ELSEVIER

Journal of Chromatography A, 721 (1996) 69–74

JOURNAL OF CHROMATOGRAPHY A

Postcolumn excitation of aflatoxins using cyclodextrins in liquid chromatography for food analysis ☆

A. Cepeda[a,*], C.M. Franco[a], C.A. Fente[a], B.I. Vázquez[a], J.L. Rodríguez[a], P. Prognon[b], G. Mahuzier[b]

[a] *Laboratorio de Higiene e Inspección de Alimentos, Dpto. de Química Analítica, Nutrición y Bromatología, Facultad de Veterinaria, Universidad de Santiago de Compostela, 27002-Lugo, Spain*

[b] *Laboratoire de Chimie Analytique II, Faculté de Pharmacie, rue J.B. Clèment, 92290 Chatenay-Malabry, France*

First received 1 December 1994; revised manuscript received 28 April 1995; accepted 10 May 1995

Abstract

Measurement of fluorescence increase was used for the comparative quantification of the effect that several cyclodextrins (α-, β-, heptakis-2,6-β-*o*-dimethyl- and γ-) produce on the fluorescent response of aflatoxins B_1 and G_1. This constitutes a new chromatographic method with stability of the mobile phase, and shows general improvements in the chromatographic conditions with respect to other methods (especially those using an iodine reservoir as a postcolumn reactor). A C_{18}-type column was used, with methanol–water (60:40, v/v) as the mobile phase. The excitation phase of the natural fluorescence of aflatoxins, a 10^{-2} *M* solution of each cyclodextrin, was introduced postcolumn. The determination of the elution order aflatoxin $G_2 > G_1 > B_2 > B_1$ was performed for each phase in less than 15 min. As expected using an aqueous–alcoholic medium, an increase in the fluorescence response of aflatoxins with an unsaturated furanic ring was found to occur with all the cyclodextrins studied, except γ-cyclodextrin. The observed increase was larger for heptakis-2,6-β-*o*-dimethyl- than for β-cyclodextrin (to our knowledge, the only cyclodextrin previously described in the literature to serve for the determination of aflatoxins). The difference is of the order of 70.1-fold in the case of aflatoxin G_1 and 45.2-fold in the case of aflatoxin B_1. The detection limit in the mobile phase used was determined (for aflatoxin B_1) for β-cyclodextrin and 2,6-β-*o*-dimethylcyclodextrin (signal-to-noise ratio 1:3) to be 4 and 9 mg l^{-1}, respectively.

1. Introduction

The aflatoxins (Af) B_1, B_2, G_1 and G_2 (Fig. 1) are carcinogenic toxins produced by *Aspergillus flavus* and *Aspergillus parasiticus* fungi under favourable environmental conditions [1–6]. Nowadays, an increasing number of countries have established controls of the Af content in both animal and human foods [7]. This great interest in the determination and control of Af was the reason for the development of a number of detection methods (mostly based on chromatography and immunology) at the ppb level [8–11]. Some reviews on this methodology are available [12].

The natural fluorescence of Af arises from their oxygenated pentaheterocyclic structure. The fluorescence capacity of Af B_2 and G_2 is

* Corresponding author.

☆ Presented at the *23rd Annual Meeting of the Spanish Chromatographic Society, Peñiscola, 19–21 October 1994.*

0021-9673/96/$15.00
SSDI 0021-9673(95)00566-8

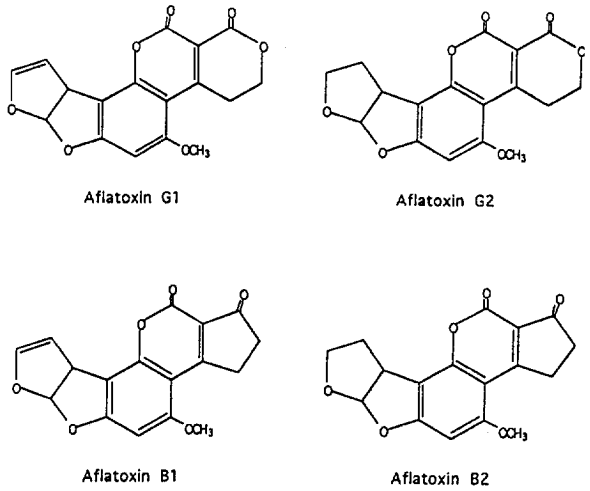

Fig. 1. Structures of the aflatoxins.

