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Advantages and drawbacks of immunoaffinity columns in analysis of mycotoxins in food

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A number of countries are setting legislations on mycotoxins. In order to reduce dispute between importing and exporting countries, the analytical data should be as comparable as possible, especially when levels are close to the regulatory limits. The present trend in the analysis of mycotoxins is to use immunoaffinity column (IAC) as a clean-up and enrichment technique, and Association of Official Analytical Chemists and European Union have validated methods which address a few food commodities. This study describes our experience using both conventional and IAC approaches in the analysis of three mycotoxins. *Aflatoxins* (AFs): Aflatoxin G_1 has been detected by liquid–liquid partitioning methods with HPLC detection as false-positive in some maize. On IACs, this compound behaves as an AF, lowering the amount of the AFs trapped. The problem was solved using either TLC or HPLC with detection in the Kobra cell®. Depending on the additives to food during the processing and cooking, the AFs might appear as an opened ring not recognised by the antibody. *Fumonisins* (FB): Compounds interfering with the FB's antibodies were also observed while analysing breakfast cereals leading to underestimation of FB. *Ochratoxin A* (OTA): Depending on the food composition and extraction techniques, OTA is underestimated with IAC in some breakfast cereals and coffee. These data strengthen the necessity to validate methods using IAC for each complex matrix.

Keywords: Aflatoxins / Analytical problem / Fumonisins / Immunoaffinity columns / Mycotoxin analysis

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1 Introduction

Mycotoxins develop either in the field or during storage. Aflatoxins (AFs) are produced by a series of *Aspergilli*, mainly *A. Flavus* and *A. parasiticus* and can contaminate raw material such as ground nuts, pistachios, Brazil nuts, cereals, maize, fruits, figs, spices and all other food commodities derived from these [1–3 for review]. Fumonisin B_1 (FB_1) is produced in the field by some *Fusarium* species growing not only on maize, oat or rice but also on other cereals like wheat, millet and sorghum [2, 3 for review]. During storage, wheat, oat, barley, raisins, *etc*., contaminated by Penicillia (*Penicillium verrucosum*, *P. aurantiogriseum*, *P. citrinum* and *expansum*) or Aspergilii (*A. ochraceus, carbonarius*, *niger*) can contain ochratoxin A (OTA). All these three mycotoxins can be transferred to the final products such as breakfast cereals, *i. e.* FB which has already been detected in numerous samples [4, 5] during the food process. OTA has been recently reported [6, 7].

From the recent survey of food and agriculture organisation (FAO)/Rijkinstituut voor Volksgezondheid en Milieu (RIVM) [8], it has been observed that there is a strong increase in the number of countries who are setting legislations on mycotoxins and particularly on AFs. In order to reduce dispute between importing and exporting countries, the analytical data should be as comparable as possible, especially when levels are close to the regulatory limits. The present trend in analysis of mycotoxins is to use immunoaffinity column (IAC) as a clean-up and enrichment tech-

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Abbreviations: AF, aflatoxin; **AFB_1, aflatoxin B_1; AFB_2,** aflatoxin B_2; **AFG_1, aflatoxin G_1; AFG_{2a},** aflatoxin G_1 hemiacetal; **EU,** European Union; **FB,** fumonisin; **IAC,** immunoaffinity column; **OTA,** ochratoxin A; **OP-OTA,** open-ring OTA; **OTB,** dechlorinated OTA; **AOAC,** Association of Official Analytical Chemists

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nique, and Association of Official Analytical Chemists (AOAC) and European Union (EU) have validated several methods which addresses only a few food commodities. Until now, no methods are validated for analysis of mycotoxins in complex matrices such as complex breakfast cereals. There is, therefore, a tendency to extrapolate the use of AOAC- or EU-validated methods to the analysis of mycotoxins in such commodities and uses IACs as clean-up/concentration procedures. However, the laboratories do not always perform all that is necessary to validate the methods for each new matrix.

The use of these columns is, however, not completely devoid of problems: (1) the complex matrices contain thousands of compounds, some of them may be able to interfere with the antibodies, thus limiting the column capacity for the adsorption of the toxin; (2) in addition, the composition of the matrices may interfere with the toxin structure making them not extractable and/or not recognisable by the antibodies.

