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Note

Fluorimetric determination of aflatoxins in foodstuffs by high-performance liquid chromatography with flow injection analysis

F. LÁZARO, M. D. LUQUE DE CASTRO and M. VALCÁRCEL*
Department of Analytical Chemistry, Faculty of Sciences, University of Córdoba, Córdoba (Spain)
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Although high-performance liquid chromatography (HPLC) and flow injection analysis (FIA) have significant differences (working pressure, presence of interfaces, cost), they involve a number of common components (liquid reservoirs, pumps, injection valves and continuous detectors) and are complementary in nature. Hence attempts to use HPLC and flow injection analysis in conjuction are justified[1]. A flow injection system has been used as the precolumn reactor of an HPLC unit (Fig. 1A) to determine Zn^{II} through its activation of the catalytic effect of carboxypeptidase A on the decomposition of hippuryl-L-phenylalanine[2], and as a post-column reactor–detector in two different manners: (a) by inserting successive aliquots of the eluate from an HPLC system at high frequency into the same or different flow injection systems[3] (Fig. 1B) and (b) by applying the merging zones approach to merge the eluate from the HPLC system with a large reagent plug (Fig. 1C)[4–6].

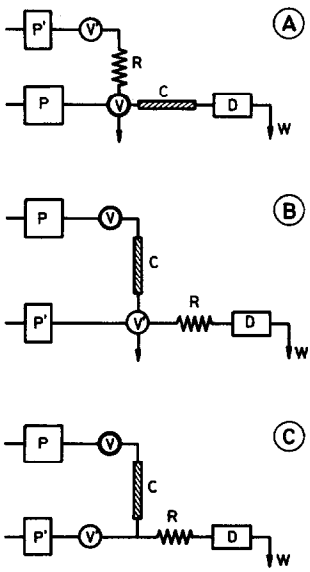

Fig. 1. Types of HPLC–FIA configurations. P, HPLC pump; P', FIA pump; V, HPLC injection valve; V', FIA injection valve; R, reactor; C, analytical column; D, detector; W, waste.

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This paper presents a new approach to the joint use of HPLC and flow injection analysis (Fig. 1C) in which the flow injection sub-system allows the total determination of several related compounds; the individual analysis for each analyte is performed by the HPLC sub-system while the flow injection sub-system acts as a post-column reactor–detector, thereby enhancing the information obtained from the sample. The best field for the joint use of these techniques is the analysis of large numbers of samples of which only a few may be of interest because their toxicity or their role as disease indicators. An example is the analysis of foods for aflatoxins. A study performed on about 300 food samples for these carcinogenic compounds revealed their presence in only 6% of them[7]. Among the different aflatoxins known, the most frequent are B_1, B_2, G_1 and G_2. Fluorimetry is the detection technique most commonly used with these substances because of their native fluorescence (λ_{ex} = 360 nm for all them, λ_{em} = 440 nm for B_1 and B_2 and 470 nm for G_1 and G_2). The maximum allowed content of aflatoxins is of the order of a few micrograms per kilogram[8], hence methods for their determination must be very sensitive. The fluorescence of aflatoxins B_1 and G_1, which is lower than that of B_2 and G_2, can be increased by using strong acids[9] or oxidants such as chloramine T[10] iodine[11] or bromine[12]. Bromine was used as the derivatizing agent in this work. It acts by adding itself to the double bond of the furan ring of aflatoxins B_1 and G_1[13], thereby increasing their fluorescence by a factor of 20 or more.

EXPERIMENTAL

Apparatus

The experimental set-up is shown in Fig. 2. The system was built from a high-pressure dual-piston pump (Model 64; Knauer, Berlin, F.R.G.), a Knauer variable-volume rotary injection valve, a small-bore, low-volume mixing Y-piece (Omnifit 2407), a 200 mm × 4 mm I.D. analytical column filled with Nucleosil 120 Å, 5 μm, C_{18}, and a Perkin-Elmer LS-1 fluorescence detector equipped with a low-volume flow cell (inner volume 4 μl) and connected to a Perkin-Elmer R100 recorder. All connections were made from 0.25 mm I.D. stainless-steel capillary tubing fitted with laboratory-constructed hand-tight Kel-F nut–ferrule combinations. The flow injection sub-system was made up of a four-channel Gilson Minipuls-2 peristaltic pump

