per duplicate portion (individual exposure assessment)

[1] FMS = Food Monitoring Study, TDS = Total Diet Study, DDS = Duplicate Diet Study.
[2] Scope = number of mycotoxins included in one analysis method: + = multiple mycotoxins, – = one or few mycotoxins.
[3] LOQ limit of quantification: range from ++ = very low, to – = relatively high; resulting in higher or lower number of numerical data.
[4] Food processing can be taken into account via food processing factors.

The Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2011) used this approach to calculate for example the chronic and acute exposure to DON and its acetylated derivatives. For chronic exposure, the average consumption data from 10 different regions (GEMS/food consumption cluster diets) were coupled with the weighted mean levels of DON and its acetyl metabolites in different food categories, and summed to obtain an overall estimate of the chronic exposure to DON and its acetyl metabolites per geographic region. In this example, for acute exposure, a wheat intake of 9 g/kg bw (around the P97.5) was coupled to a high mycotoxin level of 10 mg/kg wheat.

In point estimates, often conservative estimates of the input variables are applied, resulting in high-end estimation of the exposure. The main advantage of this approach is that it is easy to perform and often used for a first conservative risk identification.

Observed individual mean

With the observed individual mean approach, a mean mycotoxin concentration per food product is combined with the food consumption per day per consumer, averaged over the days available in the survey and, in most cases, divided by the individual's body weight (Boon and Van der Voet, 2015). This results in the average exposure per kg bw per person per day. Results from a food monitoring study or a total diet study are used as input for mycotoxin concentration.

For chronic exposure, the mean concentration of mycotoxins is used, because in the long run it is unlikely that a consumer will always consume products with only high or only low levels. Typically, information on food consumption is available over a period of 2 to 7 days per individual. With this approach, a distribution of daily average exposures for different individuals within a population is generated. From this distribution the mean and median chronic exposure and upper percentiles (e.g. 95th or 97.5th) for that population can be estimated. This method is currently used by EFSA to assess the chronic exposure to food contaminants and additives (EFSA, 2012a,c; 2015c). In the case of chronic exposure to mycotoxins it was applied for ochratoxin A (EFSA, 2006), aflatoxins (EFSA, 2007), *Alternaria* toxins (EFSA, 2011a), T2/HT-2 toxins (EFSA, 2011b), zearalenone (EFSA, 2011c), ergot alkaloids (EFSA, 2012d), nivalenol (EFSA, 2013a), sterigmatocystin (EFSA, 2013b), DON (EFSA, 2013d), fumonisins (EFSA, 2014a) and beauvericin/enniatins (EFSA, 2014b). Occurrence data from a total diet study were recently used by to assess the exposure of consumers in the Netherlands to 48 mycotoxins, including patulin, aflatoxins, ochratoxin A, fumonisins, zearalenone, trichothecenes, ergot alkaloids, *Alternaria* toxins, beauvericin and enniatins (Lopez *et al.*, 2016a; Sprong *et al.*, 2016a,b).

Probabilistic approach

With the use of the probabilistic approach, both the acute and chronic exposure via food can be estimated. The acute exposure is estimated by combining daily individual consumption patterns from a food consumption survey with randomly selected levels per food product from a databank with mycotoxin levels in individual samples. The resulting individual exposures per food product are summed to obtain the exposure per day and subsequently divided by the individual's body weight. This procedure is repeated multiple times (Monte Carlo simulation) resulting in individual daily exposure estimates that reflect all plausible combinations of daily consumptions and concentrations in a population. The upper part of the distribution represents consumers with a high intake of the compound (high consumers), which is important for assessing the acute risk.

In the case of ergot alkaloids, which may exert acute toxic effects, EFSA combined individual consumption data with a high mycotoxin level (P95 or the mean of the upper quartile) for the four main contributing food groups and an average level for the remainder (EFSA, 2012d). By summing up the exposure per individual per day, a distribution of so-called 'acute' exposure estimates was generated. Both the mean and P95 values for these distributions in the various food consumption surveys were calculated and used to assess acute risks. Since no individual mycotoxin levels in food are used in this study, this approach is merely a combination of the point estimate (fixed levels) and a probabilistic approach (distribution of consumption).

