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Screening of aflatoxins in feed samples using a flow system coupled to capillary electrophoresis

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Abstract

A method is proposed which presents a new approach to the joint use of capillary electrophoresis (CE) commercial equipment and a flow system. This flow system allows the total determination of several compounds by using a fluorimetric screening system. The individual determination for each analyte is performed by the CE proposed method. The screening procedure uses simple equipment and operations and provides a yes/no binary response that occasionally requires confirmation. A fast, simple, and reliable method has been developed in order to determine the most frequent mycotoxins in feed samples using micellar electrokinetic capillary chromatography (MECC). An extraction step followed by a purification step was carried out on the samples in order to remove interference substances before analysis. A C_{18} column was chosen to concentrate the mycotoxins, and the analytes were eluted from C_{18} using methanol. The MECC method allows the separation of six mycotoxins within 50 min with a reproducibility as RSD between 7.45 and 13.06%, and a limit of detection (LOD) between 0.02 and 0.06 mg l^{-1} for all the mycotoxins. These LODs were clearly below legal limits (0.05 mg l^{-1}).

Keywords: Aflatoxins; Mycotoxins

1. Introduction

Aflatoxins are extremely toxic metabolites of fungi such as *Aspergillus*, and these fungi occur in a variety of foodstuffs and feeds intended for both livestock and human consumption [1]. They are extremely potent mutagens and are suspected human carcinogens. Determination of mycotoxins is a difficult task, because only trace amounts of toxin are present in the samples, especially in animal feeds. However, rapid progress in the development of new analytical methodologies have been made during the last few years [2].

Most of the methods available for the determination of mycotoxins involve two steps: (a) isolation from the matrix (this step includes slow extraction processes with a large variety of organic solvents and expensive Sep-Pak cartridges); (b) quantitative determination by different analytical techniques, which are, in general, slow, complex and expensive. Moreover, almost all the mycotoxin methods currently used by the US Food and Drug Administration (FDA) to accomplish the agency's regulatory mission are methods that use liquid–liquid extraction, open bed column or solid-phase extraction (SPE)

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clean-up procedures prior to the final determination of the analytes [3].

Sample matrix problems are more severe in the determination of mycotoxins in feed because more than one commodity contains other additives. Generally, the quantitative analysis of these compounds involves toxin extraction from the sample matrix, followed by laborious and very extensive clean-up treatments to remove interference substances before analysis is done [4,5]. The typical extraction methods are based on the methods recommended by the AOAC [6]. In recent years, supercritical fluid extraction (SFE) was also employed, but with serious limitations because poor recoveries were obtained from natural contaminated samples [7]. For the clean-up step solid-phase extraction on Florisil/C_{18} silica gel cartridges, immunoaffinity cartridges or Mycosep columns have been used. The solid-phase extraction has several advantages, namely reduction in the amount of hazardous solvent used per analysis, shorter analysis time, and amenable automation. The use of Mycosep columns to clean-up is well established and offers several advantages over other clean-up procedures (speed, solvent efficiency, and, in some cases, increased recovery) [8–13].

For the final separation and quantification step, several methods are used and include thin layer chromatography (TLC), high-performance liquid chromatography (HPLC) with laser induced fluorescence detection or diode array UV detection [2,5,7,14], gas chromatography (GC) with electron capture detection (ECD) or mass selective detection (MS) [5] and supercritical fluid chromatography (SFC) coupled with MS or flame ionisation detection (FID) [15]. Immunochemical methods are also used for the determination of aflatoxins [16]. HPLC methods for mycotoxins are a good alternative. However, the disadvantages are that the analyses are long, complex gradient mobile phases are needed, and large quantities of organic solvents are necessary. The emergence of capillary electrophoresis, as an analytical technique, with its advantages of rapid analysis, high column efficiency, simplicity and, in particular, minimal use of organic solvents, led the authors to investigate CE as a potentially more convenient method for determination of mycotoxins. Micellar electrokinetic capillary chromatography (MECC) is a highly efficient liquid phase separation technique, that has recently been applied to the determination of aflatoxins B1, B2, G1 and G2 [1,17–20], in which most of the published methods have relied upon UV–Vis absorbance detectors. In many cases, this means that the methods are useful for helping to determine the purity of standard solutions or concentrated mixtures, but it presents insufficient sensitivity for the determination of the low levels of mycotoxins in foods [19].

A sample screening system can be used to minimise the need for permanent use of instruments with high purchase and maintenance cost. The screening systems can be described as a system that filter samples to select those with analyte content levels similar to or higher than a previously established threshold. A very fast and sensitive method for the determination of total aflatoxins in food was proposed by Lazaro et al. [21].