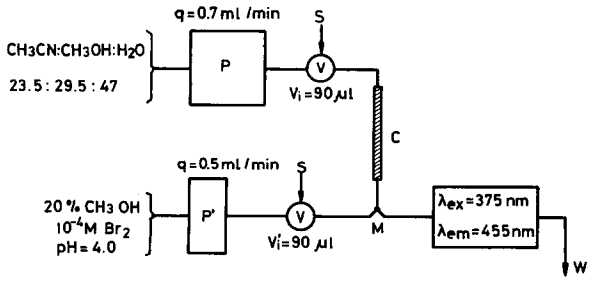

Fig. 2. Integration of HPLC–FIA for the determination of total aflatoxins by FIA and individual aflatoxins by HPLC. P, P', V, V' and C as in Fig. 1; S is the sample and M is the point of mixing of the eluent and derivatizing reagent. V' can be removed and a $1.3 \cdot 10^{-3}$ *M* bromine solution flow-rate of 0.18 ml/min must be used when only the HPLC system is employed.

and a Rheodyne 5041 variable-volume injection valve. All connections were made from 0.3 mm I.D. PTFE tubing. The temperature of the analytical column was kept at 25°C by using a thermostated water-bath.

Reagents

Stock solutions of each of aflatoxins B_1, B_2, G_1 and G_2 (Serva, Heidelberg, F.R.G.) (50 μg/ml in methanol) were prepared by weighing and checked by following the AOAC recommendations[14]. The stock solutions were diluted with methanol–water (1:4) to the desired level prior to analysis. Stock and diluted solutions were kept in the dark at 4°C.

The eluent was prepared by mixing doubly distilled water, HPLC-grade methanol and HPLC-grade acetonitrile (Scharlau, Barcelona, Spain) (47:29.5:23.5) and was microfiltered through a nylon 66 (0.45-μm pore size) filter disk and degassed *in vacuo* in an ultrasonic bath (Sonorex TK 52, Bandelin, Berlin, F.R.G.) prior to use.

Bromine solution (0.2 *M*) was prepared by pouring the contents of a 78.0-g vial of bromine (Merck, Darmstadt, F.R.G.) into 2.5 l of water. After stirring, the solution was filtered and titrated with ammonium iron(II) sulphate (using diethyl-*p*-phenylenediamine as indicator[14]) prior to storage at 4°C in a topaze flask.

All other reagents used in the extraction of aflatoxins (chloroform, benzene, hexane, sodium chloride and ammonium sulphate) were of analytical-reagent grade (Merck).

All glassware in contact with aflatoxins was cleaned with 5% sodium hypochlorite solution. Clean glassware was rinsed with dilute nitric acid (1:4) and doubly distilled water.

Extraction procedures

The procedures used were based on the methods recommended by the AOAC[14], slightly modified for use in flow injection analysis, which usually involves aqueous solutions.

Peanuts. In a 1000-ml separating funnel 100 g of peanuts were extracted with a mixture of 500 ml of methanol–water (55:45, v/v), 200 ml of hexane and 4 g of sodium chloride. After shaking and separating the layers, 25 ml of the methanol–water phase was pipetted into a 100-ml separating funnel and extracted for 1 min with 25 ml of chloroform. The chloroform phase was evaporated to dryness on a steam plate under a stream of nitrogen. The residue was dissolved in 5 ml of methanol and made up to 10 ml with doubly distilled water. The solution was filtered through a nylon 666 (0.45 μm) disc filter and extracted with 10 ml of chloroform. The chloroform phase was again evaporated to dryness and the residue was dissolved in 1 ml of methanol and made up to 5 ml with doubly distilled water.

