Overview on Analytical Methods for Ochratoxin A (Part B: Techniques)

Methods routinely used today for mycotoxins are mainly based on high-performance liquid chromatography with fluorescence detection (LC/FLD), thin-layer chromatography (TLC) or enzyme-linked immunosorbent assay (ELISA) although fluorometry, flow lateral devices (FLD), fluorescence polarization immunoassay (FPIA), among others, have been used. However, new analytical approaches to rapid, low cost screening methods, such as those based on biosensors and dip-stick-like kits, are a direction in which innovation can be expected (Monaci & Palmisano, 2004). Liquid chromatography coupled with electro-spray ionisation mass spectrometry (MS) and tandem mass (MS/MS) or sequential spectrometry has been employed.

The key characteristics, advantages and limitations of each of the main types of methods are described below.

Liquid Chromatography (LC):

Reverse phase liquid chromatography is widely used for separation and detection of ochratoxin A (OTA) in foods, feeds and biological fluids (Scott, 2002). In coffee, the analysis of OTA had a substantial improvement with the introduction of liquid chromatography as a modification of the AOAC official method quantification step (Cantáfora *et al.,* 1983) by reducing the detection limit of the methods by 10-20 times.

The great improvement in 1980s in the analysis of OTA was the combination of immunoaffinity (IAC) as clean-up step (Nakajima *et al.*, 1990) with reversed-phase LC. It has been since then the most attractive approach to assess OTA contamination in coffee (Pittet *et al.*, 1996; Nakajima *et al.*, 1997; Patel *et al.*, 1997; Scott & Trucksess, 1997; Jorgensen, 1998; Trucksess, 1999), giving clean extracts, and well defined chromatograms with no interference at OTA retention time (Figure 1). Nevertheless, interferences such as caffeine and/or an OTA diastereomer are still reported in the analysis of roasted coffee by IAC with LC, and the use of phenyl silane and aminopropyl as solid phase in combination with IAC has been proposed (Entwisle *et al.*, 2001; Sibanda *et al.*, 2002; Lombaert *et al.*, 2002), indicating that some interferences counts to higher incidence of OTA in samples analysed by IAC when compared to phenyl silane/IAC.

However, no decrease of OTA recoveries from spiked green coffee samples fortified with three levels of caffeine and cleaned up on IAC was observed (Santos & Vargas, 2002). Acidic mobile phases have been preferable for separation of OTA by liquid chromatography. Because OTA is a polar compound, which has a carboxyl group in the structure, it must be chromatographed in an ionised form. Usually mixtures of acetonitrile or methanol with aqueous o-phosphoric acid or acetic acid have been used, with detection generally by fluorescence (Scott, 2002). By increasing the pH value, the fluorescence intensity increases but OTA is not retained under neutral and alkaline conditions from reversed phase chromatography that usually cannot stand pH higher than 8 (Terada *et al.*, 1986). The use of an ion-pair chromatography enables the high

polar compounds to act as weak polar compounds. Capcell Pak C_{18} (silicone coated C_{18} column) has been reported to stand pH as high as 10, allowing the sensitivity to increase 80-fold (Nakajima *et al.*, 1990).

The main advantage of LC lies in its possibility of automation, separation power, selectivity and low detection limit achievement (Santos & Vargas, 2002) of f 0.12-0.2 μg/kg (Pittet *et al.*, 1996; Santos & Vargas, 2002) and 0.5-2 μg/kg (Terada *et al.*, 1996; Tsubouchi *et al.*, 1988; Studer-Rohr *et al.*, 1994; Studer-Rohr *et al.,*1995). However, LC is expensive in initial capital investment and requires skilled and experienced staff to operate and maintain the equipment (Gilbert & Anklam, 2002).

Confirmation of OTA by LC has been carried out mostly by OTA methyl ester formation using boron trifluoride (Cantáfora *et al.,* 1983; Pittet, *et al.*, 1996) or sulphuric (Terada *et al.*, 1996; Tsubouchi *et al.*, 1988) and chloride acids (Studer-Rohr *et al.*, 1994) and diazomethane (Studer-Rohr *et al.*, 1995). The use of sep-pack NH_2 cartridge with LC as a confirmatory procedure for contaminated samples already cleaned up by liquid partition in combination with C18 sep-pak has been reported (Tsubouchi *et al.*, 1988).

