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Efficacy of Different C_{18} HPLC Analytical Columns in the Analysis of Fumonisins B_1 and B_2 in Different Matrices

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Abstract: Fumonisins B_1 and B_2 are carcinogenic and commonly contaminate corn and corn-based products. Analysis of such toxins using C_{18} HPLC column is officially accredited but still unknown if all column types can effectively separate FB_1 and FB_2 or not. The present study evaluated the efficiency of 5 analytical columns with different dimensions, particle sizes, and porosities to determine these toxins in both agar cultures of *Fusarium verticillioides* and cornflakes. Interestingly, the traditional column 150mm of length with 5µm porous particles had close retention times to those of the short-fused core column 75mm of length with 2.7 µm reflecting in time and solvents saving. Using Sep-Pack C_{18} for clean-up played an important role in enhancement the limit of quantification (LOQ) for cornflake samples (5-13.7 and 16.1-39 µg kg^{-1} for FB_1 and FB_2, respectively). However, it was relatively higher for fungal culture samples that were not passed through the cleaning-up step (11.5-16 and 28.1-46.3 µg kg^{-1} for FB_1 and FB_2, respectively). Overall, the lowest LOQ was obtained using the shorter fused core column. Finally, using such clean-up in the extraction of FB_1 and FB_2 from spiked cornflakes samples gave good recoveries (>80%) using all tested columns.

Keywords: fumonisin; particle size; column length; solid core; cornflakes.

1. Introduction

Fumonisins are mycotoxins produced mainly by *Fusarium verticillioides* (formerly: *F. moniliforme*) and *Fusarium proliferatum* and are considered a global serious problem in corn crops [1-5].

Fumonisin B1 (FB_1) is 2-amino-12, 16-dimethyl-3,5,10,14,15-pentahydroxyeicosane esterified at C-14 and C-15 to propane-tricarboxylic acid, and fumonisin B2 (FB_2) is 10-deoxy-FB1 (Figure 1, [1]). FB_1 has been classified as a group 2B carcinogen (possibly carcinogenic in humans) [6, 7]. It is found to cause equine leukoencephalomalacia and porcine pulmonary edema and a non-genotoxic kidney or liver cancer in rats and mice [8-11]. Several studies noticed a relation between FB_1 and human esophageal cancer [12, 13].

Fumonisins are mainly found as natural contaminants of corn and corn-based foods [1, 14-16]. However, there is evidence that they can occur in other crops and derived foods: sorghum [17] and sorghum syrup [18]; white beans, adzuki beans, and mung beans [19]; wheat, barley, and soybean [20, 21]; black tea and medicinal plants [22, 23]; rice [24].

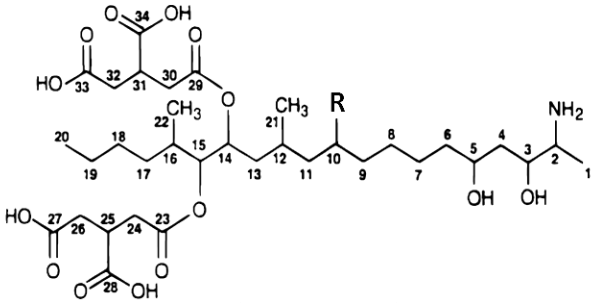

Figure 1. Structures of FB_1 (R=OH) and FB2 (R=H).

Finding the appropriate method for fumonisins determination in foodstuff is the first step to establish a good control strategy since the severity of the occurrence needs to be determined. Such a method should also meet the international acceptance criteria limits [25].

The European Commission set up permissible limits of 4000 µg kg^{-1} for unprocessed maize, 1000 µg kg^{-1} for maize intended for direct human consumption, 800 µg kg^{-1} in snacks & breakfast cereals, and 200 µg kg^{-1} processed maize-based foods and babies and young children foods [26].

Based on its hydrophobicity properties, reversed-phase HPLC columns (RP, C_{18}) are used to analyze most of the common toxins belonging to the fumonisins group [27]. Although several methods have been developed, either using mass spectrometry [1,28-32] or diode array [33] detectors, the fluorescence detector (FLD) is still the most common and appropriate for most matrices and still is, the official recommended method [34-36]. In addition, the analysis cost using HPLC equipped with FLD is cheaper than that using LC/MS, which is not available in many laboratories around the world.

Before the analysis, different clean-up techniques have been used to extract and purify fumonisins B_1, B_2 from food samples. Most of these approaches have used strong anion exchange, SAX [14, 33], immunoaffinity column, IAC [37], or RP-C18 solid-phase extraction (SPE) [20, 34, 38). The lack of a native fluorophore in the fumonisin structure makes derivatization prior to the analysis necessary. Currently, the most common derivatizing agent used is o-phthaldialdehyde (OPA) [36].

On the other hand, using new C18 packing materials with fused solid core particles was found to improve the separation of aflatoxins [39] and ochratoxin A [40] by reducing the time and solvents used in the analysis with no effect on the performance.