Maize. In a 1000-ml separating funnel 50 g of maize were mixed with 10 g of diatomaceous earth and extracted with 150 ml of acetone–water (85:15, v/v) for 5 min. The supernatant solution was filtered through a Whatman 2V flutted paper and 50 ml of the filtrate solution were mixed with 20 ml of saturated ammonium sulphate solution, 130 ml of water and 10 g of diatomaceous earth. After agitation, the mixture was filtered and 100 ml of the filtrate solution were extracted with 3 ml of benzene for 30 s. The benzene phase was evaporated to dryness on a steam plate under a stream of nitrogen. The residue was treated as in the extraction of peanuts.

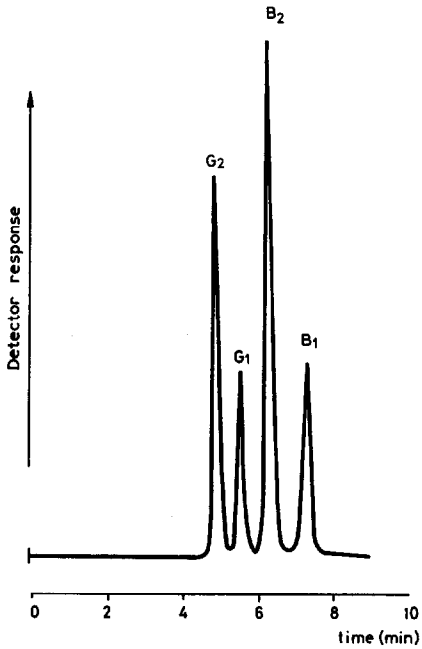

Fig. 3. Chromatogram of a test mixture of standard solutions of aflatoxins G_2, G_1, B_2 and B_1 (10 ng/ml each). Conditions as in Fig. 2.

RESULTS AND DISCUSSION

We recently reported[10] the flow injection determination of total aflatoxins. This work concerned the optimization and implementation of the determination of aflatoxins by the joint use of HPLC and flow injection analysis.

Optimization of the HPLC conditions

A previous study[10] of derivatizing reagents for aflatoxins was performed by the univariate method. The experiment considered the chemical and flow injection variables involved in order to determine the individual influence of each; thus, the modified simplex method (MSM)[15] was used in HPLC to optimize interrelated variables. The response function favours not only the fluorescence intensity of the four peaks, but also less overlapping between peaks and the minimum analysis time. Watson and Carr's response function[16], slightly modified, was used.

The optimum composition of the mobile phase found by the MSM was acetonitrile–methanol–water (23.5:29.5:47), allowing the complete resolution of the four peaks.

The concentration and flow-rate of the bromine solution and the flow-rate of the mobile phase were interrelated; the MSM was also applied and gave optimum values of 0.7 ml/min for the mobile phase (column pressure 120 atm), 0.18 ml/min for the bromine solution (four times lower to avoid excessive dilution of the eluted

TABLE I

FEATURES OF THE HPLC METHOD

Aflatoxin	*Residence time (min)*		*Calibration graph*				
	Mean value	*R.S.D. (%)*	*Intercept, % I_f*	*Slope, % I_f/ (ng/ml)*	*Correlation coefficient*	*Determination range (ng/ml)*	*R.S.D. (%) (n = 11)*
G_2	5.2	1.0	0.02	0.432	0.9998	0.5–200.0	1.7
G_1	5.8	1.1	0.14	0.199	0.9998	0.5–200.0	1.0
B_2	6.5	1.1	0.37	0.560	0.9998	0.5–200.0	1.7
B_1	7.5	0.9	0.13	0.206	0.9999	0.5–200.0	1.8

analytes) and $1.3 \cdot 10^{-3}$ *M* bromine solution (the pH of the aqueous phase was 4.0, *i.e.*, the optimum for this determination).

The methanol content of the bromine solution did not affect the analytical signal up to 20–30%; therefore, the bromine and sample solutions contained 20% of methanol to facilitate the dissolution of aflatoxins and to avoid disturbance of the signal due to changes in the refractive index and dielectric constant.

The maximum analytical signal was obtained by directly connecting the Y connector (point M in Fig. 2) to the detector, the heights of all peaks decreasing with increase in the length of the post-column reactor. This is accounted for by the high rate of the bromine–aflatoxin reaction and the increasing dilution of the sample plug with increasing reactor length.

