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Short communication

Comparison of three different C_{18} HPLC columns with different particle sizes for the optimization of aflatoxins analysis

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ABSTRACT

In this work we compared the performance of chromatography columns with particles of 5 and 3 μm with the new 2.7 μm solid core particles for the analysis of aflatoxins B1, G1, B2, and G2 using trifluoroacetic acid pre-column derivatization. Three different columns have been used and chromatographic parameters as retention time, resolution, limit of detection (LOD), limit of quantification (LOQ) were obtained from all of them and compared. The results show that comparing with the traditional columns, shorter columns (100 mm × 4.6 mm) with the new solid core particles are suitable for the analysis of these mycotoxins and allowed the reduction of the analysis time by 45.5% and 33.3% with respect to columns with particle size 5 μm (150 mm × 4.6 mm) and 3 μm (150 mm × 4.6 mm) respectively, without any detrimental effect on performance. This leads to the reduction of the analysis costs by saving on organic solvents and increasing the total number of analyses per day. The capability of these columns for analyzing samples, in different culture media, was assessed by analyzing different samples from: yeasts extract sucrose medium, corn meal agar medium and fresh hazelnut media.

1. Introduction

Aflatoxins B1 (AFB1), G1 (AFG1), B2 (AFB2), and G2 (AFG2), are secondary metabolites produced by members of the *Aspergillus* section *flavi* such as *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nomius*. There is worldwide concern of the contamination of a wide range of agricultural foodstuffs for both human and animal consumption with aflatoxins because they are potent carcinogens. High carbohydrate and fatty acid containing foodstuffs including maize, oilseeds, tree nuts and nut products, peanuts, dried figs and raisins, cottonseed, milk, and dried spices are particularly at risk and often can be contaminated [1–8].

Aflatoxin B1 is one of the most potent human carcinogens known [9], and because it contaminates such a wide range of food and feed-based commodities there is strict regulation in the EU, which sets limits of 2 μg/kg in foods for direct human consumption [10].

Current research is integrating traditional ecological and physiological experiments with molecular biology data as new systems biology approaches to understand the contamination of foodstuffs with mycotoxins. These experiments require high numbers of replicates and usually involve a wide range of environmental conditions increasing the relative numbers of samples for analysis [11–13]. Thus, a cheap, rapid and accurate analysis of mycotoxins from culture media (including synthetic media and media containing natural cereal/nut extracts) is required.

An agar plug method was introduced by Frisvad and Filtenborg in 1983 [14] and currently is one of the commonest and quickest methods for the characterisation of toxin/no toxin production. This has been used in numerous studies to identify the relative ability of strains to produce specific mycotoxins and the amounts produced [15–17].

The commonest technique used for the separation of the aflatoxins has been the use of HPLC [18]. Although separation can be achieved with both normal and reversed phase columns, reversed-phase systems are more commonly used. The optimum mobile phase was found to be a ternary mixture, composed of water, methanol and acetonitrile, tailored to the individual characteristics of the HPLC column [19].

Among the different detection technologies, fluorescence detection is most often used in the analysis of aflatoxin analogues [20]. Although the aflatoxins exhibit natural fluorescence, various analogues have shown solvent-dependent quenching. Using aqueous mixtures for reversed-phase chromatography, the fluorescence of AFB1 and AFG1 appears to be significantly quenched [21]. The solution to this problem is achieved by derivatization of these two analogues at the reactive 8,9-double bond of the dihydrofuran moiety.

Pre-column derivatization using trifluoroacetic acid (TFA), which causes hydration of the 8,9-bond, was used to produce the

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hemiacetals AFB2a and AFG2a. These two compounds have similar fluorescence properties to AFB2 and AFG2 [19,20].

In recent years significant improvements have been introduced in the analysis of aflatoxins especially in column chromatography technology [19,22]. The use of packing materials with particle sizes <2 μm and the use of chromatographs that can support very high pressures led to a dramatic reduction of analysis times. Although promising results have been obtained with these modifications the technology is not often available in many laboratories due to the higher cost implications.