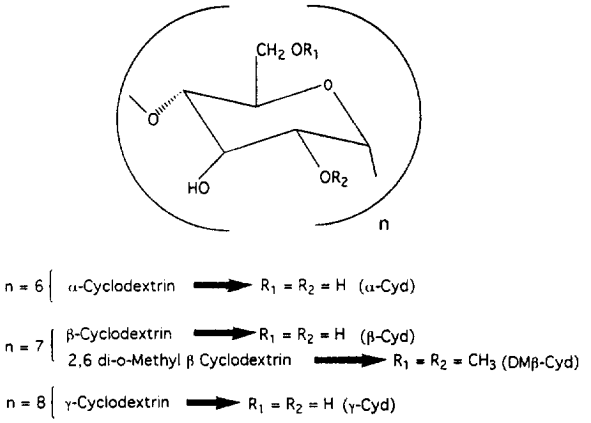

Fig. 2. Structures of the cyclodextrins.

much larger (ca. ten times) than that of Af B_1 and G_1, probably owing to the structural difference, namely a double bond on the furanic ring. Such a double bond seems to be very important for the photophysical properties of these derivatives measured just after spectroscopic studies [13–15]. Owing to the high toxicity of Af B_1, it has been widely investigated, and the notable difference in the fluorescence signal has been used to develop sensitive and specific HPLC methods.

The excitation of the natural fluorescence of Af B_1 (and correspondingly of Af G_1) can be promoted in many different ways, the postcolumn iodination method being the most often used [16–18]. This method was also adopted as a reference throughout the European Community (EC) Standards, Measurements and Testing Programme. However, we consider that the halogenation method has several drawbacks: extended time for the establishment of the mobile phase (at least 1 h), heating to 75°C and, above all, the physical and mechanical deterioration that the postcolumn reservoir pump suffers owing to its contact with iodine.

An alternative way of avoiding these inconveniences, allowing at the same time excitation of the fluorescence response of Af B_1 and G_1, is the use of cyclodextrins. Cyclodextrins (Cyd; see Fig. 2) are molecules with a chiral–toroidal configuration, formed by action of the enzyme Cyd-transglycolase on dextrans. These cyclic oligomers contain 6–8 glucose units in an α-union [1–4]. Several kinds of cyclodextrins are commercially available, including α-, β-, γ-, hydroxypropyl- and 2,6-*o*-methyl-β-), and their physical and chemical properties are well described in the literature [19].

These oligomers are capable of enclosing a large number of organic and inorganic species in their cavity. To our knowledge, only in one study [20] was β-Cyd introduced into an RP-HPLC system via a postcolumn method to increase the fluorescence response of Af B_1 and G_1. However, even there [20], no reference was made to the spectroscopic characteristics of the interaction that takes place during the formation of the Af–Cyd inclusion complex. The study of the reactions between various Cyd and coumarin derivatives (which have a structure similar to Af) was of interest to us, especially for their subsequent application as a detection method in RP-LC [21,22]. In view of the similarity between the coumarin derivatives and Af, we studied the spectroscopic properties originating from the interaction of several Cyd with the Af of interest [23]. This study allowed us to demonstrate that high Cyd concentrations are necessary to evaluate the excitation effect, and that the response produced by 10^{-2} *M* 2,6-β-*o*-dimethyl-Cyd (DMβ-Cyd) is 2–3 times larger than that by β-Cyd, 8 times larger than that by α-Cyd and 16

times larger than that by γ-Cyd (which showed no noticeable effect).

The interaction between Cyd and Af in the chromatographic system was performed as in a previous study [24]. The thermodynamic parameters that govern the interaction permit the evaluation of Cyd as versatile fluorophores for their postcolumn use in an RP-HPLC system. The use of a high Cyd concentration together with a mobile phase containing methanol indicates that only partial inclusion of the aflatoxins takes place, since methanol molecules are competitors for the Cyd cavity.

In this work, we intended to apply the previous knowledge to a comparative study of the effect of several Cyd eluents on the fluorescent response of Af B_1 and G_1. An analytical method was developed, based on the postcolumn use of the Cyd solution that promotes the greatest excitation of the fluorescence of Af B_1 and G_1. Such an excitation should be comparable to that promoted by halogenation, and the other conditions of use (stability of the solution, identical ratio between the Af B_1 and G_1 responses, etc.) should be maintained.

2. Experimental

2.1. Reagents

All the reagents used were of analytical-reagent grade. Methanol was distilled before use in chromatography and water was obtained with a Milli-Q system (Millipore, Waters Chromatography, Bedford, MA, USA). Cyclodextrins (α-, β-, γ-, and heptakis-2-,6-β-*o*-dimethyl-) and aflatoxins B_1, G_1, B_2 and G_2 were purchased from Aldrich (Milwaukee, WI, USA).