The manuscript reports our experience in the analysis of three mycotoxins using IACs and more conventional clean-up/enrichment techniques: AFs in a matrix as common as maize FB_1 and OTA in a more complex matrix such as breakfast cereals.

2 Materials and methods

2.1 Origin of the samples

Maize samples for analysis of AFs were collected during a community-based intervention study on hepatocellular carcinoma in Guinea [9]. Breakfast cereals for FBs and OTA analysis were collected in the French market [10, 11]. Roasted coffees were collected in the French market.

2.2 IAC

Aflaprep, fumoniprep and ochraprep were from Rhône Diagnostic technologies (RDT, France).

Reagents were of analytical quality grade.

AFs were extracted with methanol/water from 50 g of sample according to the method described in Stoloff *et al.* [12]. The extract is defatted by liquid partition with *n*-hexane. Then they are extracted into chloroform. The objective being that the technique be transferred and performed in Guinea, the samples were first analysed by TLC on 20×20 cm silica gel 60 plates with diethyl ether-methanol-water (96-3-1 v/v/v) as running a solvent and visualisation under UV light (365 nm). Positive samples were confirmed by TLC under the same conditions after derivatisation in presence of TFA of aflatoxin B_1 (AFB_1) and aflatoxin G_1 (AFG_1) into the hemiacetals AFB_{2a} and AFG_{2a}, respectively [13]. The Rf of AFB1 and AFG1 shifted from 0.69 to 0.24 and from 0.29 to 0.16 for the respective hemiacetals. Quantification was compared to HPLC after derivatisation with detection by spectrofluorimetry (excitation, 365 nm; emission 430 nm). HPLC conditions: column, Zorbax® C18 5 µ, 150×4.6 mm; solvent, water-methanol-ACN (69-19-12 v/v/v); flow rate 1 mL/min. Finally underivatised AFB_1 and AFG_1 were also separated by HPLC and detected by spectrofluorimetry after post-column derivatisation in the Kobra cell® where they are converted into their 9,10-dibromo derivatives.

FBs were analysed as described by Solfrizzo *et al.* [14]. In short, FBs were extracted twice with ACN/methanol/water (25/25/50) and the combined extract were diluted with PBS and applied to the IAC column. After washing the column with PBS, the FBs were extracted with methanol. After derivatisation with o-phtaladehyde/2-mercapoethanol to form a fluorescent derivative they were analysed by HPLC spectrofluorimetry.

OTA was analysed in breakfast cereals using a newly developed method for the simultaneous extraction/clean-up of OTA and CIT [11]. In brief, 20 g of sample was acidified with an aqueous solution of potassium chloride (4%) acidified to pH 1.5 with sulphuric acid. The mixture was homogenised and extracted with ACN. After filtration on Whatman No. 4 paper, the sample is defatted twice with *n*-hexane and extracted with chloroform. The sample was then purified by liquid-liquid partition.

Two methods using immunoaffinity clean-up, of Entwisle *et al.* [15] and of Rhône Diagnostic Technologies [16], were used. Entwisle *et al.* [15] extract OTA from cereals in ACN/water (60 : 40). The extract is filtered on Whatman No 4 filter paper. Forty-four millilitres of PBS is added to 4 mL of filtrate, and the mixture is transfered to the IAC. After washing the column with PBS, the OTA is extracted with methanol/acetic acid (98 : 2) and analysed by HPLC spectrofluorimetry. In Rhône Diagnostic Technologies method [16] OTA is extracted by a 1% sodium bicarbonate solution, and the sample is transferred to the IAC column conditioned with a solution of 1% sodium bicarbonate.

From coffee, OTA was extracted from ground coffee beans by three methods, two using IAC clean-up [17, 18] and the later using toluene extraction in acidic conditions [19, 20] (See brief description below). In addition, OTA was also analysed in coffee as beverage prepared as follows. Twenty-five grams of ground coffee was put on a filter and extracted with 300 mL boiling water. Twenty millilitres of this extract was then added to 80 mL of PBS and passed entirely through the IAC.

