DETERMINING AFLATOXINS $B_1$, $B_2$, $G_1$ AND $G_2$ IN MAIZE USING FLORISIL CLEAN UP WITH THIN LAYER CHROMATOGRAPHY AND VISUAL AND DENSITOMETRIC QUANTIFICATION

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SUMMARY
A method for determining aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$ in maize with florisil clean up was optimised aiming at one-dimensional thin layer chromatography (TLC) analysis with visual and densitometric quantification. Aflatoxins were extracted with chloroform: water (30:1, v/v), purified through florisil cartridges, separated on TLC plate, detected and quantified by visual and densitometric analysis. The in-house method performance characteristics were determined by using spiked, naturally contaminated maize samples, and certified reference material. The mean recoveries for aflatoxins were 94.2, 81.9, 93.5 and 97.3% in the range of 1.0 to 242 µg/kg for AFB$_1$, 0.3 to 85mg/kg for AFB$_2$, 0.6 to 148mg/kg for AFG$_1$, and 0.6 to 140mg/kg for AFG$_2$, respectively. The correlation values between visual and densitometric analysis for spiked samples were higher than 0.99 for AFB$_1$, AFB$_2$, AFG$_1$ and 0.98 for AFG$_2$. The mean relative standard deviations (RSD) for spiked samples were 16.2, 20.6, 12.8 and 16.9% for AFB$_1$, AFB$_2$, AFG$_1$ and AFG$_2$, respectively. The RSD of the method for naturally contaminated sample (n = 5) was 16.8% for AFB$_1$ and 27.2% for AFB$_2$. The limits of detection of the method (LD) were 0.2, 0.1, 0.1 and 0.1mg/kg and the limits of quantification (LQ) were 1.0, 0.3, 0.6 and 0.6mg/kg for AFB$_1$, AFB$_2$, AFG$_1$ and AFG$_2$, respectively. (218 words)

Keywords: aflatoxins; florisil; maize; thin-layer chromatography (TLC); densitometry.

RESUMO
DETERMINAÇÃO DE AFLATOXINAS $B_1$, $B_2$, $G_1$, $G_2$ EM MILHO UTILIZANDO PURIFICAÇÃO COM FLORISIL, SEPARAÇÃO POR CROMATOGRAFIA EM CAMADA DELGADA E QUANTIFICAÇÃO VISUAL E DENSITÔMÉTRICA. Um método para determinação de aflatoxinas $B_1$, $B_2$, $G_1$, $G_2$ em milho utilizando florisil na etapa de purificação foi otimizado com vistas a cromatografia em camada delgada (CCD) unidimensional com quantificação visual e densitômética. As aflatoxinas foram extraídas com solução de cloroformio: água (30:1, v/v), purificada em cartuchos de florisil, separada em placas cromatográfica de sílicagel, detectadas e quantificadas por análise visual e densitômética. As características do método foram determinadas utilizando amostras de milho natural e artificialmente contaminadas e material de referência certificado. Os valores de recuperação média para cada aflatoxina obtidos na faixa de 1.0 a 242 µg/kg para AFB$_1$, 0.3 a 85mg/kg para AFB$_2$, 0.6 a 148mg/kg para AFG$_1$, e 0.6 a 140mg/kg para AFG$_2$ foram respectivamente 94,2; 81,9; 93,5 e 97,3% por análise densitômética. As correlações obtidas entre análise visual e densitômética para amostras artificialmente contamina-

Aflatoxins are produced by three species of fungi namely: Aspergillus flavus, A. parasiticus and A. nomius in agricultural products that are susceptible to mould infection. Aflatoxin $B_1$ - agenotoxic substance - is hepatotoxic in humans and animals and immunosuppressive in animals [22]. Because of the high toxicity of aflatoxins, regulatory limits for aflatoxins in food and feed have been laid down in many countries including Brazil [16,17,18,19,20], aiming at reducing human and animal exposure to aflatoxins.

The monitoring of aflatoxins – which are present in food and feed usually inmg/kg - depends on precise, reliable and efficient methods for their accurate determination [10].

Thin layer chromatography has been the most widely used and established separation and quantification technique since its development, in the early 1960s, and it is still recommended by AOAC for the aflatoxin analysis [5, 36]. Low cost is the main advantage associated with TLC-based procedures as long as purified final extracts are obtained by an adequate clean-up procedure. Visual TLC has been the method of choice in the countries where other expensive instruments and the infrastructure for immunoassays are not available, though it is criticised for high degree of variation due to individual acuity. Visual TLC estimation is simple and reliable, as long as the analyst ensures the validity of the method by acceptable recovery tests [31,33]. The disadvantage about poor repeatability, associated with visual analysis could be decreased by the use of densitometric methods to automate the plate interpretation [14,33]. Two-dimensional TLC is required for that extracts with the presence of interference substances but this technique causes elongation of sample spots compared to aflatoxin standard spots that is not suitable for densitometric measurement [28] apart from the consuming of time involved in the analysis.
The major problem associated with most analytical methods for aflatoxins determination is the presence of co-extracted sample interferences, which requires multiple extraction and clean-up steps before quantification [2]. Solid-phase extraction (SPE) has been used in clean-up procedures saving a lot of time, being solvent-efficient and economical [7,37]. The use of SPE cartridges is simple and usually involves three steps: loading the sample, washing the impurities and eluting the toxin to be determined [13]. A variety of solid phase have been used in aflatoxin analysis, such as silica gel, surfaced-modified bonded silica like florisor and octadecil, a mixture of reversed-phase, ion exclusion, and ion exchange adsorbents (ISOLUTE and ROMER multifunctional column) and other minicolumns [1,3,6,7,25,27].