With the use of the probabilistic approach, also the chronic exposure can be estimated using statistical models which use the same input as the observed individual mean approach (see previous section). The largest uncertainty of the use of the observed individual mean approach to assess the long-term exposure is equalling the distribution of mean exposures over the person-days per person, typically two days, to the 'true' long-term exposure distribution of a given population. Given the limited number of person-days present in a food consumption database per person and the variation in daily food consumption patterns within an individual, the distribution of mean exposures over individuals obtained with the observed individual mean approach will often be too wide in comparison to distributions of 'true' long term exposures (Goedhart *et al.*, 2012). These distributions contain both the variation in exposure between individuals and between days for the same individual (the within individual variation), whereas for the long-run the variation within individuals is not relevant by definition (the long-term exposure distribution is the variation between individuals, not within individuals) (Boon and Van der Voet, 2015). Statistical models that separate these two sources of variation, and subsequently remove the within individual variation from the long-term exposure distribution, have proven to be useful for the estimation of long-term exposure (Dodd *et al.*, 2006; Hoffmann *et al.*, 2002; Slob, 1993).

The resulting long-term exposure distribution will be less broad than the distribution obtained with the observed individual mean approach, resulting especially in lower estimates of the higher percentiles of exposure and higher estimates of the lower percentiles. For example, the mean exposure assessed over just two days is more variable than the mean exposure assessed over more (up to hundreds) days that constitute a longer period of time.

Dietary exposure using duplicate diet studies

In duplicate diet studies (described in detail in Section 2) daily portions are analysed for the compound(s) in question, resulting in an actual exposure level per day for that individual. The food consumption data collected in accompanying food diaries can be used to evaluate the possible sources of exposure. When duplicate portions are collected on only one day per individual, these data can only be used to assess acute exposure. If duplicate portions are collected on multiple days, chronic exposure can be estimated by either averaging the exposure over the collection days per individual (see observed individual mean) or by removing the within individual variation using statistical models (see probability approach). Duplicate diets have been collected and analysed for estimating the

exposure to various mycotoxins, like aflatoxin B_1, aflatoxin M_1, ochratoxin A, trichothecenes, fumonisins and T-2/HT-2 toxins (Bakker *et al.*, 2009; Gilbert *et al.*, 2001; Jekel and Van Egmond, 2014; Sizoo and Van Egmond, 2005).

4. Human biomonitoring studies

An alternative way to assess exposure to a compound is the use of biomarkers to quantify the internal dose. Characteristics of the data collection studies and subsequent exposure assessment strategy are mentioned in Table 3 and 4.

Exposure can be assessed by biomarkers of exposure. This includes the detection of the parent compound (mycotoxins) and/or its main phase I and phase II metabolites (e.g. glucuronide conjugates), measured in accessible body fluids (blood, urine) or body specimen (like hair). In contrast, biomarkers of effect can be used to assess the outcome after exposure to mycotoxins and include, e.g. changes in level of specific proteins (including diagnostic enzymes), cellular metabolites (metabolomics), or gene expression profiles (toxicogenomics) resulting from the specific alteration in metabolic or signalling pathways (Valencia-Quintana *et al.*, 2014). Since these changes can also results from inter-individual differences, effects from other compounds in food or underlying diseases, it is difficult to link them exclusively to the intake of mycotoxins. Human biomarker research related to mycotoxins started in the early 1990's to gain insight into the mechanisms of action of aflatoxin B_1 and to control the outcome of intervention strategies. Aflatoxin M_1 was the predominant biomarker of exposure, detected in milk and urine during exposure assessments (Gambacorta *et al.*, 2013; Routledge *et al.*, 2014). Changes in the sphingosine/sphinganine ratio were used as biomarker of exposure and effect for fumonisins (Eaton and Gallagher, 1994; Gilbert *et al.*, 2001; Guengerich *et al.*, 1998; Mclean and Dutton, 1995; Shephard *et al.*, 2007).

The measurement of biomarkers of exposure is the only available strategy that integrates exposure from all sources, (e.g. food, inhalation, skin contact) and reflects the internal biological active fraction (Choi *et al.*, 2015). The exposure can be assessed by converting urinary mycotoxin concentrations to intake levels, taking into account their kinetics, such as rate of excretion (i.e. % of the ingested mycotoxins excreted as the parent compound or metabolites in urine), daily urine production (defined by e.g. creatinine)

Table 3. Characteristics of: (1) study design; (2) sample number and analysis; (3) ethical issues; (4) costs; and (5) ability to use provided samples for other contaminants.

Data collection study	Characteristics
Human biomonitoring studies	1. Study design is complex; knowledge on relevant biomarkers and toxicokinetics is required. 2. Collection and analysis involves handling of body fluids; high costs for sample preparation; standardised analytical methods not readily available; dedicated highly sensitive analysis required to achieve quantifiable results due to low concentrations in urine. 3. A representative group of reliable volunteers must be identified; ethical approval is required and privacy must be managed. 4. Relatively high analysis costs per sample/mycotoxin. 5. Samples can be used for analysis of multiple contaminants; allows estimation of chronic and acute exposure to contaminants.

