
**REVIEW ARTICLE**

# Sampling feed for mycotoxins: acquiring knowledge from food

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## ABSTRACT

The occurrence and control of mycotoxins in feed and food are items of great interest to researchers, producers, manufacturers and regulatory agencies. In order to implement knowledge of control measures for mycotoxins in the entire food production chain, coordinated inspection programmes aimed to check the presence and concentration of mycotoxins in feedingstuffs are recommended by the Commission of the European Communities. Reliability of measured levels of mycotoxins in feed and food is greatly affected by the collection of representative samples. Because of the heterogeneous distribution of mycotoxins, the variability associated with a mycotoxin test procedure usually depends heavily on the sampling plan. European legislation dealing with sampling plans for mycotoxins in foodstuffs has been recently revised. The aim of the following overview is to discuss the role of sampling in mycotoxin-contaminated feed by considering the evolution of legislation dealing with sampling plans for food. A sampling procedure is a multistage process and consists of three distinct phases: sampling, sample preparation and analysis. The variability associated with each step of a sampling procedure and the aspects related to feedstuffs, matrix/mycotoxin combination and level of contamination are discussed.

*Key words:* Feed analysis, Mycotoxins, Sampling.

## RIASSUNTO

### MICOTOSSINE NEL *FEED* E *FOOD*: PROBLEMATICHE DI CAMPIONAMENTO ED ASPETTI NORMATIVI A CONFRONTO

*La valutazione della presenza di micotossine negli alimenti per animali ed in quelli destinati all'alimentazione umana rappresenta un problema di dimensione globale e di estremo interesse per ricercatori, operatori del settore agro-alimentare ed organismi di controllo e legislativi. Annualmente la Commissione della Comunità Europea raccomanda agli stati membri di attuare un programma coordinato di controlli nel settore dell'alimentazione animale al fine di verificare la presenza e la concentrazione di micotossine nei mangimi. La raccolta di dati sulla presenza delle micotossine è utile per valutare la situazione in vista dell'elaborazione della legislazione in questo campo. Poiché la concentrazione di micotossine varia da un anno all'altro, risulta opportuno raccogliere dati riguardanti più anni consecutivi. Le micotossine*

*sono caratterizzate da una distribuzione non omogenea e pertanto il campionamento è una fase critica. Ne deriva che la standardizzazione delle metodologie di campionamento ed analisi per la determinazione delle micotossine negli alimenti è un aspetto primario e assolutamente essenziale ai fini della garanzia della affidabilità del dato analitico. In relazione alle modalità di campionamento ed analisi degli alimenti destinati all'uomo, la normativa europea è stata recentemente revisionata. In relazione a tale evoluzione normativa, l'obiettivo di questa review è quello di approfondire alcuni aspetti critici del campionamento per la valutazione della presenza di micotossine negli alimenti per animali, aspetti utili al fine di migliorare e validare protocolli di campionamento specifici per i mangimi. Una procedura di campionamento deve essere considerata un processo costituito da diverse fasi: raccolta di campioni rappresentativi, preparazione del campione e successiva fase analitica. I fattori che influenzano la variabilità associata alle tre diverse fasi della procedura di campionamento verranno discussi in relazione a specifiche problematiche per gli alimenti per animali, alle interazioni matrice/micotossine ed ai livelli di contaminazione.*

Parole chiave: *Analisi alimenti per animali, Micotossine, Campionamento.*

## Introduction

The knowledge and control of mycotoxin contamination and distribution in food and feed is a worldwide objective of producers, manufacturers, regulatory agencies and researchers due to the high economic and sanitary impact on food safety and human/animal health. Since it is impossible to fully eliminate the presence of undesirable substances, maximum concentrations should be set at a strict level which is reasonably achievable considering the risk related to the consumption of the food. Since sampling is the critical step in obtaining reliable results on mycotoxin content, the Commission of the European Communities brought together in one single legal act (European Commission, 2006c) the sampling methods and the performance criteria for the methods of analysis to be used for the official control of mycotoxins in foodstuffs. There are significant differences in the distribution of mycotoxins in commodities (Whitaker *et al.,* 1996; Hart and Schabenberger, 1998; Larsen *et al*., 2004; Whitaker *et al.,* 2004; Miraglia *et al*., 2005), and Commission Regulation (EC) 401/2006 (European Commission, 2006c) provides different sampling plans according to the type of food product. All these aspects have a strong impact on animal feeding, due to the awareness that high food quality standards cannot be reached unless such high standards are applied to feed. In this context, the "White Paper on Food Safety", issued in 2000, represented a revolutionary document and clearly proposed the "from producer to consumer" approach. This approach addresses the importance of integration among primary production, feed manufacturing and quality of feed production *per se* to ensure food product safety.

The aim of the following review is to discuss the role of sampling in mycotoxin analysis by examining the evolution of legislation dealing with sampling plans for food, pointing out the critical points to be considered for the further development of legislation for feedstuffs. A sampling procedure is a multistage process and consists of three distinct phases: sampling, sample preparation and analysis. The variability associated with each phase of a sampling plan, the aspects related to feedstuffs, matrix/mycotoxin combination and level of contamination and their effects on the reliability of mycotoxin contamination level results are discussed.