Rapid test kits are available for screening of aflatoxins, such as enzyme linked immunosorbent assay (ELISA) and antibody – based immunoaffinity column clean-up coupled with fluorometry or florisor tip. These methods do not normally allow for simultaneous monitoring of both individual and total aflatoxin levels although the use of immunoaffinity columns with TLC and HPLC [4,34] for the individual determination of the aflatoxins has been reported [30].

In this paper, the use of florisor as clean-up [2,24,25,27] step was investigated in order to improve and assess separation, recovery, reliability and sensitivity of the method, aiming at one-dimensional TLC analysis with visual and densitometric quantification.

Additional tests were carried out to optimise the clean-up, separation, detection and quantification steps. The determination of the method characteristics – accuracy, precision, linearity and limits of quantification were carried out by means of recovery tests for spiked maize samples containing five levels of contamination of aflatoxins and by analysis of a naturally contaminated sample (n = 5) and certified reference material.

2 - MATERIAL AND METHODS

2.1 – Standard solutions

Stock standard solutions of AFB₁, AFB₂, AFG₁, and AFG₂ (~10µg/mL) were prepared by dissolving the solid standard (Sigma, Sigma Chemical Co - St. Louis, MO) in benzene: acetonitrile (98:2, v/v) and the exact concentration were measured by spectrophotometer (Shimadzu UV-1601PC, Shimadzu Scientific Instruments, Japan) [5]. A working standard solution of AFB₁ (0.12µg/mL), AFB₂ (0.04µg/mL), AFG₁ (0.07µg/mL), and AFG₂ (0.07µg/mL) in benzene: acetonitrile (98:2, v/v), for spiking and quantification purposes, was prepared by diluting appropriate aliquots of stock solutions of each toxin.

2.2-Extraction

Finelyground (~20mesh) maize samples (50g), at room temperature, were weighed into a suitable flask (Mason Jar) and extracted with 10mL distilled water and 300mL chloroform [25] for 5 minutes in an Omni mixer (Omni 17105, Omni International, USA) at a medium velocity. The extract was filtered under vacuum through paper filter Whatman 4 and the filtrate were added 20g anhydrous sodium sulphate. Subsequently, the filtrate was filtered under vacuum through a Whatman fibreglass membrane (GF/B 1mm). The filtrate (100mL) was evaporated (40-50°C) in rotary evaporator (Buchi B-481, Labortechnik AG, Switzerland).

2.3 – Clean-up

The residue was transferred with 6mL chloroform to a florisor cartridge (1g, 6mL, vac, Supelcleanan, Supelco, USA), previously conditioned with 6mL chloroform, fitted to plastic stopcock, and connected to a vacuum system (Varian®, Vac Elut, SPS 24, Varian Associates Inc, USA). The cartridge was washed with 6mL chloroform: hexane (1:1, v/v) followed by 6mL chloroform: methanol (9:1, v/v), without allowing air to pass through the cartridge. The aflatoxins were eluted with 30mL acetone: water (97:3, v/v) [39], in small portions of 5mL, allowing air to pass through the cartridge between every aliquot of acetone: water (97:3, v/v). The eluate was evaporated until dryness in a water bath at 40°C (Yamato Water Incubator BT 25, Yamato, Japan) under nitrogen flow. Clean up and TLC analyses were carried out in subdued light.

2.4 – Thin-layer chromatography

The residues were re-dissolved in 150mL benzene: acetonitrile (98:2, v/v) homogenised and 5mL spotted on a commercially pre-coated silicagel 60 TLCglass plate, 20 x 20cm, 0.25mm thickness, (E. Merck,Germany) along with a working standard calibration curve containing 0.4 to 1.2ng of AFB₁, 0.1 to 0.4ng of AFB₂, 0.2 to 0.7ng of both AFG₁ and AFG₂. The plate was first eluted with anhydrous ethyl ether [32], dried up in a fume hood for 5 minutes, and developed with chloroform: acetone (9:1, v/v) [21,32] at same direction. The TLC plate was visually examined under ultraviolet light at 366nm (Chromato-Vue C-70G, Ultra-violet Products, USA). The aflatoxins levels in the samples were calculated by comparing the area of chromatographic peak of the samples with those of standard calibration curve by densitometric analysis (Shimadzu Densitometer, CS9301PC, Shimadzu Scientific Instruments, Japan) 200-300nm, mercury lamp l = 366nm, photo mode: fluorescence, beam size: 0.4 x 5.0mm, reference wave: 360nm, scan mode: linear, fluorescence sensitivity: high. Visual analyses were carried out by comparing the fluorescence intensity of sample spots with those of standard solution.