To overcome this problem, recently, many manufacturers are producing packing materials with solid core particles, coated with an outer layer of porous material. Based on these characteristics, separations can be achieved much faster and more efficiently than with standard columns but without the need for expensive high-pressure instrumentation, as they are able to work at standard pressures (up to 600 bar) and can be used on all HPLC systems. Furthermore, increase in separation speed and efficiency could result in better sensitivity and lower limits of detection.

The aim of this study was to assess the performance of 3 different particle sizes, including the use of new solid core particles, for the analysis of AFB1, AFB2, AFG1 and AFG2. The limit of detection (LOD), the limit of quantification (LOQ), resolution between the peaks and other chromatographic parameters (number of theoretical plates (N), height equivalent to a theoretical plate (HEPT) and reduced plate height (h)) were calculated. The different columns were assessed for the analysis of the target analytes in different matrices including yeast extract sucrose, corn meal agar media and those prepared from natural cereal compounds.

2. Materials and methods

2.1. Reagents and standards

Acetonitrile, methanol and hexane were purchased from Fisher Scientific (Fisher Scientific UK Ltd., UK). Trifluoroacetic acid was purchased from Sigma (Sigma–Aldrich, UK). Aflatoxins standard mix containing AFB1 and AFG1 1.0 μg/mL and, AFB2 and AFG2 0.3 μg/mL in methanol was purchased from Supelco (Sigma–Aldrich, UK).

All solvents were HPLC grade. Pure water was obtained from a Milli-*R*/Q water system (Millipore, Billerica, MA, USA) and used when water was required.

2.2. Standards preparation

Different amounts of the aflatoxins stock standard were placed in 2 mL volume safe-lock Eppendorf tubes and were let to dry under a gentle stream of N_2. After drying, samples were derivatized using trifluoroacetic acid (TFA) as described by the AOAC [27].

With all the columns, linearity was checked for all toxins between 0.0263 and 2.625 ng injection^{-1} for AFB1 and AFG1, and from 0.0079 to 0.79 ng injection^{-1} for AFB2 and AFG2 using the previously prepared standards.

2.3. Chromatographic equipment and aflatoxins analysis

The HPLC system used for AF analysis was an Agilent 1200 series system (Agilent, Berks., UK) with a fluorescence detector (FLD, G1321A, Agilent), an auto sampler (ALS, G1329, Agilent), autosampler thermostat (G1330B, Agilent), Thermostatted Column Compartment (G1316A, Agilent), on-line degasser (G1379B, Agilent), and binary pump (G1312A, Agilent). Analysis was performed in the isocratic mode and the mobile phase was ethanol/water/acetonitrile (30:60:10, v/v/v) using a flow rate of 1 mL min^{-1}. FLD detection was performed using 360 nm and 440 nm excitation and emission wavelengths respectively.

Three different columns were used:

(1) Phenomenex Luna C_{18}, (5 μm, 150 mm × 4.6 mm) column joined to a pre-column (security guard, 4 mm × 3 mm cartridge, Phenomenex Luna).
(2) Phenomenex Gemini C_{18}, (3 μm, 150 mm × 4.6 mm) column joined to a pre-column (security guard, 4 mm × 3 mm cartridge, Phenomenex Gemini).
(3) Agilent Poroshell 120, C_{18}, (2.7 μm, 100 mm × 4.6 mm). An in line universal (Agilent, Berks., UK) solvent filter was fitted before the column in order to protect the column.

Signals were processed by Agilent ChemStation software Ver. B Rev: 03.01 [317] (Agilent Technologies, Palo Alto, CA, USA).

2.4. Chromatographic parameters calculation

The limit of detection (LOD) and the limit of quantification (LOQ) were calculated using Eqs. (1) and (2) respectively [23]:

$$\mathrm{LOD} = \bar{X}_n + 3 \cdot S_{n-1} \quad (1)$$

$$\mathrm{LOQ} = \bar{X}_n + 10 \cdot S_{n-1} \quad (2)$$

where $\bar{X}$ is the mean concentration and S_n is the standard deviation.