The objective of this work has been the development of a method for the extraction and determination of mycotoxins in feed samples incorporating a screening system to obtain a rapid response of the presence or not of these analytes. The use of CE for the identification and quantification of the mycotoxins present in feed samples were dramatically reduced because only those samples giving positive responses in the screening system were analysed. The extraction and preconcentration steps were reduced incorporating a C_{18} minicolumn in the loop of the injection valve of a flow system before the CE instrument. As an alternative, Mycosep columns could be used for cleaning feed samples.

2. Experimental

2.1. Reagents

All reagents were of analytical grade and purified water (18 MΩ cm^{-1}) was obtained using a Millipore Milli-Q water purification system. Aflatoxins B1, B2, G1, G2 and ochratoxins A and B were the analytes determined. These compounds were supplied by Sigma–Aldrich. Stock standard solutions of 2000 μg ml^{-1} of each compound were prepared following the AOAC recommendation [6]: the stock solutions of aflatoxins were prepared dissolving the appropriate quantity in methanol. The methanol solvent was

evaporated to dry film, and then the residue redissolved in benzene–acetonitrile (98:2) and stored in the refrigerator.

To prepare 100 μg ml^{-1} of each stock standard solution in water, 0.5 ml from 2000 μg ml^{-1} was taken. This volume was evaporated and the residue was redissolved in 10 ml of water. Working standard solutions were prepared daily by diluting with purified water. Sodium dodecyl sulfate (SDS; Aldrich), sodium dihydrogenphosphate, sodium borate and γ-cyclodextrin were purchased from Riedel-de Häen, and HPLC-grade acetonitrile was used for preparing the buffer. Bromine (0.2 *M*; Merck) solution was prepared in water and stored at 4 °C in a topaz flask. Mycosep 226 multifunctional columns for sample clean-up were supplied by Romer Labs (Austria). C_{18} minicolumns (Varian) were used to clean up the feed extracts.

2.2. Apparatus

A Beckman P/ACE 5500 capillary electrophoresis system provided with a diode array detector was used for the separation of the analytes. Beckman capillary tubing of 67 cm×75 μm I.D.×375 μm O.D. was used. System control and data processing were carried out with PACE software.

A Kontron spectrofluorimeter furnished with an 18-μl flow cell and equipped with a Knauer recorder was used. Two Gilson Minipuls-2, four-channel peristaltic pumps with rate selectors, two Reodyne 5041 injection valves and PTFE tubing of 0.5 mm I.D. were also used.

2.3. Operating conditions

The experimental conditions selected, after previous optimisation, were the following.

(A) Screening system: a single flow injection analysis (FIA) channel design was used to insert 20 μl of sample into the reagent stream (4×10^{-5} *M* Br_2). The maximum signals (excitation wavelength of 360 nm and emission wavelength of 470 nm) were obtained by connecting the injection valve directly to the flow cell. The flow-rate of the bromine solution was 1.5 ml min^{-1}.

(B) Capillary electrophoresis: the running buffer used was prepared mixing the appropriate amount of SDS, γ-cyclodextrin and acetonitrile with phosphate–borate buffer to obtain an aqueous solution with concentrations of 50 m*M* SDS, 7 m*M* γ-cyclodextrin, 5% acetonitrile, 10 m*M* of NaH_2PO_4 and 6 m*M* $Na_2B_4O_7$ adjusted at pH 8.02 with H_3PO_4. The applied voltage was 10 kV (average current 35.5 μA) at 20 °C and the selected wavelengths were 214 and 362 nm. Samples were hydrodynamically injected (by high pressure) for 15 s.

In order to maintain the capillary under good conditions, its surface was regenerated once a day by consecutive washing with water (5 min), 0.1 *M* sodium hydroxide (5 min), and water (1 min) followed by the running buffer (15 min). When used under the conditions mentioned above, the capillary showed good performance for a long time without losing its initial efficiency. The capillary was rinsed between separations with 0.1 *M* sodium hydroxide (1 min), water (1 min), buffer (3 min) and as a final step, a voltage of 10 kV was applied for 2 min.

2.4. Extraction and purification procedures

The extraction and purification procedures used were based on the methods recommended by the AOAC [6], slightly modified for the analysis of aqueous solutions by micellar electrokinetic capillary chromatography.

For the extraction, 50 g of feed sample were weighed into a 250-ml blender container, then 100 ml of acetonitrile–water (9:1) solution were added and shaken for 1 h. The extraction solution was filtered through a pre-pleated filter paper to remove solid material and the extract was collected and saved for analysis. In order to achieve a sample compatible with the MECC method, 1 ml of this extract was evaporated at room temperature with N_2, and the residue was dissolved in 1 ml of water.

The sample cleaning and the preconcentration of the analytes were carried out in a first step in batch and finally to automate this step of the analytical methodology it was incorporated in a continuous flow system.

