Overview of Analytical Methods for Ochratoxin A (Part A: Sample Preparation, Extraction and Clean-Up)

As food legislation calls for methods of control, reliable analytical methods have to be available to make the enforcement of regulations possible. The reliability of analysis data can be improved through the use of methods that fulfil certain performance criteria (demonstrated in interlaboratory studies) and the application of analytical quality assurance (AQA) procedures is recommended, including the use of (certified) reference materials, especially when high degree of comparison and accuracy are required. Good analytical methodology and AQA are prerequisites for adequate food law enforcement (FAO, 2004). For more information on validation refer to the additional supporting documentation in this Section on 'Overview of Official Methods for Analysis of Ochratoxin A [.pdf]'.

Sample Preparation for OTA Analysis:

There are five discrete stages for any analytical scheme for mycotoxins, *sampling, sample preparation, extraction, clean-up, separation and determination* (Monaci & Palmisano, 2004).

Sample preparation is a critical step in the analytical procedure and contributes to the variability of the test result. Sample preparation is time consuming and involves grinding and homogenisation, or slurry preparation, before proceeding with extraction of ochratoxin A (OTA). OTA concentration levels in coffee samples are dependent on particle size. The analysis of the green coffee fractions (14-28 mesh) showed that the highest contamination was determined in the finest fraction of green coffee (28 mesh) (Vargas, Santos & Castro, 2001).

Besides factors such as accuracy and reproducibility, analytical procedures are characterized by three very practical criteria: the speed with which the analysis can be performed, the level of technical skill required to perform the assay and whether the assay provides a qualitative or quantitative fundamental importance, because all of them determine the length of the analytical result (Gilbert, 2000). Sample size, sample extraction and clean-up are of procedure, accuracy, recovery and achievable limits (Scott, 2002).

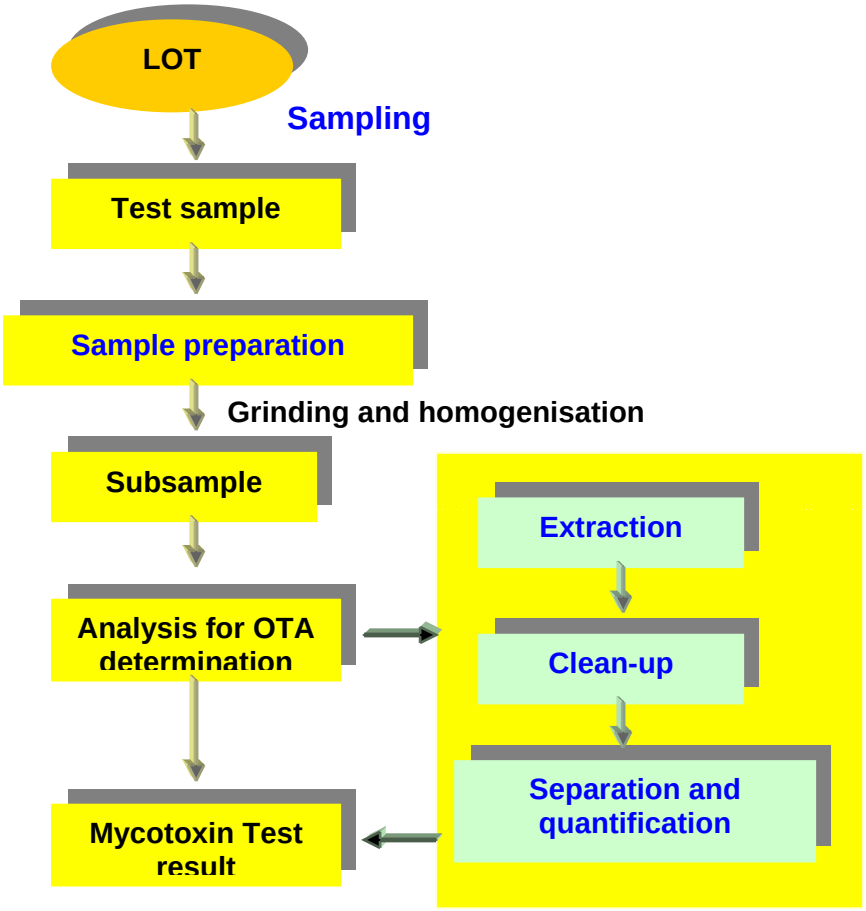

Diagram 1: *General steps involved in sampling, sample preparation, and analysis of mycotoxins in agricultural commodities*

Extraction:

All methods for the determination of mycotoxins require preliminary extraction of the mycotoxins from the commodity into a suitable solvent (Council for Agricultural Science and Technology, 2003), which is considered an essential stage (Gilbert, 2000). The choice of solvents depends on the matrix and on the choice of purification and quantification step (van Egmond, 1996). Performances of extraction solvents are usually compared to each other, but recovery of the true concentration of mycotoxin can only be achieved with the use radio labelled mycotoxins (Gilbert, 2000). Procedures for extraction of OTA from solid foods and feeds generally use an organic solvent in the presence of an acid, or an extraction solvent containing aqueous sodium bicarbonate followed by shaking or high speed blending (Scott, 2002).

The classic extraction solvent mixture is chloroform-0.1 M-*o*-phosphoric acid (10+1) with or without the addition of diatomaceous earth during the blending. Ethanol has been included in the mixture of dichoromethane-ethanol - 0.1M-o-

phosphoric acid (8+2+1) and other acids such as citric acid (dichloromethane) have also been used (Scott, 2002).

