

# COMPARISON OF METHODS BY TLC AND HPTLC FOR DETERMINATION OF AFLATOXIN M<sub>1</sub> IN MILK AND B<sub>1</sub> IN EGGS<sup>1</sup>

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## SUMMARY

Milk and egg matrixes were assayed for aflatoxin M<sub>1</sub> (AFM<sub>1</sub>) and B<sub>1</sub> (AFB<sub>1</sub>) respectively, by AOAC official and modified methods with detection and quantification by thin layer chromatography (TLC) and high performance thin layer chromatography (HPTLC). The modified methods: Blanc followed by Romer, showed to be most appropriate for AFM<sub>1</sub> analysis in milk. Both methods reduced emulsion formation, produced cleaner extracts, no streaking spots, precision and accuracy improved, especially when quantification was performed by HPTLC. The use of ternary mixture in the Blanc Method was advantageous as the solvent could extract AFM<sub>1</sub> directly from the first stage (extraction), leaving other compounds in the binary mixture layer, avoiding emulsion formation, thus reducing toxin loss. The relative standard deviation (RSD%) values were low, 16 and 7% when TLC and HPTLC were used, with a mean recovery of 94 and 97%, respectively. As far as egg matrix and final extract are concerned, both methods evaluated for AFB<sub>1</sub> need further studies. Although that matrix leads to emulsion with consequent loss of toxin, the Romer modified presented a reasonable clean extract (mean recovery of 92 and 96% for TLC and HPTLC, respectively). Most of the methods studied did not performed as expected mainly due to the matrixes high content of triglycerides (rich on saturated fatty acids), cholesterol, carotene and proteins. Although nowadays most methodology for AFM<sub>1</sub> is based on HPLC, TLC determination (Blanc and Romer modified) for AFM<sub>1</sub> and AFB<sub>1</sub> is particularly recommended to those, inexperienced in food and feed mycotoxins analysis and especially who cannot afford to purchase sophisticated (HPLC, HPTLC) instrumentation.

**Keywords:** aflatoxin; AFM<sub>1</sub>; AFB<sub>1</sub>; TLC; HPTLC; milk; egg.

## RESUMO

COMPARAÇÃO DE METODOLOGIA PARA ANÁLISE DE AFLATOXINA M<sub>1</sub> EM LEITE E AFLATOXINA B<sub>1</sub> EM OVOS POR CCD E CCDAE. Aflatoxinas M<sub>1</sub> (AFM<sub>1</sub>) e B<sub>1</sub> (AFB<sub>1</sub>) foram analisadas em leite e ovos respectivamente, por diferentes métodos oficiais da AOAC e modificações usando detecção por cromatografia em camada delgada (CCD) e CCD de alta eficiência (CCDAE). Os métodos modificados: Blanc e Romer, apresentaram-se mais apropriados para análise de AFM<sub>1</sub> em leite. Ambos reduziram formação de emulsão, produziram extratos limpos, sem formação de caudas. Inclusive, a precisão e acuidade aumentaram, especialmente quando a quantificação foi realizada por CCDAE. O uso de solventes ternários, no método de Blanc, foi vantajoso. Este solvente extrai AFM<sub>1</sub> diretamente na primeira fase (extração), deixando outros compostos na camada binária, evitando emulsão, reduzindo assim, perda da toxina. O RSD% foi muito baixo com 16 e 7%, respectivamente. Quanto ao ovo, AFB<sub>1</sub> e extrato final, ambos os métodos necessitam mais estudos. Embora esta matriz induza a formação de emulsão com consequente perda de toxina, o método de Romer modificado apresentou extrato razoavelmente limpo com recuperação de 92 e 96% para CCD e CCDAE, respectivamente. A maioria dos métodos estudados não apresentou o desempenho esperado porque as amostras possuem conteúdo elevado de triglicerídios, colesterol, caroteno e proteínas. Embora, a maioria das metodologias para AFM<sub>1</sub> seja baseada em CLAE; o uso de CCD para de determinação de AFM<sub>1</sub> e AFB<sub>1</sub> é particularmente recomendada para aqueles, inexperientes em análises de alimentos e micotoxinas e especialmente, laboratórios que não podem adquirir equipamento sofisticado.