2.4.1. Batch procedure

For the determination of mycotoxins at low levels

of concentrations, it was necessary to introduce a purification step in the proposed methodology. Two different pretreatments of the samples, using Mycosep or C_{18} columns, were carried out and the results were compared.

Procedure 1: in the first procedure the Mycosep 226 columns were used. The purification step was carried out with 5 ml of extract sample. The filtrate was transferred to a culture tube of 10 ml and the Mycosep column was placed flanged-end into the culture tube [8]. One ml of the purified extract was collected from the top of the column and was evaporated at ambient temperature with N_2 in order to eliminate acetonitrile, which was incompatible with the electrophoretic method. The residue was dissolved in 0.5 ml of water to be analysed by MECC.

Procedure 2: the C_{18} minicolumn was preconditioned by sequentially passing 2 ml water, 2 ml methanol and 2 ml water followed by a 1-min drying time with air passing through the tube via aspiration. Five ml of extracted sample were evaporated at room temperature with N_2 because the extract solvent (acetonitrile–water) did not allow aflatoxins to be retained in the C_{18}. The residue was dissolved in 5 ml of water and passed through the column, and after a drying period of 2 min, the aflatoxins were eluted with 1 ml of methanol. The elution solvent was evaporated and the remaining residue dissolved in 0.5 ml of water prior to injection in the capillary electrophoresis equipment.

2.4.2. Continuous flow system

In Fig. 1 is shown the continuous flow system in which a minicolumn was incorporated in the loop of the injection valve to clean the samples and retain the analytes of interest. Two different columns were tested, the C_{18} and the Mycosep, respectively. In both cases the flow system used was the same but when the C_{18} minicolumn was incorporated in the system the analytes were retained in the solid-phase, however when the Mycosep columns were used the particles retained were the interference and the mycotoxins passed through the column.

In the first procedure, the Mycosep 226 columns were used. Only 0.1 g of the solid Mycosep material of a commercial column (2 g) was used to build the minicolumn incorporated in the flow system. This minicolumn cannot be used for the purification of more than one sample, because the minicolumn could not be regenerated with any of the organic solvent tested. The flow-rate of sample was 1.25 ml min^{-1}. The purification step was carried out with 0.5 ml of extract sample. And, in the second procedure, the C_{18} minicolumn was preconditioned in the flow system with the same volumes used in batch. Five ml of the aqueous sample were passed through the column, and after a drying period of 1 min, the mycotoxins were eluted with 0.5 ml of methanol. The elution solvent was evaporated and the remaining residue dissolved in 200 μl of water prior to injection into the capillary electrophoresis equipment.

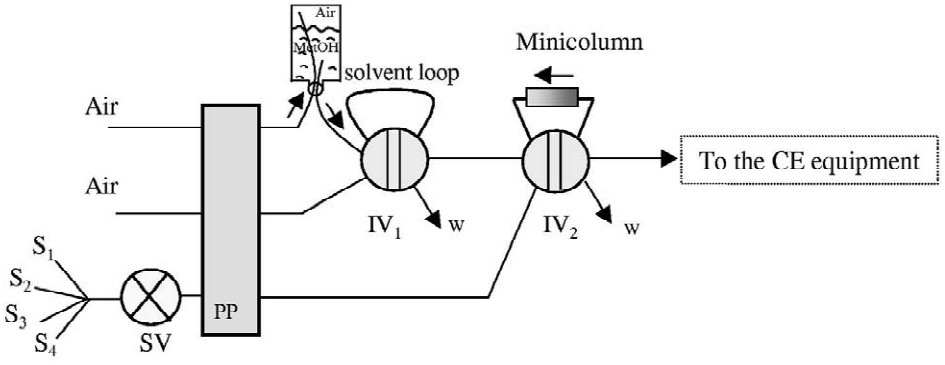

Fig. 1. Continuous flow system used for the pretreatment of the samples. IV, Injection valve; SV, selection valve; W, waste; S, sample; PP, peristaltic pump.

2.5. Safety precautions

The standard solutions were prepared in the septum vial in which they were received by injecting the solvent. The solutions were prepared under a vented hood and all the work areas and all glassware in contact with mycotoxins were cleaned with 5% sodium hypochlorite solution (bleach). Waste was also disposed of in bleach. Disposable latex gloves were worn at all times when working with the toxins.

3. Results and discussion

The determination of mycotoxins in feed samples can be focused in two different ways, depending on the analytical information needed. The use of only a screening system could be enough to detect the presence or absence of mycotoxins in real samples. The use of a separation technique such as capillary electrophoresis is only justified if there is an interest in knowing the different mycotoxins present in the samples.

