Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1059 (2004) 13–16
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Simultaneous determination of aflatoxins and ochratoxin A in food using a fully automated immunoaffinity column clean-up and liquid chromatography–fluorescence detection

D. Chan*, S.J. MacDonald, V. Boughtflower, P. Brereton

Central Science Laboratory, Sand Hutton, York YO41 1LZ, UK

Received 31 December 2002; received in revised form 22 April 2004; accepted 29 September 2004

Abstract

An automated HPLC method for the simultaneous detection of aflatoxins (AF) and ochratoxin A (OA) has been developed. The method uses an immunoaffinity column containing antibodies specific to both AF and OA. The samples were extracted with an acetonitrile/water mixture and diluted with phosphate buffer saline (PBS). The aqueous extracts were then transferred to an ASPEC HPLC system for automated clean-up using AflaOchra™ immunoaffinity columns. OA and AF were quantified using HPLC with fluorescence detection, with a run time of approximately 40 min. Limits of quantification were estimated as 0.2 μg/kg for OA and AFB_1, AFB_2, AFG_1 and AFG_2. Initial validation of this method gave average recoveries of 72–101% for OA and AF for a range of food products (maize cereal products and peanut butter). Within laboratory RSD_r and RSD_R for a 5.0 μg/kg spike level in maize cereals was found to be 7.6–10.1% (AF and OA) and 10.2–13.8%, respectively.

Keywords: Food analysis; Cereals; Nuts; Mycotoxins; Aflatoxins; Ochratoxin A

1. Introduction

Aflatoxins (AF) and ochratoxin A (OA) are fungal metabolites which can occur in food. Aflatoxins are known potent carcinogens, whilst ochratoxin A is regarded as nephrotoxic and carcinogenic [1]. The European Scientific Committee for Food has advised that levels of these contaminants should be reduced to the lowest levels technologically achievable. To attain this goal, a new Commission Regulation (EC) No. 466/2001 came into force on 8th March 2001, limiting levels of aflatoxins to 2 μg/kg aflatoxin B_1 and 4 μg/kg total aflatoxin in nuts, dried fruit and cereals intended for direct human consumption. Limits for OA of 5 μg/kg for raw cereals, 3 μg/kg for cereals intended for human consumption and 10 μg/kg for dried vine fruit were subsequently introduced in March 2002. Other commodities such as cocoa, wine and spices will be reviewed and added to future legislation if required. A variety of well established methodologies already exist for analysing OA and AF in many different food commodities [2]. One of the most widely used tools for OA and AF detection in food employs immunoaffinity clean-up columns, allowing for a much lower limit of detection compared to SPE column clean-up (e.g. C_{18}) and has been demonstrated to give accurate and reproducible results [3]. However, until more recently most immunoaffinity columns were specific to only one type of toxin (i.e. either to OA or AF only). This often requires separate extractions, clean-up and/or detection methods if quantification of both toxins is required. In view of the legislation for OA and AF, it is desirable to have a single method of analysis for both toxins, using a single extraction and detection method. This will increase sample throughput and reduce consumable costs. To overcome this, a technique using linked OA and AF immunoaffinity clean-up columns has been previously reported by Scudamore et al. [4] for cereal based pet foods. This method allows for

* Corresponding author. Tel.: +44 1904 462507; fax: +44 1904 462111.
E-mail address: d.chan@csl.gov.uk (D. Chan).

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.09.096

the simultaneous detection of OA and AF in the same analytical run (after manual column clean-up), with recoveries averaging 100% for AF, but below 60% for OA. In order to improve the efficiency and performance of this previous method, we have developed a fully automated clean-up and HPLC analysis method using a new type of immunoaffinity column with anti-bodies specific to both AF and OA. We report here the development and performance characteristics of this new method for maize cereals and peanut butter.

2. Experimental

2.1. Materials and reagents

2.1.1. Immunoaffinity columns

AflaOchra™ HPLC immunoaffinity columns were obtained from Vicam (Labtech Int. Ltd., Sussex). These columns (single use only) have a quoted range of 0.25–100 μg/kg with at least 70% recovery [5].

All solvents employed were of glass distilled or HPLC grade. All other reagents were of AR grade or equivalent. Aflatoxins and ochratoxin A were purchased from Sigma (purity not stated). Phosphate buffered saline was prepared by adding potassium chloride (0.2 g), potassium dihydrogen phosphate (0.2 g), anhydrous disodium hydrogen phosphate (1.16 g) and sodium chloride (8.0 g) to water (900 mL). The pH was adjusted to 7.4 ± 0.1 with 0.1 M HCl or 0.1 M NaOH as appropriate, and the volume made to 1 L with water. Pyridinium hydrobromide perbromide (Sigma) solution (50 mg/L) was made fresh when required.