**Palavras-chave:** aflatoxina; AFM<sub>1</sub>; AFB<sub>1</sub>; CCD; CCDAE; leite; ovos.

## 1 - INTRODUCTION

Aflatoxin M<sub>1</sub> (AFM<sub>1</sub>), a metabolite of aflatoxin B<sub>1</sub> (AFB<sub>1</sub>), was first detected in milk of lactating cows that had ingested AFB<sub>1</sub> contaminated feed. The AFM<sub>1</sub> was also detected in the animals' urine and faeces. As for cows, when chicken are fed with contaminated feed, AFB<sub>1</sub> can be transferred, to the egg, in that case, without modification on its chemical structures [1,2,7,11].

Studies have indicated that the toxicity of AFM<sub>1</sub> is of the same order of magnitude as that of AFB<sub>1</sub> [2,15,16,34] and that AFM<sub>1</sub> is also a potent hepatocarcinogen in laboratory animals [28]. Therefore, the contamination of dairy products with AFM<sub>1</sub> has been recognised as a significant human health hazard,

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particularly for babies, since milk is their basic food and the young of most animal species is more susceptible than the adult animal to its effect. The conversion ratio of the AFB<sub>1</sub> ingested to the AFM<sub>1</sub> found in the milk is between 0.5 and 3% [4]. As far as the Regulations and maximum residue level (MRL) are concerned, the United States have declared a risk assessment for AFM<sub>1</sub> with an MRL in milk for human consumption of 0.5µgkg<sup>-1</sup> (US Food and Drug Administration Guidelines for Acceptable Levels of Aflatoxin in Food and Feed), the same for Brazil [9] and the Economic European Community (EEC) proposal on MRL of AFM<sub>1</sub> is 0.05µgkg<sup>-1</sup> for milk and milk products [29, 33], on the other hand, there is not legislation for AFB<sub>1</sub> contamination in eggs.

The detection of mycotoxins often involves a combination of adsorption and fluorescence properties, being therefore, the AFM<sub>1</sub> and AFB<sub>1</sub> detected by their inherent fluorescence by thin layer chromatography (TLC), high performance thin layer chromatography (HPTLC) or high performance liquid chromatography (HPLC). The physico-chemical properties based

techniques for mycotoxins, TLC and HPTLC, are simple, sensitive and less subject to interference by co-extractives than HPLC. Two dimensional thin layer chromatography (2D TLC) has greatly improved the separation ability of the thin layer techniques. However, one should take into account that the presence of a spot on the TLC/HPTLC plate can only be considered as presumptive evidence of identification, and further confirmation tests are required. Although several HPLC methods have been developed for AFM<sub>1</sub> analysis [10, 12, 30, 31, 32], and by immunoassay [8] still TLC methods can be recommended when no HPLC equipment is available [17, 29].

Considering that (a) feeding milking cows and laying hens with highly AFB<sub>1</sub> contaminated feed cause its transfer as AFM<sub>1</sub> to milk and AFB<sub>1</sub> to eggs, respectively, (b) many laboratories still do not have HPLC equipment, (c) the high lipid or protein content of the milk and egg matrixes that lead to emulsion formation, thus loss of the compost of interest, and (d) contamination of animal feed and its ingredients with AFB<sub>1</sub> in Brazil has been reported [13, 14, 19, 20, 21, 24, 25, 26, 27], a study on the official methodology available in the literature and modified methodology for whole fluid milk and eggs were carried out in order to find out a method that could be fast, simple, reduce emulsion formation, produce a clean extract, as well as sensible and accurate. In addition a comparison on the final extracts behaviour on TLC and HPTLC plates under UV light also was carried out.