Features of the calibration graphs

A chromatogram of a test mixture containing four aflatoxins obtained with the HPLC system is shown in Fig. 3. Complete resolution of the peaks was achieved

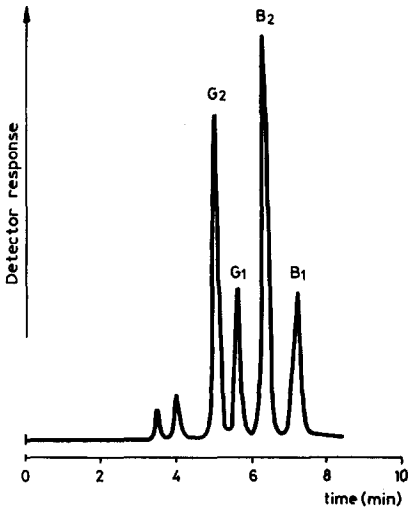

Fig. 4. Chromatogram of an extract of maize spiked with aflatoxins G_2, G_1, B_2 and B_1 (16.4 ng/ml each). Conditions as in Fig. 2.

TABLE II

RECOVERIES OF AFLATOXINS ADDED TO PEANUT AND MAIZE SAMPLES OBTAINED BY THE HPLC METHOD

Sample	*Added (ng)*	*Recovery (%)*			
		G_2	G_1	B_2	B_1
Peanut 1	16.4	104.1	106.9	99.6	100.9
	32.8	101.9	103.2	98.6	100.3
Peanut 2	16.4	106.1	107.2	99.4	99.7
	32.8	106.3	107.5	100.7	99.3
Maize 1	16.4	104.4	104.3	104.7	93.2
	32.8	103.8	101.7	100.2	102.9
Maize 2	16.4	99.2	99.4	101.9	100.5
	32.8	99.7	100.5	98.9	98.4

in 7.5 min. Relevant data for the analytical system are summarized in Table I. The precision was studied with a solution containing 30 ng/ml of each aflatoxin. The within-day repeatabilities for both retention times and peak height showed that the HPLC system was not subject to large fluctuations. The calibration graphs have excellent correlation coefficients (greater than 0.999) and a determination range of 0.5–200.0 ng/ml in all instances.

Application of the HPLC method to maize and peanut samples

A study of the recovery of aflatoxins B_1, B_2, G_1 and G_2 from extracts of maize and peanut (the two matrices in which these toxins occur most frequently) by the proposed method was performed. The concentrations of the analytes in the samples were well above the lower limit of the method (0.5 ng/ml). The analysis was subject to no interferences from other compounds present in the sample (see Fig. 4). The average recovery obtained on addition of 16.4 and 32.8 ng of each aflatoxin to different samples of maize and peanut was 101.7%, with an average deviation of ±2.6% from 100% (see Table II, which shows the reliability of the proposed method).

TABLE III

FEATURES OF THE CALIBRATION GRAPHS FOR THE INTEGRATED HPLC–FIA METHOD

Aflatoxin	*Intercept, % I_f*	*Slope, % I_f/(ng/ml)*	*Correlation coefficient*	*Determination range (ng/ml)*	*R.S.D. (%) (n = 11)*
$G_2+G_1+B_2+B_1$ (FIA)	0.18	0.468	0.9999	1.0–200.0	1.7
G_2 (HPLC)	0.26	0.375	0.9998	1.0–200.0	1.8
G_1 (HPLC)	0.12	0.121	0.9999	1.0–200.0	2.0
B_2 (HPLC)	0.26	0.327	0.9998	1.0–200.0	1.9
B_1 (HPLC)	0.13	0.132	0.9998	1.0–200.0	2.8

TABLE IV

RECOVERIES OBTAINED BY FLOW INJECTION ANALYSIS (INTEGRATED HPLC–FIA) OF TOTAL AFLATOXINS ADDED TO PEANUT AND MAIZE SAMPLES