## Sampling: definitions and objectives

A sampling plan for mycotoxins may be defined as a "test procedure combined with

a sample acceptance limit" (Johanson *at al.*, 2000a). Therefore, among the purposes of mycotoxin control programmes, the setting of maximum levels for these contaminants in feed and food and the definition of effective sampling plans represent important targets for European legislation. Maximum concentrations should be set at a strict level which is reasonably achievable by following good agricultural and manufacturing practices and considering the risk related to the consumption of the food. In order to achieve the general objective of a high level of protection of human health and life, food law is based on risk analysis. The European Food Safety Authority (EFSA) was established to assess risks associated with the food chain. EFSA's risk assessment work contributes to improving food safety in Europe and to building public confidence in the way risk is assessed. Risk assessment is a specialised field of applied science that involves reviewing scientific data and studies in order to evaluate risks associated with certain hazards. Therefore, maximum acceptable levels for mycotoxins have been established based on literature data, scientific opinions, assessment of the dietary intake by the population, reports of Scientific Committee for Food. The Commission of the European Communities fixed maximum levels for mycotoxins in foodstuffs through Commission Regulation 466/2001. This regulation was frequently and substantially amended and definitely replaced by Commission Regulation (EC) 1881/2006 of 19 December 2006 (European Commission, 2006b) further amended by Commission Regulation (EC) 1126/2007 of 28 September 2007 (European Commission, 2007). In the field of animal nutrition, specific indications on mycotoxins and other undesirable substances in animal feed are considered in the Commission Directive 2003/100/EC (European Commission, 2003) and in the Commission Recommendation 2006/576/EC (European Commission, 2006a). The maximum content for aflatoxin B1 and the guidance levels for deoxynivalenol, zearalenone, ochratoxin A and fumonisins in products intended for animal feeding are reported in Table 1 and Table 2.

Regarding the methods of sampling and analysis for the official control of mycotoxins and undesirable substances in feed, two legal acts are to be considered: First Commission Directive 76/371/EEC "Establishing Community methods of sampling for the official control of feedingstuffs" (European Commission, 1976) and Commission Regulation (CE) 401/2006 "Laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs" (regulation criteria are in accordance with the opinion of the Standing Committee on the Food Chain and Animal Health (European Commission, 2006c)), that repeal Directives 98/53/EC, 2002/26/EC, 2003/78/EC and 2005/38/CE. Among the issues addressed by the Commission Directive and Regulation, definitions and quantitative requirements related to the control of undesirable substances, uniformly distributed in different commodities or supposed to be heterogeneously distributed throughout products, are listed in order to supply standardized information for representative sampling. In relation to sampling protocols, the most relevant definitions are reported in Table 3 and Table 4.

In order to obtain reliable analytical results, sampling procedures represent the foremost critical point. Although they are usually underestimated in food/feed analysis, they are accountable for the largest source of variation associated with the quality of the final analytical result (Whitaker, 2003). The sampling protocol adopted is of critical relevance since fungal development and mycotoxin production are "spot processes" significantly affected by crop variety, agronomic practices, weather conditions during growing and harvest, storage and process-

Table 1. Maximum content of aflatoxin B1 in feed materials, complete and complementary feedingstuffs (European Commission, 2003).

| Products intended for animal feed | Aflatoxin B1: maximum content in mg/kg (ppm) relative to a feedingstuff with a moisture content of 12% |
|---|---|
| All feed materials | 0.02 |
| Complete feedingstuffs for cattle, sheep and goats with the exception of: | 0.02 |
| - complete feedingstuffs for dairy animals | 0.005 |
| - complete feedingstuffs for calves and lambs | 0.01 |
| Complete feedingstuffs for pigs and poultry (except young animals) | 0.02 |
| Other complete feedingstuffs | 0.01 |
| Complementary feedingstuffs for cattle, sheep and goats (except complementary feedingstuffs for dairy animals, calves and lambs) | 0.02 |
| Complementary feedingstuffs for pigs and poultry (except young animals) | 0.02 |
| Other complementary feedingstuffs | 0.005 |

ing conditions and toxigenic potential of the different mould species. For instance, bulk moisture can facilitate the development of localized clumps particularly rich in moulded kernels (Shotwell *et al.,* 1974). These small percentages of extremely contaminated portions ("hot spots") are randomly distributed in the lot (average value usually registered 0.1%) (Johanson *et al*., 2000c). This condition can lead to an underestimation of the real level of mycotoxin if a too small sample size without contaminated particles is analysed or, instead, to an overestimation of the true level in the case of a too small sample size featuring one or more contaminated particles. Furthermore, due to the ecology of moulds, aspects related to specific matrix/mycotoxin combination and level of contamination must be considered when planning a sampling protocol. Moreover, screening, monitoring, controlling, exposure studies or targeted purposes may require specific sampling approaches (Miraglia *et al*., 2005). Therefore, a scientifically based sampling protocol has to consider the variability and heterogeneous distribution of mycotoxins, the specific matrix/mycotoxin combination and the sampling target. In order to perform this purpose, statistical and mathematical models, verified by simulations, have been developed or are in study (Whitaker *et al*., 1998; Johanson *et al*., 2000a; Macarthur *et al*., 2006; Whitaker, 2006). Since legal limits for aflatoxins have been established in many countries, through the past several years, focus has mainly been on aflatoxins in food and feed (Whitaker *et al*., 1994, 1996; Gilbert and Vargas, 2003; Macarthur *et al.*, 2006; Whitaker, 2006), not on other mycotoxins, for which there are still not enough