2.2. General procedures

Samples were extracted and analysed as described below. Aqueous sample extracts (50 mL) were loaded onto an ASPEC XL system with Unipoint HPLC control software (Gilson, Anachem Luton) for automated immunoaffinity column clean-up and on-line HPLC analysis [6,7].

Peanut butter samples were prepared as a homogenised slurry (5 parts nut, 4 parts water by weight) in a food processor. Cereal samples were ground (through a 2 mm screen) and mixed thoroughly on a tumbler prior to extraction.

Sub-samples were extracted for AF and OA (36 g for nut slurries, 20 g for cereals) with an acetonitrile/water mixture (84 mL, 60:24 (v/v) for nut slurry samples and 100 mL, 60:40 (v/v) for cereals) by homogenising with an Ultra-Turrax blender for 3 min. The mixture was filtered through a Whatman 113V paper and the filtrate (10 mL) was diluted to 150 mL with PBS. AF and OA were cleaned-up and detected simultaneously on an ASPEC XL system.

The automated clean-up step was carried out in a similar way as for individual aflatoxins and ochratoxin A [6,7]. The immunoaffinity column (AflaOchra, Vicam) was conditioned with PBS (20 mL) at a rate of approximately 3–6 mL/min. The aqueous sample extract (50 mL) was loaded onto the column at a rate of no more than 3 mL/min. The column was washed with water (10 mL) and then dried by passing air through the column (approximately 3 mL) in order to remove any remaining water prior to quantitative elution. Ochratoxin A and aflatoxins were eluted with methanol (2 mL). An aliquot of the eluate (1 mL) was collected for storage. The remaining eluate (1 mL) was diluted with 2% aqueous acetic acid (2 mL) for HPLC analysis. Automated clean-up normally takes approximately 30 min per sample, during which HPLC analysis takes place (see below).

HPLC conditions used a modified method previously described by Scudamore et al. [4], a mobile phase switching device (Janitor unit) is used here instead of a gradient mobile phase. Two different mobile phases, (A) methanol/acetonitrile/0.1% phosphoric acid (24:24:52) and (B) methanol/acetonitrile/0.1% phosphoric acid (54.4:14.4:31.2) were used on a Spherisorb ODS1 Excel HPLC column (250 mm × 4.6 mm i.d.). A Janitor unit was programmed to run mobile phase (A) (0–15 min), then (B) (15–30 min) and finally (A) again (30–40 min). The mobile phase was pumped at 1.0 mL/min (Gilson 307 pump). Injections were carried out after automated clean-up, using an automated Rheodyne switching valve and partial loop fill method. Post-column derivatisation of AFB_1 and AFG_1 was achieved through a zero dead volume T-piece and 30 cm × 0.3 mm i.d. stainless steel reaction tube. Pyridinium hydrobromide perbromide solution (50 mg/L) was added at 0.3 mL/min (Gilson 307 pump). The mycotoxins eluted in the order of G_2, G_1, B_2, B_1 (aflatoxins) and OA at ca. 10, 12, 13, 15 and 25 min, respectively. Detection was via a JASCO FP1520 fluorescence detector and was time programmed to detect aflatoxins at 364 nm (excitation) and 440 nm (emission) (0–18 min) and ochratoxin A at 333 nm (excitation) and 477 nm (emission) (18 min onwards).

2.3. Method validation

This method was initially validated by analysis of replicate spiked samples (AF and OA, $n = 5$ or 6) at 5.0 μg/kg for a variety of maize matrices and peanut butter. Additionally, peanut butter was also validated at a low spiking level of 0.2 μg/kg. Spiked samples were allowed to equilibrate for 30 min prior to extraction. In addition, a matrix blank was also analysed to determine any residual mycotoxin levels.

As well as assessing within batch variation, batch to batch variation for dry maize cereals was also determined. Overall, nine batches of duplicate spike samples (5.0 μg/kg) and one blank sample were analysed.

3. Results and discussions

Samples spiked at 5.0 μg/kg gave average recoveries (within batch) of 72–94% for OA and 73–101% AF. Peanut butter spiked samples at the limit of quantification of 0.2 μg/kg of each toxin gave average recoveries of 94% and

Table 1
Initial validation data of joint OA and AF method

	n	OA		B_1		B_2		G_1		G_2	
		Rec	CV	Rec	CV	Rec	CV	Rec	CV	Rec	CV
Maize cereals	5	76	7	83	2	87	3	84	3	80	3
Whole corn	5	84	3	82	5	86	5	84	4	83	3
Maize snacks	6	93	12	95	14	97	12	90	11	93	10
Corn flour	5	91	19	82	8	82	6	73	7	75	7
Polenta	6	72	7	90	6	93	5	79	6	84	7
Peanut butter	6	90	4	87	5	101	2	100	9	98	6
Peanut butter (low)	5	94	6	98	6	95	6	84	11	83	7

Recovery (Rec) and in-batch coefficient of variance (CV) in %.