2.5 – Method characteristics

The in-house performance characteristics of the method in terms of accuracy, precision, linearity, limits of detection and quantification were determined by using spiked maize samples in 5 levels of contamination (Table 1), a naturally contaminated sample (n=5) and certified reference material (Series IV, FAPAS, MAFF, UK).
The linearity of standard calibration curve by densitometry was determined by the application of aflatoxin working standard solution on silicagel 60 TLC plates - 0.24 to 84ng of AFB\textsubscript{1}, 0.08 to 28ng of AFB\textsubscript{2}, 0.14 to 49ng of both AFG\textsubscript{1} and AFG\textsubscript{2}. The TLC plate was developed with anhydrous ethyl ether, dried up, and further developed with chloroform: acetone (9:1, v/v) at same direction (approximately 15-17cm).

The absolute amounts of aflatoxins detectable on a TLC plate were determined by spotting volumes from 0.5 to 5µL of the working standard solution on TLC plates. For visual analysis, the LD was considered the lower amount of each aflatoxin in the spiked sample that could be detectable on TLC plate. For densitometric analysis, the LD of the method was considered the lower amount of each aflatoxin in spiked samples that could be detectable on TLC plate with signal three times the baseline noise of blank sample extract. The limit of quantification for each aflatoxin (LQ) was considered the level of contamination with recoveries rates between 70% and 120%, with RSD lower than 30% [12].

2.6 – Additional tests

Other aspects were investigated during the study, concerned the improvement of separation, resolution and fluorescence intensity of aflatoxins on TLC plates by using different commercially available TLC plates and developing solvent systems. Commercially pre-coated silicagel 60TLCglass plate 20 x 20cm, 0.25mm thickness, (Merck, E. Merck,Germany) and pre-coated silicagel 60TLCglass plate 20 x 20cm, 0.25mm thickness, with pre-adsorbent layer and 19 channels (Whatman, Whatman INC., USA) were spotted with the same aflatoxin standard (0.1 and 0.2ng AFB\textsubscript{1}) solution and sample extracts; developed with 4 pairs of TLC usual solvent systems at same direction as follows: a) ethyl ether anhydrous [32] followed by ether: methanol: water (96:3:1, v/v, unsaturated tank) [5]; b)ethyl ether anhydrous [32] followed by chloroform: acetone, (9:1, v/v, saturated tank) [21,32]; c)ethyl ether anhydrous [32] followed by toluene: ethyl acetate: chloroform: formic acid (7:5:5:2, v/v, saturated tank) [21] and d) ether: methanol: water (96:3:1, v/v) [5] followed by chloroform: acetone (9:1, v/v, saturated tank) [21,32].

The elution profile patterns of aflatoxins on florisil cartridges with 30mL of acetone: water (97:3, v/v) [39] by collecting every 5mL portion [11].

### Table 1. Levels of aflatoxin contamination (mg/kg) in spiked maize samples

<table>
<thead>
<tr>
<th>Levels Studied</th>
<th>Aflatoxin contamination levels (µg/kg)</th>
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<tbody>
<tr>
<td></td>
<td>AFB\textsubscript{1}</td>
</tr>
<tr>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>2</td>
<td>1.0</td>
</tr>
<tr>
<td>3</td>
<td>2.4</td>
</tr>
<tr>
<td>4</td>
<td>24.1</td>
</tr>
<tr>
<td>5</td>
<td>242</td>
</tr>
</tbody>
</table>

The analysis of spiked maize samples gave an overall mean recoveries for AFB\textsubscript{1}, AFB\textsubscript{2}, AFG\textsubscript{1} and AFG\textsubscript{2}, for levels 2 to 5 (Table 1) of 90.6, 90.3, 90.5 and 90.5% for visual analysis and 94.2, 81.9, 93.5 and 97.3% for densitometric analysis, respectively. Individual recoveries values varying from 78.6% to 101% for all aflatoxins were achieved in the range above the quantification limit. The lowest mean recovery was obtained to the lowest level studied – Level 1. The over all mean of relative standard deviation (RSD) for spiked samples were 5.1% for AFB\textsubscript{1}, AFG\textsubscript{1} and AFG\textsubscript{2} and 5.3% for AFB\textsubscript{2}, by visual analysis and 16.2, 20.6, 12.8 and 16.9% for AFB\textsubscript{1}, AFB\textsubscript{2}, AFG\textsubscript{1} and AFG\textsubscript{2}, respectively, by densitometric analysis.