A novel procedure GC - negative ion chemical ionisation (NICI), MS and multiple ion detection (MID) modes using the hexadeutered O-methyl-d_3-OTA methyl-d_3 ester derivative, as internal standard for confirmation of OTA in contaminated food by converting into its O-methylochratoxin A methyl ester derivative (OA-Me_2) at level of 0.1 μg/kg has been demonstrated (Jiao *et al.*, 1992).

The combination of diazomethane methylation with GC/MS (CCI/MID) for the confirmation of OTA identity in roasted coffee has also been reported (Studer-Rohr *et al.*, 1995).

Liquid chromatography tandem mass spectrometry (LC-ESI-MS-MS) in combination with SRM has been employed as confirmation procedure for OTA in coffee (Becker *et al.*, 1998; Ventura *et al.,* 2003). Good agreement between LC-MS-MS and LC has been reported (Lombaert *et al.*, 2002). Method for simultaneous detection of several mycotoxins including OTA in a building material matrix using HPLC with tandem mass spectrometric identification and quantification using ESI-MS-MS have been reported (Tuomi *et al.*, 2001).

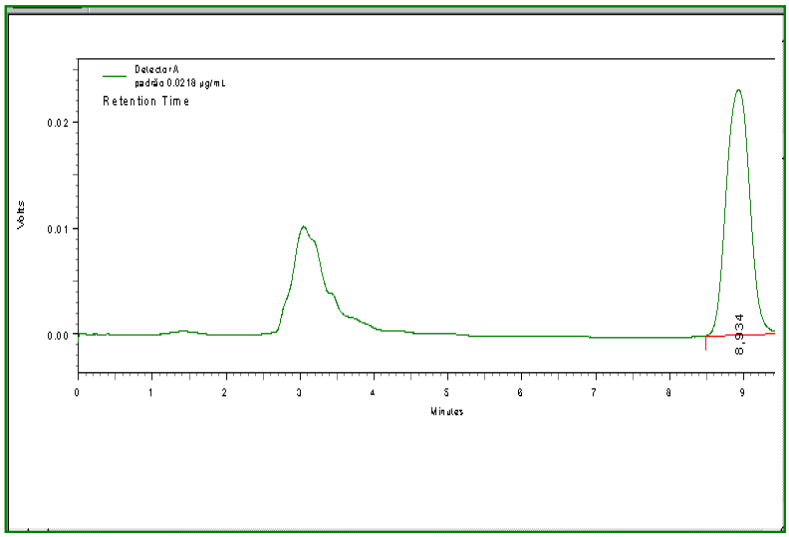

Figure 1: *Chromatogram of OTA standard solution at concentration 0.0218 µg/mL. Reverse Phase 250x4.6 mm – 0.5µm. Fluorescence detector, MobilePhase: acetonitrile: methanol: water: acetic acid (35+35+29+1). Flow rate: 0.8mL/min.*

Figure 2: *View of ASPEC-XL sample automatic processor and Shimadzu liquid chromatograph used for clean-up, separation, detection and quantification of OTA in green coffee.*

Thin-layer chromatography (TLC):

TLC is a simple, robust technique, which is relatively inexpensive to establish in a testing laboratory. Many laboratories in developing countries have considerable expertise and experience (Gilbert & Anklam, 2002). TLC has been the most widely used and established separation and detection technique for aflatoxins since its development in the 1960s. The same, however, does not apply to OTA analysis. The AOAC official method dates back to 1975, and was extensively used until the 1980s when liquid chromatography was introduced.

