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High-performance liquid chromatographic determination of ochratoxin A in artificially contaminated cocoa beans using automated sample clean-up

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Abstract

A HPLC method is described for the analysis of ochratoxin A at low-ppb levels in samples of artificially contaminated cocoa beans. The samples are extracted in a mixture of methanol–water containing ascorbic acid, adjusted to pH and evaporated to dryness. Samples in this state are then placed onto a Benchmate sample preparation workstation where C_{18} solid-phase extraction operations are performed. The resulting materials are evaporated to dryness and analyzed by reversed-phase HPLC with fluorescence detection. The method was evaluated for accuracy and precision with R.S.D.s for multiple injections of sample and standard calculated to be 1.1% and 2.5% for sample and standard, respectively. Recoveries of ochratoxin A added to cocoa beans ranged from 87–106% over the range of the assay.

Keywords: Cocoa; Sample preparation; Food analysis; Ochratoxins; Toxins

1. Introduction

Ochratoxin A is a secondary metabolite produced by *Penicillium* and *Aspergillus* species. It is a phenylalanine derivative of a substituted isocoumarin with carcinogenic, nephrotoxic, teratogenic and immunotoxic properties. While there are a substantial number of ochratoxin derivatives that have been isolated in the laboratory, it is ochratoxin A that is found primarily in extracts of moldy plants and is the cause for the most concern [1–5].

There have been a number of methods reported on the analysis of this compound in various agricultural products and biological samples. Some of the agricultural products included are cereals, coffee, milk, cheese, egg, corn and cocoa while methods for serum, urine, liver, kidneys and rumen fluid have also been developed. Earlier methods relied on thin-layer chromatography (TLC) while many of the present methods rely on the use of reversed-phase high-performance liquid chromatography (HPLC) with an acidic mobile phase and fluorescence detection since this affords the best sensitivity and selectivity for this assay. The sample preparation protocols have varied and ranged from extraction of the material of interest in chloroform followed by a series of liquid–liquid extraction steps, to a direct extraction with more polar materials such as acetonitrile before a solid-phase extraction (SPE) sample clean-up [6–11].

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There have been analyses and commodity surveys reported for the determination of this compound in cocoa [12,13], and previously a method was developed in our laboratory where samples were extracted in a mixture of chloroform–methanol and hexane followed by a liquid–liquid extraction with hydrogen carbonate followed by chloroform. After evaporation to dryness, samples were reconstituted in mobile phase for analysis by reversed-phase HPLC [14].

The purpose of these studies was to apply current analytical technology in automated sample preparation and HPLC analysis to a method for the determination of ochratoxin A in artificially contaminated cocoa beans.

2. Experimental

2.1. HPLC and sample preparation equipment

The HPLC system used in these studies consisted of a Shimadzu Model 9A HPLC pump, a Shimadzu Model 551 fluorescence detector set at an excitation wavelength of 330 nm and emission wavelength of 460 nm (Shimadzu, Columbia, MD, USA). Data were acquired with a Waters 845 Data System operating on a VAX 3100 Workstation running Version 5.2 of Expert Ease (Waters, Milford, MA, USA). The HPLC column used was 5 μm Spherisorb ODS-2 (150×4.6 mm) with a mobile phase of acetonitrile–water–trifluoroacetic acid (TFA) (40:59.7:0.3, v/v/v) containing 0.2 m*M* β-cyclodextrin (β-CD) flowing at 1.5 ml/min. All solvents were HPLC grade and the water was obtained from an Elga Maxima HPLC water purification system. A Zymark Benchmate I equipped with SPE, filtration and HPLC injection capabilities was used for sample clean-up and HPLC injection while a Zymark TurboVap LV was used for solvent removal (Zymark, Hopkinton, MA, USA).

2.2. Ochratoxin A

Ochratoxin A was purchased from Mycolab (Washington, MO, USA) at an initial concentration of 100 μg/ml and stored at −20°C until used. When it is ready to use, an aliquot was withdrawn, evaporated to dryness and dissolved in HPLC mobile phase at a final concentration of 200 pg/μl.