Thus, this study aimed to examine the efficiency of 5 analytical columns with different particle sizes and lengths and C_{18} chemistry to analyze FB_1 and FB_2 in synthetic culture media and natural and spiked cornflakes samples. The limit of detection (LOD), the limit of quantification (LOQ), and other chromatographic parameters (Peak width (W), number of theoretical plates (N), height equivalent to a theoretical plate (HEPT), and reduced plate height (h)) were used to perform the evaluation.

2. Materials and Methods

2.1. Reagents and standards.

A Fumonisins mixture (FB_1 and FB_2, 50 µg ml^{-1} Acetonitrile (CAN): water (1:1, v/v), Orthophythaldehyde (OPA), orthophosphoric acid, sodium tetraborate ($Na_2B_4O_7$), and 2-mercaptoethanol were purchased from Sigma-Aldrich, UK. Acetonitrile (AcN) and methanol (MeOH) were purchased from Fisher Scientific (Fisher Scientific UK Ltd., UK). C_{18} Sep-Pak

solid-phase extraction (SPE) cartridges, 500 mg, were purchased from Variant (Union, Missouri, USA). All solvents were HPLC grade. The water used was obtained from a Milli-*R*/Q *water* system (Millipore, Billerica, MA, USA).

2.2. Working standards.

Calibration curves of FB_1 and FB_2 were prepared using a range of concentrations from 0.05 to 10 µg ml^{-1} ACN:H_2O, 1:1.

2.3. Media and sample preparation.

The medium used in this study was a fumonisin-inducing solid agar medium (FIM) previously used [41]. It contained for each liter 0.5 g malt extract, 1 g yeast extract, 1 g peptone, 1 g KH_2PO_4, 0.3 g $MgSO_4 \cdot 7H_2O$, 0.3 g KCl, 0.05 g $ZnSO_4 \cdot 7H_2O$, 0.01 g $CuSO_4 \cdot 5H_2O$, 20 g fructose, and 15 g bacteriological agar. The culture medium was autoclaved for 20 minutes at 121°C, vigorously shaken, and poured into 9 cm diameter sterile Petri dishes.

FIM plates were centrally inoculated with 3 µl spore suspensions ($1x10^6$ spores ml^{-1}) of two strains *of F. verticillioides*, S30 and S40 (kindly provided by Dr. Sejakoshi Mohale, Cranfield). Four replicates of each strain were incubated in the dark at 25°C for 10 days. Afterward, 5 agar discs, including the fungus and agar, were removed from these cultures using a cork borer (0.8 cm), transferred to a pre-weighed 2 ml Eppendorf tube, weighed again, and frozen at -20°C until further fumonisins analysis by HPLC-FLD.

Cornflakes samples (500g package, Kellogg's, UK) were purchased from a retail shop in the UK, mixed well, and split into two subsamples. The first subsample represented the control samples, and the second was spiked using the fumonisin B_1 and B_2 standards at different concentrations (0.625, 1.25, and 2.5 mg kg^{-1}). Each subsample was subsequently divided into smaller samples (25g) for later analysis.

2.4. FB1 and FB2 extraction.

Toxins extraction from media was performed according to the method of Lazzaro *et al.* [42] by adding 1ml acetonitrile/water (1:1 v/v) to the plugs, shaking for 1h, and centrifugation for 5 min. The supernatant was subsequently filtered through a Nylon Filter 13 mm 0.22µm (Jaytee) in a new Eppendorf in preparation for derivatization.

Cornflakes samples were extracted according to Dombrink-Kurtzman and Dvorak [34]. Twenty-five grams of ground cornflakes were extracted with 100 ml of acetonitrile/water (1:1 v/v), shaken for 1 h, and filtered. A C_{18} cartridge was preconditioned by passing through 5 ml of methanol and then 5 ml of water at 1-3 ml min^{-1} flow rate. Two milliliters of sample filtrate were diluted with 5 ml of water and applied to the cartridge Sep-Pak. It was washed with 5 mL of water, followed by 2 ml of acetonitrile/water (1:9 v/v). The fumonisins were eluted with 4 ml of acetonitrile/water (7:3 v/v) at a flow rate of <1 ml min^{-1}. The eluent was evaporated to dryness (under nitrogen), dissolved in 1 ml acetonitrile/water (1:1 v/v), filtered, and derivatized with OPA as described by AOAC [36].

2.5. Derivatization and LC analysis.

Fifty microliters aliquots of the sample extracts or standards were transferred to 250µl vial inserts (Agilent, Berks., UK), and 100µl of OPA reagent (40 mg OPA+1 ml methanol for dissolving+5ml 0.1M $Na_2B_4O_7$ +50µl 2-mercaptoethanol) were added and mixed by pipetting and injected to the HPLC system within 1 min.

2.6. Chromatographic equipment and fumonisin analysis.

The HPLC system used for FB_1, FB_2 analysis was an Agilent 1200 Series system (Agilent, Berks., UK) equipped with a fluorescence detector (FLD, G1321A, Agilent), an autosampler (ALS, G1329, Agilent), autosampler thermostat (G1330B, Agilent), thermostatted column compartment (G1316A, Agilent), on-line degasser (G1379B, Agilent), and binary pump (G1312A, Agilent). The separations were performed in the isocratic mode. A slight modification of the mobile phase recommended by the AOAC official method [36] was used and contained 25% 0.1M NaH_2PO_4:75% methanol adjusted to pH 3.35 using phosphoric acid. The flow rate was set at 1 ml min^{-1}. FLD detection was performed at 335nm excitation and 440nm emission wavelengths.