## 2 - MATERIALS AND METHODS

### 2.1 - Materials

**Sample:** (a) whole fluid cow milk and (b) egg from white Leghorn laying hen samples spiked at level of 0.5µg kg<sup>-1</sup> of (a) AFM<sub>1</sub> and (b) AFB<sub>1</sub>, respectively.

**Standards:** AFB<sub>1</sub> and AFM<sub>1</sub> (0.1mg) pure standards from Sigma.

**Reagents and solvents:** all reagent and solvents used for the TLC and HPTLC methods were *p.a. grade*.

**Aluminium TLC and HPTLC plates:** coated with silica gel G60. Dimensions for *TLC*: 200 x 200 and/or 100 x 100mm and for *HPTLC*: 100 x 200mm (length x width), Merck.

**Apparatus:** *Centrifuge*: international size 2, Vortex. *Heating block*: Aluminium 254mm thick x 254mm wide. Drilled 190mm deep on 762mm centres to accommodate vials. *High speed blender*: 1 L jar, drill 32 mm hole ca. 1cm from centre of lid to permit escape of vapours. *Chromatographic tubes*: 22 x 500 (id) (width x length). *Microcaps*: 1, 2, 5 and 10µL, Drummond Scientific Company. For *HPTLC*: *autosampler* Camag TLC model 27220; *Camag HPTLC linear developing tank* (5 ml solvent capacity); *fluorodensitometer*: capable of scanning in reflectance mode by fluorescence, equipped with Hg lamp, monochromator for adjustment to excitation 366nm and emission cut off filter 420nm – Camag TLC Scanner II;

and Hewlett Packard *integrator*. Part of this work was carried out in England at Natural Resources Institute (NRI), the former Tropical Development Research Institute (TDRI).

### 2.2 - Methodology of analysis for AFM<sub>1</sub> in milk

The methods studied for analysis of AFM<sub>1</sub> in milk were: AOAC I (*AFM<sub>1</sub> in Milk and Cheese - Final Action*), AOAC II (*AFM<sub>1</sub> in Dairy Products - Final Action*), AOAC II/NRI (AOACII modified by NRI), AOAC III (*Peanut and Peanut Products BF Method - Final Action*), Romer/NRI, and modified Blanc Method [3,4,5,6,18]. *Table 1* summarises the characteristics of those methods studied. The final extracts from all methods were quantified by TLC and HPTLC. The AFM<sub>1</sub> spiked samples of milk and AFB<sub>1</sub> spiked eggs were analysed ten times using each method.

**TABLE 1.** Summary of the methods compared for AFM<sub>1</sub> in milk

Method	Extraction Solvent	Cleanup		Separation <sup>a</sup>	
		Chemical	Column	TLC	HPTLC
• AOAC I	CHCl <sub>3</sub>	-	Silica gel	1,2D <sup>b,c</sup> TLC	✓ <sup>d</sup>
• AOAC II	Acetone	Lead acetate	Cellulose	1D <sup>b</sup> TLC	✓
• AOAC II/NRI	Acetone	Lead acetate	-	2D <sup>c</sup> TLC	✓
• Romer/NRI	Acetone	Ferric gel	-	2DTLC	✓
• AOAC III	MeOH/H <sub>2</sub> O	-	-	1DTLC	✓
• Modified Blanc	CHCl <sub>3</sub> /MeOH	-	Silica gel	1DTLC	✓

TLC = thin layer chromatography  
 HPTLC = high performance thin layer chromatography  
<sup>a</sup> UV detection at 365nm  
<sup>b</sup> one dimensional  
<sup>c</sup> two dimensional  
<sup>d</sup> HPTLC used in all methods

#### 2.2.1 - AOAC official methods

The materials and methods for the analytical procedure of the AOAC Method I, AOAC Method II and AOAC III, were essentially the same as given by Methods of Analysis of the AOAC [3]. *Table 1* shows the solvent used for extraction, the chemicals and column packing for the cleanup step and the type of TLC used for separation. For the quantification step the AOAC I method can be carried out either by visual or densitometric reading.