On Table 2 method performance characteristics of various methods can be observed. The recoveries of aflatoxins from spiked maize samples achieved in the present study, in the range above the limit of quantification, are similar to the results reported for aflatoxins in maize by TLC [25], HPLC [2,37,41] using different clean-up procedures such as florisil [2,25], multifunctional column [37,41], and silica [35]. Although similar recoveries were reported by THEAN et al [35] using silica sep-pak cartridge and HPLC determination, loss of aflatoxins has been reported when silica is used as solid phase due to weak interaction between silica surface and aflatoxins [25] and the effect of small amount of ethanol in the chloroform usually used during loading and washing steps [31]. Lower recoveries for CB and BF method in the analysis of maize samples were reported by KAMIMURA et al [25].

The RSD for visual analysis achieved in this study are comparable to those achieved by KAMIMURA et al [25] and ALI, HASHIM, YOSHIZAWA [2], THEAN et al [35], WILSON and ROMER [41], TRUCKSESS et al [38], and SOARES and RODRIGUEZ-AMAYA [32] using florisil, silica, mycosep column, immunoaffinity column and liquid-liquid partition respectively, as shown on Table 2. The RSD for densitometry were higher than those determined by ALI, HASHIM, YOSHIZAWA [2] and KAMIMURA et al [25] using ISOLUTE multimode column clean-up and HPLC, and florisil clean up with densitometry, respectively; and similar to those determined by BARSMAK and LARSSON [9] and KAMIMURA et al [25] using immunoaffinity column and HPLC, and liquid-liquid partition (CB and BF method), respectively (Table 2). The higher RSD achieved in this study compared to the ones reported by KAMIMURA et al [25] using florisil and densitometry may be due to the wider range of aflatoxin contamination studied in the present paper (Table 2).

Analysis of a naturally contaminated maize sample (n=5) by densitometry gave agood repeatability of 16.8% for AFB\textsubscript{1} (30.5 µg/kg) and 27.2% for AFB\textsubscript{2} (2.7µg/kg). The naturally contaminated sample used in this study

### 3 – RESULTS AND DISCUSSION

3.1 – Method characteristics

The analysis of spiked maize samples gave an overall mean recoveries for AFB\textsubscript{1}, AFB\textsubscript{2}, AFG\textsubscript{1} and AFG\textsubscript{2}, for levels 2 to 5 (Table 1) of 90.6, 90.3, 90.5 and 90.5% for visual analysis and 94.2, 81.9, 93.5 and 97.3% for densitometric analysis, respectively. Individual recoveries values varying from 78.6% to 101% for all aflatoxins were achieved in the range above the quantification limit. The lowest mean recovery was obtained to the lowest level studied – Level 1. The over all mean of relative standard deviation (RSD) for spiked samples were 5.1% for AFB\textsubscript{1}, AFG\textsubscript{1} and AFG\textsubscript{2} and 5.3% for AFB\textsubscript{2}, by visual analysis and 16.2, 20.6, 12.8 and 16.9% for AFB\textsubscript{1}, AFB\textsubscript{2}, AFG\textsubscript{1} and AFG\textsubscript{2}, respectively, by densitometric analysis.

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The RSD for visual analysis achieved in this study are comparable to those achieved by KAMIMURA et al [25] and ALI, HASHIM, YOSHIZAWA [2], THEAN et al [35], WILSON and ROMER [41], TRUCKSESS et al [38], and SOARES and RODRIGUEZ-AMAYA [32] using florisil, silica, mycosep column, immunoaffinity column and liquid-liquid partition respectively, as shown on Table 2. The RSD for densitometry were higher than those determined by ALI, HASHIM, YOSHIZAWA [2] and KAMIMURA et al [25] using ISOLUTE multimode column clean-up and HPLC, and florisil clean up with densitometry, respectively; and similar to those determined by BARSMAK and LARSSON [9] and KAMIMURA et al [25] using immunoaffinity column and HPLC, and liquid-liquid partition (CB and BF method), respectively (Table 2).
was not contaminated with AFG<sub>1</sub> and AFG<sub>2</sub>. Repeatability (RSD) of 21.0% for AFB<sub>1</sub>, 15.8% for AFB<sub>2</sub>, in naturally contaminated maize using multifunctional clean up with HPLC has been reported [37]. TRUCKSESS et al [38] reported an RSD of 19.7 and 21.4% for the determination of total aflatoxin in maize by immunoaffinity column clean up with fluorometry and HPLC respectively; and 24.8% for AFB<sub>1</sub>, with immunoaffinity column and HPLC.

The use of florisil cartridge as clean-up step gave an extract free of interference and suitable for one-dimensional TLC visual and densitometric analysis. Figure 1 shows typical chromatograms of spiked, naturally contaminated maize samples and aflatoxin working standard solution. The chromatogram shows a characteristic profile with no interferences in the retention factor (RI) of the aflatoxins (AFB<sub>1</sub>:0.7, AFB<sub>2</sub>:0.6, AFG<sub>1</sub>:0.5, AFG<sub>2</sub>:0.35) andgood separation indicating thegood specificity and selectivity of the method. Florisil has been reported as an effective solid phase in cleaning extracts for the aflatoxin analysis [24,27] although in some cases another clean-up step and two dimensional TLC analysis has been necessary such as the analysis of aflatoxins in citrus pulp and other feedstuffs [27].