The chromatographic resolution (R_s) between the peaks was calculated using Eq. (3):

$$R_s = \frac{t_{R_2} - t_{R_1}}{0.5(t_{w_1} + t_{w_2})} \quad (3)$$

where t_R is the retention time and t_w are the tangents' width of the peak at the base line.

The number of theoretical plates (N) was calculated using Eq. (4):

$$N = 16\left(\frac{V_e}{W_b}\right)^2 \quad (4)$$

where V_e is the elution volume and W_b is the width of the peak at the base line.

The Van-Deemter equation [24] describes the height equivalent to a theoretical plate (HEPT) and was calculated in order to compare between different columns using Eq. (5):

$$\mathrm{HEPT} = \frac{L}{N} \quad (5)$$

where L is the length of the column and N is the number of theoretical plates.

And finally, the reduced plate height (h), that is a dimensionless parameter that allows the direct comparison of the efficiency of two or more columns packed with different particle size packing materials, was calculated using Eq. (6) [25]:

$$h = \frac{\mathrm{HEPT}}{d_p} \quad (6)$$

where d_p is the mean particle size (μm).

2.5. Media preparation

Yeast extract sucrose (YES) (20 g/L yeast extract, 150 g/L sucrose, 15 g/L agar) and corn meal agar media (CMA) (2 g/L corn meal extract (from 50 g whole maize), 15 g/L agar) were prepared.

For preparing a hazelnut medium, milled hazelnuts were prepared by homogenisation for 5 min in a Waring laboratory science homogeniser model 7009G (Waring Laboratory Science, CT, USA). Mixtures of 2% (w/v) hazelnut flour in water were prepared and 1.5% (w/v) agar added.

Table 1
Comparison of AFB1, AFB2, AFG1 and AFG2 the retention times (t_R) obtained with columns with different particle sizes. Average values from 3 different injections with different concentrations.

Column particle size	5[a] μm $t_R \pm SD$	3[b] μm $t_R \pm SD$	2.7[c] μm $t_R \pm SD$
Aflatoxin derivative			
AFG1	4.412 ± 0.012	4.114 ± 0.093	2.095 ± 0.005
AFB1	6.158 ± 0.020	5.538 ± 0.109	2.789 ± 0.004
AFG2	8.243 ± 0.030	7.024 ± 0.103	3.610 ± 0.005
AFB2	12.367 ± 0.053	10.173 ± 0.123	5.280 ± 0.004

[a] Phenomenex Luna C_{18}, 5 μm, 150 mm × 4.6 mm column.
[b] Phenomenex Gemini C_{18}, 3 μm, 150 mm × 4.6 mm column.
[c] Agilent Poroshell 120, C_{18}, 2.7 μm, 100 mm × 4.6 mm column.

The culture media were prepared by autoclaving for 20 min at 121 °C. The medium was vigorously shaken and poured into 9 cm diameter sterile Petri dishes.

2.6. Sample preparation, aflatoxins extraction and derivatization

Ten days old, centrally inoculated cultures of *A. flavus* (NRRL3357) and *A. parasiticus* (kindly provided by Sejakhosi Mohale) were used. From 4 to 6 discs of fungal culture, weighing approx. 0.75 g, were removed from these cultures using a cork borer. They were placed in previously weighed 2 mL volume safe-lock Eppendorf tubes [26]. A total of 3 replicates per treatment were collected, weighed, and immediately frozen at −20 °C and stored.

For aflatoxin extraction 800 μl chloroform was added to each Eppendorf and shaken well for 1 h. The chloroform extract was transferred to a new vial and dried gently under air. Afterwards samples were derivatized using TFA as described by the AOAC [27].

3. Results and discussion

3.1. Retention times

The different retention times obtained, for different concentrations of aflatoxins and with the different columns, were studied. Examples of the chromatograms obtained by the injection of aflatoxins with the different columns can be seen in Fig. 1. Retention times for all four toxin derivatives and for the three columns studied are shown in Table 1. Comparison between retention times obtained from different particle sizes showed that reduction of particle size from 5 (Phenomenex Luna) to 3 μm (Phenomenex Gemini) led to a slight reduction of the retention times. The average reduction was 12.3% (AFG1 6.76%, 10.07% AFB1, 14.78% AFG2, and 17.74% AFB2). This allowed the reduction of total analysis time by around 4 min changing the total time from 22 to 18 min (including column cleaning).