Prior to their chromatographic use, each solution was filtered through a filter of 0.45-μm pore size (HA) under vacuum (Millipore, Waters Chromatography).

Stock standard solutions of each aflatoxin were prepared by dissolving 5 mg in 20 ml of methanol–chloroform (1:1 v/v). The solutions were stored in amber-coloured flasks to protect them against light.

Working aflatoxin solutions were prepared by diluting the stock standard solutions with a solution identical with that used for the mobile phase. The concentrations of each aflatoxin were B_1 45, G_1 227, B_2 90 and G_2 227 ng/l.

2.2. Apparatus

For liquid chromatography, the following apparatus and conditions were used: column, Sphery, 5 μm (220 mm × 4.6 mm I.D.) (Brownlee); mobile phase, methanol–water (40:60, v/v); flow-rate, 1 ml/min; pump, LC9A (Shimadzu, Kyoto, Japan); injector, Rheodyne Model 7125 equipped with a 20-μl loop; fluorescence detector, RF-530 (Shimadzu), with λ_{ex} = 365 nm and λ_{em} = 418 nm; and integrator, LC5A (Shimadzu).

For excitation of the fluorescent response, an LC9A pump (Shimadzu) and a VALCO T micromixer connected from the exit of the chromatographic column to the detector by means of steel tubes with I.D. of 0.016 mm were used. The tube length from the mixer to the detector was 30 cm. Post-column reagents were 10^{-2} *M* aqueous solutions of each cyclodextrin. The flow-rate was 1 ml/min.

3. Results and discussion

From the results obtained, we can conclude that DMβ-Cyd and β-Cyd provide suitable cavities for exciting the fluorescent response of Af B_1 and G_1 through the formation of inclusion complexes.

The spectroscopic consequence of the formation of the inclusion complex is an increase in fluorescence. The mechanism involved in this process is not yet completely understood, but seems to be induced by the rigid matrix formed at the molecular level S_1 (particularly excited level). Such a matrix reduces the vibrational relaxation of the aflatoxin, at the same time increasing the transparency of Cyd molecules to UV radiation, which facilitates the excitation of the enclosed molecule [22].

The excitation and emission wavelengths were

selected to agree with the experimental results measured for Af B_1 [23] and literature values [20].

For the mobile phase, a methanol–water (60:40, v/v) at a flow-rate of 1 ml/min was chosen. Under these conditions, one analysis can be performed in less than 15 min. For the comparison between the different Cyd, a concentration of 10^{-2} *M* was used owing to the solubility limit of β-Cyd in aqueous solution. The order of elution in the mobile phase of the aflatoxins used was $G_2 > G_1 > B_2 > B_1$ (see Fig. 3).

Table 1 summarizes the response ratios measured for each Cyd with respect to their natural fluorescence. It can be observed that the fluorescence increase of Af B_1 and G_1 promoted by each Cyd was different. DMβ-Cyd promoted the largest excitation, which was 2.7 times larger than that promoted by β-Cyd for both aflatoxins. The signal ratio between β-Cyd and α-Cyd is in disagreement with previous studies [24], from which one should expect a larger response by α-Cyd with a similar concentration (in a pure aqueous solution). This result reinforces the idea that methanol plays an important role, on the one hand as a competitor with the aflatoxins for the Cyd cavity, and on the other diminishing the K_f value. For this reason, the methanol content in the mobile phase was chosen as a compromise

Table 1
Fluorescence signal ratio for the different aflatoxins excited by different cyclodextrins

Fluorescence signal ratio[a]	Aflatoxin[b]			
	G_2	G_1	B_2	B_1
$F_\alpha F_0$	1.08	5.30	1.10	5.6
$F_\beta F_0$	1.16	26.3	0.98	17
F_{DM}/F_0	1.10	70.1	0.96	45.2
F_β/F_α	1.08	4.9	0.90	3.0
F_{DM}/F_β	1.09	2.69	0.88	2.7

[a] Precolumn mobile phase, methanol–water (40:60, v/v), flow-rate 1 ml/min; postcolumn mobile phase, 10^{-2} *M* solution of each cyclodextrin.
[b] Averages of three measurements.