With an appropriate pre-column, 5 different columns were examined for their performance in FB_1 and FB_2 analysis. Three of them contained fully porous particles [Cronus Nucleosil 100 C18 4.6x150mm, 5µm; Agilent Zorbax Eclipse plus C_{18} 4.6x150mm, 3.5µm and Agilent Zorbax Eclipse plus C_{18} 4.6x100mm, 3.5µm], whereas Agilent Poroshell 120 EC-C_{18} 4.6x100mm, 2.7µm and Agilent Poroshell 120 SB-C_{18} 4.6x75mm, 2.7µm were packed with fused-core silica particles.

The injection volume was set at 20 µl. Agilent ChemStation software Ver. B Rev: 03.01 (Agilent Technologies, Palo Alto, CA, USA) was used to analyze the output signals.

2.7. Column efficiency.

The efficiency of all tested columns was assessed. Along with different runs, the backpressure of each column was recorded. In triplicate injections, the linearity of standard solutions was measured at concentrations of 0.66, 6.6, 66, and 134 ng of either FB_1 or FB_2 $injection^{-1}$. Calibration lines were generated by plotting the peak area against the concentrations. Linear regression was obtained using Microsoft®Excel® in order to establish the correlation coefficient.

Based on the peak width (W) of either FB_1 or FB_2 obtained using Agilent ChemStation software, parameters of each column were calculated as such equations:

1) $N = 16\left(\frac{V_e}{W_b}\right)^2$, where N is the number of theoretical plates, V_e is the elution volume, and W_b is the width of the peak at the baseline.

2) $HEPT = L/N$, where HEPT is the height equivalent theoretical plate and L is the column length

3) h= HEPT/*d*p, where h is the reduced plate height (h), and *d*p is the particle size (µm). That parameter is dimensionless, which facilitates the comparison of different columns packed with different particle sizes [43].

The sensitivity of the tested columns was assessed by the calculation of LOD and LOQ of FB_1 and FB_2 for standards, fungal cultures, and cornflake samples. LOD and LOQ were considered the fumonisins concentration that provided S/N ratio 3:1 and S/N ratio 10:1, respectively [44].

2.8. Statistical analyses.

Statistica Version 10 (StateSoft, Tulsa, Okla., USA) was used for data analysis. Analysis of variance (ANOVA, one way at $p<0.05$) was applied to fumonisin B_1 and B_2 concentrations in fungal cultures and cornflakes samples. Fisher's LSD method was used for the comparison between columns' parameters.

3. Results and Discussion

3.1. The enhancement of FB1 and FB2 separation.

The first improvements were made using the Cronus 150x4.6mm-5µm column, and then the modified mobile phase was used with the other columns for the comparison in FB_1 and FB_2 separation (Figure 2).

FB_1 was totally separated, and no interference with any close peaks was noticed. The peak that appeared closer to FB_2 was also completely separated, lifting a completely resolute FB_2 peak.

3.2. Inter-column comparison of the different chromatographic parameters.

Table 1 shows the data of column backpressure, total time of analysis, the retention time of both FB_1 and FB_2, and the most important chromatographic parameters of the tested columns.

In general, the backpressures of all tested columns were within the safe range advised for most Agilent HPLC 1200 series (<400 Bar). An increase in the backpressure was noticed by decreasing the particle size of the same length columns (150mm: 5 and 3.5 µm). Contrarily, a decrease in backpressure was observed by decreasing the length of the same particle size columns (100 and 75 mm: 2.7µm).

Similarly, the shorter the column was, the less the retention times obtained at the same particle sizes (150, 100mm: 3.5µm -100, 75mm: 2.7µm). At the length of 150 mm columns, the reduction in porous particle size from 5 to 3.5 µm led to increased retention time (+3 and 11 min for FB_1 and FB_2, respectively). However, the reduction from 3.5µm porous particles to 2.7 µm solid core particles in columns of 100mm slightly decreased the retention times from 5.5 and 14.4 to 5 and 13.2 min for FB_1 and FB_2, respectively.