The best results were obtained when aflatoxin derivatives were analysed using the shorter solid core 2.7 μm particle column (Agilent Poroshell 120). The average reduction of retention time as compared with the 3 μm Phenomenex Gemini was 48.9% (AFG1 49.08%, AFB1 49.64%, AFG2 48.61%, AFB2 48.09%). This reduction in retention times allowed an even higher decrease in the total analysis time per sample from 18 to 11 min (including column cleaning).

3.2. Linearity and limits of detection and quantification

Calibration lines were built with data obtained from all the columns and the data points fitted to a straight line using Microsoft® Excel® for Mac 2011. For all columns and all aflatoxin derivatives R^2 values were >0.999, showing an excellent linear response of the FLD detector in the range of the concentrations tested.

Table 2
Comparison of the LOD and LOQ (ng injection^{-1}) obtained with the columns assayed.

	LOD			LOQ		
	5[a] μm	3[b] μm	2.7[c] μm	5 μm	3 μm	2.7 μm
Aflatoxin derivative						
AFG1	0.042	0.040	0.038	0.131	0.124	0.116
AFB1	0.015	0.016	0.012	0.045	0.048	0.035
AFG2	0.023	0.025	0.021	0.072	0.076	0.067
AFB2	0.012	0.013	0.011	0.032	0.038	0.031

[a] Phenomenex Luna C_{18}, 5 μm, 150 mm × 4.6 mm column.
[b] Phenomenex Gemini C_{18}, 3 μm, 150 mm × 4.6 mm column.
[c] Agilent Poroshell 120, C_{18}, 2.7 μm, 100 mm × 4.6 mm column.

LOD and LOQ (ng injection^{-1}) were calculated according to Long and Winefordner [23] and the results are shown in Table 2. Even though there was a slight improvement in both LOD and LOQ with the decrease of particle size, there were no significant differences between them.

3.3. Columns performance

The chromatographic parameters calculated after the injection of various aflatoxin standard concentrations with columns containing different particle sizes are shown in Table 3.

3.3.1. Columns resolution

Regarding the peak resolution, the best results for all the toxins were obtained with the column containing particles of 3 μm and 150 mm of length (Phenomenex Gemini). Resolution values ≥1.5 are considered appropriate for a good analysis, demonstrating that the peaks will be effectively separated. Table 3 shows that all values obtained between the different aflatoxins are >6.87 showing that with all the stationary phases used in this experiment the peaks were effectively resolved.

3.3.2. Number of theoretical plates and height equivalent to a theoretical plate

Generally, an increase in the number of theoretical plates can be observed with the reduction of the particle size. The effect is less evident with AFG1 due to the short retention time (see Table 3). Special attention should be paid to the results obtained with the column containing 2.7 μm solid core particles (Agilent Poroshell 120) as the length of this column was 33% shorter than the other columns. That means that, although the length of the column is shorter, this material is able to provide even higher number of theoretical plates with less packed material.

This results were confirmed when we compared the HEPT between the different columns, with the Poroshell 120 column having a smaller HEPT for all the aflatoxin derivatives.

3.3.3. Comparison of the reduced plate heights

Giddings [25] introduced the reduced plate height dimensionless parameter that allows the direct comparison of the efficiency of two or more columns packed with different particle size packing materials. According to the theory, a well-packed column should have a reduced plate height (h) in the range of 2–3. It also depends on other factors, such as the velocity of the mobile phase through the porous matrix. The results obtained from the analysis of the standard solutions show that further development of the method would be possible by increasing the flow rate. With this approach h would have been optimized. According to Van-Deemter's curve for 3 μm and 2.7 μm particles in 4.6 mm column, the optimum flow rate range should be between 1.5 and 1.7 mL min^{-1}. However, it should be noted that we propose the analysis of four compounds in a single run, with the first compound having a short retention