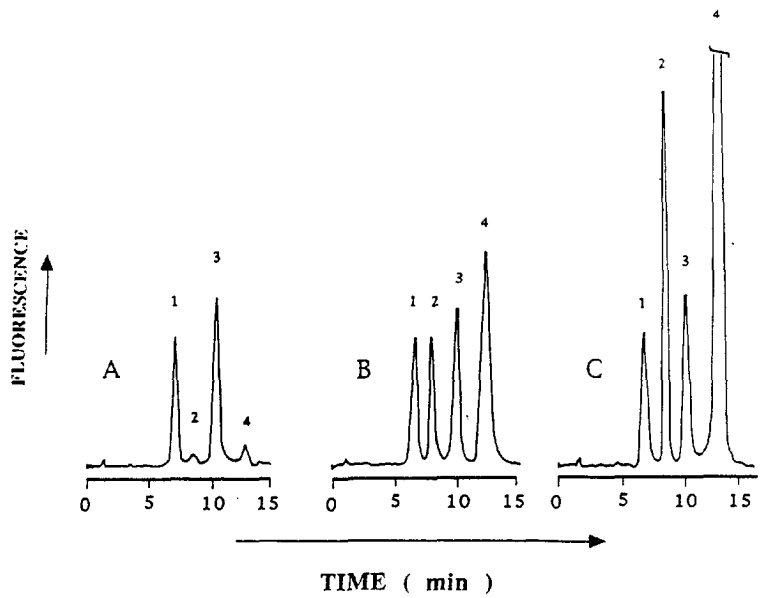

Fig. 3. Comparison of the different chromatograms: (A) without Cyd; (B) with addition of β-Cyd, 10^{-2} *M*; (C) with addition of DMβ-Cyd, 10^{-2} *M*. Peaks: 1 = Af G_2; 2 = Af G_1; 3 = Af B_2; 4 = Af B_1.

Table 2
Fit parameters of linear regressions to the chromatograms measured for Af B_1 and G_1 using β-Cyd and DMβ-Cyd (10^{-2} *M* postcolumn)

Postcolumn reagent	Af G_1			Range (ppb)	Af B_1			Range (ppb)
	a	*b*	*r*		*a*	*b*	*r*	
Without Cyd	0.1081	−0.6453	0.997	60.25–1000	0.2476	−7.2145	0.991	60.25–1000
β-Cyd	0.8767	8.1347	0.9997	6.25–1000	1.7668	1.2932	0.997	6.25–1000
DMβ-Cyd	2.1795	−1.6832	0.9999	6.25–1000	4.6532	−0.2198	0.9996	4.2–1000

which should minimize the competition for the Cyd cavity, while providing the lowest acceptable analysis time and correct sensitivity [$\alpha_s(G_1/G_2) = 1.23$; $\alpha_s(B_2/G_1) = 1.25$; $\alpha_s(B_1/B_2) = 1.51$].

Another interesting point is the large fluorescent excitation produced by DMβ-Cyd with respect to the natural fluorescence of Af B_1 and G_1 (ca. 45 and 70 times, respectively).

The ratio between the signals measured for Af B_1 and G_1 was 0.43 for DMβ-Cyd and 0.44 for β-Cyd. The close similarity between these results confirms the assumption that the introduction of Af inside each Cyd is only partial and occurs at a position as yet unknown. In the case of α-Cyd, however, the signal ratio was lower (0.31). In all cases the measured ratios were the same for at least eight successive injections.

Table 2 shows the fit parameters of the linear regressions performed on the chromatograms. The reproducibility of the chromatographic conditions was checked for β-Cyd. Eight independently prepared solutions were injected and the signals recorded. The statistics of the results are presented in Table 3, for both reproducibility and repeatability, which corresponds to nine consecutive injections of the same solution. The R.S.D. calculated from measurements of the height of the chromatographic peaks was about 3% in all cases, except for G_2, for which the R.S.D. was slightly lower. As can be observed in Table 3, the use of β-Cyd resulted in a more stable signal than the use of DMβ-Cyd.

The detection limit (expressed in mg l^{-1}) of each aflatoxin was determined with DMβ-Cyd and β-Cyd at a concentration of $5 \cdot 10^{-3}$ *M* (as

Table 3
Reproducibility (injections of eight different solutions) and repeatability (nine consecutive injections of the same solution) of the chromatographic conditions

Parameter[a]	Aflatoxins							
	G_2		G_1		B_2		B_1	
	β-Cyd	DMβ-Cyd	β-Cyd	DMβ-Cyd	β-Cyd	DMβ-Cyd	β-Cyd	DMβ-Cyd
X (1)	29.9		33.1		62.8		19.7	
S_{n-1} (1)	0.53		1.08		2.03		0.65	
R.S.D. (%) (1)	1.82		3.27		3.23		3.31	
X (2)	34.5	34.8	31.9	84.6	73.7	75.9	24.5	62.6
S_{n-1} (2)	1.2	1.73	0.94	4.70	2.87	4.60	0.76	0.79
R.S.D. (%) (2)	3.46	4.91	2.95	5.60	3.89	7.64	3.09	12.5

[a] (1) Reproductibility: *X* = average of the values (height in mm) of the eight chromatographic peaks. (2) Repeatability: *X* = average of the values (height in mm) of the nine chromatogrphic peaks.