Some of the factors affecting the acceptance of TLC as a quantitative method, such as its lack of resolution and poor sensitivity (AOAC, 2000; Pittet & Royer,

2002), have been overcome in recent years. The combination of an efficient and robust immunoaffinity column clean-up with this advantageous, low-cost based procedure has been published (Santos & Vargas, 2002). This is a promising analytical approach for the determination of OTA in green coffee by providing sample extracts free of major matrix interferences, and suitable for one dimensional TLC analysis. This makes the TLC method more straightforward and amenable to automation, and improves the sensitivity, separation, resolution and visualisation of the mycotoxins on TLC plates (Figure 6). The published method presents detection limit of 0.5 µg/kg and the possibility of quantification by using a densitometer (Figure 7), and meets the method performance characteristics standards required by international organisations (CEN, 1999; Santos & Vargas, 2002) (Table 1).

***Table 1**: Methods' Performance Criteria for Ochratoxin A analytical methods adopted by CEN (Lombaert et al., 2002).*

Level (µg/kg)	Recovery (%)	RSD_r	RSD_R
< 1	50 to 120	40	60
1 – 10	70 to 110	20	30

TLC should always be considered an important tool as it is fast, cost effective and can be used in routine applications (crude extract analysis), is versatile in using different solvent systems, and applicable to different visualisation system using the same sample extract. TLC allows those in developing countries to assess OTA contamination irrespective of the purpose of the assessment, be it qualitative (Pitter & Royer, 2002) or quantitative (Santos & Vargas, 2002).

It is essential that TLC is not always seen as being inferior to LC and that, when combined with modern clean-up techniques and low-cost plate scanner methods, are validated methods (Gilbert & Anklam, 2002).

However, quantification is still a limiting factor due to the high cost of commercial fluorodensitometers, which could otherwise decrease the variability associated with the ability of individual analysts to visually quantify the toxin (Vargas, Santos & Castro, 2001; Pittet & Royer, 2002; Santos & Vargas, 2002). Successful attempts have been made (Stroka & Anklam, 2000) to develop alternative devices to the expensive commercial TLC densitometers for aflatoxin analysis that could be validated for OTA. These developments could be extremely helpful in building the ability to analyse OTA in developing countries, especially if the densitometers could be available on a semi-commercial scale.

In addition, the TLC method is limited by the necessity of efficient clean-up (Gilbert & Anklam, 2002), and acidic substances co-extracted with OTA have been reported as limiting factor when liquid-liquid partition and solid-phase extraction are used as clean-up (Levi, Trenk & Mohr, 1974; Levi, 1975). One of the disadvantages of TLC is its lack of separation and power, and thus an inability to discriminate any possible co-extracted interference from the toxins of interest (Gilbert & Anklam, 2002).

Confirmation of OTA by TLC has been achieved by spraying the TLC plates with aluminium chloride and sodium bicarbonate or by exposing the plates to ammonia vapour (Levi, 1975; AOAC, 2000; Santos & Vargas, 2002). RP TLC has been reported as a confirmation method for OTA in coffee appearing in normal-phase TLC (Santos & Vargas, 2002), or as preparative chromatography for LC (Frohlich, Marquardt & Bernatsky, 1988) for matrices other than coffee.

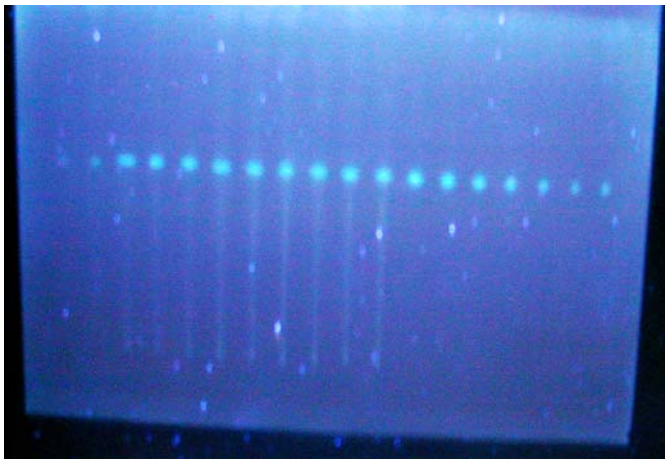

Figure 3: *OTA standard and naturally contaminated samples dissolved with toluene-acetic acid (99+1, v/v) and spotted on normal TLC plate.*