2.3. Sample preparation

One of the methods proposed by Seidel et al. [15] with slight modifications was used for extraction. Sample (5 g) and ascorbic acid (2 g) were extracted with 50 ml of methanol–water (90:10, v/v) for 60 min on an laboratory shaker. The methanolic extract was then filtered and adjusted to an apparent pH of approx. 7.0±0.2 with NaOH. A 5-ml volume of the extract was withdrawn and evaporated to dryness using a stream of nitrogen and redissolved in 2 ml of a mixture of methanol–5% acetic acid (1:1, v/v). This reconstituted sample was then placed on the Benchmate which was equipped with 300 mg Varian C_{18} SPE cartridges (Varian, Harbor City, CA, USA). SPE was accomplished in an automated fashion with the Benchmate program given in Fig. 1 but repeated here for completeness. A 5-ml volume of 5% aqueous acetic acid was added to the tube containing the

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                    Zymark BenchMate 2.51

  Zymark BenchMate Procedure : OCHRATOXIN A SPE ISOLATION

Step  1 : Add 5 ml of 5% Acetic Acid
Step  2 : Mix by cycling 4.5 ml in tube 4 times
Step  3 : Condition column with 4 ml of Methanol
Step  4 : Condition column with 4 ml of 5% Acetic Acid
Step  5 : Load 6 ml of sample onto column
Step  6 : Rinse column with 2.5 ml of 5% Acetic Acid
Step  7 : Collect 6 ml fraction into next tube using 80/15/5 Elution
Step  8 : Wash syringe with 4 ml of 80/15/5 Elution
Step  9 : Wash syringe with 3 ml of Methanol
Step 10 : Mix by cycling 5.5 ml in tube 4 times
Step 11 : Wash syringe with 3 ml of 80/15/5 Elution
Step 12 : END
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Fig. 1. Benchmate sample preparation program.

sample and mixed for 30 s. The SPE cartridges are pretreated with 4 ml of methanol which is followed by 4 ml of 5% acetic acid. A 6-ml sample volume was then loaded onto the SPE column which is then washed with 2.5 ml of the 5% acetic acid solution. The ochratoxin containing fraction is eluted with 6 ml of methanol–water–5% acetic acid (80:15:5, v/v/v) and mixed using the vortex on the workstation. The resulting materials were then placed into the Turbovap where they are again evaporated the dryness using a stream of nitrogen.

2.4. HPLC analysis

After the extracts have reached dryness, they are then placed back on the Benchmate where 1 ml of HPLC mobile phase was automatically added and the samples vortexed for 1 min. After that operation, a 50-μl aliquot of the extract was injected onto the HPLC system in duplicate and compared with the standard compound made at concentrations of 0.2 ng/μl.

As an additional component of this study, Self-Pak columns from Perseptive Biosystems were evaluated for this determination to investigate the chromatography of this compound using perfusion chromatography. The column with dimensions of 150×3.9 mm I.D. was packed with 10 μm Poros RP-2, a reversed-phase material, according to instructions provided with the Self-Pak unit [16]. The same mobile phase as the standard analytical columns was used but the flow-rate was tripled.

2.5. Confirmatory studies

While the samples analyzed were artificially contaminated, methods were evaluated to allow for the confirmation of the peak for ochratoxin A. Extracts were evaporated to dryness, redissolved in acetonitrile and treated with BF_3 in methanol to form the methyl ester of ochratoxin A. This compound have different elution time when compared to standard ochratoxin A [17]. Additionally, positive samples were also analyzed using an enzyme-linked immunosorbent assay (ELISA)-based Ochratoxin EZ Screen assay card (Diagnostix, Mississauga, Canada) [18].

3. Results

Figs. 2–4 provide sample chromatograms of standard, sample with no ochratoxin A and sample artificially contaminated with ochratoxin A at a concentration of approximately 35 μg/kg. Fig. 5 provides an illustration of the chromatography for standard ochratoxin A at 10 ng injection, while Fig. 6 provides an example of a cocoa bean extract spiked with ochratoxin A to illustrate the application of this material in an authentic sample spiked at 50 μg/kg. The lower limit of detection at $S/N=3$ was calculated to be 100 pg which translated to a lower limit