Table 4. Main characteristics of exposure assessments via biomarkers.

Exposure assessment strategy	Collection of data on mycotoxin contamination[1]	Volunteers involved in sampling	Takes consumer's food processing into account	Typical samples analysed	Analysis method (scope)[2]	Analysis method (LOQ)[3]	Assessing chronic and/or acute exposure	Characteristics of exposure assessment strategies
Human biomonitoring	HBS	Yes	Yes	Urine, blood	–/+	++	Chronic and acute	Input: concentrations in biological fluids Output: estimation of uptake or intake

[1] HBS = human biomonitoring study.

[2] Scope = number of mycotoxins included in one analysis method: + = multiple mycotoxins, – = one or few mycotoxins.

[3] LOQ limit of quantification: range from ++ = very low, to – = relatively high; resulting in higher or lower number of numerical data.

and body weight (Gratz *et al.*, 2014; Rodriguez-Carrasco *et al.*, 2014; Sarkanj *et al.*, 2013; Solfrizzo *et al.*, 2014; Turner *et al.*, 2008; Wallin *et al.*, 2013; Warth *et al.*, 2012). This re-calculation is only possible for toxins showing a rapid elimination and short mean residence time (like for example DON). Since most of the toxicokinetic studies for mycotoxins have been performed in animals and specific data for humans are often lacking, exposure assessment using biomarkers of exposure is up to now a quantitative approach with high uncertainties.

Nevertheless, human biomonitoring was recently used to assess exposure of the Belgian population to the mycotoxins aflatoxins, citrinin, fumonisins, trichothecenes, ochratoxin A, zearalenone and their metabolites or modified forms (Heyndrickx *et al.*, 2015). This study showed the very high prevalence of exposure to toxins such as DON. Moreover, citrinin was found in 50% of the urine samples while this mycotoxin was detected at only low levels and incidence in monitored food samples. This apparent discrepancy may result from variations in kinetic factors influencing the excretion of citrinin in humans, but may also indicate the need to identify as yet neglected sources of human citrinin exposure (Heyndrickx *et al.*, 2015).

A human biomonitoring study may also give insight in the bioavailability of mycotoxins from unexpected or unknown sources and the presence of modified forms in food (e.g. glucosides, sulphates), which may become available for absorption after conversion to the parent compound in the gastrointestinal tract (EFSA, 2014a; Solfrizzo *et al.*, 2014). These modified mycotoxins are those forms that may not be included in the currently applied analytical methods, partly since analytical standards and reference materials are not yet available or because the (modified) mycotoxins are not extracted with the applied methods.

In summary, although biomarkers for mycotoxin exposure can be measured in various biological fluids and tissues, such as (breast)milk, plasma, saliva, faeces, hair, nails, liver, kidneys or lungs, urine has become the preferred matrix (Sewram *et al.*, 2001; Shephard *et al.*, 2007). Human biomonitoring can identify vulnerable groups and can reduce the assumptions regarding consumption rates (Choi *et al.*, 2015). Results of epidemiological studies on mycotoxins using biomonitoring have been reported in the past two decades (Duarte *et al.*, 2012; Ediage *et al.*, 2013; Gilbert *et al.*, 2001; Pena *et al.*, 2006; Rubert *et al.*, 2011; Turner *et al.*, 2010; Van der Westhuizen *et al.*, 2011). These studies were based on only one urine sample per person, and thus the collection can be fairly easy (Heyndrickx *et al.*, 2014; Solfrizzo *et al.*, 2014). However, authors of a recent study, whereby the exposure to DON in three EU countries using these urinary biomarkers was assessed, strongly advice to collect 24-hours urine (Brera *et al.*, 2015).

In order to compare the urinary concentrations within individuals, inter-individual variability in urine production needs to be circumvented. For this reason, urinary creatinine concentrations are widely used to adjust urinary concentrations of chemicals or their metabolites (Barr *et al.*, 2005; Turner *et al.*, 2010). Furthermore, the analysis of urine samples requires low LOQs, which can be reached by applying targeted analysis. Moreover, urine samples are usually treated with enzymes like β-glucuronidase/sulfatase to convert conjugates into the parent compound (Solfrizzo *et al.*, 2014). Costs for human biomonitoring studies are high due to the involvement of large numbers of volunteers, a complicated study design and sample collection. Analysis of the samples is costly. Finally, human biomonitoring involve volunteers and, therefore, ethical issues must always be addressed and a consensus of the volunteers is needed.