#### 2.2.2 - AOAC and romer methods modified

The AOAC Method II modified by NRI, follows the AOAC procedure for the initial extraction and clean up, but the cellulose column chromatography clean up step was taken out. The quantification was carried out by two dimensional thin layer chromatography (2DTLC) using the solvent systems: ethanol: methanol: water (EMW) (94:4.5:1.5) in the first direction and chloroform: acetone: isopropanol (CAI) (86:4:10) in the second direction. The Romer/NRI Method had some slight modifications from the published Romer Method [18] and these were: (a) the amount of water added at the sample preparation step, (b) no addition of basic copper

carbonate at the cleanup step and (c) no addition of the potassium hydroxide/potassium chloride washing at the final extraction step. Also 2DTLC was used instead of one dimensional (1DTLC) and the solvent systems were EMW in direction I and CAI in direction 2.

### 2.2.3 - Modified blanc method

The basic principle of the Blanc Method [4] is the extraction of AFM<sub>1</sub> using a ternary mixture\* of water, methanol and chloroform [28]. The proportion of each solvent depends on the actual material to be extracted. The extraction occurs in the chloroform monophase. After extraction the equilibrium is changed to diphasic by adding more chloroform. The whole of the chloroform layer is recovered and it is assumed that the total aflatoxin is contained in that layer. Modifications were made to the method in order to make it easier and cheaper whilst maintaining its sensitivity and speed. It was taken out the use of pressure during the column clean-up step by using a wider chromatographic column (diameter of 2cm) with twice the amount of the packing material i.e., 10g of silica-gel used in the original column. The one dimensional TLC was substituted by 2DTLC and HPTLC [23].

### 2.3 - Methodology of analysis for AFB<sub>1</sub> in eggs

The methods compared for AFB<sub>1</sub> analysis in eggs were the official method AOAC (*AFB<sub>1</sub> in Eggs - Final Action*) and Romer modified by NRI which, as for AOAC I method from milk, the quantification can be done either by visual or densitometric reading. Details of the first method are found in the AOAC [3] and for the Romer/NRI are as in Section 2.2.1. Those methods are outlined in *Table 2*.

**TABLE 2.** Summary of the methods compared for AFB<sub>1</sub> in eggs

Method	Extraction	Cleanup		Separation <sup>a</sup>	
	Solvent	Chemical	Column	TLC	HPTLC
1. AOAC	Acetone	Lead acetate	Silica gel	2D <sup>b</sup> TLC	✓ <sup>c</sup>
2. Romer/NRI	Acetone	Ferric gel	Silica gel	1D <sup>d</sup> TLC	✓

TLC = thin layer chromatography

HPTLC = high performance thin layer chromatography

<sup>a</sup> UV detection at 365nm

<sup>b</sup> one dimensional

<sup>c</sup> two dimensional

<sup>d</sup> HPTLC used in all methods

### 2.4 - Final extract application on plates

*For TLC:* the extracts were applied *manually* on 200 x 200 and 100 x 100mm plates for one dimensional (1D) and two dimensional (2D), respectively, as well as with standards at different concentrations, inclusive internal standard [3], using *microcaps* (see details on Section 2.1).

*For HPTLC:* extract and standards were applied *automatically* on plates 100 x 200mm, using a TLC autosampler (Camag). The final extract spot size (diameter) produced by the HPTLC autosampler was consistently of 1.5 to 2mm, different of that *manually* applied on 1DTLC

that presented various spot sizes (ca. 5 to 10mm diameter), depending on the extracts appearance (clean, oily or dirty), as they take longer to dry.