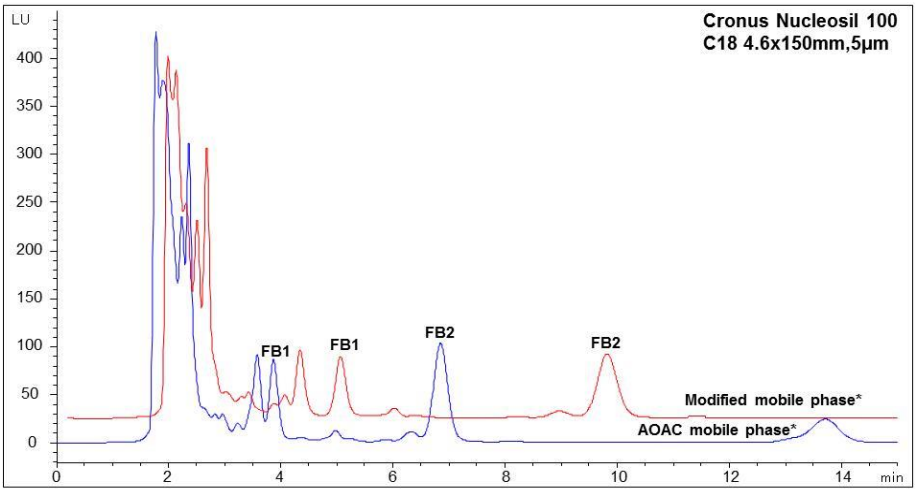

Figure 2. Enhancement of Fumonisin B_1 and B_2 separation in *Fusarium* culture medium using modified mobile phase.

Table 1. Backpressures, Fumonisins B_1 and B_2 (FB_1, FB_2) retention times (t_r), and chromatographic parameters calculated for the different columns. Values obtained from 3 different injections at low, medium, and high concentrations. SE: Standard Error.

C_{18} Column	Column pressure (Bar)	Toxin type	t_r±SE	W±SE	N±SE	HEPT±SE	h±SE	Total analysis time (min.)	
Cronus 150x4.6mm-5µm	180±2	FB_1	4.759±0.002	0.2068±0.0038	8488.16±316.23	17.72±0.67	3.54±0.03	Cornflakes	105
		FB_2	9.269±0.007	0.3826±0.0041	9395.49±214.47	15.98±0.37	3.20±0.07	Culture	90

C_{18} Column	Column pressure (Bar)	Toxin type	t_r±SE	W±SE	N±SE	HEPT±SE	h±SE	Total analysis time (min.)	
Zorbax Eclipse 150x4.6mm-3.5µm	190±2	FB_1	7.791±0.006	0.2313±0.0034	18173.18±557.00	8.27±0.25	2.36±0.07	Cornflakes	115
		FB_2	20.649±0.019	0.5328±0.0087	24066.30±760.83	6.24±0.19	1.78±0.055	Culture	100
Zorbax Eclipse 100x4.6mm-3.5µm	132±2	FB_1	5.469±0.018	0.1988±0.0024	12126.33±374.39	8.26±0.25	2.36±0.07	Cornflakes	110
		FB_2	14.350±0.020	0.4329±0.0026	17587.01±249.09	5.69±0.08	1.63±0.02	Culture	95
Poroshell 100x4.6mm-2.7µm	272±2	FB_1	4.993±0.006	0.1581±0.0019	15937.37±406.72	6.28±0.16	2.33±0.06	Cornflakes	110
		FB_2	13.156±0.016	0.3476±0.0007	22923.11±45.76	4.36±0.01	1.62±0.00	Culture	95
Poroshell 75x4.6mm-2.7µm	232±2	FB_1	3.234±0.004	0.1241±0.0004	10825.42±58.83	6.93±0.04	2.57±0.01	Cornflakes	105
		FB_2	7.662±0.017	0.2402±0.0028	16295.46±397.59	4.61±0.11	1.71±0.04	Culture	90

W: peak width (min); N: number of theoretical plates; HEPT: height equivalent to a theoretical plate (µm); h: reduced plate height.

Figure 3 shows examples of the FLD chromatograms obtained by injecting both FB_1 and FB_2 standards, fungal culture extracts, and spiked and unspiked (control) cornflakes using the set of columns tested.