Figures 2 and 3 show the linear regression equations for the recovery tests for aflatoxins in the range studied, by visual and densitometric analysis using florisil cartridge clean up. The equations show coefficient of correlation (R<sup>2</sup>) for the visual analysis higher than 0.99 for all aflatoxins indicating good linearity (Figure 2). Lower R<sup>2</sup> was obtained for the densitometric analysis with values varying from 0.96 to 0.98 (Figure 3).

![Figure 1. Chromatogram of working standard solution (a), naturally contaminated (b) and spiked maize sample (c) with AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub> and AFG<sub>2</sub> (peaks 4 to 1, respectively) obtained with silicagel 60 TLC glass plate, 20 x 20cm, 0.25mm thickness. Mobile phase: First development: ethyl ether anhydrous (unsaturated tank), second development: chloroform: acetone (9:1, v/v, saturated tank), at same direction.](image)

Good linearity (R<sup>2</sup> = 0.99) between emitted fluorescence and aflatoxin concentration was obtained for the standard calibration curves over a range of 0.24 to 84ng of AFB<sub>1</sub>, 0.08 to 28ng of AFB<sub>2</sub>, 0.14 to 49ng of both AFB<sub>1</sub> and AFG<sub>1</sub>, and of AFG<sub>2</sub>. AYRES and SÉNHEUSER [8] have reported linearity in a narrower range of 0.25 to 1.5ng of AFB<sub>1</sub>, while PONS andGoldblatt [29] have demonstrated linearity over a range of 0.2 to 10.5ng of AFB<sub>1</sub>, KAMIMURA [14] over a range of 0.5 to 20ng of AFB<sub>1</sub>, and of AFG<sub>1</sub>, and of AFG<sub>2</sub>, 0.5 to 10ng of AFB<sub>2</sub> and AFG<sub>2</sub>.