### 2.5 - Confirmation methods

All the methods studied were checked at the end of their procedures for confirmation of the fluorescent spots detected, if they were of AFB<sub>1</sub>, AFM<sub>1</sub> or impurities: (a) aflatoxin derivatisation (AFB<sub>2a</sub>) prior to development of the TLC plate using hydrochloric acid or trifluoroacetic acid (AFM<sub>1</sub>), (b) using AFB<sub>1</sub> or AFM<sub>1</sub> internal standards and (c) spraying the TLC plates with sulphuric acid solution that changes AFM<sub>1</sub> or AFB<sub>1</sub> spot color [3, 22].

## 3 - RESULTS AND DISCUSSION

### 3.1 - Methodology of analysis of AFM<sub>1</sub> in milk

The recovery of AFM<sub>1</sub> from the spiked milk samples using the six different methods, as well as the comparison of their different parameters (time, emulsion formation, extract appearance, plate background, and type of TLC used) are shown in *Tables 3 and 4*, respectively. The official AOAC I Method, which does not has a cleanup step (*Table 1*), presented the greatest disadvantage of all methods studied since it consistently gave a stable emulsion and an oily final extract leading to the lowest mean recovery (40 and 65% for TLC and HPTLC, respectively) and the highest RSD of all methods. The quantification using either, TLC or HPTLC plates, was difficult due to co-extractives in the final extract, producing spot streaking even when using the alternative: 2DTLC for visual reading. The densitometric quantification of 1DTLC provided reading error of the spot fluorescence (detection of the fluorescence from co-extractives with similar wavelength to the toxin's, together with AFM<sub>1</sub>), different of that obtained on the 2DTLC cleaner spots by visual reading. The RSD% for both readings, visual and densitometric, were high with 48 and 39, respectively, indicating variation on reading: min. 35; max. 82 and min. 39; max. 88% mean recovery for TLC and HPTLC, respectively, the last being lower than the previous as that technique provides a better spot shape and much smaller size than TLC leading to a more accurate fluorescence detection than the visual (the extract is manually applied on the plate).

On the other hand, the AOAC II was too time consuming due to its cleanup step: an open column (22 x 500mm) packed with cellulose (10g) that lead to a reduced sample extract flow rate, as well as of the washing and elution solvent systems through its packing material. Apart from that, the AOAC II provided a very clean extract and a quite good TLC separation of the toxin (no streaking and good spot shape) with a better recovery than the previous method, either for visual or densitometric reading, (mean recovery of 72

\* Ternary mixtures have particularly to be mono or diphasic, depending on the proportion of the components.

and 79% and RSD 26 and 15%, respectively). No spot interference by co-extractives was observed. Although it is still among the AFM<sub>1</sub> methodology, this method retains its official *status* but is carried out only by reference [3].

When the AOAC II was modified by NRI, in order to improve time and material, the recovery changed. The use of that modified Method II without the cellulose column cleanup step, greatly reduced the time and materials required for analysis, although, that meant some emulsion formation, dirty final extract and the need of two dimension (2DTLC) for purifying/cleaning the AFM<sub>1</sub> spot. That technique (2D) was excellent on separating AFM<sub>1</sub> from other impurities, on the other hand, it tends to give diffused and sometimes distorted (i.e., not round) spots which lead to difficulties on the quantification step, hence, inaccuracies can arise when visual reading is used. The same can occur for HPTLC technique, as the toxin is eluted only at one direction on the plate (1DHPTLC) thus, some co-extractives are still present and one may get over-reading results leading to error. Furthermore, the 2DTLC has the disadvantage of only one sample can be tested per plate, different to those several (*ca.* 3) samples per plate on 1DTLC and *ca.* of 60 on HPTLC plates (30 spots each side of the HPTLC plate plus standards). The mean AFM<sub>1</sub> recovery was higher than AOAC II with 80% (min. 70; max. 87%) and 92% (min. 87; max. 96%) and RSD of 25 and 12% for TLC and HPTLC, respectively.