In Fig. 2 is shown the entire arrangement to combine the screening flow system for the binary response with the identification electrophoretic technique. The screening method proposed includes a previous treatment (official method recommended by the AOAC [8]). Following the official method, the analytes were preconcentrated 4.5 times. The sensitivity of the screening system was enough to determine mycotoxins present in feed samples. The samples with a content in mycotoxins above the legal limit (0.05 mg l^{-1}) were analysed by CE to determine the different mycotoxins present in real samples.

Samples containing concentrations of mycotoxins above 0.1 mg l^{-1} were analysed at 362 nm by CE without previous treatment, because at this wavelength any interferences present in the matrix samples overlapped with the peaks of the analytes of interest. When the content of mycotoxins was lower than 0.1 mg l^{-1} the samples were preconcentrated before the analysis by CE and the analytes were determined at 214 nm. At this wavelength, the sensitivity obtained was better than at 362 nm.

3.1. Optimisation of the screening system

The screening system was designed to provide a

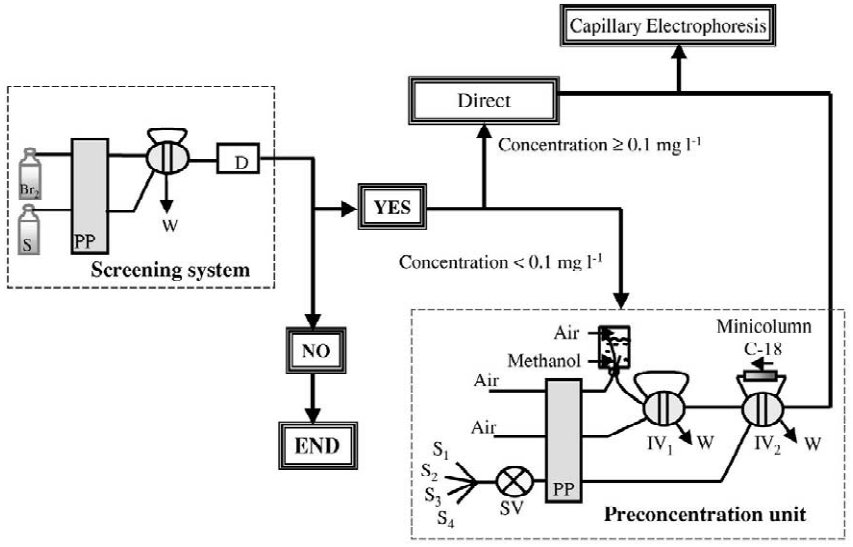

Fig. 2. Different methodology used in this work to determine mycotoxins present in feed samples. IV, Injection valve; SV, selection valve; W, waste; S, sample; PP, peristaltic pump.

rapid response to the presence or not of aflatoxins at a present concentration level. This system is based on the fluorescence of the oxidized aflatoxins. The fluorescence of aflatoxins B1 and G1 can only be increased upon reaction with bromine, while the fluorescence of B2 and G2 remains unaltered. The conditions optimised by Lazaro et al. [21] were used to run calibration graphs to determine the aflatoxins studied. The graphs were constructed by introducing 20 μl of each solution, using three replicates for each standard and five standards for each calibration graph. The results obtained are summarised in Table 1. The determination of ochratoxins was not possible using this screening system due to their very low native fluorescence and the fact that they do not react with bromine solution.

The crucial requirements for measuring the total aflatoxin content of a feed sample with fluorescence measurements is that all aflatoxin components presented the same fluorescence properties, and, thus, the fluorescence peak containing the overall response for the total amount of aflatoxins in the sample can be obtained. However, there are some differences in the response factor (i.e. slope of the calibration curves). Table 1 shows that G1 was the less fluorescent analyte and G2 was the most fluorescent analyte and therefore it has the highest response factor. To quantify total mycotoxin content the ''best-worst-case scenario'' approach can be used, by alternatively assuming that either the whole fluorescence signal comes from the compound with the highest response (G2) or from the compound with the lowest response (G1). To avoid false negatives, the G1 calibration curve was chosen.

Table 2
Determination of aflatoxins in synthetic samples

Sample	Concentrations added					G_1[a]
	Total	B_1	B_2	G_1	G_2	
1	0.05	0	0	0	0.05	0.3399
2	0.05	0	0	0.05	0	0.0567
3	0.05	0	0.05	0	0	0.1122
4	0.05	0.05	0	0	0	0.4257
5	0.005	0.005	0	0	0	0.0486
6	0.005	0	0.005	0	0	0.0173
7	0.005	0	0	0.005	0	0.0117
8	0.005	0	0	0	0.005	0.0401
9	0.01	0.005	0.005	0	0	0.0592
10	0.01	0.005	0	0.005	0	0.0536
11	0.01	0.005	0	0	0.005	0.0819
12	0.01	0	0.005	0.005	0	0.0223
13	0.01	0	0.005	0	0.005	0.0506
14	0.01	0	0	0.005	0.005	0.0451
15	0	0	0	0	0	0.0067
16	0.0059	0.0049	0.001	0	0	0.0498
17	0.0069	0.0049	0	0.002	0	0.0498
18	0.0052	0.0049	0	0	0.0003	0.0498
19	0.008	0.002	0.002	0.002	0.002	0.0430
20	0.012	0.003	0.003	0.003	0.003	0.0612

[a] Concentration in mg l^{-1} using G1 calibration curve.