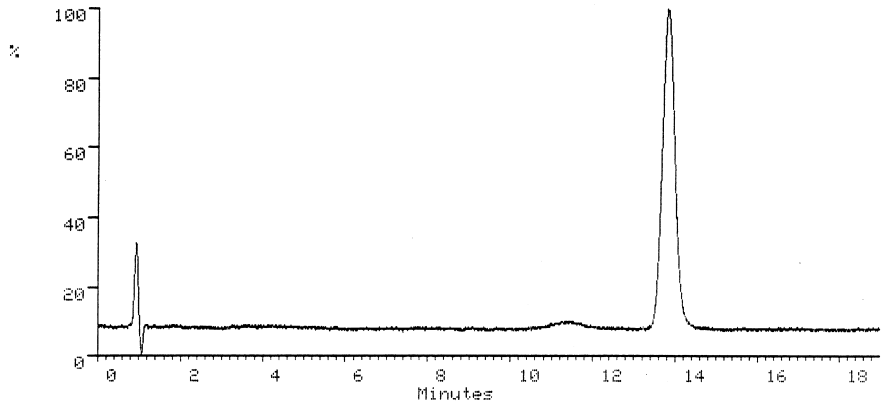

Fig. 2. Chromatogram of 10-ng injection of ochratoxin A standard. Column: Spherisorb ODS-2 (150×4.6 mm); mobile phase: acetonitrile–water–TFA (40:59:1, v/v/v) containing 0.2 m*M* β-CD; flow-rate: 1.5 ml/min; detection: fluoresence at 330 nm excitation and 460 nm emission.

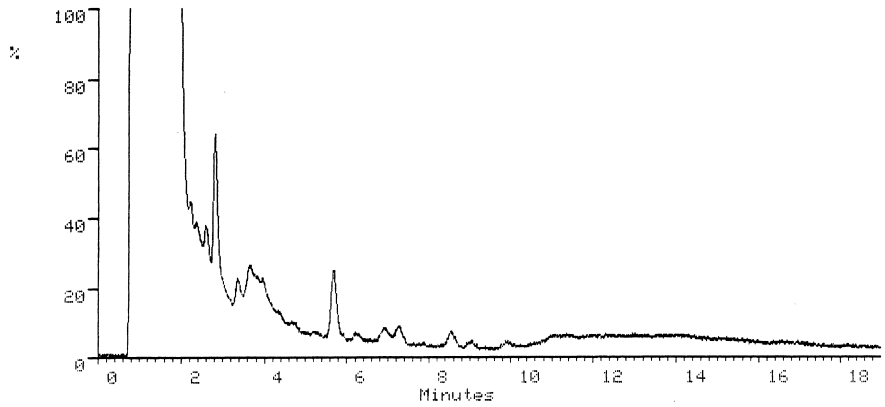

Fig. 3. Chromatogram of cocoa bean extract with no ochratoxin A. Column: Spherisorb ODS-2 (150×4.6 mm); mobile phase: acetonitrile–water–TFA (40:59:1, v/v/v) containing 0.2 m*M* β-CD; flow-rate: 1.5 ml/min; detection: fluoresence at 330 nm excitation and 460 nm emission.

in the sample of approx. 2 μg/kg. Standard material was injected in duplicate from 2 to 50 ng and a regression calculated to be 0.984. Additionally, both standard at 10 ng and sample spike at 20 μg/kg were injected 10 times and the R.S.D.s were calculated at 1.1% and 2.4% for the standard and sample, respectively. Recoveries were conducted at 10, 20, 50 and 100 μg/kg and determined to range from 87–106% with $n=3$ at each level. Corresponding data for the Poros RP-2 column were not developed but one was able to determine that the limit of detection for ochratoxin A under these conditions was poorer than those achieved by the 5 μm C_{18} column.

4. Discussion

The results indicate that the method described is suitable for the determination of ochratoxin A in samples of cocoa beans. The serial processing of the