**TABLE 3.** Recovery of AFM<sub>1</sub> from spiked milk samples

Method	Extraction Solvent	Cleanup		Separation <sup>a</sup>	
		Chemical	Column	TLC	HPTLC
1. AOAC I	CHCl <sub>3</sub>	-	Silica gel	1,2D <sup>b</sup> TLC	✓ <sup>d</sup>
2. AOAC II	Acetone	Lead acetate	Cellulose	1D <sup>b</sup> TLC	✓
3. AOAC II/NRI	Acetone	Lead acetate	-	2D <sup>c</sup> TLC	✓
4. Romer/NRI	Acetone	Ferric gel	-	2DTLC	✓
5. AOAC III	MeOH/H <sub>2</sub> O	-	-	1DTLC	✓
6. Modified Blanc	CHCl <sub>3</sub> /MeOH	-	Silica gel	1DTLC	✓

<sup>a</sup> n = 10 from TLC separation and visual readings

<sup>b</sup> minimum

<sup>c</sup> maximum

<sup>d</sup> relative standard deviation

<sup>e</sup> carried out 5 times only (column cleanup = time consuming)

**TABLE 4.** Comparison of method parameters – milk

Method	Time (Hours)	Emulsion Formation	Extract Appearance	Plate Background		TLC/ HPTLC <sup>a</sup>
				Streaking	Size	
1. AOAC I	3½ - 4	+	Oily	Yes	Good	1D <sup>b</sup> /+ <sup>d1</sup>
2. AOAC II	4½ - 5	+/-	Clean	No	Good	1D/+++ <sup>d3</sup>
3. AOAC II/NRI	3 - 3½	+	Dirty	Yes	Spread	2D <sup>c</sup> /+ <sup>d1</sup>
4. Romer/NRI	2½	+/-	Clean/dirty	Yes/no	Spr/good	2D/++ <sup>d2</sup>
5. AOAC III	3	+	Dirty	Yes	Good	1D/+ <sup>d1</sup>
6. Modified Blanc	3	-	Clean	No	Good	1D/+++ <sup>d3</sup>

TLC = thin layer chromatography

HPTLC = high performance thin layer chromatography

<sup>a</sup> UV detection at 365 nm

<sup>b</sup> one dimensional

<sup>c</sup> two dimensional

<sup>d</sup> densitometer quality of fluorescence reading (<sup>d1</sup> = bad, <sup>d2</sup> = good, <sup>d3</sup> = very good)

The Romer/NRI Method showed a substantial reduction of the time spent for the analysis with a mean recovery of 90% (min. 79%; max. 95%) and 96% (min. 90; max. 96%) and of a quite good RSD of 15 and 8% for TLC and HPTLC, respectively. However, with the disadvantage of the extracts not been always clean and needed 2DTLC (as for AOAC/NRI). The alkaline and ferric chloride cleanup greatly improved the quality of the final extract, when compared to the previous method. From the methods studied for milk, the Romer/NRI method was the shortest among them.

The AOAC III (Method developed for peanuts and its products) presented a reasonably good method for milk with a recovery of 75% and RSD of 27% for TLC. The same occurred, as for some of the previous methods studied, due to the fact that milk tends to form a stable emulsion when mixed with the solvents, it led to a difficult separation of AFM<sub>1</sub> (even using centrifugation) and a dirty final extract. That method has not got an efficient cleanup procedure especially for matrixes rich on protein such as milk.

The mean recovery for the modified Blanc Method was the best among the methods studied (was 97% with a RSD of 7% for HPTLC and 94% with RSD of 16% for TLC), although the time taken per sample was slightly longer (3h.) than that for the Romer/NRI Method (2½h). By using the Blanc ternary solvent mixture for AFM<sub>1</sub> extraction, no emulsion is formed leading to a reduction of AFM<sub>1</sub> loss. In addition, using an HPTLC for detection in the modified Blanc Method, the accuracy of fluorescence intensity reading improves. All that leading to a limit of detection (LOD) and limit of quantification (LOQ) of 0.001µgkg<sup>-1</sup>. The HPTLC equipment's cost will reduce, as a large number of samples can be quantified per HPTLC plate run. It is important to emphasize that the RSD% for the HPTLC is lower because in an complete HPTLC procedure either, the autosampler improves the extract application quality and the linear development tank leads to a high quality toxin separation improving the repeatability, reproducibility, accuracy of the scanner detection and quantification.