In order to verify this assumption, 20 synthetic samples with different concentrations of aflatoxins were analysed (see Table 2). As has been mentioned, the G1 calibration curve was chosen to translate the fluorescence of a sample into total concentration of aflatoxins because it was the less sensitive of the four aflatoxins studied. The cut-off was fixed at a concentration of 0.05 mg l^{-1} (legal limit). A second cut-off was defined at 0.049 mg l^{-1} to analyse the samples with a content of aflatoxins very close to the legal limit.

Table 1
Figures of merit for the screening method[a]

Mycotoxins	$y = a + bx$	r	R^2 (%)	$S_{y/x}$	LOD	LOQ
G2	$a = 0.0 \pm 0.2$ $b = 986 \pm 5$	0.9996	99.93	0.96	0.0006	0.002
G1	$a = 0.9 \pm 0.3$ $b = 148 \pm 1$	0.9991	99.82	1.16	0.006	0.02
B2	$a = 1.2 \pm 0.3$ $b = 312 \pm 1$	0.9998	99.96	1.10	0.003	0.01
B1	$a = -0.2 \pm 0.4$ $b = 1240 \pm 10$	0.9994	99.89	1.55	0.001	0.003

[a] a, Intercept; b, slope; r, regression coefficient; R^2, curve fitting level (in %) obtained by analysis of variance (ANOVA) for the validation of the model; $S_{y/x}$, standard deviation of residuals; LOD, limit of detection; LOQ, limit of quantification (LOD and LOQ in mg l^{-1}).

By definition, a false negative arises when a feed sample with a concentration of analyte higher than the cut-off value gives a fluorescence measurement lower than the cut-off signal. A false positive is produced when a feed sample with a concentration of analyte lower than the cut-off value gives a fluorescence measurement higher than the cut-off signal. As can be observed in the results presented in Table 2, by using this approach, false negatives are practically avoided, due to the use of the G1 calibration curve which gives the concentration ''in the worst case''. Only 13 of the 20 samples analysed were checked by CE to determine the content of aflatoxins and only four of the 13 samples analysed by CE were false positive (samples 5, 16, 17 and 18 in Table 2).

Once the screening system was optimised with synthetic samples, real samples were processed. The direct analysis of real samples in the screening system was not possible due to the high colour of the matrix of the samples. The clean up of the samples was conducted by the method recommended by the AOAC [8]. As an alternative to the official method, the use of minicolumns for solid-phase extraction, such as Mycosep or C_{18}, was tested but the results obtained were not good enough to obtain comparable results with those obtained by following the official method. One of the reasons could be that the use of a non-separative technique such as FIA resulted in the occurrence of interference owing to the fluorescence of substances extracted with the analytes, and hence in spurious results. This problem was not observed with a combined SPE–CE system to separate the analytes of interest.

3.2. Optimisation of the separation system

By using a simple phosphate–borate buffer, the mycotoxins cannot be separated by CE since they are neutral substances. On the contrary, when the SDS anionic surfactant is used to form micelles, different migration behaviour can be obtained, due to the different interactions between the mycotoxins and the micelles. The SDS micelle being slightly polar results in stronger associations with the neutral, hydrophobic aflatoxins. Hydrophobic compounds tend to completely associate with the micelles and probably are not resolved, but in such situations, increasing the organic content of mobile phase will improve the resolution of these compounds.

It is reported that cyclodextrins (CDs) are cylinder-shaped with an axial void apolar cavity and an outer hydrophilic surface, which allows certain molecules, with the appropriate size and spatial configuration, to form CD inclusion complexes. The structure of the mycotoxins studied appears to meet these criteria for γ-CD and could be inserted into the cavity of a γ-CD being carried along at the same rate as electroosmotic flow which implies a decrease in the retention times [17].