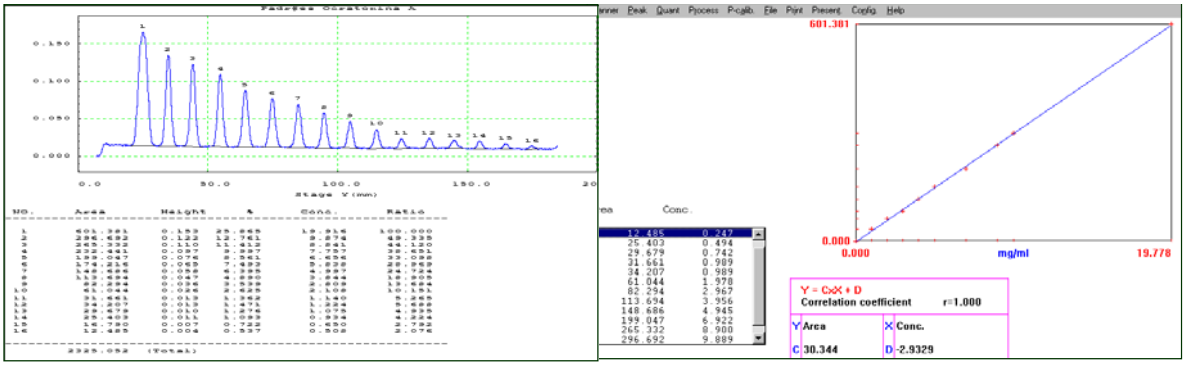

Figure 4: *Chromatograms of OTA standard solution in toluene: acetic acid (99+1) and spotted on normal TLC silica gel 60 TLC plate, calibration curve of OTA standard solution and densitometer Shimadzu used to scan the TLC plate.*

Screening Techniques:

Methods of screening are typified as being rapid test methods. In general, they are qualitative methods frequently giving a yes/no answer above a predetermined threshold limit, although they may be qualitative or semi-quantitative. The kits are usually simple to use and analysis can be undertaken in the field without the need for recourse to a laboratory environment (Gilbert, 2000), and most of them are basically designed as visual tests that require only low-cost instrumentation and offer an advantage speed (Lobeau *et al.*, 2005).

The use of these kits is limited by qualitative answers: yes/no contamination, false negative results and the need of confirmation of positive results by more rigorous analytical methods. The disadvantages of mycotoxins test kits can be the cost, the lack of attention with regard to sample extraction condition and a tendency to disregard sample requirements (Gilbert, 2000). A number of test kits for OTA detection in coffee are detailed in Table 2.

Table 2: *Some available test kits for detection of OTA in coffee.*

ANALYTE	COMPANY	KIT NAME	RECOGNITION	PRIMARY MATRICES
Ochratoxin	r-Biopharm GmbH	**RIDASCREEN OTA Column**	-	**Coffee beans**
Ochratoxin	VICAM	**OchraTest**	-	**Coffee beans**
OTA	TECNA S.r.l.	**Immunoscreen OCHRA**	-	Cereals, feeds, wine, **green coffee** and cocoa
Ochratoxin	Tepnel BioSytems Ltd.	**BioKits OTA Assay**	-	Cereal, dried fruits, **green coffee**, and white wine
OTA and B	Romer Labs	**AgraQuant OTAssay (2-40ppb)**	-	Barley, corn, **green coffee,** maize, soybeans and wheat
OTA and B	Romer Labs	**AgraQuantR Ochratoxin (2-40 ppb) ELISA Test Kit**	-	Corn, maize, barley, **green coffee**, wheat,

Source: **AOAC**

ELISA Assays:

Enzyme-linked immunosorbent assay (ELISA) has become a popular and useful screening tool thanks to the availability of polyclonal and monoclonal antibodies against OTA (Lobeau *et al.*, 2005). Most commercial ELISAs for mycotoxins rely on a competitive, heterogeneous ELISA format, in that the toxin from the sample competes with a labelled toxin (such as a toxin-enzyme conjugate) for a limited number of antibody-binding sites. If any factor (as structurally related constituents and matrix constituents) that diminishes the binding between the labelled toxin and the antibody can be mistaken for the presence of toxin (Council for Agricultural Science and Technology, 2003).