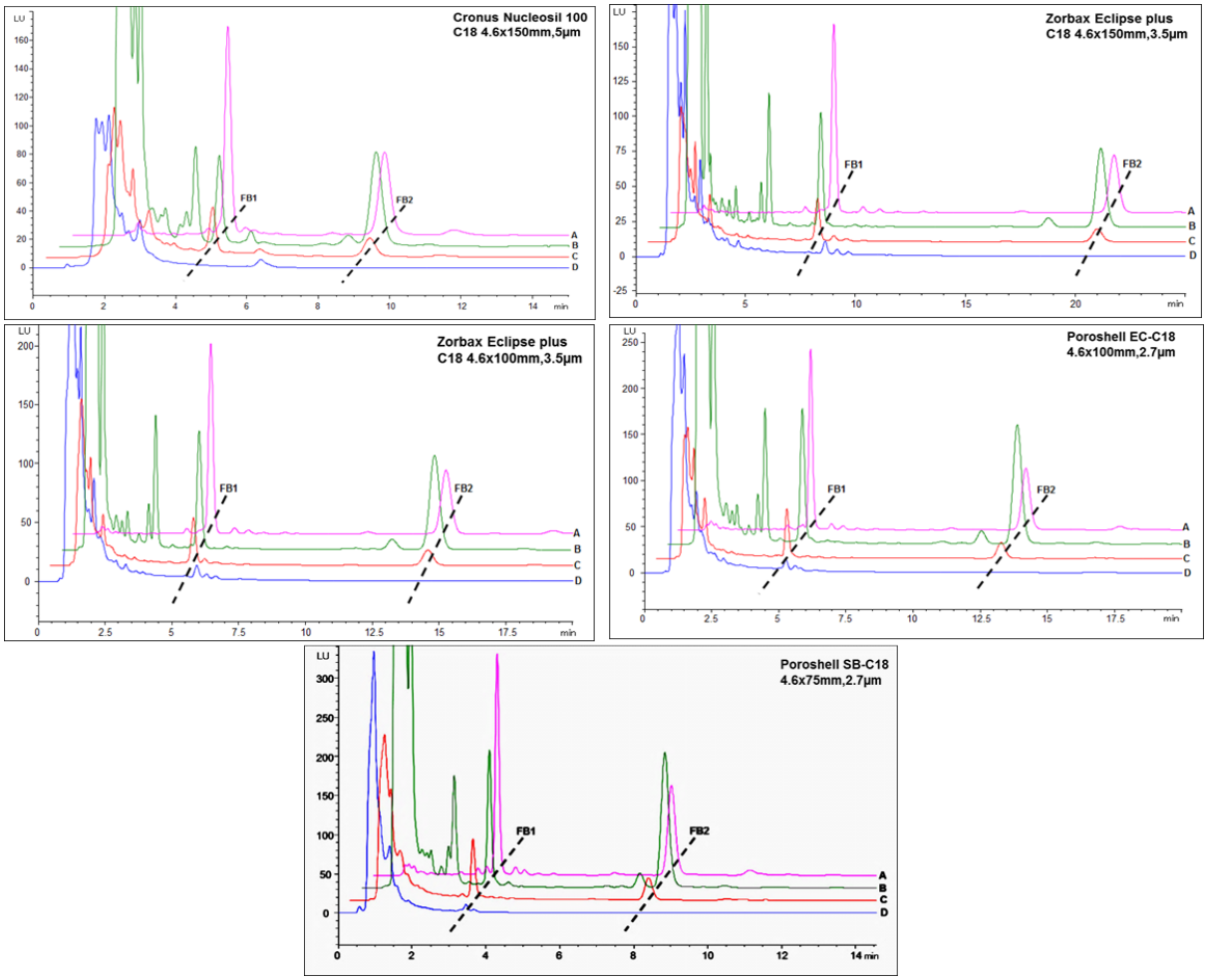

Figure 3. HPLC-FLD chromatograms of different matrices contaminated with fumonisins B1 and B2 using all tested columns at wavelengths λex 335nm and λem 440nm (**A**) mix of fumonisins B_1 and B_2 standard ; (**B**) fumonisins B_1 and B_2 produced by *Fusarium verticillioides* grown on FIM; (**C**) fumonisins B1 and B2 in spiked cornflakes sample; (**D**) fumonisins B_1 and B_2 in control cornflakes sample (Non Detected).

The shortest retention times were obtained using the new Agilent Poroshell 120 (75 mm, 2.7µm). Retention times were 3.2 and 7.7 min for both FB_1 and FB_2, respectively. In contrast, Poroshell columns showed the highest backpressure when compared with Nucleosil and Zorbax eclipse columns.

Generally, the peak width of FB_2 was around twice wider than that of FB_1 using all selected C_{18} columns. Poroshell columns, notably the shorter one (75mm) showed the narrowest peaks of both FB_1 and FB_2 (0.12 and 0.24 min), referring to how sharp the peaks were. The reductions in particle size (Cronus Nucleosil 100, 5 µm to Zorbax Eclipse plus, 3.5 µm) led to 114% and 156% increase in N of FB_1 and FB_2 respectively and subsequently 53% and 61% reduction in the HEPT. Also, the length increases led to an increase in the number of theoretical plates at the same particle sizes (N)(Table 1).

Results for the reduced plate height (h) are also shown in Table 1. The highest h value was for Cronus Nucleosil 100, 5 µm recording >3.2 for both FB_1 and FB_2. However, Zorbax and Poroshell columns had close h values with only differences <0.25. Finally, the shortest time of whole analysis for either cornflakes or culture samples was obtained using both Cronus 150 mm and Agilent 75 mm (105 and 90 min, respectively).

3.3. Linearity and sensitivity.

Table 2 illustrates the linear regression equations and the coefficient of determination (R^2) of FB_1 and FB_2 standards analyzed by different columns. In general, the slope of FB_1 (16.5-17.5) was higher than that of FB_2 (11-13.2) for all columns, referring to the higher FLD response. The coefficient of determination (R^2) of both fumonisin types using the five columns was very near from 1 (>0.997), indicating an excellent linear response of the FLD detector.

Table 2. Linear regression of FB_1 and FB_2 standards using FLD detector and different HPLC C18 columns.