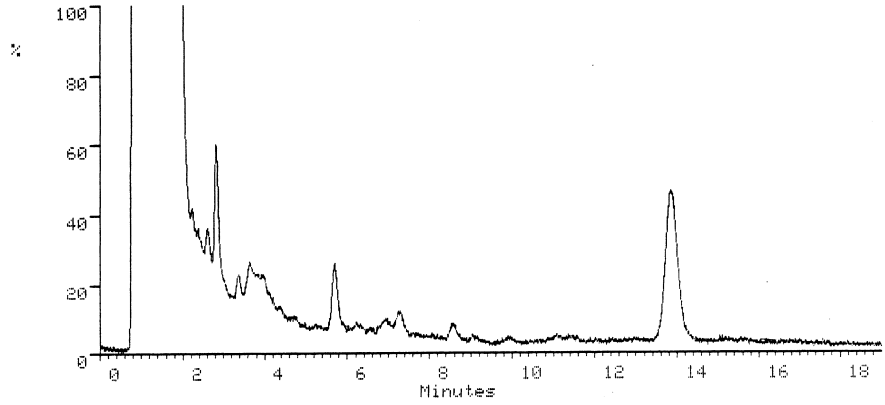

Fig. 4. Chromatogram of artificially contaminated cocoa bean extract at 35 μg/kg. Column: Spherisorb ODS-2 (150×4.6 mm); mobile phase: acetonitrile–water–TFA (40:59:1, v/v/v) containing 0.2 m*M* β-CD; flow-rate: 1.5 ml/min; detection: fluoresence at 330 nm excitation and 460 nm emission.

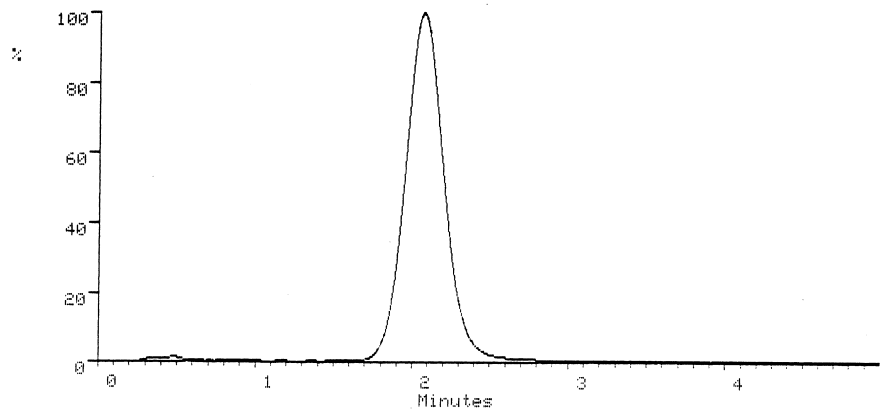

Fig. 5. Chromatogram of 10-ng injection of ochratoxin A standard. Column: 10 RP-2 Poros (150×3.9 mm); mobile phase: acetonitrile–water–TFA (40:59:1, v/v/v) containing 0.2 m*M* β-CD; flow-rate: 3.0 ml/min; detection: fluoresence at 330 nm excitation and 460 nm emission.

samples by the Benchmate followed by evaporated to dryness in a controlled atmosphere using the Turbovap provides the same treatment for all samples thereby eliminating many variables in this determination. The use of the Benchmate also allows for the unattended SPE of samples, freeing up laboratory personnel and lessening their exposure to hazardous compounds. Obviously, these steps can be performed in a manual mode. There have been some other reports on the use of automation for this determination but have focused on the use of immunoaffinity columns for sample clean-up [19]. This method describes the use of standard C_{18} SPE columns for the sample clean-up step. A small amount of ascorbic acid was added to the extraction solution and served to convert the ochratoxin A into its unionized from and to protect the phenolic groups from oxidation which might cause the extraction on interfering compounds.