ELISA methods have advantages due to their simplicity, and number of samples that can be analysed at the same time (Scott, 2002). However, ELISA is less accurate and sensitive than conventional chromatographic assays and very few

correlations were found between traditional types of techniques (Vargas, Santos & Castro, 2001). In addition, false positive or negative results are observed because of cross-reactions among molecules or interferences.

Therefore, ELISA kits should not be used as a quantitative method (Gilbert & Anklam, 2002), and should only be used with foods for which they have been extensively tested and demonstrated to work. Additionally, sufficient controls must be employed for each test, to ensure the validity of the quantification (Council for Agricultural Science and Technology, 2003).

Parameters such as accuracy, precision and linearity of a non-competitive, quantitative commercial ochratoxin ELISA kit, for screening and quantitative determination of OTA in green coffee was evaluated, by means of recovery (R%) and coefficient of variation (CV), interwell and interassay CV values in validation studies according to an intra-laboratory validation protocol (Vargas, Santos & Castro, 2001; Oliveira, Santos & Vargas, 2000). In additional the correlation between ELISA (Levi, 1975) and LC (Frohlich, Marquardt & Bernatsky, 1988) recoveries of OTA from spiked green coffee was evaluated (Figure 5 and 6).

As can be seen in Figures 5 and 6, results show that the ELISA kits evaluated were not efficient in recovering OTA from spiked green coffee samples (~60% recovery), considering the LC method as a reference and the amount of OTA spiked in the green coffee sample. Considering that, in general, it is easier to extract mycotoxins from spiked samples, the use of this kit can result in false negatives results.

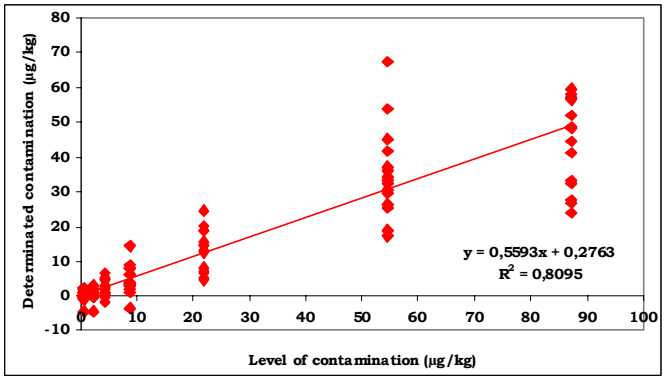

***Figure 5**: Recoveries (µg/kg) of OTA from spiked green coffee samples, by ELISA, (4 kits, 7 levels, 16 replicates).*

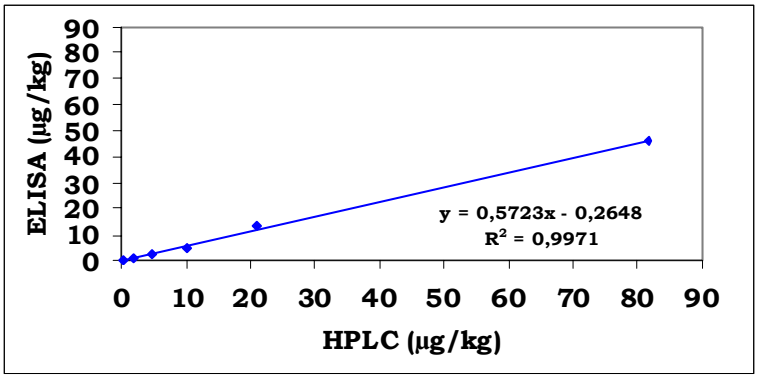

Figure 6: *Linear correlation between LC and ELISA recoveries of OTA from spiked green coffee samples.*

Fluorometric Kits:

The use of immunoaffinity columns coupled with a fluorometer has been proposed as quick and specific method for routine analysis of mycotoxins (Scott & Trucksess, 1997; Shim *et al.*, 2004). However, for coffee, an in-house validation study was carried out (CEN, 1999), according to an intra-laboratory validation protocol using commercial fluorometry (Shim *et al.*, 2004). The precision, linearity, correlation with LC method, limit of detection and false positive/false negative rate was evaluated.