Table 2. Guidance values for the acceptability of compound feed, cereals and cereal products for Commission Recommendation 2006/576/EC (European Commission, 2006a).

| Mycotoxin/Products intended for animal feed | Maximum content in mg/kg (ppm) relative to a feedingstuff with a moisture content of 12% |
|---|---|
| Deoxynivalenol | |
| Feed materials: | |
| - Cereals and cereal products with the exception of maize by-products | 8 |
| - Maize by-products | 12 |
| Complementary and complete feedingstuffs with the exception of: | 5 |
| - complementary and complete feedingstuffs for pigs | 0.9 |
| - complementary and complete feedingstuffs for calves (<4 months), lambs and kids | 2 |
| Zearalenone | |
| Feed materials: | |
| - Cereals and cereal products with the exception of maize by-products | 2 |
| - Maize by-products | 3 |
| Complementary and complete feedingstuffs: | |
| - Complementary and complete feedingstuffs for piglets and gilts (young sows) | 0.1 |
| - Complementary and complete feedingstuffs for sows and fattening pigs | 0.25 |
| - Complementary and complete feedingstuffs for calves, dairy cattle, sheep (including lambs) and goats (including kids) | 0.5 |
| Ochratoxin A | |
| Feed materials: | |
| - Cereals and cereal products | 0.25 |
| Complementary and complete feedingstuffs: | |
| - Complementary and complete feedingstuffs for pigs | 0.05 |
| - Complementary and complete feedingstuffs for poultry | 0.1 |
| Fumonisin B1+B2 | |
| Feed materials: | |
| - Maize and maize products | 60 |
| Complementary and complete feedingstuffs for: | |
| - pigs, horses (Equidae), rabbits and pet animals, | 5 |
| - fish | 10 |
| - poultry, calves (<4 months), lambs and kids | 20 |
| - adult ruminants (>4 months) and mink | 50 |

Table 3. Definitions (European Commission, 1976).

| | |
|---|---|
| Sampled portion | A quantity of product constituting a unit, and having characteristics presumed to be uniform |
| Incremental sample | A quantity taken from one point in the sampled portion |
| Aggregate sample | An aggregate of incremental samples taken from the same sampled portion |
| Reduced sample | A representative part of the aggregate sample, obtained from the latter by a process of reduction |
| Final sample | A part of the reduced sample or of the homogenized aggregate sample |

Table 4. Definitions (European Commission, 2006c).

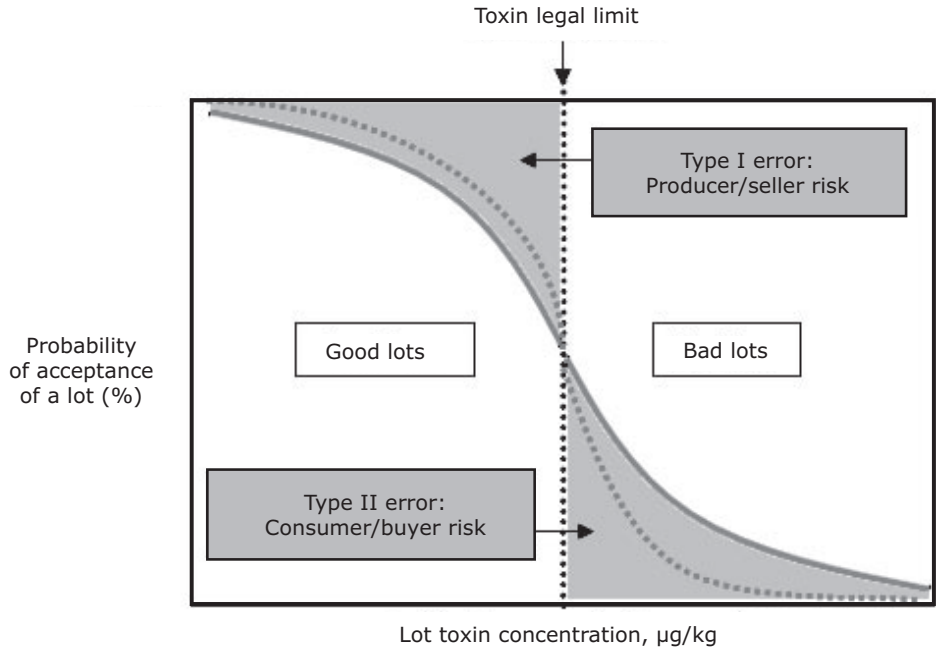
| | |
|---|---|
| Lot | An identifiable quantity of a food commodity delivered at one time and determined by the official to have common characteristics, such as origin, variety, type of packing, packer, consignor or markings |
| Sublot | A designated part of a large lot in order to apply the sampling method on that designated part; each sublot must be physically separate and identifiable |
| Incremental sample | A quantity of material taken from a single place in the lot or sublot |
| Aggregate sample | The combined total of all the incremental samples taken from the lot or sublot |
| Laboratory sample | A sample intended for the laboratory |

data available (Hart and Schabenberger, 1998; Whitaker *et al*., 2000). Existing data indicate that ochratoxin A and deoxynivalenol are less heterogeneously distributed than aflatoxins, and therefore sampling procedures could be less difficult (Larsen *et al*., 2004; Miraglia *et al*., 2005).