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3 Results and discussion

3.1 IAC problems associated with a compound from the matrix being recognisable by the antibodies

3.1.1 AFs

One hundred and sixty-four samples of food collected in four villages of lower Guinea were analysed by TLC, 30 were detected positive for AFB_1 or for all four AFs (AFB_1, AFB_2, AFG_1, AFG_2). The results corroborated with those determined by TLC on the samples after derivatisation to the hemiacetals.

While comparing data from the TLC method and from HPLC spectrofluorimetry obtained with the same samples extracts it was observed that HPLC data were generally higher, demonstrating a probable quenching effect of the fluorescence of the sample spots separated on the TLC plates. However, for two maize samples AFG_1 detected by HPLC as AFG_{2a} was not detected with the TLC of the toxin itself and of the hemiacetal; in addition, in these samples no AFG_2 was detected.

In order to solve this problem, the HPLC solvent was modified. The apparent AFG_{2a} peak could not be separated from the AFG_{2a} standard. The samples were then purified on an Aflaprep IAC following the manufacturer's information, ensuring that the column binding capacity was not exceeded. After the IAC clean-up of the underivatised sample, the apparent AFG_{2a} was still detected after derivatisation. The reduction of amount of samples added on to the IAC resulted in an increase of AFB_1 and AFB_2, while the apparent AFG_{2a} was nearly constant (Table 1).

Table 1. Evolution of the concentration of the apparent AFG_1/AFG_{2a} and the other three mycotoxins by passing through decreasing amount of the same extract

Amount of extract added to the IAC (mL)	Apparent AFG_1 content (AFG_{2a}) (μg/kg)	AFB_1 (μg/kg)	AFG_2 (μg/kg)	AFB_2 (μg/kg)
1	23	84	n.d.	7
0.75	26	95	n.d.	9
0.5	22	70	n.d.	9
0.25	26	163	n.d.	31
0.1	29	258	n.d.	37

A possible explanation to this is that a large amount of an unknown compound had some affinity to the antibodies directed against AFs, thus saturating the binding sites on the IAC and reducing the binding capacity for AFs. When reducing the amount of compound passed onto the column, more sites became available for the AFs, resulting in increased retention and further elution/detection of AFB_1 and AFB_2.

Finally, after extraction, the AFs without prior derivatisation were separated by HPLC and detected using postcolumn derivatisation in a Kobra cell. The samples contained no AFG_1 and AFG_2.

These data demonstrate that false-positive can be obtained for AFG1 after IAC together with reduced levels of AFB_1 and AFB_2.

3.1.2 FBs

The aim of the study described below was to analyse some breakfast cereals for the content of OTA, citrinin and FBs. No method had been validated for the analysis of these toxins in such complex mixtures.

In order to test the recovery of the method validated for maize and corn flakes [14, 21] on complex matrices such as breakfast cereals, four samples (containing different ingredients) were spiked with 200 μg/kg FB_1 extracted and analysed using this method. Depending on the composition of the breakfast cereals, the recoveries ranged from 40 to 74% [10].

Recovery for corn flakes alone was in the range of the EU study: 70–75%. With other types of samples (*i.e.* containing, fruits, oat, rice, sugar chocolate, *etc.*), recoveries could be under the criteria for recovery set by EU (<60%) [22].

To investigate the potential cause of these losses, we enriched nine extracts with equivalent to 300 μg/kg FB_1, just before the IAC purification. The unspiked samples were run in parallel. The recovery was between 54.4 and 79.1 at this step. Up to 45% losses are observed at the IAC clean-up step (Table 2).

This can be explained by the presence of some compound(s) which are recognised by the antibodies, thus blocking the sites and reducing the trapping efficiency for FBs. This may be due to the presence of high levels of some

Table 2. Recovery tests for FB1 added to the extract just before the IAC to extracts of various samples of breakfast cereals

Sample code	A	B	C	D	E	F	G	H	I
Recovery from spike of 300 μg FB_1/kg breakfast cereals before IAC (%)	74.9	69.5	74.8	54.4	78.4	61.7	79.1	59.0	67.0

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Table 3. Recovery tests for OTA added to the extract just before the IAC to extracts of various samples of breakfast cereals

Sample code	28	51	52	53	54	55
Main ingredients	Dried fruits, wheat flour and bran, barley	Maize	Cocoa, rice, maize, oats, wheat flour	Dried fruits, oats, wheat bran and flour	Dried fruits, oats, wheat flour	Oat
Recovery from spike of 3 μg OTA/kg breakfast cereal before IAC (%)	65.0	60.9	75.0	78.2	90.0	80.5

fatty acids which have some similarities in structure with FBs and could probably interfere with the antibody.