The major problems found in the utilisation of fluorometric quantification were the false positive/false negative rates (Figure 7), inconsistent results regarding recovery (Figure 8) and linearity of standard calibration curve (data not published).

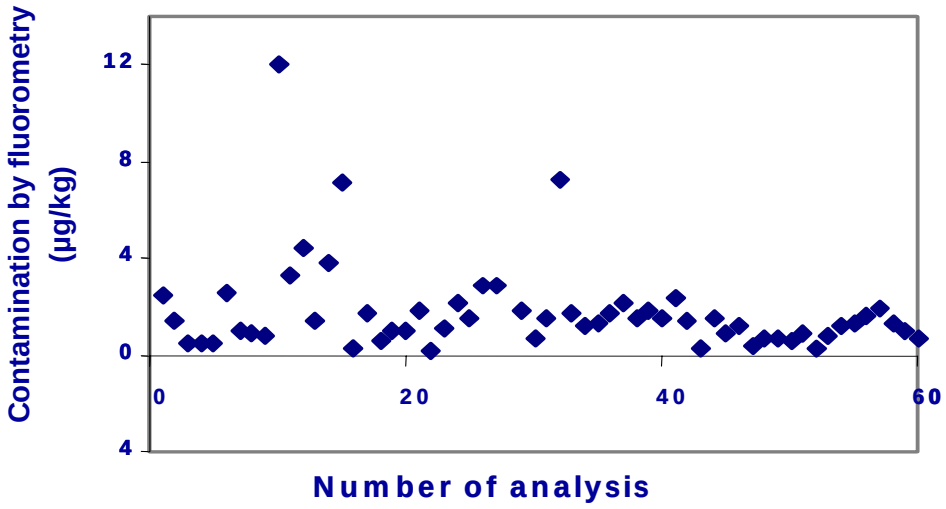

Figure 7: *False positive and dispersion of OTA contamination determined by fluorometry for blank green coffee sample (nd<0.12 µg/kg, determined by HPLC).*

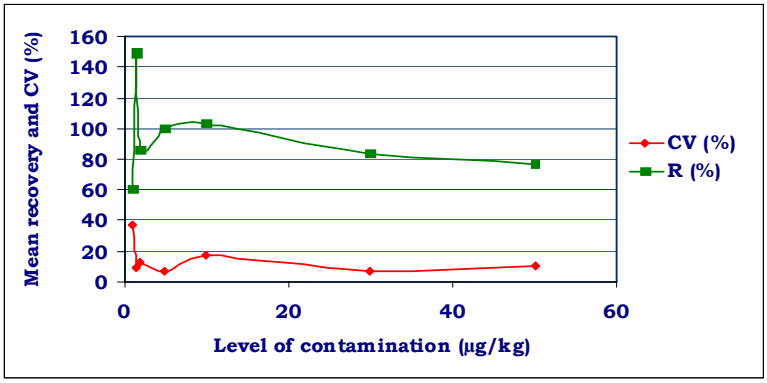

Figure 8: *Mean recovery and coefficient of variation (CV%), obtained for OTA from spiked green coffee samples by fluorometry (in 5 replicates).*

Inconsistent results have been found even for non-contaminated samples (i.e. blank samples) making usual bench control laboratory procedures worthless. This indicates that matrix effects have been a restraint for the use of the immunoassays for screening of coffee (for examples of matrix effect see Figure 5, 6 (ELISA) and Figures 7, 8 (fluorometer)), especially when the proposed regulatory limits are very low and close to the detection limits of the kits. In additional to these analytical difficulties, the high cost of these kits makes their use unattractive for developing countries.