### TABLE 2. Methods performance characteristics for aflatoxin analysis in spiked maize samples.

<table>
<thead>
<tr>
<th>Reference method</th>
<th>Cleanup</th>
<th>Detection/Quantification</th>
<th>(% Mean recovery (B&lt;sub&gt;i&lt;/sub&gt;, B&lt;sub&gt;j&lt;/sub&gt;, G&lt;sub&gt;i&lt;/sub&gt;, G&lt;sub&gt;j&lt;/sub&gt;))</th>
<th>(% Mean relative standard deviation (B&lt;sub&gt;i&lt;/sub&gt;, B&lt;sub&gt;j&lt;/sub&gt;, G&lt;sub&gt;i&lt;/sub&gt;, G&lt;sub&gt;j&lt;/sub&gt;))</th>
<th>Range of contamination (µg/kg)</th>
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<tbody>
<tr>
<td>THIS METHOD</td>
<td>Florisil cartridge (1.0g)</td>
<td>TLC/Visual and densitometry</td>
<td>90.6; 90.3; 90.5; 90.5 (n=5) 5.1; 5.1; 5.1; 5.3</td>
<td>16.2; 20.6; 12.8; 16.9; 16.9</td>
<td>B&lt;sub&gt;i&lt;/sub&gt;: 0.5-242, B&lt;sub&gt;j&lt;/sub&gt;: 0.2-85</td>
</tr>
<tr>
<td>KAMIMURA et al. [25]</td>
<td>Florisil columns (0.7g)</td>
<td>TLC/Densitometry</td>
<td>92, 88, 99, 78 (n=5) 7.3; 4.6; 8.7; 7.6</td>
<td>14.9; 22.2; 13.1; 25.7 (RSD &lt;sub&gt;fr&lt;/sub&gt;)</td>
<td>G&lt;sub&gt;i&lt;/sub&gt;: 0.3-148, G&lt;sub&gt;j&lt;/sub&gt;: 0.3-140</td>
</tr>
<tr>
<td>THEAN et al. [35]</td>
<td>Silica sep-pak cartridges</td>
<td>TLC/Fluorescence</td>
<td>94.3; 88.3; 88.9; 90.5* (n=4) 2.2; 2.5; 4.2; 1.5*</td>
<td>5 to 125 AFB&lt;sub&gt;i&lt;/sub&gt; and G&lt;sub&gt;i&lt;/sub&gt;: 1.5 to 37.5 AFB&lt;sub&gt;j&lt;/sub&gt; and G&lt;sub&gt;j&lt;/sub&gt;</td>
<td></td>
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<tr>
<td>WILSON &amp; ROMER [41]</td>
<td>Mycosep multifunctional cleanup column</td>
<td>TLC/Fluorescence</td>
<td>106.5 and 95.2 (n=3) 5.7 and 3.6</td>
<td>4 (4:1:4:1 B&lt;sub&gt;i&lt;/sub&gt;:B&lt;sub&gt;j&lt;/sub&gt;:G&lt;sub&gt;i&lt;/sub&gt;:G&lt;sub&gt;j&lt;/sub&gt;)</td>
<td>20 (1:3:1 B&lt;sub&gt;i&lt;/sub&gt;:B&lt;sub&gt;j&lt;/sub&gt;:G&lt;sub&gt;i&lt;/sub&gt;:G&lt;sub&gt;j&lt;/sub&gt;)</td>
</tr>
<tr>
<td>ALI, HAMISH, YOSHIZAWA [2]</td>
<td>Silica column</td>
<td>TLC/Fluorescence</td>
<td>79; 93; 135; 100 (n=3) 12.7; 11.6; 5.9; 5*</td>
<td>4:4:6:3:3:6:5*</td>
<td>B&lt;sub&gt;i&lt;/sub&gt;: 20; B&lt;sub&gt;j&lt;/sub&gt;: 10, G&lt;sub&gt;i&lt;/sub&gt;: 20; G&lt;sub&gt;j&lt;/sub&gt;: 10</td>
</tr>
<tr>
<td>TRUCKSESS et al. [37]***</td>
<td>Multifunctional column</td>
<td>TLC/Fluorescence</td>
<td>98.8; 94.7; 105.0; 112.3* 3.7;10.2;9.6;17.5 (RSD&lt;sub&gt;f&lt;/sub&gt;) 17.3; 17.2; 27.5; 34.3 (RSD&lt;sub&gt;d&lt;/sub&gt;)</td>
<td>20 (5:1:3:1 B&lt;sub&gt;i&lt;/sub&gt;:B&lt;sub&gt;j&lt;/sub&gt;:G&lt;sub&gt;i&lt;/sub&gt;:G&lt;sub&gt;j&lt;/sub&gt;)</td>
<td>10, 20 and 30 (7:1:3:1 B&lt;sub&gt;i&lt;/sub&gt;:B&lt;sub&gt;j&lt;/sub&gt;:G&lt;sub&gt;i&lt;/sub&gt;:G&lt;sub&gt;j&lt;/sub&gt;)</td>
</tr>
<tr>
<td>TRUCKSESS et al. [38]***</td>
<td>Immunofinity column</td>
<td>TLC/Fluorescence</td>
<td>82.7; 61.0; 89.3; 42.3* 7.3 (RSD&lt;sub&gt;f&lt;/sub&gt;) and 22.4 (RSD&lt;sub&gt;d&lt;/sub&gt;)</td>
<td>113° (total aflatoxin) 16.6 (RSD&lt;sub&gt;f&lt;/sub&gt;) and 22.5 (RSD&lt;sub&gt;d&lt;/sub&gt;)</td>
<td>10, 20 and 30 (7:1:3:1 B&lt;sub&gt;i&lt;/sub&gt;:B&lt;sub&gt;j&lt;/sub&gt;:G&lt;sub&gt;i&lt;/sub&gt;:G&lt;sub&gt;j&lt;/sub&gt;)</td>
</tr>
<tr>
<td>SOARES &amp; RODRIGUEZ-AMAYA [32]**</td>
<td>Clarifying agent</td>
<td>TLC/Fluorescence</td>
<td>95.3° (mean AFB&lt;sub&gt;i&lt;/sub&gt;) 6.3° (mean AFB&lt;sub&gt;j&lt;/sub&gt;)</td>
<td>14.9:22.2:13.1:25.7 (RSD&lt;sub&gt;f&lt;/sub&gt;)</td>
<td>B&lt;sub&gt;i&lt;/sub&gt;: 4; B&lt;sub&gt;j&lt;/sub&gt;: 1.8; G&lt;sub&gt;i&lt;/sub&gt;: 3.4; G&lt;sub&gt;j&lt;/sub&gt;: 2.2</td>
</tr>
<tr>
<td>BARSMAK &amp; LARSSON [9]**</td>
<td>Immunofinity column</td>
<td>TLC/Fluorescence</td>
<td>72.3;70.0;76.5;91.8 (n=16) 14.9:22.2:13.1:25.7 (RSD&lt;sub&gt;f&lt;/sub&gt;)</td>
<td>33.9:50.0:33.1:57.9 (RSD&lt;sub&gt;d&lt;/sub&gt;)</td>
<td>B&lt;sub&gt;i&lt;/sub&gt;: 4; B&lt;sub&gt;j&lt;/sub&gt;: 1.8; G&lt;sub&gt;i&lt;/sub&gt;: 3.4; G&lt;sub&gt;j&lt;/sub&gt;: 2.2</td>
</tr>
<tr>
<td>AOAC – CB Method (KAMIMURA et al. [25])</td>
<td>Silica column</td>
<td>TLC/Fluorescence</td>
<td>74, 81, 87, 71 (n=5) 14.4; 6.9; 6.5; 11.4</td>
<td>12.2; 7.1; 19.1; 14.8</td>
<td>B&lt;sub&gt;i&lt;/sub&gt;: 20; B&lt;sub&gt;j&lt;/sub&gt;: 10, G&lt;sub&gt;i&lt;/sub&gt;: 30; G&lt;sub&gt;j&lt;/sub&gt;: 10</td>
</tr>
<tr>
<td>AOAC – BF Method (KAMIMURA et al. [25])</td>
<td>Liquid-Liquid partition</td>
<td>TLC/Fluorescence</td>
<td>78, 72, 31, 64 (n=5) 12.2; 7.1; 19.1; 14.8</td>
<td>12.2; 7.1; 19.1; 14.8</td>
<td>B&lt;sub&gt;i&lt;/sub&gt;: 20; B&lt;sub&gt;j&lt;/sub&gt;: 10, G&lt;sub&gt;i&lt;/sub&gt;: 30; G&lt;sub&gt;j&lt;/sub&gt;: 10</td>
</tr>
</tbody>
</table>