Toxic chlorinated solvents for extraction of ochratoxin A (Levi, Trenk & Mohr, 1974; Levi, 1975; Patel *et al.*, 1997) have been replaced by alternative extractants. OTA is usually extracted from green, roasted and soluble coffee with organic solvent and water, or a mixture of both, containing a small amount of acid (Studer-Rohr *et al.*, 1995; Pittet *et al.*, 1996; van der Stegen *et al.*, 1997; Trucksess, 1999).

The combination of aqueous methanol and bicarbonate has been the preferred extraction solvent (Pittet *et al.*, 1996; Entwisle *et al.*, 2001a). Methanol 5% aqueous sodium bicarbonate is the solvent of extraction of the two AOAC official methods (Entwisle *et al.*, 2001b; Vargas, Santos & Pittet, 2005) (Figure 1).

Acetonitrile acidic, 1% aqueous sodium bicarbonate with ultrasonication and even water alone have been used for OTA extraction from coffee powder (Scott, 2002).

Figure 1: *OTA extraction in green coffee sample with methanol 5% aqueous sodium bicarbonate.*

Clean-Up:

The clean-up stage of the analysis essentially involves preliminary separation of the mycotoxin from other co-extracted substances and an initial concentration step (Monaci & Palmisano, 2004).

At the turn of the millennium, the use of conventional clean up procedures such as liquid-liquid partition (Pittet & Royer, 2002) has been reported along, or in combination with, solid–phase extraction, in particular, in combination with the laborious and time-consuming celite column chromatography (Levi, Trenk & Mohr, 1974; Levi, 1975; Cantafora *et al.*, 1983; Micco *et al.*, 1989; Studer-Rohr *et al.*, 1994; Studer-Rohr *et al.*, 1995).

Other conventional solid phase materials such as the surface-modified bonded silica like C_{18}, (Terada *et al.*, 1986), aminopropyl, trimethyl aminopropyl, n-propyl-ethylene-diamine, cyanopropyl and diol (Sibanda, de Saeger & van Peteghem, 2002), DEA - anion exchange column (Akyama *et al.*, 1997) have been introduced

as a clean-up step for OTA analysis. Among the solid-phases (aminopropyl, n-propyl-ethylene-diamine, cyanopropyl and diol) studied by Sibanda *et al.* (2002) only aminopropyl was efficient to remove the brown interferences from roasted coffee. Neither false positive nor false negative was determined in the analysis of OTA by HPLC and flow-through enzyme immunoassay when aminopropyl was used as a clean up step. The method is recommended to screen roasted coffee samples using a cut off point of 4 μg/kg.

Solid-phase microextraction (SPME) has been a popular extraction technique for GC and LC in food analysis but not mycotoxins, for which the use has largely not been explored. Detection of 4 μg/kg of OTA was achieved with Diode Array – UV for ochratoxin A (Monaci & Palmisano, 2004).

The introduction of immunoaffinity solid-phase extraction (SPE) sorbents as clean-up step for the analysis of OTA in coffee (Nakajima *et al.*, 1990) was considered a major advance in cleaning techniques in OTA and, since then, has been used in the development of new methods for OTA determination in food (Scott, 2002). The immunosorbents, through improved selectivity in the SPE step, have allowed the development of highly selective methods with detection limits as low as 0.1-0.2 ng/g (Pittet *et al.*, 1996; Santos & Vargas, 2002).

Immunoaffinity column clean up has been shown to be a robust technique for purification, separation and concentration of OTA in green, roasted and soluble coffee (Pittet *et al.*, 1996) (Figure 2). Immunoaffinity columns operates under biomimetic conditions thus permitting direct processing of aqueous solutions, with benefits in terms of operational simplicity, rapidity and reduction of organic solvents (Maier *et al.*, 2004). Methods using IAC can be easily automated (Vargas, Santos & castro, 2001), making possible a high throughput of samples per run of analysis. Properly handled, immunoaffinity column clean-up technique allows extracts of the highest purity to be obtained, which can be separated either using normal or reversed-phase (RP) TLC plates, detected and quantified by visual and densitometric analysis (Santos & Vargas, 2002), or used for HPLC fluorescence quantification (Vargas, Santos & Pittet, 2005). The time saved during daily routine analysis counts as a distinct advantage over a number of commercially available SPE columns.

However, there are some limitation of IACs. Due to the high molecular weight of OTA the number of immobilized antibodies is generally low and may lead, especially under sub-optimal conditions, to poor binding conditions. IAC tolerates limited amounts of organic solvents. For optimal performance of IACs, packing materials often require specific environmental conditions such as aqueous media, well-controlled PH, ionic strength and concentration levels of matrix components), which limit the optimization space for analytical method development. Another aspect is the susceptibility of SPE materials to biodegradation, leading to short shelf life (Maier *et al.*, 2004). The cost is also a disadvantage associated with immunoaffinity columns.

Figure 2: *Immunoaffinity column clean-up of green coffee.*

Attempts to replace the biorecognition element of IAC by a less expensive and more stable biomimetic counterpart have recently been described (Monaci & Palmisano, 2004; Maier *et al.*, 2004). Molecularly imprinted polymer-assisted (MIP) are chemically and thermally stable allowing for considerable flexibility in terms of solvents and additives in the course of SPE method development. The enhanced chemical resistance also may permit MIP cleaning and regeneration under harsh condition. Another advantage of MIP are the ease preparation and the low prices of materials (Maier *et al.*, 2004). The applicability of MIP to determination of red wine has been evaluated however, their applicability to real matrices needs to be demonstrated (Monaci & Palmisano, 2004; Maier *et al.*, 2004).

A clean-up tandem assay column for detection of ochratoxin A in roasted coffee has also been developed. The analysis results are a binary yes/no indicating whether ochratoxin A is present or not above 6 μg/kg with less than 10% of false positive/negative results (Lobeau *et al.*, 2005).

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