83–98% for OA and AF, respectively. Table 1 shows a summary of the initial validation data, and Fig. 1 shows a typical chromatogram of a naturally contaminated and a spiked peanut butter sample. The chromatograms show no interferences, all peaks clearly resolved, with no major disturbance to the baseline during the mobile phase or detector wavelength changes.

The within laboratory relative standard deviation of repeatability (RSD_r, within batch precision) and reproducibility (RSD_R, between batch precision) for the joint OA and AF method were calculated using a one-way analysis of variance (ANOVA) approach from nine batches of replicate spike samples (5 μg/kg) in maize cereals [8]. Within laboratory RSD_r and RSD_R were found to be 7.6–10.1% and 10.2–13.8%, respectively. Between and within batch precision data for maize cereals for the joint method are summarised in Table 2. We can compare the precision of this method against the predicted precision (Horwitz value) for this level of concentration by calculating the Horwitz ratio (HORRAT or Ho) [9–11]. In order for the method to be "fit for purpose", Ho_R (HORRAT value for reproducibility, $Ho_R = RSD_R/RSD_{R\text{-predicted}}$) will need to be <2 to be satisfactory, which is the case for this method (see Table 2). Furthermore, this joint method falls well within CEN's (European Committee for Standardization) criteria for analytical methods for OA and AF analysis, with RSD_R <30%, RSD_r <20% and recovery of 70–110% for a 1–10 μg/kg range [12].

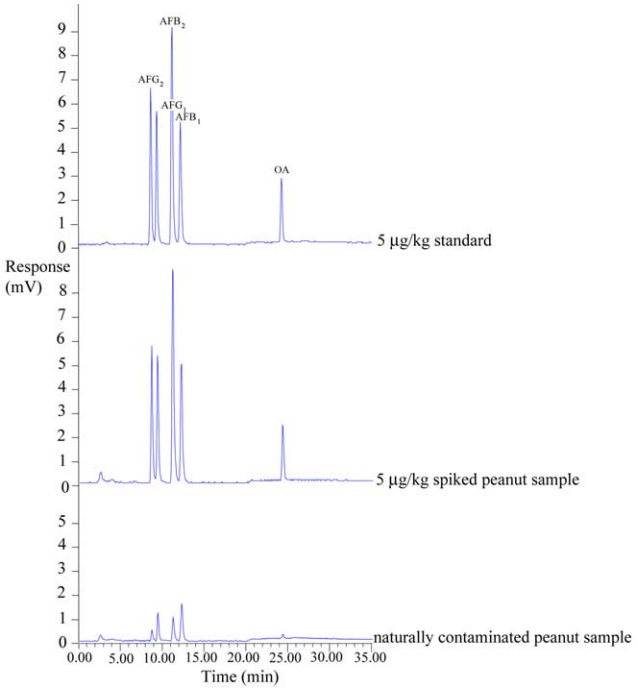

Fig. 1. Chromatograms of a 5 μg/kg standard (top), a 5 μg/kg spiked peanut sample (middle) and a naturally contaminated peanut sample (bottom).

The performance of the joint OA and AF method for cereals was compared against in-house data (from routine analysis) using the individual methods (cereal samples spiked at 5 μg/kg) (Table 3). Average recoveries were slightly higher for the joint method (87–96%) compared to the individual methods (78–88%), although standard *F*-test [13] showed no significant between batch differences in terms of precision (where $F < F_{crit}$), for these cereal samples.

The capacity and cross reactivities of these joint immunoaffinity columns have not been fully examined in this study, however the joint immunoaffinity columns used have a quoted recovery of at least 70% and a capacity of up to 100 μg/kg in grain and feed [5]. No problems were apparent in this laboratory during our validation study and day to day sample analysis, where hundreds of these joint OA/AF columns have been used.