3.1.3 OTA

Six samples of breakfast cereals were extracted under acidic conditions by the technique developed in our laboratory [10] and part of the extract enriched with the equivalent of 3 μg/kg OTA in the extract just before the IAC clean-up. The nonspiked sample was run in parallel. The recovery of the spike was calculated by subtracting for each sample the natural concentration from that of the spiked one. The results are presented in Table 3.

As can be observed, depending on the composition of the breakfast cereals, large amounts of the spiked OTA can be lost (recoveries from the spike ranging from 60.9 to 90%). An amount of OTA equivalent to the 3 μg/kg breakfast cereals was passed onto the same batch of column and the recovery was 93%. We, therefore, observe losses most probably due to compounds coextracted under acidic conditions from the breakfast cereals with the OTA which bind the IAC thus blocking the IAC capacity to retain OTA.

3.2 IAC problems associated with a modification of the toxin structure that make it not extractable and/or recognisable by the antibody

3.2.1 OTA

In the case of OTA, the trends in the extraction has changed, going from extraction by an acidified medium to a neutral medium [15] or even an alkaline one [16, 17].

According to the data obtained in our laboratory [11], acidic conditions improve the recovery of OTA from wheat and wheat products (Fig. 1). In this study we devised a method for simultaneous extraction of OTA and citrinin from the above matrices. As can be seen from Fig. 1, reducing the pH from 4 to 1.5 improves not only the recovery of citrinin (21 – 80%) but also that of OTA (66.5 – 78.9%) and the variability of the recovery.

Table 4 compares the results of analysis of different samples of breakfast cereals by three methods [11, 15, 17]. It can be observed that for positive samples, the OTA amount detected decreases with increasing pH of extraction. There are two reasons for this: (i) extraction from a neutral medium gives lower recoveries than from an acidic medium, the optimum pH for good extraction being pH 1.5 [10], (ii) in alkaline medium, OTA is converted to an open-ring OTA (OP-OTA) (Fig. 2) which could not be anymore recognised by the antibodies.

Extracting without rather than after acidification leads to a loss of 20% (sample 28) to 50% (samples 16 and 53). These two latter samples were processed with a corrector of acidity such as sodium carbonate or sodium bicarbonate, and thus extraction without prior acidification leads to higher losses (sample 16). When extracting OTA with an alkaline reagent [17] and purifying the extract by immunoaffinity, the effect is even more drastic and greater losses are observed (Table 4, last line).

To confirm this, we have passed through an Ochraprep®, a solution containing 60 ng OTA in water and in 1 M sodium bicarbonate. After 1 h of contact of OTA and the sodium bicarbonate solution, only 40% of the OTA was recovered through the column. When the contact time with sodium

Table 4. Comparison of OTA concentration (μg/kg) in function of three different methods of purification

Sample codes Method	16[a)]	25	28	51[a)]	52[a)]	53	54	55
Acid extraction and partition [11]	3.4	<0.2	8.8	ND	ND	1	ND	<0.2
Neutral extraction and IAC [15]	1.6	ND	7.2	ND	ND	0.45	ND	ND
RDT (application note N° A9-P14.V1) (alkaline extraction and IAC) [17]	0.2	ND	5.6	ND	ND	<0.2	ND	ND

a) Samples containing a corrector of acidity (sodium carbonate or sodium bicarbonate).