Obtaining a precise and accurate estimation of mycotoxin content in a commodity bulk lot is fundamental for products intended for food/feed uses. The trade volumes of commodities for feed use contaminated with aflatoxins are lower than matrices characterised by other mycotoxin contamination, e.g. deoxynivalenol (Larsen *et al.,* 2004). In addition, EU regulations and legal limits have effects on EU products and the associated trade, although, as a matter of fact, a great part of contaminated commodities come from third countries with different legislation (Stroka *et al*., 2004). All these reasons justify the need for reliable and validated sampling methods, which also need to be feasible and purpose-fitted in order to support the official control of different mycotoxin/matrix combinations. This goal is also of great importance for feedstuff because although knowledge may be drawn from foodstuff research, mycotoxin contamination in by-products or raw materials mainly designated for animal consumption are less studied and not well-known. Results from official control of mycotoxin content on animal feed are at present very limited (European Commission, 2002). In this context, Annual Commission Recommendations on

coordinated inspection programmes in the field of animal nutrition have been issued in the Official Journal of the European Union since 2001. The Recommendations state that gathering information on the presence of mycotoxins through random sampling could provide useful data for assessing the situation in function of legislation development. Further concerns to take into account during sampling procedure development and planning are the consequences of economic relevance and consumer health benefits after procedure application. Even in the case of protocols for official control, in any circumstance it is not possible to obtain a quantitative value for the analyte associated with 100% certainty. However, for a given sampling plan, it is possible to calculate the acceptance probability to reject a good lot (type I error, false positive sample) or to accept a wrong lot (type II error, false negative sample) as a function of the toxin concentration and the risk associated with a specific sampling plan. The two different conditions depict an economic loss or a health consumer risk increase, respectively. The effect of each sampling protocol, for the determination of type I or II errors may be described by the probability of accepting a lot as a function of lot quality level, graphically explained by the plot of mycotoxin concentration versus acceptance probability. This plot takes the name of Operating Characteristic Curve (OC curve) (Figure 1)

Figure 1. Representation of Operating Characteristic (OC) curves. Portions of an accepted lot and producer/seller and consumer/buyer risks are indicated by the shape of the curve. Change in curve slope (dotted line, e.g. by increasing sample and subsample size, number of aliquots analysed) indicate better classification of a lot and reduction of risks.



(Whitaker, 2006). By plotting an OC curve, the limit level ("sample acceptance limit") of mycotoxin concentration is specified beforehand (legal or product quality needs) by the operator. The areas delimited by the curve and the limit level describe, respectively, economic (area above the curve) or consumer risk (area below the curve) magnitude. Each sampling plan is described by a unique OC curve. Since the slope of the OC curve has high economic and health relevance, it is crucial, when designing a sampling plan, to increase the slope of the OC in order to reduce the two areas associated with consumer and producer risks (Figure 1) (Johansson *et al*., 2000a; Whitaker, 2006). This can be achieved, as described in detail in the following paragraphs, by using specific sampling plans capable of reducing the uncertainty associated with the analytical value. To the best of our knowledge, specific OC curves have been reported only for few specific feedstuffs, such as copra and palm meal and cake contaminated with aflatoxin B1 (Cocker *et al.,* 2000), while are missing for other by-products for animal feeding.

**The sampling procedure: uncertainty**

A mycotoxin-sampling plan is a multistage process and consists of three distinct phases: sampling, sample preparation and analysis (Whitaker, 2006). Each phase of a sampling plan is associated with a specific level of uncertainty and therefore, as mentioned above, in no circumstance is it possible to obtain a quantitative value for the mycotoxin contamination associated with 100% certainty (Whitaker, 2006). Two main sources of uncertainty can be associated with all mycotoxin test procedures: accuracy (also defined as "bias") and precision ("variability") (Cochran and Cox, 1957; Whitaker, 2006). Bias is described as a deviation of the measured value of a sample from the true value. Precision represents the closeness of the measured value to the average value and may be described by standard deviation (SD) and coefficient of variation (CV, usually reported as a percentage) of the average final analytical values.

It is intuitive that each step of a sampling protocol specifically contributes to the final uncertainty of the procedure. The total variance of a specific sampling plan (TV, also indicated as "Total error"), may be expressed by using statistic variance as a measure of variability and may be described as the sum of sampling variance (SV), sample preparation (SPV), and analytical variance (AV) as follows: TV=SV+SPV+AV. TV and variance distribution during different steps of the sampling protocol give indications on sampling plan efficiency and effectiveness of different sampling plans to the final purpose.