Table 4
Detection limits ($\mu g\ l^{-1}$) for each aflatoxin with β-Cyd and DMβ-Cyd at a concentration of both of $5 \cdot 10^{-3}$ *M* Cyd measured after the T-mixer

Aflatoxin	Without Cyd	β-Cyd	DMβ-Cyd
G_2	3	4	4
G_1	200	20	6
B_2	1	2	4
B_1	125	9	4

determined after the T-mixer). These results are given in Table 4.

Acknowledgement

Special appreciation is extended to Dr. Luís Liz for assistance with the manuscript.

References

[1] P.M. Scott, J. Assoc. Off. Anal. Chem., 70 (1987) 276.
[2] J. Leitas, G. de Saint Blanquat, J.R. Bailly and Ch. Paillas, J. Chromatogr., 435 (1988) 229.
[3] S.J. van Rensburg, P. Cook-Mozaffari, D.J. van Schalkwyk, J.J. van Der Watt, T.J. Vincent and J.F. Purchase, Br. J. Cancer, 51 (1985) 713.
[4] W.F. Busby and G.N. Wogen, in C.E. Searleed (Editor), Aflatoxins, American Chemical Society, Washington, DC, 1985, pp. 1–15.
[5] F.Z. Kiermeir, Z. Lenensm.-Unters.-Forsch., 180 (1985) 389.
[6] V.L. Diener, R.E. Petit and R.J. Cole, in H.E. Pattee and C.T. Young (Editors), Peanut Science and Technology, American Peanut Research and Education Society, Yoakum, TX, 1982.
[7] H.P. van Egmond, Food Addit. Contam., 6 (1985) 139.
[8] R.B. Shashidhar, Y. Ramakrishna and V. Ramesh Bhat, Anal. Lett., 21 (1988) 507.
[9] P.J. Cotty, Appl. Environ. Microbiol., 54 (1988) 274.
[10] A.P. Wilkinson, D.W. Denning and M.R.A. Morgan, Hum. Toxicol., 7 (1988) 353.
[11] B.L. van Duren, C. Tze-Lock and F.M. Irani, Anal. Chem., 40 (1968) 2024.
[12] M. Holcomb, D.M. Wilson, M.W. Trucksess and H.C. Thompson, Jr., J. Chromatogr., 624 (1992) 341.
[13] M.N. Dirr, Int. J. Biochem., 19 (1987) 1137.
[14] D.L. Orti, J. Grainger, D.L. Ashley and R.H. Hill, J. Chromatogr., 462 (1989) 269.
[15] P.G. Thiel, S. Stockenstro and P.S. Gathercole, J. Liq. Chromatogr., 9 (1986) 103.
[16] L.G.M.T. Tuinstra and W. Haasnoot, J. Chromatogr., 282 (1983) 457.
[17] H. Jansen, R. Hansen, U.A.Th. Brinkman and R.W. Frei, Chromatographia, 24 (1987) 555.
[18] R.W. Beaver, D.M. Wilson and M.W. Truckness, J. Assoc. Off. Anal. Chem., 73 (1990) 579.
[19] S. Sozsef, Cyclodextrin Technology, Kluwer, Dordrecht, 1988.
[20] O.J. Francis, Jr., G.P. Kirschenheuter, G.M. Ware, A.S. Carman and S.S. Kuan, J. Assoc. Off. Anal. Chem., 71 (1988) 725.
[21] J. Blais, P. Prognon, G. Mahuzier and P. Vigny, J. Photochem. Photobiol., 21 (1988) 455.
[22] A. Cepeda Saez, P. Prognon, G. Mahuzier and J. Blais, Anal. Chim. Acta, 211 (1988) 331.
[23] M.L. Vazquez, A. Cepeda Saez, P. Prognon, G. Mahuzier and J. Blais, Anal. Chim. Acta, 255 (1991) 343.
[24] M.L. Vazquez, C.M. Franco, A. Cepeda Saez, P. Prognon and G. Mahuzier, Anal. Chim. Acta, 269 (1992) 239.