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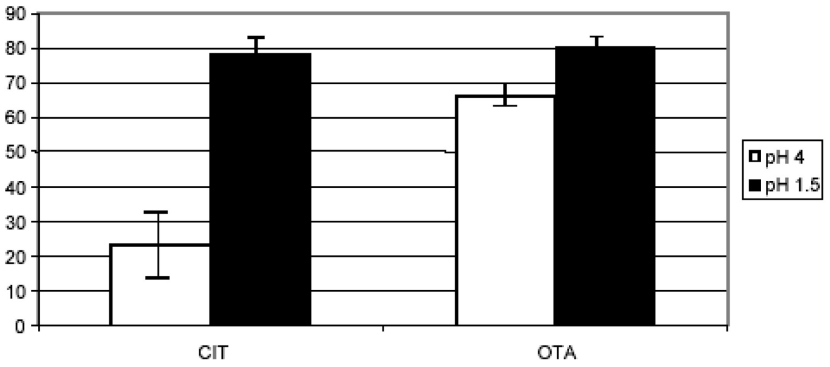

Figure 1. Recoveries of OTA and citrinin from wheat according to the pH of extraction.

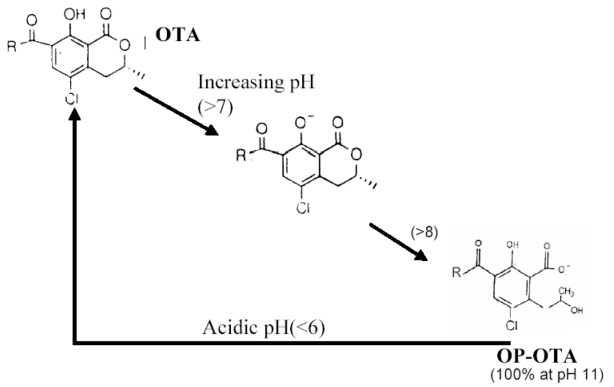

Figure 2. Ring opening of OTA under alkaline conditions and reversibility when returning to acidic conditions.

bicarbonate was longer, the losses are even more pronounced. This is due to the opening of the lactone ring of OTA (OP-OTA) under alkaline conditions [23, 24], and due to OP-OTA not being recognised by the antibodies against OTA from the IAC.

The knowledge of the opening of the lactone ring in alkaline media explains, at *posteriori*, the "strange" results obtained by Chelkowski *et al.* [25, 26]. These authors, treated by ammoniation at ambient temperature a rat feed contaminated by OTA. They did not detect any OTA in the ammoniated feed but found similar levels of OTA in the kidneys of animals receiving the ammoniated feed than in the group fed with the original nonammoniated feed. The acidic condition of the stomach allows the reformation of OTA [27] (Fig. 2).

3.2.2 AFs

As was reported in the subsection on OTA, some foods are conditioned in presence of sodium carbonate and bicarbonate. In the case of food commodities contaminated by AFs, these two alkaline agents will open the AF lactone ring during the process and no AF will be detected using IACs in the samples because open AFs are not recognised by the antibodies.

However, part to all of the AFs will be recycled when going through the acidic conditions of stomach of animals and human consumer.

This problem of recyclisation of AFB has been observed with feed decontaminated by ammoniation. Ammoniation at ambient temperature and pressure leads to opening of the lactone ring of AF and the reaction is reversible after acidification [28–31]. Decarboxylation of this open ring AF, needs high temperature and high pressure (Fig. 3) to give AF D. In both case no AF is detected by the conventional AOAC and IUPAC methods in which the extraction is performed from nonacidified media. Toxic effects have nevertheless been observed in animal is fed with feed decontaminated by ammoniation at low temperature and low pressure [32].

The food production processes, mainly the addition of alkaline such as sodium bicarbonate and sodium carbonate, must therefore be closely scrutinised in order to adapt the extraction procedure of AFs in order to add an acidification step of the matrix before extraction and subsequent analysis using an IAC.

3.3 IAC problems associated both with a modification of the structure of the toxin and compounds from the matrix being recognisable by the antibodies

Similarly, we observed some discrepancies in the results when we analysed roasted coffee beans by three methods run in parallel [17–20], at least in triplicates. The main steps of each method are presented in parallel in Table 5.

The recovery by Entwisle's method increased with the amount of OTA in roasted coffee beans. With low concentration of OTA, almost all OTA is converted into OP-OTA and not recognised by the antibodies. The recovery by the Pittet method is relatively constant, because the dilution of extract before IAC column buffers the pH, thus allowing reconversion of almost all OP-OTA into OTA before IAC.

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In the case of an extract, coffee beverage is prepared by passing hot water over ground coffee beans. Losses are no more observed, because no alkalinisation is made before OTA extraction from the beverage.