		Cronus 150x4.6mm-5µm	Zorbax 150x4.6mm-3.5µm	Zorbax 100x4.6mm-3.5µm	Poroshell 100x4.6mm-2.7µm	Poroshell 75x4.6mm-2.7 µm
FB_1	Equation	y = 16.634x - 6.5329	y = 16.524x - 16.345	y = 17.489x - 16.421	y = 16.916x - 22.72	y = 17.016x - 5.0586
	R^2	0.9996	0.9988	0.9981	0.9983	0.9997
FB_2	Equation	y = 12.46x - 5.3169	y = 11.079x - 19.252	y = 12.577x - 6.6159	y = 12.91x - 22.395	y = 13.173x - 19.07
	R^2	0.9998	0.9966	0.9996	0.9982	0.9981

3.4. Limit of detection and quantification.

LOD and LOQ of FB_1 and FB_2 in both standards solution (µg l^{-1}) and sample matrices (µg kg^{-1}) were calculated according to Miller and Miller [43], and the data are illustrated in Table 3. Generally, both LOD and LOQ values of FB_2 were more than twice times higher than those of FB1, referring to the lower response by FLD detector. The lowest values of both parameters were observed in the case of standards followed by cornflakes and culture. Poroshell 75x4.6mm-2.7µm was the most sensitive column notably with FB_2 type recording LOD<1.2 µg l^{-1} for standard and <8.5 for culture samples. The same trend was observed regarding to the LOQ parameter. On the other hand, Cronus 150x4.6mm-5µm recorded the highest LOD and LQD of FB_1 and FB_2 in standard. Otherwise, these limits were very close in other substances using the tested columns.

Table 3. Comparison of the LOD and LOQ (µg kg^{-1}) of Fumonisins B_1 and B_2 (FB_1, FB_2) obtained with the columns assayed.

			Cronus 150x4.6mm-5µm	Zorbax 150x4.6mm-3.5µm	Zorbax 100x4.6mm -3.5µm	Poroshell 100x4.6mm-2.7µm	Poroshell 75x4.6mm-2.7 µm
LOD	**FB_1**	**Std. (µg l^{-1})**	1.172	0.53	0.94	0.74	0.49
		Cornflakes (µg kg^{-1})	1.90	1.49	2.64	4.10	1.99
		Culture (µg kg^{-1})	3.87	3.55	4.66	4.79	3.41
	FB_2	**Std. (µg l^{-1})**	2.99	1.73	2.81	2.10	1.180
		Cornflakes (µg kg^{-1})	4.83	4.88	7.89	11.70	4.85
		Culture (µg kg^{-1})	9.87	11.64	13.89	13.65	8.43
LOQ	**FB_1**	**Std. (µg l^{-1})**	3.91	1.76	3.15	2.46	1.62
		Cornflakes (µg kg^{-1})	6.32	4.96	8.8	13.67	6.64
		Culture (µg kg^{-1})	12. 94	11.83	15.52	15.96	11.54
	FB_2	**Std. (µg l^{-1})**	9.97	5.76	9.40	7.00	3.93
		Cornflakes (µg kg^{-1})	16.10	16.27	26.26	39.00	16.16
		Culture (µg kg^{-1})	32.89	38.79	46.28	45.51	28.09

3.5. Analysis of FB_1 and FB_2 in fungal cultures and cornflakes samples.

Table 4 shows fumonisins levels in cultures of 2 isolates of *F. verticilloides* and in cornflakes samples separated by different columns. In general, the isolate S40 was able to produce a high amount of FB_1 and FB_2 (>27 and >64 µg g^{-1} media, respectively) whereas, a small amount of FB_1 (<2.6 µg g^{-1} media) and FB_2 (<1.3 µg g^{-1} media) were produced by the isolate S30. The records of both FB_1 and FB_2 levels in S30 culture had no significant Cronus 150x4.6mm-5µm was statistically lower than that of Poroshell 100 at P <0.05. Control samples of cornflakes contained only low levels of FB_1 (<0.14ppm), which varied among the tested columns.

Table 4. Fumonisin B_1 and B_2 levels in fumonisins producing cultures and cornflakes samples.

C18 Column type		µg g^{-1} media ± SE			Spiked cornflakes (mg kg^{-1}± SE)					
					0.625		1.25		2.5	
		F. verticillioides S30	*F. verticillioides* S40	Control	Calculated Conc.	Recovery%	Calculated Conc.	Recovery%	Calculated Conc.	Recovery%
Cronus 150x4.6mm-5µm	FB_1	2.12^{a}±0.425	26.9^{b}±2.50	ND	0.67^{b}±0.06	107.2	1.13^{c} ±0.07	90.4	2.19^{c}±0.10	87.6
	FB_2	0.94^{a}±0.219	64.38^{a}±2.81	ND	0.71^{b}±0.07	113.6	1.24^{a}±0.05	99.2	2.40^{b}±0.11	96.2
Zorbax 150x4.6mm-3.5µm	FB_1	2.27^{a}±0.45	30.51^{ab}±2.04	0.032^{b}±0.005	0.73^{b}±0.07	112.0	1.33^{b} ±0.08	104.0	2.42^{ab}±0.12	95.6
	FB_2	1.17^{a}±0.133	67.07^{a}±2.95	ND	0.68^{a}±0.80	108.8	1.26^{a}±0.13	100.8	2.51^{ab}±0.11	100.4
Zorbax 100x4.6mm-3.5µm	FB_1	2.57^{a}±0.51	30.35^{ab}±1.64	0.038^{b}±0.007	0.56^{c}±0.07	84.0	1.36^{b} ±0.10	105.8	2.34^{b}±0.09	93.6
	FB_2	1.29^{a}±0.32	71.58^{a}±3.84	ND	0.50^{c}±0.03	80.0	1.33^{a}±0.0.7	106.4	2.70^{a}±0.18	108
Poroshell 100x4.6mm-2.7µm	FB_1	2.55^{a}±0.46	35.6^{a}±1.85	0.021^{c}±0.003	0.75^{ab}±0.10	116.6	1.49^{ab}±0.08	117.5	2.56^{a}±0.06	101.6
	FB_2	1.26^{a}±0.28	72.6^{a}±0.40	ND	0.74^{ab}±0.04	118.4	1.28^{a}±0.03	102.4	2.36^{b}±0.05	94.4
Poroshell 75x4.6mm-2.7µm	FB_1	2.47^{a}±0.51	30.74^{ab}±1.67	0.140^{a}±0.04	0.77^{a}±0.13	101.0	1.57^{a} ±0.12	114.8	2.55^{a}±0.15	96.5
	FB_2	1.20^{a}±0.22	65.51^{a}±2.12	ND	0.73^{b}±0.06	116.8	1.30^{a}±0.12	104	2.56^{ab}±0.09	102.4