The individual OA and AF methods have the same extraction procedure but clean-up and HPLC analysis is carried out separately [6,7]. Furthermore, the clean-up columns em-

Table 2
Performance data for duplicate spiked (5 μg/kg) maize cereals using the joint OA and AF method

	OA	B_1	B_2	G_1	G_2
Number of analytical batches ($n = 2$)	9	9	9	9	9
Mean value (μg/kg)	4.37	4.66	4.81	4.26	4.33
(Recovery, %)	(87)	(93)	(96)	(85)	(87)
Standard repeatability (S_r)	0.33	0.44	0.44	0.43	0.42
Standard reproducibility (S_R)	0.55	0.48	0.49	0.53	0.60
Relative repeatability (RSD_r, %)	7.59	9.42	9.06	10.13	9.67
Relative reproducibility (RSD_R, %)	12.50	10.31	10.22	12.50	13.79
Predicted S_R (Horwitz)	0.96	1.03	1.06	0.94	0.95
Ho_R	0.57	0.47	0.46	0.57	0.63

Table 3
Performance data of duplicate spiked samples (5 μg/kg) for cereal samples using individual OA and AF methods

	OA	B_1	B_2	G_1	G_2
Number of analytical batches ($n = 2$)	8	7	7	7	7
Mean value (μg/kg)	4.19	3.91	4.41	4.10	4.27
(Recovery, %)	(84)	(78)	(88)	(82)	(85)
Standard repeatability (S_r)	0.28	0.17	0.07	0.15	0.09
Standard reproducibility (S_R)	0.53	0.49	0.77	0.69	0.79
Relative repeatability (RSD_r, %)	6.75	4.40	1.69	3.70	2.20
Relative reproducibility (RSD_R, %)	12.58	12.59	17.43	16.75	18.62
Significance tests on reproducibility					
F (against the joint method)	1.08	1.04	2.47	1.69	1.73
F_{crit} (97.5% confidence)	4.90	4.65	4.65	4.65	4.65

ployed are specific only to either aflatoxins or ochratoxin A in the individual methods. For a fully automated clean-up and analysis run, the individual methods would require nearly twice as long to complete with one ASPEC instrument or twice the resources in the same amount of time as the joint method. This is one of the biggest advantage in the joint method, where it frees up instrument time for a higher throughput of samples in a busy analytical laboratory, in addition to the staff time saved in instrument set up. Furthermore, the cost of one joint OA/AF clean-up column is normally 30% cheaper compared to two individual immunoaffinity clean-up columns, plus the costs of other consumables (e.g. HPLC vials) are reduced by half.

The possibility of expanding this technique to other types of food matrices (e.g. dried fruit and spices) is currently under investigation.

4. Conclusions

We have shown that aflatoxins and ochratoxin A can be simultaneously determined in maize cereals and peanut butter using a fully automated procedure with a new type of immunoaffinity column. This method has been found to be fit for purpose, and falls well within CEN's method performance criteria for OA and AF analysis. No significant differences were found when compared to the individual OA and AF methods (i.e. batch to batch variation of the joint method is not significantly different to the individual method).

This joint method has already been in routine use in this laboratory for the analysis of hundreds of cereal and nut samples and has been found to give performance characteristics equivalent to the established individual methods whilst reducing the cost of analysis in terms of both time (staff and instrument) and resource (consumables, e.g. IAC columns and HPLC vials).

References

[1] J. Fink-Gremmels, A. Jahn, M.J. Blom, Nat. Toxins 3 (1995) 214.
[2] M.W. Trucksess, A.E. Pohland (Eds.), Methods in Molecular Biology, vol. 157: Mycotoxin Protocols, Humana Press, Totowa, NJ, 2001.
[3] J. Gilbert, E. Anklam, Trends Anal. Chem. 21 (2002) 468.
[4] K.A. Scudamore, M.T. Hetmanski, S. Nawaz, J. Naylor, R. Rainbird, Food Addit. Contam. 14 (1997) 175.
[5] http://www.vicam.com/products/aflaochra.html.
[6] M. Sharman, S. MacDonald, J. Gilbert, J. Chromatogr. 603 (1992) 285.
[7] M. Sharman, J. Gilbert, J. Chromatogr. 543 (1991) 220.
[8] M. Thompson, R. Wood, Pure Appl. Chem. 65 (1993) 2123.
[9] W. Horwitz, Anal. Chem. 54 (1982) 67A.
[10] M. Thompson, Analyst 125 (2000) 385.
[11] J.T. Peeler, W. Horwitz, R. Albert, J. Assoc. Off. Anal. Chem. 72 (1989) 784–806.
[12] European Committee for Standardization, Food analysis – Biotoxins – Criteria of analytical methods of mycotoxins, CEN Report-CR 13505, February 1999.
[13] T.J. Farrant, Practical Statistics for the Analytical Scientist – A Bench Guide, Royal Society of Chemistry, Cambridge, UK, 1997.