The separation conditions of mycotoxins were optimised as follows. In the first place, optimisation of chemical variables were carried out. For this purpose a phosphate–borate buffer was used. The buffer pH was studied in the range between 7.5 and 10.2, and a pH of 8.0 proved to be the optimum value, because it provided the best sensitivity in the analyses. Different solutions of 1–100 m*M* of NaH_2PO_4 and 0–100 m*M* $Na_2B_4O_7$ were prepared at pH 8.0 in order to achieve the best resolution and sensitivity of the test mixture. NaH_2PO_4 (10 m*M*) and 6 m*M* $Na_2B_4O_7$ were the values chosen as buffer concentrations due to the best sensitivity achieved. A similar sensitivity was obtained by using 20 m*M* NaH_2PO_4, but the time of analysis was increased.

When the concentration of SDS in the buffer system was studied it was observed that a concentration of 50 m*M* was the optimum value. Lower concentrations of SDS did not produce separations between the studied compounds and with major concentrations the time of analysis was greater. To improve the results the influence of the acetonitrile concentration was studied, and it was proven that increasing the organic content of the mobile phase will improve the resolution between the G1 and B2 aflatoxins, but unfortunately, micelles are not stable in mobile phases with a high organic content [17]. A 5% acetonitrile level was chosen to achieve the appropriate resolution among the analytes. γ-CD with a concentration of 7 m*M* was chosen for the buffer concentrations due to the best sensitivity obtained in the analyses carried out. No significant variations were observed in the retention times.

The instrumental variables were also optimised. The characteristic UV–Vis spectra of the aflatoxins

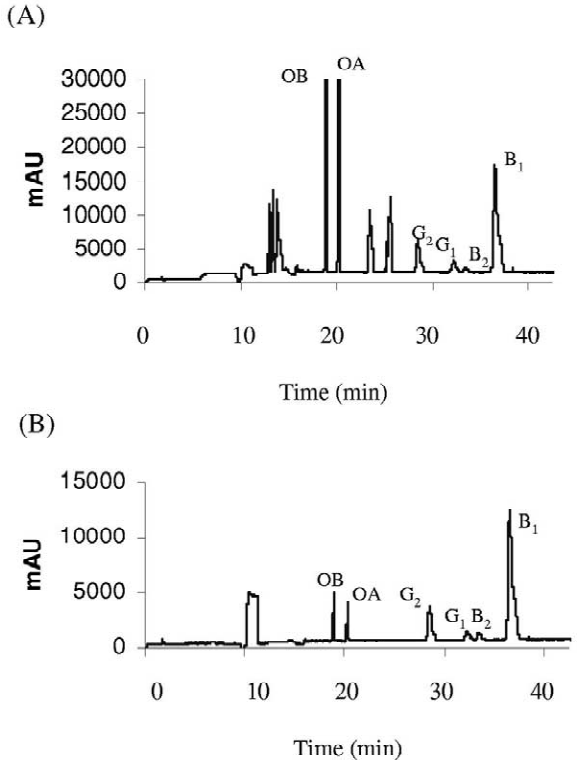

Fig. 3. Electropherograms of a spiked feed sample. The electrolyte comprised 50 m*M* SDS, 7 m*M* γ-cyclodextrin, 5% acetonitrile, 10 m*M* of NaH_2PO_4 and 6 m*M* $Na_2B_4O_7$ adjusted to pH 8.0. The separation was performed at 10 kV. (A) 214 nm and (B) 362 nm.

are helpful in confirming the presence of the toxins when they are isolated from complex matrices. The spectrums of the four aflatoxins show three maximums of absorbance at 214, 254 and 362 nm. As it is well known, there are more substances absorbing at 214 nm than at 362 nm. Fig. 3 shows two electropherograms of an extract of feed sample spiked with a mixture of six mycotoxins after the clean-up with the C_{18} minicolumn measured at 214 nm and 362 nm, respectively. At 214 nm the sensitivity obtained was better than at 362 nm, but at 362 nm the electropherogram of a feed sample was recorded free of interference. Finally, 214 nm was chosen to determine the low concentration of mycotoxins in feed samples because the interference peaks were not overlapped with the mycotoxin peaks.

Values between 10 and 25 kV of applied voltage were tested. A lower voltage provided advantages in sensitivity and selectivity, but not in analysis time. A voltage of 10 kV was chosen because it provided a good separation between G1 and B2 and the analysis time was not very long. Hydrostatic injection was used as it showed better results in comparison with the electrokinetic injection. An injection time of 15 s was the value necessary to obtain low determination limits. A capillary of 67 cm in length was used to improve the resolution between peaks in real samples. With this capillary the total time for the

Table 3
Figures of merit for the electrophoretic method[a]