* data calculated from author’s paper data
** for yellow corn meal
*** interlaboratory study
**** collaborative study

Coefficients of correlation of 0.94 for AFB₁, 0.88 for AFB₂, 0.82 for AFG₁ and 0.20 for AFG₂, for naturally contaminated maize samples have been reported by OLIVEIRA, PRADO, JUNQUEIRA [26]. According to these authors, the lower correlation obtained for AFG₂ in that study was due to very low levels of contamination of this toxin in the analysed samples.

Good linearity between visual and densitometric analysis of 0.99 for AFB₁, AFB₂, AFG₁, and 0.98 for AFG₂ for spiked maize samples were achieved in the present study with an overall $R^2$ of 0.98 (Figure 4). Lower coefficients of correlation of 0.94 for AFB₁, 0.88 for AFB₂, 0.82 for AFG₁ and 0.20 for AFG₂, for naturally contaminated maize samples have been reported by OLIVEIRA, PRADO, JUNQUEIRA [26]. According to these authors, the lower correlation obtained for AFG₂ in that study was due to very low levels of contamination of this toxin in the analysed samples.

Low LD and LQ for the aflatoxins on TLC plates by densitometric analysis were achieved in this study as can be observed on Table 3. The low limits LD and LQ determined by the authors are due to a combination of the efficient clean-up procedure and good separation and resolution of the aflatoxins on TLC plates. Higher limit of detection of 1mg/kg for AFB, and 5mg/kg for AFG, in maize by using a florisisol open column clean up coupled with HPLC has been reported by ALI, HASHIM, YOSHIZAWA [2]. TRUCKSESS et al [37] also reported higher limits of detection for AFB₁ (3mg/kg), AFB₂ (11mg/kg), AFG₁ (2mg/kg) and AFG₂ (1mg/kg) in maize by using multifunctional column clean-up with HPLC in an international collaborative study. SOARES and RODRIGUEZ-AMAYA [32] have determined higher limit of detection of 2mg/kg for AFB, and a limit of quantification of 4mg/kg by using clarifying agents and liquid-liquid partition with visual analysis.

This method has been submitted to a proficiency testing scheme (FAPAS, MAFF, UK) for two years giving satisfactory z-score results of $2 < z$-score < 2.

**TABLE 3. Absolute amount of aflatoxins detectable on TLC plate on silicagel 60 TLC plates, LD and LQ of the method for AFB₁, AFB₂, AFG₁, AFG₂ and total aflatoxin by densitometric analysis**

<table>
<thead>
<tr>
<th>Aflatoxin</th>
<th>Absolute amount of aflatoxins detectable on TLC plate (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LD (µg/kg)</td>
</tr>
<tr>
<td>AFB₁</td>
<td>0.10</td>
</tr>
<tr>
<td>AFB₂</td>
<td>0.04</td>
</tr>
<tr>
<td>AFG₁</td>
<td>0.04</td>
</tr>
<tr>
<td>AFG₂</td>
<td>0.24</td>
</tr>
<tr>
<td>Total</td>
<td>0.4</td>
</tr>
</tbody>
</table>

The method has shown to be accurate and precise for determining aflatoxins in maize. The limit of detection and quantification of the method comply with the actual regulatory limits for aflatoxin in maize and maize products for human and animal consumption in Brazil [16,17,18]. The performance characteristics in terms of recoveries and repeatability determined in the present study are consistent with the criteria of analytical methods for AFB, and total aflatoxins established by the European Committee for Standardisation [12] and the European Union Directive 98/53 [15]. This method has been in routine for two years and has been adopted as Official Method of Analysis of the Ministry of Agriculture and Supply, Brazil [19].

### 3.2 Additional tests

It was noticed that the development of TLC plates with ethyl ether anhydrous followed by a second development using a solvent system containing ethyl ether increased the $R_f$ values and it was not efficient to remove the usual fluorescence interference present in maize extracts that is observable near AFB₂ $R_f$. The elution of aflatoxins on TLC plates with ethyl ether anhydrous followed by the use of a solvent system containing formic acid quenched the AFB₁ fluorescence and cause decreasing of AFB₁ and AFG₁, and increasing of AFB₂ and AFG₂ fluorescence intensity as also reported by JAIN and HATCH [23], apart from not being efficient to separate the interference spot near AFG₂ $R_f$, making the visual...
and densitometric analysis of difficult resolution. The best separation of aflatoxins on TLC plates for standard and maize sample extracts was obtained by developing the plate with ethyl ether anhydrous followed by a second development with chloroform: acetone (9:1, v/v) saturated tank) giving an easily recognised profile with no fluorescence interference and streaking in the aflatoxin RfS (AFB₁:0.7, AFB₂:0.6, AFG₁:0.5, AFG₂:0.4) making the one dimensional TLC with densitometric analysis possible (see Figure 1). The elution of the toxins on TLC plates with ether: methanol: water (96:3:1, v/v) followed by chloroform: acetone (9:1, v/v, saturated tank) at same direction, did not give good separation and resolution of the aflatoxins by eluting them to solvent front line.