As in the case of breakfast cereal, some compounds interfered with the OTA extraction and analysis. Indeed, the amount of OTA found is generally higher when using the Pittet's method [18] than with Entwisle's method [17, 20],

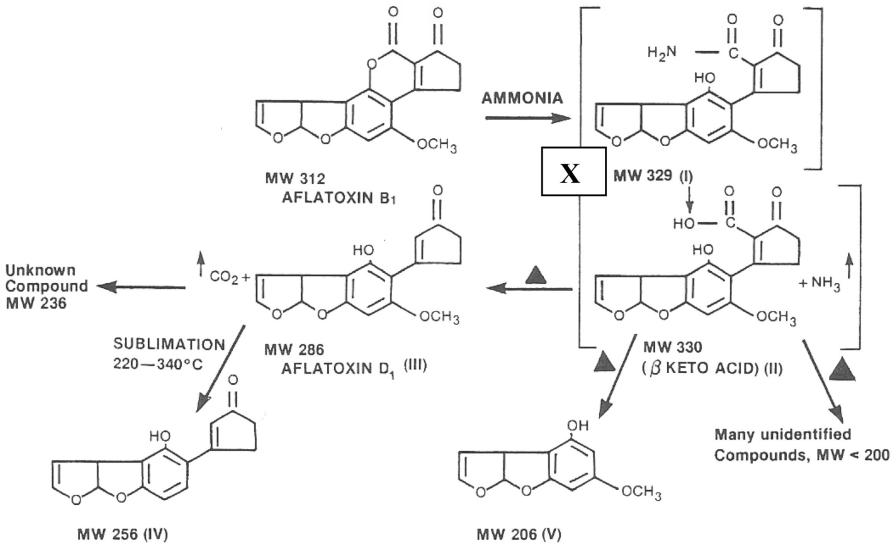

Figure 3. Decomposition pathway of AFB_1 in presence of ammonia [31].

Table 5. (A) Comparison of methods used for analysis of OTA in coffee. (B) Percent recoveries from ground roasted coffee beans. (C) Percent recovery from coffee beverage used as extract

(A)						
Reference of the method used	NF EN 14132 [17]		[18]		NF EN ISO 15141-1, modified by addition of a chloroform step	
Analytical step	Sample size		2 (g)		0.5 (g) 10 (g)	
Extraction	Methanol/bicarbonate 3% (1/1 v/v); dilution of 10 mL extract with 30 mL PBS pH 7.4		Methanol/bicarbonate 3% (1/1 v/v); dilution of the 4 mL extract to 100 mL with PBS pH 7.4		HCl 2 M, MgCl2 0.4 M, Toluene (3/5/10 v/v/v)	
Clean-up	SEP-PACK® (elution methanol/water, 7/93) followed by IAC (elution methanol)		IAC (pass through the total 100 mL of dilution)		Sep-Pack, re-extraction by chloroform	
Detection	Fluorimetry after HPLC		Fluorimetry after HPLC		Fluorimetry after HPLC	
(B)						
Contamination method	0.5	1.0	2.0 Recovery (%)	2.5	5.0	10.0
Entwisle 14132	16.6 – 18.7	24.5 – 27.0		33.0 – 34.0	41.0 – 49.0	63.0 – 70.0
Pittet	LOD		62 – 65		70 – 72	80 – 82
15141-1	50 – 55	53 – 55		57 – 55	57 – 57	
(C)						
Contamination method	0.5	1.0	2.0 Recovery (%)	2.5	5.0	10.0
Entwisle 14132	68 – 72	75 – 73		72 – 70	80 – 73	68 – 70
Pittet		72 – 70		75 – 76	80 – 80	