Results obtained from the FAO Technical Cooperation Project in Thailand, comparing HPLC and fluorimetric analysis (Figure 9), where the same extract was analysed by each technique, appears to have the same tendency observed by LACQSA/MAPA. In conclusion, fluorometry has shown itself incapable of reliably identifying OTA free samples (FAO Global Project Communication).

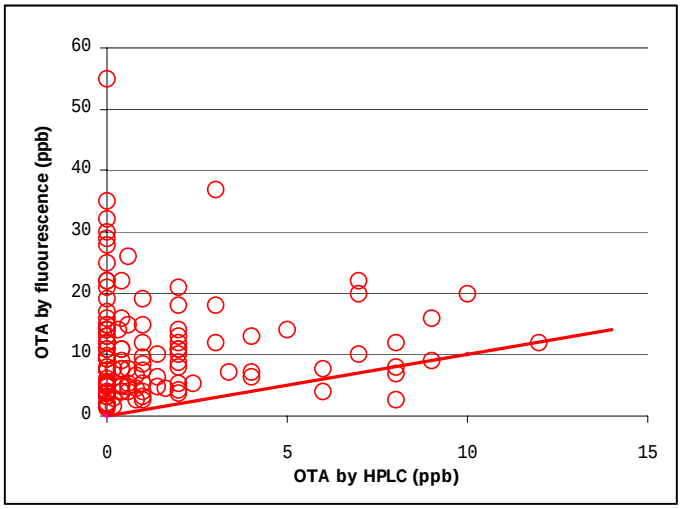

Figure 9: *Scatter plot of OTA content of 120 samples of Thai robusta collected from farmers as determined by HPLC and fluorometry after immunoaffinity column clean up.*

Fluorescence Polarization Immunoassay (PFIA):

PFIA is a competitive immunoassay method based on the increase in the fluorescence polarization of a small fluorescent labelled hapten (tracer) when bound by a specific antibody. If the sample contains a free (unlabelled) analyte, its molecules will compete with the tracer for antibody-binding sites and polarization signal will decrease.

This technique, which is a hugely promising way for the simplification of immunoassays for routine applications, is a shift from heterogeneous methods (with separation) to homogeneous assays (without separation). FPIA meets requirements of a simple, reliable, fast and cost effective analysis and has been used for determination of fumonisins, deoxynivalenol and aflatoxins in grains.

PFIA using monoclonal antibody for OTA in barley was developed and optimised (Shim *et al.*, 2004). The PFIA appeared to meet the performance criteria for OTA screening and should be carefully evaluated for coffee quantification, once the coffee matrix presents interferences.

Chemiluminescent (CL) and Bioluminescent Assays (BL):

Bioluminescence and chemiluminescence have been investigated for mycotoxin analysis. CL is based on a chemical reaction that can be described as follows:

$$A + B + C^* + D = C + light$$

, where * indicates an electronically excited state.

To amplify and prolong this signal, a compound known as an enhancer is added to the reaction medium. Enhanced CL reaction is one of the most sensitive and rapid detection methods in medical and analytical biochemistry.

This luminescence reaction can be used for the detection of antigen-antibody binding at the final stage of an immunoenzymatic assay. A solid-phase chemiluminescent immunoassay (CIA) was used to analyse OTA. Results comparing CIA and a conventional ELISA test show that an excellent correlation was observed between the ELISA and CIA results, however the results with OTA spiked corn gave a recovery of less than 70%, indicating that this CL immunoenzymatic assay cannot yet be used in the routine testing for agricultural commodities (Sarter & Zakhia, 2004).

Bacterial bioluminescent (BL) assays as a toxicological assay for mycotoxins have also been investigated. This method measures a total toxicity rather than just the mycotoxin effect. It is proposed to use it as a rapid screening method for rejecting grain bathes in production.

These two methods constitute an innovative shift in techniques for mycotoxin analysis. However they need to be improved before they can be applied to large-scale food production testing (Sarter & Zakhia, 2004).

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