**Sampling: collecting the incremental samples**

The first step of a sampling protocol is the sampling phase. In order to perform reliable sampling, each unit within a lot must have the same probability of being chosen. If this is the case, sampling is defined random sampling and will be theoretically characterized by the absence of systematic errors. Random sampling strongly contributes to the reduction of variability and is able to transfer reliable information from the samples to the entire lot. Two main aspects of this sampling step are critical for reducing uncertainty: number/size of incremental samples selected by the commodity bulk lot and techniques applied for physical sample selection. A reduced number of samples and/or a too small sample (inadequate mass) represent the two most frequent and important errors responsible for total uncertainty amplification. Kernel (or particles) dimensions and distribution of

mycotoxin contamination for single kernel, described above as "spot contamination", induce variation among samples taken from the same bulk. In the majority of cases, only few kernels, dispersed in the lot, have high levels of contamination (Johansson *et al*., 2000c). Due to this, granulometry of particles and/or seed size (number of seeds per weight unit) of the commodity under examination, mass and number of samples need to be considered when planning incremental sample collection (Whitaker *et al*., 2002). For example, Macarthur *et al*. (2006) performed a simulation statistical model in order to assess the minimum number of incremental samples and reduce the variability associated with the sampling step. The model worked well and gave reliable results for the estimation of ochratoxin A content in dry fruit, but did not work for aflatoxin B1 in peanuts. Specific studies regarding quantification of the number/size of incremental samples to be collected for feed analysis are not reported in literature. In this context, the only contribution has been given by Coker *et al.* (2000) who selected copra meal pellets, copra cake, palm kernel cake and cottonseed cake, collected from large shipments, as matrices for aflatoxin B1 sampling plans evaluation. Incremental sample size was found to be less critical for oilseed meals and cakes compared to oilseed kernel. The authors conclude that these findings are related to differences in granulometry of particles and/or seed size and reflect a relatively homogeneous distribution of aflatoxin B1 in oilseed by-products compared to the highly skewed distribution of the same mycotoxin in particulate commodities such as groundnuts kernels. Therefore, precise sampling plans for by-products for animal consumption need to be developed. In order to make objective choices of sampling strategy, specific knowledge of the form and the parameters of the statistical distribution of the concentration of each mycotoxin in feedstuffs, granulometry of particles and/or seed size must be available and their relationship understood.

The techniques used for physically collecting and selecting samples are an important source of uncertainty associated with mycotoxin analysis. There is generally agreement that larger bias is observed in cases of static sampling (sampling of resting bulk, for instance, by probes) than in cases of dynamic sampling (sampling of a moving stream of material). Sampling from a moving stream makes it possible to obtain a high number of incremental samples with regular frequency along the entire flow. Sampling planning is easier and automated sampling is facilitated in dynamic conditions. Finally, in the majority of cases, incremental sample aggregates are more representative in dynamic sampling than in static sampling. Other bias sources of uncertainty, as a condition for the selection of undesirable samples, are the tools used for collecting samples, for example, sampling probes. If the particles are not thoroughly mixed, these tools are able to influence particle size selection, non-uniform sampling distribution within shipment lots and low number of sampling points (Whitaker, 2006). A quantification of the variability associated with the use of different sampling probes is reported by Park *et al*. (2000). These authors used three different probes for sampling cottonseed lots and found a significantly different CV associated with the samplings, respectively 13%, 28% and 43% in relation to the decreasing size of the incremental sample collected by each probe.

The contribution of SV to TV has been evaluated and quantified in several products (Table 5). Data from Table 5 indicate that the sampling phase is the largest source of variability associated with the mycotoxin test procedure for several commodities, such as

Table 5. Distribution of variability associated to each sampling step: sampling variance (SV), sample preparation variance (SPV) and analysis variance (AV).

| Matrix, mycotoxin and test procedure | SV %TV | SPV %TV | AV %TV | Reference |
|---|---|---|---|---|
| Shelled corn, 0.91 kg sample, Romer mill, 50 g subsample, 1 aliquot analysed, aflatoxin 20 ng/g | 75.6 | 15.9 | 8.5 | Whitaker, 2006 |
| Shelled corn, 4.54 kg sample, Romer mill, 100 g subsample, 2 aliquots analysed, aflatoxin 20 ng/g | 55.2 | 29.1 | 15.7 | Whitaker, 2006 |
| Shelled corn, 1.13 kg sample, Romer mill, 50 g subsample, 1 aliquot analysed, aflatoxin 20 ng/g | 77.8 | 20.5 | 1.7 | Johanson *et al.*, 2000b |
| Wheat, 0.454 kg sample, Romer mill, 25 g subsample, 1 aliquot analysed, Deoxynivalenol 5 ppm | 22 | 56 | 22 | Whitaker *et al.*, 2002 |
| Shelled corn, 5 kg sample, Romer mill, 100 g subsample, 1 aliquot analysed, aflatoxin 20 ng/g | 59.8 | 34.5 | 5.7 | Johanson *et al.*, 2000b |
| Peanut, 2.27 kg sample, 100 g subsample, aflatoxin 100 ppb | 92.7 | 7.2 | 0.1 | Whitaker *et al.,* 1994 |
| Shelled corn, 1.1 kg sample, 25g subsample, 1 aliquot analysed, fumonisin 2 mg/kg | 61 | 18.2 | 20.8 | Whitaker *et al.*, 1998 |

corn and peanuts when contaminated with aflatoxins and fumonisins (Johanson *et al*., 2000c; Whitaker, 2004, 2006). On the contrary, a lower variability associated with the sampling of wheat contaminated with deoxynivalenol, 22% of the total, is reported by Whitaker *et al*. (2002) (Table 5). The same authors suggest that these results may be related to differences in seed size as well as to a less heterogeneous distribution of deoxynivalenol compared to aflatoxins. Seed size would significantly contribute to lower variability associated with the sampling because it influences the number of seeds per weight unit. In fact, there are 10 to 30 fold more wheat seeds per unit weight compared to corn and peanuts, respectively. Results reported by Biselli *et al*. (2005) confirm these

conclusions. These authors investigated the sampling variability associated with testing wheat for deoxynivalenol and ochratoxin A. Samples were collected from a truck in accordance with the sampling plan 2005/38/EC directive. They found that the distribution of deoxynivalenol revealed some stratification effects, possibly related to the lower weight of infected kernels, while ochratoxin A distribution was highly heterogeneous.