Sample	*Added (ng)*	*Found (ng)*	*Recovery (%)*
Peanut 1	10.0	10.5	105.0
	20.0	19.2	96.0
Peanut 2	10.0	10.6	106.0
	20.0	18.6	93.0
Maize 1	10.0	9.8	98.0
	20.0	20.8	104.0
Maize 2	10.0	9.5	95.0
	20.0	19.0	95.0

Integrated HPLC–flow injection analysis

The development of this integrated method requires a compromise to be made between the optimum non-coincident values of the variables that affecting the two methods. The emission wavelength chosen was 455 nm [intermediate between the optimum values for flow injection (470 nm) and HPLC (440 nm)]. The most important of the non-coincident variables was the flow-rate of the bromine solution; by using the optimum value for flow injection (1.5 ml/min), the dispersion suffered by the chromatographic peaks was excessive, whereas the optimum flow-rate for HPLC (0.2 ml/min) dramatically reduced the sensitivity of the flow injection method. An intermediate flow-rate (0.5 ml/min) was used with a bromine concentration of $1 \cdot 10^{-4}$ *M*. Under these conditions, the maximum of the flow injection peak was obtained within 18 s and the complete chromatogram was obtained in less than 8 min.

TABLE V

RECOVERIES OBTAINED BY THE HPLC METHOD (INTEGRATED HPLC–FIA) OF AFLATOXINS ADDED TO PEANUT AND MAIZE SAMPLES

Sample	*Added (ng)*	*Recovery (%)*			
		F_2	G_1	B_2	B_1
Peanut 1	16.4	100.4	100.2	100.4	97.7
	32.8	105.8	103.6	106.7	103.5
Peanut 2	16.4	97.0	99.4	98.5	101.6
	32.8	95.3	97.9	96.7	101.6
Maize 1	16.4	101.1	104.6	99.6	104.3
	32.8	102.5	105.6	101.3	105.3
Maize 2	16.4	98.4	100.1	97.3	98.6
	32.8	99.1	98.1	98.1	96.5

Features of the calibration graphs obtained by the integrated method

Table III gives the data for the calibration graphs for HPLC and flow injection analysis. The calibration graphs for the latter method was run with equimolar amounts of the four aflatoxins. The retention times and their relative standard deviations are very similar to those listed in Table I, the correlation coefficients being excellent with a determination range of 1.0–200.0 ng/ml in all instances.

Application of the integrated HPLC–flow injection method to the analysis of peanut and maize samples

The excellent recoveries achieved on addition of different amounts of aflatoxins B_1, B_2, G_1 and G_2 to maize and peanut extracts (100.7% and 99.0% for the HPLC and flow injection methods, respectively) are shown in Tables IV and V, with average deviations of ±2.8% and ±4.5% from 100%. The lower accuracy of the results obtained with the flow injection method could be due to the occurrence in the maize and peanut extracts of interferents with fluorescent properties under the working conditions adopted. These interferents are separated and eluted before the analytes in HPLC (Fig. 4), whereas in flow injection analysis the blank signal due to these compounds is in the range 0.4–2.0% I_f in the maize extracts and 4–8% I_f in the peanut extracts. This necessitates the injection of two successive aliquots of each sample into the flow injection sub-system to determine the real concentrations of the aflatoxins. One aliquot provides a signal due to blank + analytes; in the other, 5 μl of 11 g/l sodium hypochloric solution per ml of sample are added to destroy the aflatoxins present[14], the measurement of this sample providing the blank signal. Studies performed with different extracts with or without aflatoxins revealed different reactivities of the sample matrix, even in samples of the same food. This is the source of the error in the recovery. Nevertheless, the error does not exceed 7%; in addition, hazardous samples can always be analysed by HPLC.

CONCLUSIONS

The proposed integrated method combines the advantages of flow injection analysis, namely rapidity, simplicity and economy, with the separation capability of HPLC. In addition, it avoids the use of HPLC for routine control and results in major time and cost savings. At present our team is engaged in the automation of integrated HPLC–flow injection analysis configurations by using a sampler, a selecting valve and a microcomputer to control the overall process. To simplify and accelerate the extraction process, ultrasound will be applied directly to pulverized solid samples by using a suitable solvent, either off- or on-line[17].

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