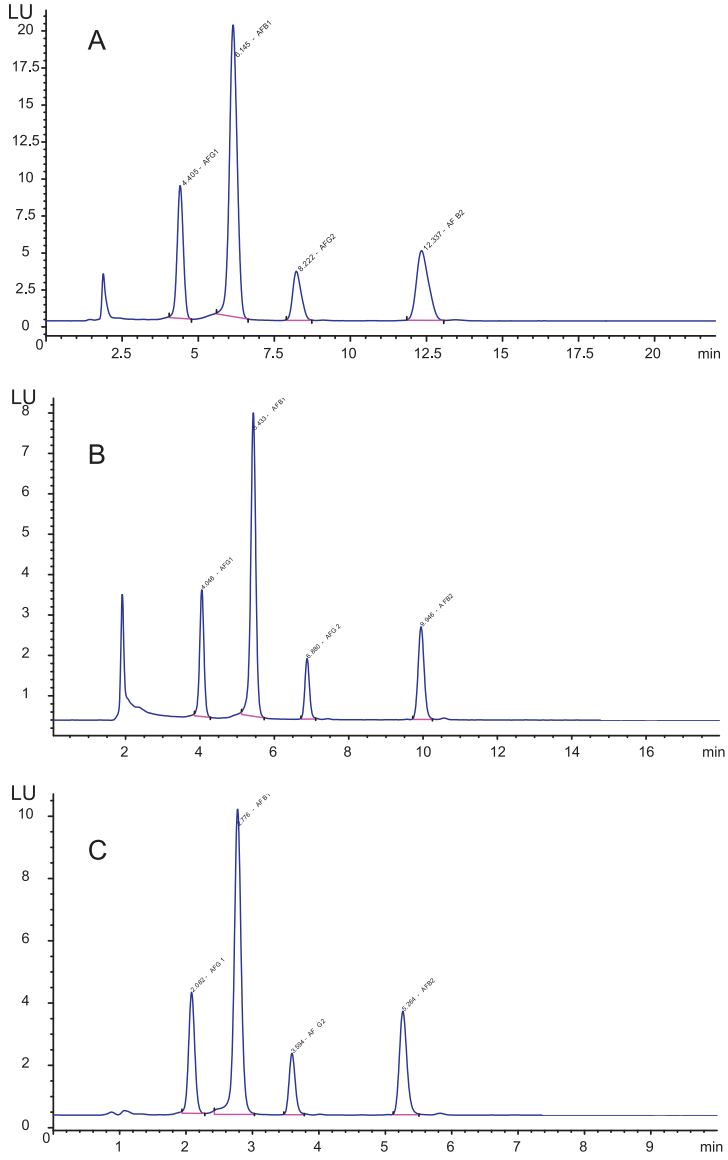

Fig. 1. HPLC-FLD chromatograms obtained after the analysis of a standard containing AFG1, AFB1, AFG2, AFB2. Wavelengths: λ_{ex} 360 nm and λ_{em} 440 nm. (A) Phenomenex Luna C_{18}, 5 μm, 150 mm × 4.6 mm column; (B) Phenomenex Gemini C_{18}, 3 μm, 150 mm × 4.6 mm column; (C) Agilent Poroshell 120, C_{18}, 2.7 μm, 100 mm × 4.6 mm column.

Table 3
Average and standard deviation of the most important chromatographic parameters obtained for the different particle size columns. Values obtained from 3 different injections at low, medium and high concentration.