In order to reduce SV, the level of contamination is another important factor to be considered when collecting samples for mycotoxin analysis. SV has been reported to be a function of mycotoxin concentration. The variability associated with sampling, as measured by variance statistics, increases with mycotoxin concentration of a bulk lot and/or a decreasing incremental sample size (Whitaker, 2006). SV magnitude is also strictly related to specific mycotoxin/matrix combination. In corn, curves describing relationships between sampling coefficient of variation (CV) *vs* mycotoxin concentration show the same pattern but with absolute values of CV higher for aflatoxin than fumonisin and deoxynivalenol (Johanson *et al*., 2000c; Whitaker *et al*., 2002). These data further confirm differences in the distribution of mycotoxins, indicating that aflatoxins are more heterogeneously distributed in corn than the other two mycotoxins tested.

In conclusion, when designing a specific sampling plan, all critical points have to be considered in order to reduce SV and increase the reliability of the final sample: collection of a sufficiently large number/size of incremental samples, choice of the sampling points, aggregate sample size properties, homogeneity of sample components in terms of size and specific weight. All these parameters must specifically consider the type of product and mycotoxin level of contamination. In this context, quantitative data are available and reported in specific regulations for foodstuffs, but are still lacking for the majority of feedstuffs. Last but not least, it is the case to point out that sampling must be feasible in order to be correctly performed and widely implemented.

**Sample preparation**

The second step of a sampling plan is the sample preparation phase for final mycotoxin quantification. This phase requires a careful mix of incremental samples in order to obtain a homogeneous "Aggregate sample". In the majority of cases, the aggregate sample must be prepared (e.g. comminuted) for the mycotoxin test procedure. Then a representative reduced part of the aggregate sample is obtained as "laboratory" sample. Milling the aggregate sample is very important for correct sample preparation. In fact increasing the degree of sample grinding leads to more uniform particle distribution and therefore to a reduced variability of the sample preparation step (Spanier *et al*., 2006). If samples are properly comminuted and mixed then it will be possible to assume that test sample and final sample contamination are similar to bulk contamination. Despite correct milling procedures, a certain degree of variation among different laboratory samples cannot be avoided and this represents the main source of variability of the sample preparation step (SPV). In a wide range of mycotoxin and commodity combinations, with reference to TV, SPV is greatly lower than SP (Table 5). SPV is described as a function of true mycotoxin concentration and comminuted sample size, so commodity, mycotoxin type, particle dimension and sample size are all factors influencing SPV value. Small particle size is associated with a reduction of SPV value as well as low level of contamination and/or big sample size.

Two key different approaches may be

used for mixing and reducing particle dimensions: dry milling or slurry mixing. When dry milling is applied, significant differences are evidenced in the variability associated with sample preparation in terms of characteristics and performance of the mills and sample size (Table 6). Data reported in Table 6 indicate that CV values associated with different mills may differ by up to two times. A problem associated with dry milling and with the achievement of uniform particle size distribution regards easily clogging samples with high oil content. In order to minimize this problem, slurry mixing (preparation of a homogeneous paste obtained by blending ground material and an appropriate amount of water at high speed in a slurry mixer) may be used as a comminuting step. Several authors report data on dry milling and slurry mixing in order to obtain uniform particle distribution and reduce subsampling variability in aflatoxin B1 analysis (Velasco and Morris, 1976; Schatzki and Toyofuku, 2004). Water slurry was found to be associated with a lower coefficient of variation of final sample preparation than dry milling (Table 7). It is interesting to point out that lower CV's (up to 3 times) are reported for seed by-products compared to whole seeds when slurry preparation is used. These results are consistent with data indicating that the application of water slurry mixing reduces clogging of samples with high oil content and produces smaller particle size and more homogeneous samples (Figure 2) (Schatzki and Toyofuku, 2003; Spanier *et al*., 2006).

In conclusion, aggregate sample mixing is a critical step in sample preparation. The application of water slurry mixing produces smaller particle size and a more homogeneous sample, enabling a reduction of subsampling variability and a better estimation of true mycotoxin content.