It was observed that silicagel plates with channels and pre-adsorbent layer were more efficient to separate and resolve the aflatoxins from sample extracts interference, when compared to normal TLC plates, may be due to efficiency of the pre-adsorbent layer in retaining the sample interferences. However, the limit of detection for the aflatoxins on these TLC plates were twice that of normal TLC plates shown in Table 3, and this fact was probably due to spread of the aflatoxin spot on TLC plates because of the type of glass coating.

The elution profile pattern of aflatoxins on 1g florisoril cartridges is shown in Figure 5.

**FIGURE 5.** Elution profile pattern of total aflatoxins with acetone: water (97:3, v/v) on 1g florisoril cartridge.

Approximately 94% of all aflatoxins were recovered from florisoril cartridge (1g) within the first 18mL of acetone: water (97:3, v/v) [11] whereas only 6% were eluted within the last 2mL of acetone: water (97:3, v/v). It was noticed the presence of a trailing in the elution profile pattern of the aflatoxins on florisoril cartridge as also reported by van RHIJN, VIVEEN and TUINSTRA [39] for different elution solvent system: acetone: water (98:2, v/v), acetone: water (97:3, v/v), and acetone: water (95:5, v/v). KAMIMURA et al [25] reported the elution of aflatoxins from florisoril cartridges with 30mL of acetone: water (99:1, v/v) while 100% recovery of AFB₁ was obtained with approximately 30mL of acetone: water (90:10, v/v) by PAULSCH, SIZOO, van EGMOND [27]. ALI, HASHIM, YOSHIZAWA [2] have reported the need of 60mL acetone: water (99:1, v/v) for the complete elution of aflatoxins from 0.7g florisoril columns.

However, van RHIJN, VIVEEN and TUINSTRA [39] reported lost of 50% of AFB₁ in the automation of the analysis of aflatoxins employing 0.1g florisoril cartridge as clean-up step and acetone: water (95:5, v/v) as elution solvent. According to PAULSCH, SIZOO, van EGMOND [27] an addition of 1% water to acetone increase the recovery of aflatoxins; and 10% of water decreases the volume necessary for the elution of the aflatoxins. However, the increase of 20% water decrease the aflatoxin recovery from florisoril probably due to decomposition of the aflatoxins caused by the alkali formed by reaction of water on florisoril. One can assume that florisoril cartridge should be kept in seal tight dissecator with low humidity content until analysis.

Although recoveries of aflatoxins from florisoril cartridges were higher than 90% within the first 18mL of acetone: water (97:3, v/v) in this study, it was decided the use of 30mL acetone: water (97:3, v/v) as a suitable volume for a proper elution of the aflatoxins, because the presence of trailing in the elution profile of standard (Figure 5) and of spiked and naturally contaminated maize samples on 1g florisoril cartridge [11].

In order to automate the clean-up step, recovery tests have been carried out. For this, different cartridges containing different amounts of florisoril (100mg, 500mg) and volumes of loading, washing and elution solvents have been evaluated.

Studies have been conduct aiming at replacing the chloroform as extraction solvent - although the high efficiency of chloroform: water (250:15, v/v) in extracting aflatoxins compared with methanol: water (55:45, v/v) and acetone: water (85:15, v/v) - due to worldwide concern of the use of hazardous chlorinated solvents [4,34]. The use of aqueous methanol, acetone and acetonitrile - reported as suitable solvents for aflatoxin extraction for a wide range of products [4,34] – has been evaluated. The limiting factor is the presence of water in the extract that should be completely removed before loading the florisoril cartridges and columns, thus avoiding lost of aflatoxins in this step.

The method has been in-house validated for the analysis of other matrices such as grains, citrus pulp and feed containing citrus pulp.

## 4 – CONCLUSIONS

- Florisoril has been shown to be an effective solid-phase for the clean-up of the aflatoxins in maize giving a final extract suitable for one-dimensional TLC analysis;
- The method developed was accurate, specific and reliable in determining the aflatoxins at levels as low as 1.0µg/kg of AFB₁, 0.3 µg/kg of AFB₂, 0.6µg/kg of both AFG₁ and AFG₂ in maize;
- The method established can comply with the strictest regulatory guidelines such as the European Union Regulation, and with the regulatory limits laid down in Brazil;
- The method established by the present study has been adopted as Official Method of Analysis of the Ministry of Agriculture, Brazil.
REFERENCES


6 – ACKNOWLEDGMENTS
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