Particle size (μm)	Toxin	Peak resolution ± SD	N ± SD	HEPT ± SD	h ± SD
5[a]	AFG1		16,847.88 ± 797.56	8.92 ± 0.43	2.55 ± 0.12
	AFB1	6.87 ± 0.11	21,218.47 ± 702.02	7.08 ± 0.23	2.02 ± 0.07
	AFG2	7.12 ± 0.13	32,889.42 ± 1910.72	4.58 ± 0.28	1.31 ± 0.08
	AFB2	11.28 ± 0.34	37,095.00 ± 2470.63	4.06 ± 0.28	1.16 ± 0.08
3[b]	AFG1		16,356.46 ± 63.97	9.17 ± 0.04	3.06 ± 0.01
	AFB1	10.32 ± 0.19	22,672.45 ± 362.43	6.62 ± 0.10	2.21 ± 0.03
	AFG2	10.44 ± 0.08	41,817.11 ± 900.96	3.59 ± 0.08	1.20 ± 0.03
	AFB2	19.90 ± 0.18	51,716.24 ± 1151.90	2.90 ± 0.06	0.97 ± 0.02
2.7[c]	AFG1		16,156.93 ± 724.43	6.20 ± 0.28	2.30 ± 0.10
	AFB1	5.99 ± 0.15	23,408.38 ± 1007.28	4.28 ± 0.18	1.59 ± 0.07
	AFG2	7.25 ± 0.03	52,526.55 ± 2959.90	1.91 ± 0.11	0.71 ± 0.04
	AFB2	14.55 ± 0.08	80,058.88 ± 1653.77	1.25 ± 0.03	0.46 ± 0.01

N: number of theoretical plates; HEPT: height equivalent to a theoretical plate (μm); h: reduced plate height.
[a] Phenomenex Luna C_{18}, 5 μm, 150 mm × 4.6 mm column.
[b] Phenomenex Gemini C_{18}, 3 μm, 150 mm × 4.6 mm column.
[c] Agilent Poroshell 120, C_{18}, 2.7 μm, 100 mm × 4.6 mm column.