Means followed by different superscript letters of the same toxin type within columns are statistically different at p<0.05. *: Recovery= (Actual concentration - Control)/prepared concentration x100.

There were significant variations in fumonisins concentrations of spiked samples. In general, the recoveries of both fumonisins types using all columns were <120%. Both Poroshell columns recorded the highest recoveries, notably at 0.625 and 1.25 levels, whereas close values to 100% were obtained at 2.5 mg kg^{-1}.

3.6. Discussion

There are no available or similar studies comparing the suitability of different chromatographic analytical columns for both fumonisins and have compared the analysis performance using different matrices. In our aim to provide optimal analysis conditions for many laboratories around the world where access to UPLC or LC/MS-MS technologies are just unaffordable and in continuation with our previous work on ochratoxin A [40], the current study has considered the suitability of 5 different C_{18} columns with different particle sizes and porosities to analyze FB_1 and FB_2. In addition to standard solutions, fungal cultures of fumonisins-producing fungi and cornflakes were chosen for column evaluation to provide evidence of how the selected methodology can be applied in a wide range of applications spanning from mycological research to the analysis of food matrices. An extensive chromatographic comparison has been carried out, exploring the separation of both FB_1 and FB_2.

The derivatization step, which needs to be performed just before each injection, was one of the challenges we faced in this study, as it hampered the potential use of autosampler devices. However, after our initial literature review, it became clear that the use of OPA was the best option in terms of LOD and LOQ [33]. Finally, OPA was selected in an attempt to save analysis time and solvents without affecting the quality of separation. Thus, this is a step where future research needs to be further developed and could be a good improvement target for analytical laboratories and research institutions.

Although the retention times of both FB_1 and FB_2 using the modified mobile phase were higher than those using AOAC mobile phase, a better resolution was observed (Figure 2). Increasing the aqueous proportion from 23% to 25% at the expense of methanol proportion increased the polarity of the mobile phase. Consequently, fumonisins were held on the column for a longer time.

The records of the tested columns' backpressure were in line with their properties. It decreased in the shorter column of the same particle size either within Zorbax or Poroshell, whereas smaller particle sizes of the same column length also had higher backpressure (150mm: from 5 to 3.5 µm).

Carbon load and surface area played an important role in the retention times of FB_1 and FB_2. Albeit Cronus 150 mm had the biggest particle sizes (5µm) among the examined columns, the separation was faster than that obtained by both Zobax Columns (150 and 100mm:3.5µm). Silica particles of the Cronus column are loaded with 14% carbon and occupy 350m^2 g^{-1} surface area exceeding those of Zorbax Eclipse Plus columns (9% and 160 m^2 g^{-1}). This made FB_1 and FB_2 passed through Cronus particles quicker than through the Zorbax bed. Separation using Cronus was even faster than the new Poroshell 120 EC-C_{18} 4.6x100mm, 2.7µm column packed with cure fused particles (0.5µm thick of porous outer layer and 1.7µm solid-core silica). This is because of the lower carbon load and surface area of the latter column, 8%, and 120 m^2g^{-1}, respectively [45]. Only the shorter poroshell column, 75 mm, was faster than Cronus in fumonisins separation (3.2 and 7.7 min for FB_1 and FB_2, respectively).

Similar to Cronus, Discovery C18 150x4.5 mm, 5 µm column was used by Solfrizzo *et al.* [37] to separate FB_1 and FB_2 after cleaning up the cornflakes samples IAC cartridge at 7 and 17.1 min, respectively. They were also eluted after 6.5 and 11.5 min using ZORBAX Eclipse® XDB C18 column 150x4.5mm, µm [12].