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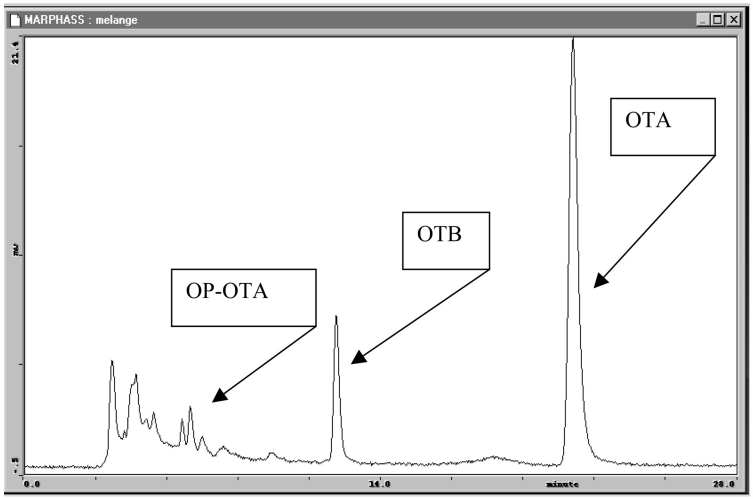

Figure 4. Example of separation of OTA, OTB and OP-OTA spiked in coffee extract before IAC column. Note: Excitation and emission wavelengths used are those of the OTA analysis, but the recoveries were calculated from the response curve of the respective standards analysed under these conditions.

both using purification on IAC columns. The data are also more reproducible and closer to those obtained with CEN 15 141-1 method [19].

The major difference between the two IAC methods is the relative proportion of PBS added to the extract. In Pittet's method 4 mL of extract containing methanol/3% bicarbonate are diluted to 100 mL with PBS pH 7.4 while in Entwisle's/AFNOR method 10 mL of the same extract is added to 30 mL of the same solution of methanol/3% bicarbonate. The former conditions [18] bring the pH much closer to neutrality than the later where the pH remains over 8 (see Fig. 2). Thus, the conditions of Pittet *et al.* [18] allow a better reconversion of OP-OTA into OTA. We have demonstrated that the OP-OTA is not recognised by the antibodies of the IAC used.

Moreover, the amount of coffee extract passed through the column, by the method of Pittet *et al.* [18] corresponds to 0.5 g of coffee and by that of Entwisle *et al.* [17] corresponds to 2 g. Therefore, the amount of compound possibly interfering with the OTA antibody is four-fold greater using this latter method.

All these experiments being run in parallel with the same batches of coffee, the compounds extracted are expected to be identical and in similar ratio. This suggests again that some of the components of the coffee bind to the antibodies, thus reducing the capability of the column to retain OTA.

In some coffee samples, we also detected dechlorinated OTA (OTB). For this reason, we tested the cross reactivity of OTA, OTB and OP-OTA (separated as standards in Fig. 4) with the antibodies from the IAC used. When 50 ng of each toxin is spiked on a coffee extract before IAC, the recoveries of OTA and OTB were, respectively, 57 and 81%, whereas recoveries of OP-OTA are not recognised (amount detected is below the LOQ).

The respective recoveries when each derivative is spiked independently were 80% for OTA, 91% for OTB and 0% for OP-OTA. This indicates that (1) OTB is recognised by the antibodies of the OTA IAC, (2) the affinity of OTB is higher than that of OTA on these columns and (3) OP-OTA is not recognised by OTA-IAC.

This explains the data obtain with the most contaminated sample for which, much higher OTA amount is found in acidic condition without IAC (15 µg/kg) than by the two other methods (9.5 µg /kg).

The loss of OTA is due to conjunction of transformation of OTA into OP-OTA and presence of OTB. In the samples which did not contain OTB, some interfering compounds are most probably coumarone derivatives, abundant in coffee and having structural analogies with OTA which is a dihydrocoumarin derivative.

4 Concluding remarks

This work demonstrated that methods using IAC which had already been validated for one or two matrices cannot be satisfactorily extrapolated to the analysis of the same toxins in very complex matrices. The presence of some components from the matrix leads to underestimation of the three mycotoxins studied.

In addition, in the case of not only OTA but also AFs, for which extraction under alkaline conditions induce opening of the lactone ring, we must keep in mind that the reaction being reversible [26, 27] in stomach of human and animals where the acidic pH induces the conversion of OP-OTA into OTA and open AF into AF, thus increasing the potential health problems due to this toxin and its underestimation.

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These analytical problems will have serious impact on the level of mycotoxin detected, especially at the levels close to those from the EU legislation. Underestimation could be highly dangerous for health, notably if this underestimation is in babyfood.

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