5. Comparison of the different approaches

Selection of the most suitable approach

In practice, a tiered approach can be used to estimate the exposure to mycotoxins within a population. In lower tiers, exposure estimates are based on limited data using a point estimate approach. When exposure seems to be significant, these estimates can be refined, in the case of chronic exposure using the observed individual mean and/or statistical methods for extrapolation to long-term exposure, and for acute exposure the probabilistic approach, resulting in the most accurate estimation of both types of intake (Pastoor *et al.*, 2014).

Advantage of observed individual mean and probabilistic approaches is that the entire food consumption database, and food concentration data, is considered which results in more realistic exposure estimates of a given population (in a country of geographic region). This supports the derivation of a health-based guidance value and identifies segments of a population (such as children) with a higher risk. Another advantage is that also the uncertainty in the exposure estimations, caused by a limited size of the database, can be quantified (EFSA, 2012b).

The effects of food processing and preparation are included in total diet studies and in duplicate diet studies. This is of particular importance for less stable compounds, as otherwise exposure could be largely overestimated. An obvious limitation is the limited number of samples and the high costs and subject burden in duplicate diets studies. To illustrate this, in a duplicate diet study in the Netherlands into the exposure to T-2/HT-2 toxins covering several years, about 125 samples were collected per year (Jekel and Van Egmond, 2014). Subsequently, the duration of the duplicate diet studies is often limited to one day enabling acute, and much less for chronic, exposure assessment (Solfrizzo *et al.*,

2014). Drawback of a total diet study is the required large amount of food items. To illustrate this, 1,617 single food items were included in a total of 88 composite samples in a total diet study on exposure to mycotoxins (Sprong *et al.*, 2016a). When assessing the exposure to mycotoxins using food consumption and food mycotoxin level data, effect of processing can be included by using processing factors.

Human biomonitoring is the only strategy that includes exposure from sources other than food, although this may be less relevant in the case of mycotoxins. Human biomonitoring requires a detailed understanding of the toxicokinetics in the human body which is still poorly understood for many mycotoxins. A clear advantage of biomarker studies is the estimation of the internal dose, determining the biological effects, and the identification of biomarkers that combine effect and exposure monitoring, such as DNA adducts of aflatoxins. Moreover, measuring the actual concentration in body fluids addresses the uncertainty of the existence of modified forms of mycotoxins that remain undetected by conventional analytical methods. Recently, EFSA concluded that glucosides of DON, nivalenol and zearalenone, and the hidden forms of fumonisins (EFSA, 2014a) may considerably contribute to the overall human exposure. In addition, EFSA proposed to apply relative potency factors for all known biologically active metabolites. This applies for example to zearalenone, as an equivalence factor of 60 needs to be considered for its metabolite α-zearalenol, in the derivation of health-based guidance levels (EFSA, 2016).

Emerging mycotoxins

Exposure assessments of mycotoxins that have no legal limit face some additional challenges. At the moment of data collection, it may not be clear which foods contribute most to the exposure, samples may not be well characterised, analytical reference standards or reference materials are lacking, method of analysis may not be harmonised or validated, LOQs required to exclude potential health risks are unknown (Mol *et al.*, 2015), and the relation between intake and excreted metabolites is unknown. Analysis of long-time stored duplicate or total diets may overcome these problems to some extent, assuming all relevant consumed foods are collected. When new mycotoxins are discovered, or new analytical methods with lower LOQs become available, or when applying trend analyses, these stored samples can provide valuable information (Jekel and Van Egmond, 2014). Human biomonitoring studies may have little relevance in this situation if relevant biomarkers of emerging mycotoxins are not yet defined.

Trends in levels and exposure

Ideally, food consumption surveys are regularly updated, to assess changes in mycotoxin exposure in time. Trends like an increased consumption of breakfast cereals containing nuts may increase the exposure to mycotoxins present in nuts, such as aflatoxins and ochratoxin A. Results from monitoring studies can be used for analysing trends in mycotoxin occurrence in (raw) food materials and may lead to an updated exposure assessment. Significant increases in mycotoxin exposure, even if below the health-based guidance value, can be detected, with subsequent identification of the source of the increased exposure and evaluation of measures for reduction.

6. Conclusions

Exposure assessments of mycotoxins are complicated since their concentrations in food: (1) are often heterogeneously distributed over the samples; (2) differ between geographic regions and production years; (3) can be affected by (food) processing and preparation; and (4) mycotoxins can be present in modified forms. Each of the five strategies for estimating the dietary exposure of a population to mycotoxins discussed in this paper has its merits and restrictions. The same applies to the studies collecting the data needed to perform these assessments. A combination of the different strategies has an added value for the risk assessment of mycotoxins, including the identification of unknown sources or modified forms.

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