The use of the β-CD as a mobile phase additive

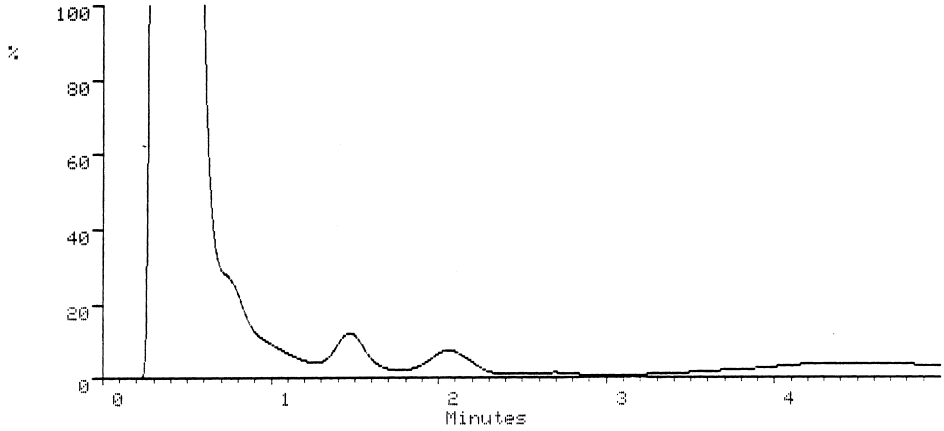

Fig. 6. Chromatogram of artificially contaminated cocoa bean extract at 50 μg/kg. Column: 10 RP-2 Poros (150×3.9 mm); mobile phase: acetonitrile–water–TFA (40:59:1, v/v/v) containing 0.2 m*M* β-CD; flow-rate: 3.0 ml/min; detection: fluoresence at 330 nm excitation and 460 nm emission.

was described by Seidel et al. [15]. The β-CD serves to form inclusion complexes allowing for an enhanced separation between the ochratoxin A and interfering compounds. In this chromatographic system, 0.2 m*M* was empirically determined to provide the best resolution.

The perfusion-based Poros RP-2 column was used to evaluate this technique for the determination of ochratoxin A and to determine if it would bring any advantages to this determination. The lower limits were not superior to the 5 μm C_{18} column but did offer a 2 min analysis time which could be useful in the determination of a large number of samples. Finally, the separation conditions could likely have been optimized to improve the lower limits.

In summary, the results indicate that this method with automated sample clean-up is suitable for the determination of ochratoxin A in samples of artificially contaminated cocoa beans. The lower limits previously published for the determination of ochratoxin A in cocoa beans were 10 μg/kg while this method has increased the sensitivity to 2 μg/kg, thereby providing superior lower limits [14]. The automation allows for increased sample throughput by being able to process samples on an unattended basis. The time to process one sample on the Benchmate is 12 min so 40 samples can be prepared for analysis in an 8 h shift. Additionally, the Benchmate provides tracking for each sample so any anomalies in the results can be compared to the this record. Samples can then be injected in a serial or batch mode. The method is accurate and precise with the automation of the sample clean-up allowing for increased sample throughput and less exposure to potentially hazardous compounds.

References

[1] K.K. Van der Merwe, P.S. Steyn, L. Fourie, J. Chem. Soc. xx (1965) 7083.
[2] A. Ciegler, Can. J. Microbiol. 18 (1972) 631.
[3] M.O. Moss, Food Addit. Contam. 13 (1996) 5.
[4] World Health Organization, Environmental Health Criteria 105, Selected Mycotoxins: Ochratoxin, Tricothecenes, Ergot, WHO, Geneva, 1990, p. 30.
[5] T. Kuiper-Goodman, P.M. Scott, Biomed. Environ. Sci. 2 (1989) 179.
[6] D. Beaker, B. Radii, J. Chromatogr. 570 (1991) 441.
[7] B.G. Osborne, J. Sci. Food Agric. 30 (1979) 1065.
[8] H. Valenta, I. Kuhn, K. Rohr, J. Chromatogr. 613 (1993) 295.
[9] P.M. Scott, Trends Anal. Chem. 12 (1993) 373.
[10] H.P. van Egmond, Food Addit. Contam. 13 (1996) 11.
[11] A. Breitholtz-Emaluelsson, M. Olsen, A. Oskarsson, I. Palminger, K. Hult, J. Assoc. Off. Anal. Chem. 76 (1993) 842.
[12] D.C. Hunt, B.R. McConnie, N.T. Crosby, Analyst 105 (1980) 89.
[13] R.M. Eppley, J. Assoc. Off. Anal. Chem. 51 (1968) 74.
[14] W.J. Hurst, R.A. Martin Jr., J. Chromatogr. 265 (1983) 353.
[15] V. Sediel, E. Poglits, K. Schiller, W. Lindner, J. Chromatogr. 635 (1993) 227.
[16] Poros Self-Pack Packing Device for High Performance Perfusion Chromatography, Perseptive Biosystems, Cambridge, MA, 1996.
[17] L.J. Vorster, Analyst 95 (1969) 136.
[18] Ochratoxin EZ Screen Card, Diagnostix, Mississauga, Canada.
[19] M. Sharman, M. MacDonald, J. Gilbert, J. Chromatogr. 603 (1992) 285.