**Mycotoxin analysis**

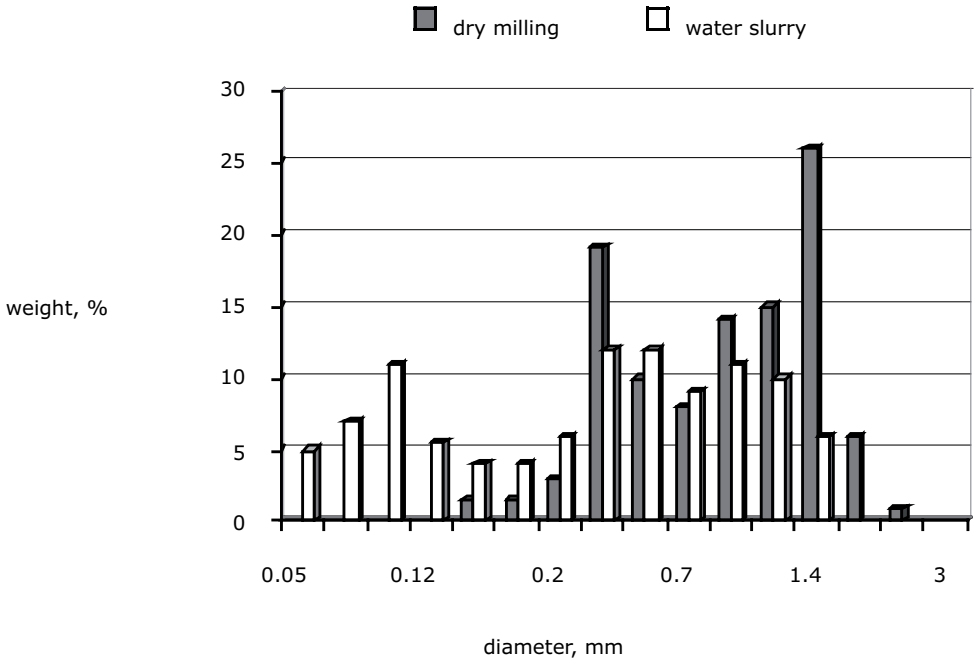
The analytical phase is the last step of the sampling procedure. Analytical variance (AV) is the variability associated with this step. As evidenced by different studies reported in Table 5, AV is the lowest contributor to TV. AV can be described as a function of mycotoxin concentration and number of aliquots analysed. For each matrix/mycotoxin combination, AV increase is associated with a high mycotoxin concentration and/or small number of aliquots analysed. Therefore, an adequate number of replicates can reduce the effect of this source of variability.

Table 6. Mycotoxin analysis: performance of different mills.

| Matrix | Sample size (kg) | Mill type | CV (average value) | Aflatoxin B1 (µg/kg) | Reference |
|---|---|---|---|---|---|
| Peanuts | 2 | DM | 38.4 | 20.4 | Dorner and Cole, 1993 |
| | | SM | 33.1 | 30.1 | |
| | | RC | 20.1 | 32.9 | |
| | 4 | DM | 42.9 | 75 | |
| | | SM | 24.4 | 82.7 | |
| | | RC | 24.3 | 69.6 | |

*DM=Dickens subsampling mill; SM=Stephan model UM-12 vertical cutter mixer; RC=Robot Coupe model RSI6Y-1 vertical cutter; CV= coefficient of variation.*

Figure 2. Ground pistachio kernels: effect of dry milling and slurry preparation on particle size distribution (Schatzki and Toyofuku, 2003).




It is also evident that the analytical method affects the variability associated with the results. The number and complexity of the analytical steps and the technology of a specific method for mycotoxin evaluation can amplify variation among results of different aliquots taken from the same analytical sample (Johansson *et al*., 2000c; Whitaker *et al.*, 2000; Whitaker, 2004, 2006). Whitaker *et al*. (1996) describe how High Pressure Liquid Chromatography (HPLC) shows less variability than Thin Layer Chromatography (TLC) and Enzyme-Linked Immuno-Sorbent Assay (ELISA). The analytical variance associated with HPLC and ELISA methods for measuring aflatoxin in corn at a level of 20 ng/g was reported to be 10.7% and 27.5% TV, respectively (Whitaker, 2006). Changing from TLC to HPLC reduces the variability associated with the analytical method from 50.1% to 20.1% (Whitaker, 2003).

The Association of Official Analytical Chemists (AOAC) and the European Standardization Committee (CEN) draw up and update a list of standardized methods of analysis that are fully validated by collaborative studies and associated with analysis performance data. Their aim is to evaluate compliance with performance criteria reported in specific EC regulations (de Vreeze, 2006). HPLC, ELISA, TLC, Gas Chromatography (GC) and Fluorimetry are considered relevant techniques for mycotoxin quantification. Given the limited number of methods of analysis, fully validated by a collaborative trial, other methods of analysis can be validated *in house*. For this reason, EC Regulation 401/2006 reports the uncertainty

Table 7. Comparison between slurry preparation and dry milling of several matrices.

| | | Aflatoxin (µg/kg) / CV | | |
|---|---|---|---|---|
| Matrix | Sample size (kg) | Dry milling | Water slurry | Reference |
| Corn | 5-10 | 49.6 / 7.6 | 49.8 / 2.6 | Mod. from Velasco and Morris, 1976 |
| Cottonseed | | 65.2 / 14.8 | 66.4 / 4.5 | |
| Cottonseed meal | | 71.9 / 5.7 | 75.3 / 4.5 | |
| Copra | | 53.4 / 7.5 | 49.8 / 4.4 | |
| Peanuts | | 40.9 / 20.8 | 48 / 5.2 | |
| Peanut meal | | 52.6 / 10.5 | 63.6 / 4.4 | |
| Pistachios | 10 | 66 / 0.2 | 86 / 0.095 | Schatzki and Toyofuku, 2004 |

*CV=coefficient of variation.*

function approach, specifying the maximum acceptable uncertainty that may be used to assess the suitability ('fitness-for-purpose') of the method of analysis used by a laboratory. Performance criteria are reported for each mycotoxin at different levels of contamination. In this context, certified reference materials are invaluable tools for assessing the accuracy and reproducibility of the data obtained, and for further improving comparisons between laboratories. Moreover, the use of certified reference materials is a major requirement for a laboratory accredited according to ISO (2005). Two different types of reference materials are available: pure mycotoxin standard solutions or matrix materials containing known and certified mycotoxin contents. The Institute for Reference Materials and Measurements founded by the European Union (IRMM, 2008), whose mission is to promote a common and reliable European measurement system in support of EU policies, is involved in the production and dissemination of internationally accepted quality assurance tools, including validated methods, reference materials, reference measurements, interlaboratory comparisons and training. Certified reference and matrix materials for mycotoxins for applications in the fields of food and feed analysis supplied by IRMM are reported in Table 8.