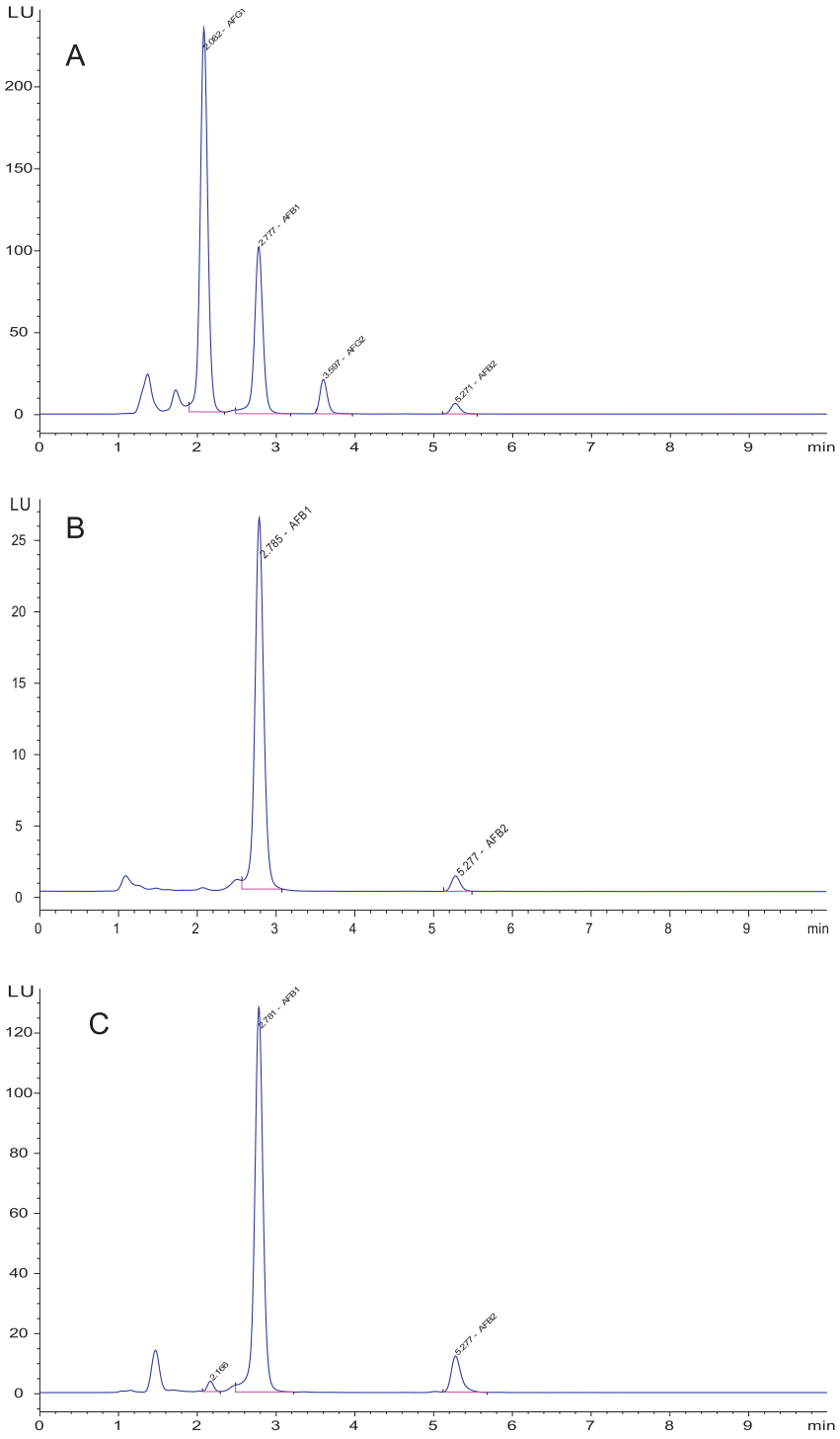

Fig. 2. HPLC-FLD chromatograms obtained after the analysis of different culture media contaminated with different aflatoxin-producing fungi using an Agilent Poroshell 120, C_{18}, 2.7 μm, 100 mm × 4.6 mm column. Wavelengths: λ_{ex} 360 nm and λ_{em} 440 nm. (A) Corn meal agar inoculated with *Aspergillus parasiticus*; (B) YES inoculated with *Aspergillus flavus*; and (C) fresh hazelnut medium inoculated with *A. flavus*.

time and $h = 3.06$ and $h = 2.3$ for the columns with 3 μm and 2.7 μm particles respectively. Thus, bearing in mind that the method is meant to analyse compounds in complex matrices, a compromise between the flow rate, that will shorten the chromatogram but make the peaks closer, and the potential appearance of interfering peaks was taken into account and the flow rate fixed at 1 mL min^{-1}. Despite the velocity of the mobile phase could have been higher and the h values obtained were not optima, the 2.7 μm particles column presented higher performance for the separation of these compounds.

3.4. Analysis of *A. flavus* and *A. parasiticus* cultures in different media

Due to its excellent performance in the analysis of aflatoxins standards the Agilent Poroshell 120, containing the new solid core particles, was selected for analyzing samples in different culture media. These were YES, CMA and fresh hazelnut media, previously contaminated with aflatoxin-producing strains of *A. flavus* (AFB1 and AFB2 producer) and *A. parasiticus* (AFB1, AFB2, AFG1 and AFG2 producer).

The chromatograms for all the samples showed very well resolved peaks for all the different media and the different fungal species, and no ghost peaks were observed. These results proved the suitability of this column to be used routinely in the laboratories to analyse aflatoxin samples from different matrixes.

Some example chromatograms from different media and different fungi are shown in Fig. 2. This shows the production of AFB1 and AFB2 in YES and fresh hazelnuts medium by *A. flavus*. It also shows that this column allowed the analysis of all four aflatoxin derivatives on CMA contaminated with *A. parasiticus*.

4. Conclusions

The use of the new 2.7 μm solid core particle columns of 100 mm length, currently produced by various manufacturers, are suitable for the analysis of AFB1, AFB2, AFG1 and AFG2 using TFA as a derivatizing agent from different matrices. These new columns allow the reduction of the analysis time by 45.5% and 33.3% with respect to columns with particle size 5 μm and 3 μm respectively (see Table 1), without any detrimental effect on performance. This leads to the reduction of the analysis costs by using less organic solvents and increasing the total number of analyses in 24 h from 57.6 and 80 for columns with particle size 5 μm and 3 μm respectively, to up to 110.77 analyses per day.

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