### 3.2 – Methodology of analysis for AFB<sub>1</sub> in eggs

For the egg samples and AFB<sub>1</sub> extraction and separation, the high content of lipids and proteins still is the main problem on the toxin extraction, leading to emulsion formation, producing an oily/dirty extract and error on recovery quantification either for the AOAC (developed for eggs First Action) and Romer/NRI methods. Despite of the use of lead acetate and ferric chloride respectively, inclusive silica gel column at the cleanup steps, the behavior of egg final extract rich on lipid co-extractives on 1DTLC plates was disastrous (spot streaking and over-reading of fluorescence from AFB<sub>1</sub> and of co-extractives), leading to error, or not being possible to read the spot fluorescence at all (Table 5 and 6). The 2DTLC improves spot purity and provides a better visual reading, however, when HPTLC is used the plate is developed on one direction only reproducing the same problem as for visual 1DTLC reading. Although

emulsion formation was a problem in the first step of the AOAC method, the recovery was 83 and 98% for TLC and HPTLC, respectively. The min. and max. recovery was 80 and 98% (RSD of 30 and 44%) and 99 and 132% (RSD 44%). Despite of that, the Romer/NRI Method extraction and detection presented a better performance than the AOAC method for eggs.

Considering that (a) even when HPTLC was used instead TLC, the performance of the final egg extract did not improve, and (b) the co-extractives interfered on the reading, leading to higher recovery than the spiked samples (for the two methods) it is necessary further study on the extraction of AFB<sub>1</sub> from egg matrix

**TABLE 5.** Recovery of AFB<sub>1</sub> from spiked egg samples

Method	Recovery (%)						RSD <sup>d</sup>	
	Mean <sup>a</sup>		Min. <sup>b</sup>		Max. <sup>c</sup>		(%)	
	TLC	HPTLC	TLC	HPTLC	TLC	HPTLC	TLC	HPTLC
1. AOAC	83	98	80	98	98	112	30	44
2. Romer/NRI	92	96	75	93	92	96	25	20

<sup>a</sup> n = 10 obtained from TLC separation and visual readings

<sup>b</sup> minimum

<sup>c</sup> maximum

<sup>d</sup> relative standard deviation

**TABLE 6.** Comparison of method parameters - eggs

Method	Time (Hours)	Emulsion Formation	Extract Appearance	Plate Background		TLC/ HPTLC <sup>d</sup>
				Streaking	Size	
1. AOAC	3½ - 4	+	Dirty	Yes	Spread	2D <sup>b</sup> / <sup>c</sup>
2. Romer/NRI	2½	+	Dirty	Yes	Spread	2D/+

TLC = thin layer chromatography

HPTLC = high performance thin layer chromatography

<sup>a</sup> UV detection at 365nm

<sup>b</sup> two dimensional

<sup>c</sup> densitometer quality of fluorescence reading (+ = bad)

As far as the choice for a chromatographic based methodology is concerned, HPLC methods with fluorescence detection are the most used and reported in the literature for AFM<sub>1</sub>. Although, when there is a lack of that equipment, laboratories must try to find out alternatives and they may use TLC in order to quantify AFM<sub>1</sub>. The Romer/NRI and Blanc methods seem to present adequate extraction and detection with good recovery. If the equipment is available, HPTLC can improve accuracy, repeatability and speed, improving therefore the quality and reliability of toxin data obtained. It is important to emphasise that the fluorescence intensity of emission for aflatoxins varies among them: the AFB<sub>2</sub> is 8 times more intense than the AFB<sub>1</sub> and AFM<sub>1</sub> and AFM<sub>2</sub> are 3 fold more fluorescent than AFB<sub>1</sub> and AFB<sub>2</sub> which is advantageous to those using the TLC detection technique in their laboratories (if AFM<sub>1</sub> is more fluorescent, it can be visible at lower concentrations than AFB<sub>1</sub>).