It is important to bear in mind that mycotoxin analysis may be performed for different purposes. In this context, if a yes/no or semi-quantitative response is considered satisfactory, the use of so called "Rapid Methods" is highly relevant for improving knowledge regarding the presence and distribution of mycotoxins in feedstuffs and for creating a reliable database (Stroka *et al.,* 2004). These low cost, simple, rapid and reliable methods may be applied in laboratory and non-laboratory environments and combine effective sampling with analysis of large number of samples for a screening approach. For example fluorimetry, dipstick and lateral flow technologies, Near Infrared Spectroscopy, micro system technology tools based on DNA arrays, electronic noses and tongues, and biosensors and chemical sensors for the detection of fungal contaminants in feed and food are available (Larsen *et al*., 2004; Maragos, 2004; Logrieco *et al*., 2005; Tognon *et al*., 2005; Zeng *et al*., 2006). The

Table 8. Certified reference and matrix material for mycotoxin analysis (IRRM, 2008).

| Reference materials | Mycotoxins |
|---|---|
| Pure mycotoxins | Aflatoxin $B_1$ |
| (solvent solutions) | Aflatoxin $B_2$ |
| | Aflatoxin $G_1$ |
| | Aflatoxin $G_2$ |
| | Aflatoxin $M_1$ |
| | Zearalenone |
| | 4-Deoxynivalenol |
| | Nivalenol |
| Mycotoxins in matrix materials | Aflatoxin $B_1$, $B_2$, $G_1$, $G_2$ in defatted peanut meal |
| | Aflatoxin B1 in compound feed |
| | Aflatoxin M1 in whole milk powder |
| | Aflatoxin $B_1$, $B_2$, $G_1$, $G_2$ in peanut butter |
| | Deoxynivalenol - maize flour very low level blank |
| | Deoxynivalenol - wheat flour very low level blank |
| | Ochratoxin A - wheat blank |
| | Zearalenone in maize |

aforementioned technologies are at various stages in the progression to be useful rapid analytical tools. Some are advanced enough for field studies and have already reached the stage of commercialization; some are at a transition phase between research and application to analysis of food/feed samples. Other are still object of research and validation studies aiming to assess their 'fitness-for-purpose' and their quality related to standard procedures.

## Conclusions

In order to evaluate feed safety and the presence of mycotoxins in feed, evaluating and planning appropriate purpose-based sampling plans are of utmost importance. Due to the variance associated with the sampling, sample preparation and analysis phases, total error in the case of non appropriate sampling protocols could lead to non reliable final analytical results with high impact on economic and health aspects. Decrease in variability in the mycotoxin test procedure reduces the number of misclassified lots and, therefore, is critical for correctly accepting or refusing a lot. As a consequence, appropriate and official sampling protocols have been designed for foodstuffs, regarding sampling collection, maximum levels admitted, performance criteria for the methods of analysis and specific mycotoxin/matrix combination. In comparison with food, the Community legislation regarding the sampling methods for the official control of feedingstuffs seems to require some improvements. The Directive

76/371/EEC (European Commission, 1976), that is focused on feed, provides for distinct requirements depending on whether the sampling purpose is to check the presence of substances or products distributed uniformly or non-uniformly throughout the lot to be sampled. However, within non-uniformly distributed substances, only aflatoxins are considered. Moreover, the quantitative (number and size) requirements for incremental, aggregate and final samples are the same for all feedingstuffs. As extensively discussed, the number and size of incremental, aggregate and test samples can differ considerably according to mycotoxin/matrix combination and particle size and uniformity. At present, these data are very limited for feedstuffs, but are of the utmost importance for designing specific sampling procedures. Therefore, gathering information on the presence of mycotoxins in the wide range of products used for animal nutrition could provide useful data for a progress in defining feed sampling plans and improving legislation.

The above-discussed official sampling approach, based on established and statistically/mathematically correct sampling plans, is quite complex and should be considered for applicability under real work conditions. This aspect is considered in Commission Regulation 401/2006 (European Commission, 2006c) reporting that: "If it is not possible to carry out the method of sampling described above because of the commercial consequences resulting from damage to the lot (because of packaging forms, means of transport, etc.) an alternative method of sampling may be applied provided that it is as representative as possible and is fully described and documented". In this context, novel non-destructive sampling approaches are object of research. For example, for the analysis of unpacked bulk material shipments, a new sampling system that collects detached surface material on filters has shown promising results (Stroka *et al*., 2004). The combination of representative non-destructive sampling procedures with validated rapid methods of analysis represents the future challenge in mycotoxin control measures.

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