Mycotoxins	$y = a + bx$	R	R^2 (%)	$S_{y/x}$	LOD	LOQ	RSD
OB	$a = 81 \pm 1239$ $b = 30712 \pm 247$	0.999	99.92	3604	0.12	0.40	7.3
OA	$a = 1459 \pm 2012$ $b = 32918 \pm 400$	0.999	99.81	5849	0.18	0.61	10.8
G2	$a = 162 \pm 263$ $b = 12428 \pm 108$	0.999	99.97	474	0.06	0.21	8.2
G1	$a = -4053 \pm 1954$ $b = 41817 \pm 805$	0.999	99.85	3524	0.14	0.47	6.1
B2	$a = 202 \pm 322$ $b = 10007 \pm 133$	0.999	99.93	581	0.10	0.32	7.0
B1	$a = -2612 \pm 1894$ $b = 45431 \pm 780$	0.999	99.88	3416	0.12	0.42	9.00

[a] *a*, Intercept; *b*, slope; *r*, regression coefficient; R^2, curve fitting level (in %) obtained by analysis of variance (ANOVA) for the validation of the model; $S_{y/x}$, standard deviation of residuals; LOD, limit of detection; LOQ, limit of quantification (LOD and LOQ in mg l^{-1}).

separation is less than 50 min, which is not excessive for one analysis because during this period the next sample is passing through the flow system in which a preconcentration step is carried out.

3.3. Performance of the method

The calibration graphs were constructed by injecting standard solutions in the 0.1–5.0 mg l^{-1} range (without any pretreatment of the standard solutions). The corresponding regression equations and other characteristic parameters for the determination of mycotoxins are shown in Table 3. Eleven replicates were performed on standards with a concentration of 5 mg l^{-1} for each analyte in order to evaluate the precision of the method. The detection limits obtained were not enough to reach the levels fixed by the Spanish legislation (0.05 mg l^{-1} for B1).

Combined MECC with SPE seemed to be an excellent way to determine mycotoxins in feed samples, which would provide the advantages of the MECC, as well as the high sensitivity achieved after SPE treatment.

Analytes were isolated from feed using one of the following procedures: acetonitrile extraction followed by clean-up on a Mycosep column (procedure 1) or acetonitrile extraction followed by clean-up on a C_{18} minicolumn (procedure 2). Both procedures were compared.

The Mycosep column allows quick sample purification within 10 to 30 s. One of the main advantages of the use of this column was that no time-consuming rinsing steps were required as in SPE. In addition, almost all analytical interfering substances were retained on the column, while the aflatoxins were not adsorbed on the packing material. The use of these columns presents the disadvantages that they cannot be re-used, increasing the cost per analysis, and their restrictive use for aflatoxins and not for ochratoxins. However, both mycotoxins (aflatoxins and ochratoxins) can be retained in the C_{18} material and eluted with methanol. The C_{18} minicolumns are cheaper than the Mycosep columns. These columns can be used during 10 extractions and the efficiency was as in the first extraction. The preconcentration with C_{18} yielded a slightly more sensitive assay. For

Table 5
Determination of aflatoxins in spiked feed samples using screening system and CE

	Sample				
	1	2	3	4	5
G1[a]	0	0.025	0	0	0.05
G2[a]	0	0	0	0	0.05
B1[a]	0	0	0.04	1	0.05
B2[a]	0	0	0	0	0.05
Screening results	−	−	+	+	+
Steps			(1+2)	(2)	(1+2)
Screening–CE results			−	+	+

[a] Concentration in mg l^{-1}. Steps: (1) preconcentration and (2) CE.

Table 4
Figures of merit for the electrophoretic method (using C_{18} minicolumn[a])

Mycotoxins	$y = a + bx$	r	R^2 (%)	$S_{y/x}$	LOD	LOQ	RSD
OB	$a = 4657 \pm 5756$ $b = 437272 \pm 12200$	0.990	98.17	22040	0.04	0.13	10.26
OA	$a = 2492 \pm 4295$ $b = 544903 \pm 10063$	0.996	99.32	16326.9	0.02	0.07	8.03
G2	$a = 7471 \pm 2239$ $b = 185043 \pm 4733$	0.994	98.84	7616.2	0.04	0.12	10.83
G1	$a = 398 \pm 416$ $b = 30276 \pm 260$	0.998	99.80	1023.7	0.04	0.13	13.06
B2	$a = 188 \pm 217$ $b = 11097 \pm 136$	0.999	99.85	533.9	0.06	0.20	11.2
B1	$a = -6351 \pm 8819$ $b = 803738 \pm 23452$	0.991	98.33	32490	0.03	0.11	7.45

[a] a, Intercept; b, slope; r, regression coefficient; R^2, curve fitting level (in %) obtained by analysis of variance (ANOVA) for the validation of the model; $S_{y/x}$, standard deviation of residuals; LOD, limit of detection; LOQ, limit of quantification (LOD and LOQ in mg l^{-1}).