Concerning to the safety of the analyst and the solvents used, the AOAC I and modified Blanc method use chloroform as the solvent for AFM<sub>1</sub> extraction. That solvent is carcinogenic being in nowadays substituted: dichloromethane can be used instead. The AOAC II and

AOAC II/NRI (for milk) and AOAC (for egg) use for cleanup lead acetate which should also be changed.

As far as international proposed methods of analysis for aflatoxin AFM<sub>1</sub> in milk and milk products are concerned, the only reference methods recommended are the AOAC methods and certified reference material for milk powder are available for methodology checking at levels of 0.05, 0.31 and 0.76µgkg<sup>-1</sup> [29].

While HPLC technique has excellent sensibility (able to detect very low amounts), reproducibility and is applicable to automation, it can bring specific problems such as: requirement of highly clean extracts, only one sample to be analyzed at time, running costs are high and highly trained personnel are normally necessary to operate the equipment and interpretation the results. The HPTLC is fast, sensible, reproducible, automatic and the most advantage: it can read ca. 60 samples per plate.

#### 4 - CONCLUSION

**Milk:** taking into account (a) the extraction step (b) emulsion formation, (c) the appearance of the extract, (d) its cleanliness and behaviour during the TLC procedure, (e) the precision, (f) cost and (g) analysis time/sample of each method, the modified Blanc and Romer/NRI were the methods that presented the best performance among all methods studied for AFM<sub>1</sub> analysis in whole fluid milk. Inclusive presenting very low and consistent LOD and LOQ (modified Blanc = 0,001 and Romer/NRI = 0.005µgkg<sup>-1</sup>) thus being able to attend the demand of most stringent international regulations for AFM<sub>1</sub> such as Germany, The Netherlands, Sweden (MRL: 0.05µgkg<sup>-1</sup> for whole milk) and Germany (MRL: 0.01µgkg<sup>-1</sup> for dairy food for infants) [9]. Especially if the modified Blanc method is used with HPTLC technique. The use of ternary mixture in the Blanc method is advantageous as the solvent can extract AFM<sub>1</sub> directly from the first stage, reducing losses through exhaustive liquid-liquid partition, a step that leads to a reduction of the LOD. The AOAC/NRI method could also be used for milk, if provided a suitable cleanup solvent system is used to substitute the cellulose column and to enable 1DTLC for quantification.

**Egg:** although, the Romer/NRI method seemed to be better than the AOAC method on the comparative study for AFB<sub>1</sub> determination in eggs, emulsion occurred on both methods assayed, leading to loss of toxin. The time required to carry out the Romer/NRI method was shorter than for the AOAC method. It is important to emphasise that the Romer method developed for peanuts (rich on triglicerides) modified by NRI was chosen for comparison of its behavior on toxin determination from milk and egg matrixes, due to the fact that they have high lipid content, either triglicerides, cholesterol and carotene. The difference is that the fatty acid in those animal samples are saturated, therefore more difficult to extract than the oil (poli unsaturated fatty acids) on the peanut samples. On the other hand, those matrixes also have high content of protein.

Despite of that, it did not performed as expected perhaps for eggs due to the emulsion formation. In addition, both matrixes are also rich on protein, which could be reduced by the Romer cleanup step.

Although the recent preference for HPLC methods, the TLC has special advantages that makes it still be very popular, such as the possibility of carrying out *in situ* derivatization procedures to confirm the presence of the toxins and the fact that the analyst has a certain contact with the result of the separation, because the human eye itself can act as a detector. Inclusive the limit of detection (LOD) for plate reading on TLC and HPTLC are similar (ca. 0.4ng) [5,6]. TLC is particularly recommended to those, inexperienced in food and feed analysis for mycotoxins and who cannot afford to purchase sophisticated (HPLC, HPTLC) instrumentation such as in developing countries.

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