Ndube *et al.* [33] separated FB_1 and FB_2 from maize samples after derivatization with naphthalene-2,3-dicarboxaldehyde and SAX column clean up. Although they used Phenomenex, Luna C18 5 µm column (75 mm × 4.60 mm), which was half-length of Cronus column, more retention time was required to elute FB_1 and FB_2 (7.2 and 17.5min).

The peak width of both FB_1 and FB_2 reflected how the sharpness of these peaks was. Poroshell columns gave the narrowest width, notably the shorter one (75mm). This is because the fumonisins molecules move a short distance through the fused core particles when compared with the completely porous particles.

Both retention time and peak width can be used to identify the number of theoretical plates, which was the highest using Zorbax Eclipse 150mm 3.5 mm. It had the broadest width and the longest retention time. Consequently, N numbers were used to calculate HEPT based on the column length, which was the highest in the Cronus column. However, none of these parameters can be used to evaluate the efficiency of the tested column alone or together. The only reduced plate height (h) parameter can be used to evaluate as it considers the column particle size. Cronus had the highest h value referring to the well-packing process with the ideal packed bed. This can be interpreted by the fact that the smaller the particle size is, the greater the difficulty in preparing a well-packed column bed is [46]. This rule is not applicable in the case of different porosity columns with different lengths like Zorbax and Poroshell columns, which had close h values for FB_1 and FB_2.

Linearity expressed by R^2 in determination for FB_1 and FB_2 is the first evaluation step of the column performance. All tested columns exhibited good linearity in addition to the small intercept values. A similar finding was observed, recording higher than 0.9997 for both toxins [12].

LOD and LOQ are important parameters for any eluents or mycotoxins like fumonisins that contaminate corn and corn products at high levels [47]. LOQ should be less than the permissible limits of FB_1 and FB_2 that reach 200 µg kg^{-1} for babies' food [26]. The extraction method and matrix type noticeably affected LOQ using all tested columns. The lowest values were for the standard solution followed by cornflakes samples which were cleaned up by C18 cartridge, then by culture samples. LOQ for both toxins in cornflakes samples was < 23 µg kg^{-1} using Cronus, Zorbax 150, and Poroshell 75 columns, whereas it recorded 35 and 53 µg kg^{-1} using Zorbax 100 and Poroshell 100, respectively. A close value was obtained by De Girolamo *et al.* [48], who estimated LOQ for both FB_1 and FB_2 in masa flour by 25 µg kg^{-1} using Symmetry Shield (150 mm×4.6 mm, 5 µm) column. In contrast, it was 400 µg kg^{-1} in milled corn using IAC for cleaning up and MS detector [1]. In general, all values in the present study are much lower than the permissible limit of fumonisins in cornflakes, 800 µg kg^{-1} [26].
Concerning the cornflake sample, it was naturally contaminated with FB_1, recording <140 µg kg^{-1} which was much lower than the permissible limits established by EC [26]. That value is very close to that obtained by Solfizzo *et al.* [37], who calculated the contamination levels of both FB_1 and FB_2 in 18 cornflake samples in the average of 0.157 and 0.036 µg kg^{-1}, respectively. Although all columns achieved good performance regarding the LOQ (<140 µg kg^{-1} for FB_1), there was a variation in the detection of the FB_1 between the tested column. A

clear peak of FB_1 was observed using Poroshell 75 column, whereas others had an unclear peak, notably using the Cronus column (Figure 3).

In general, there was a small up-shift in the obtained recoveries (<8%) than the acceptance criteria, 70%-110%, established by the European Commission [49]. This can be attributed to the matrix effect of the control cornflake samples, which chelated toxins. However, the spiking process can stimulate releasing the chelated toxin. Contrarily, Solfrizzo *et al.* [37] mentioned that low recoveries of FB_1 and FB_2 in corn-based foods as cornflakes (<30%) were obtained using the SAX column as a clean-up procedure. They explained that the existing iron in commercial cornflakes could chelate the free fumonisin at the two tricarballylic acid groups, which would not be able to hold in the SAX ion exchange column.

Except for Zorbax 100, spiked cornflake samples' recoveries at 0.625 mg kg^{-1} were higher than those at 1.25 and 2.5 mg kg^{-1}. This finding agrees with Li *et al.* [14], who reported that the recovery levels of spiked corn samples decreased by increasing the spiking levels. A similar study achieved 102.6% and 95.1% recoveries for both FB_1 and FB_2 in spiked cornflakes samples [37]. Likewise, Muscarella *et al.* [12] found that the recovery values ranged from 87 to 94% for FB_1 and 70 to 75% for FB_2 in cornflake samples.

4. Conclusions

Using C_{18} cartridge in cleaning up followed by OPA derivatization was an effective method in FB_1 and FB_2 determination in cornflakes. Albeit Nucleosil Cronus 150mmx4.6mm, 5 µm had porous particles. It had the closest results to Poroshell 75 in saving time, solvents and gave good recovery value with relatively low pressure. Meanwhile, separating FB_1 and FB_2 using solid core particle techniques in poroshell columns did not make a big difference compared with end-capping Nucleosil Cronus. Finally, column diameter and its particle type and size play an important role in fumonisin B_1 and B_2 analysis.

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Conflicts of Interest

The authors declare no conflict of interest.

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