Table 6
Determination of aflatoxins in feed samples (figures correspond to concentrations in μg ml^{-1})

Analyte	Type of sample	Concentration added	Concentration found	Recovery	Concentration[a]
G2	Feed 1[b]	0.1	0.13	130	0.16±0.04
		0.3	0.28	93	
		0.5	0.48	96	
	Feed 2	0.1	0.08	80	0.29±0.06
		0.3	0.27	90	
		0.5	0.52	104	
	Feed 3	0.1	0.12	120	nd
		0.3	0.34	113	
		0.5	0.47	94	
G1	Feed 1	0.1	0.09	90	0.09±0.006
		0.3	0.32	106	
		0.5	0.56	112	
	Feed 2	0.1	0.1	100	0.14±0.03
		0.3	0.32	106	
		0.5	0.53	106	
	Feed 3	0.1	0.12	120	nd
		0.3	0.32	106	
		0.5	0.49	98	
B2	Feed 1	0.1	0.13	130	0.08±0.03
		0.3	0.32	106	
		0.5	0.5	100	
	Feed 2	0.1	0.1	100	0.27±0.06
		0.3	0.31	103	
		0.5	0.48	96	
	Feed 3	0.1	0.09	90	nd
		0.3	0.33	110	
		0.5	0.5	100	
B1	Feed 1	0.1	0.13	130	0.39±0.08
		0.3	0.28	93	
		0.5	0.52	104	
	Feed 2	0.1	0.09	90	0.58±0.04
		0.3	0.32	106	
		0.5	0.51	102	
	Feed 3	0.1	0.1	100	nd
		0.3	0.29	97	
		0.5	0.53	106	
OB	Feed 1	0.1	0.09	90	0.21±0.03
		0.3	0.32	106	
		0.5	0.48	96	
	Feed 2	0.1	0.11	110	0.12±0.02
		0.3	0.3	100	
		0.5	0.48	96	
	Feed 3	0.1	0.09	90	nd
		0.3	0.32	107	
		0.5	0.51	102	
OA	Feed 1	0.1	0.1	100	0.19±0.02
		0.3	0.28	93	
		0.5	0.52	104	
	Feed 2	0.1	0.08	80	0.24±0.03
		0.3	0.32	107	
		0.5	0.54	108	
	Feed 3	0.1	0.13	130	n.d.
		0.3	0.32	107	
		0.5	0.5	100	

[a] Concentrations found by using the standard addition method.
[b] Feeds 1 and 2 were samples stored at room temperature for 1 year. Feed 3, fresh feed from a pig farm. nd, not detected.

these reasons the C_{18} minicolumns were chosen to clean up samples if high sensitivity is required for the determination of mycotoxins in feed samples. By using the C_{18} minicolumn the limit of detection achieved was lower than 0.06 mg l^{-1}. The results are shown in Table 4.

3.4. Analytical application to real samples

To check the screening method, five different samples were prepared spiking a feed sample (free of mycotoxins) with different amounts of aflatoxins. The obtained results are shown in Table 5.

The first sample is a feed sample free of aflatoxins and the second sample was spiked with 0.025 mg l^{-1} of G1, both samples giving negative results in the screening system. The third sample was spiked with 0.04 mg l^{-1} of B1 giving a positive result in the screening system. This sample was preconcentrated in a C_{18} minicolumn and analysed by CE checking that the result obtained with the screening system was a false positive. The last two samples gave positive results in the screening system and these results were verified in the electrophoretic system. The fourth sample was analysed directly by CE (without preconcentrating the analytes) and the last one was preconcentrated before the analysis by CE. In summary, only two of the five samples studied were preconcentrated in the FIA system and only one false positive was obtained in the screening system. Only three of the five samples analysed in the screening system were introduced in the CE equipment. With this simple example, the usefulness of the combination of a screening system with CE equipment was demonstrated.

Three different samples were analysed, two of them were samples stored at room temperature for 1 year, and the third sample was a fresh feed from a pig farm. Table 6 summarises the obtained results. As it can be seen, mycotoxins were not found in the fresh feed from a pig farm. In order to validate the method the standard addition method was carried out. The recoveries ranged between 90 and 130%. These results indicate that the clean-up procedure can be successfully applied to the analysis of very low levels of aflatoxins in a complex matrix such as feed samples.

4. Conclusions

The combination of a screening system with a confirmatory (identification) technique, such as CE, to control aflatoxins in feed samples is an advantageous methodology from a practical point of view. When a large number of samples must be processed, the screening system allows to classify them into positive and negative samples. Only positive samples need to be confirmed as true or false positive samples by using the electrophoretic method. This screening–confirmatory combination represents an interesting strategy to increase the productivity of routine laboratories, reducing time and costs. In addition, electrophoretic technique is preserved only to process the strictly necessary samples.

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