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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF THE MYCOTOXINS PATULIN, PENICILLIC ACID, ZEARALENONE AND STERIGMATOCYSTIN IN ARTIFICIALLY CONTAMINATED COCOA BEANS

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SUMMARY

A high-performance liquid chromatographic (HPLC) method has been developed to allow the determination of patulin, penicillic acid, sterigmatocystin and zearalenone in samples of cocoa beans. When this method is combined with a method that was reported earlier for the determination of ochratoxin A [W. J. Hurst and R. A. Martin, Jr., *J. Chromatogr.*, 265 (1983) 353], it allows for the determination of five mycotoxins. Samples were extracted with an acidic acetonitrile solution, partitioned with hexane to remove fat interferences and then partitioned with chloroform to remove the toxin containing fraction. Interferences were removed by the use of a bonded phase column followed by the final HPLC determination step, which uses a cyano column with a hexane–1-propanol–acetic acid mobile phase with dual chanel UV detection at 245 and 280 nm. The method exhibits good linearity, accuracy and precision.

INTRODUCTION

Since the discovery of the aflatoxins in the 1960's, there has been intense interest in methods for the determination of these and other members of the mycotoxin family. Initial methods were developed using thin-layer chromatographic (TLC) methodology. In recent years there has been significant progress using high-performance liquid chromatographic (HPLC) methods for the determination of aflatoxins and other mycotoxins in various commodities[1–5,11,12]. While the overall number of known mycotoxins is extensive, this screen centers on the determination of patulin, penicillic acid, sterigmatocystin and zearalenone in spiked cocoa beans. Fig. 1 shows the chemical structures of these compounds.

The number of mycotoxins identified in foodstuffs is increasing, and there is a potential link of these compounds to various disease states[6]. While none of the mycotoxins included in this survey have been reported in samples of cocoa beans[7], methodology was developed to allow the determination of these compounds in cocoa beans.

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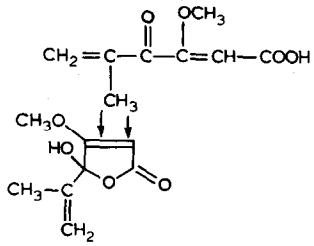

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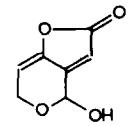

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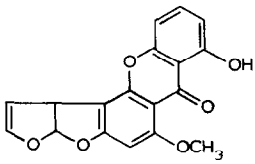

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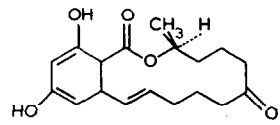

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Fig. 1. Structures of patulin (I), penicillic acid (II), sterigmatocystin (III) and zearalenone (IV). Patulin: Synonyms are clairformin, clavacin, clavatin, claviformin, expansin, expansine, gigant, leucopin, mycoin, mycoin C, mycoin C3, mycoine C3, mycosin, patuline, penatin, penicidin, tercinin; chemical name, 4-hydroxy-4H-furo[3,2-*c*]pyran-2(6H)-one; empirical formula, $C_7H_6O_4$; molecular weight, 154.1. Penicillic acid: chemical name, 3-methoxy-5-methyl-4-oxo-2,5-hexadienoic acid; empirical formula, $C_8H_{10}O_4$; molecular weight, 170.1. Sterigmatocystin: chemical name, 3*a*,12*c*-dihydro-8-hydroxy-6-methoxy-(3*aR-cis*)-7H-furo[3′2′:4,5]furo[2,3-*c*]xanthen-7-one; empirical formula, $C_{18}H_{12}O_6$; molecular weight, 324.3. Zearalenone: synonyms are 6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resorcylic acid-*n*-lactone; F-2 toxin; *trans*-zearalenone; chemical name, 3,4,5,6,9,10-hexahydro-14,16-dihydroxy-3-methyl-[S-(E)]-1H-2-benzoxacyclotetradecin-1,7(8H)-dione; empirical formula, $C_{18}H_{22}O_5$; molecular weight, 318.4.

Some mycotoxin methodologies have included cocoa as a matrix commodity but have reported recoveries of less than 50% in some cases[7]. Clearly the need for improvement in overall methodology exists for cocoa.

Current methods for individual toxins require extensive analytical time, since each compound of concern must be treated individually in the extraction, sample clean-up, quantitation and final determination and confirmation. It would be advantageous to develop methodology in which a number of these mycotoxins could be analyzed from a single prepared extract. The method described allows for the determination of patulin, penicillic acid, sterigmatocystin and zearalenone in a single extract of artificially contaminated cocoa beans.

EXPERIMENTAL

Equipment

The HPLC equipment used consisted of a Model 6000A solvent delivery system (Waters Assoc.), a Model 7125 loop injector (Rheodyne), a Model 165 UV detector (Beckman) set at 245 and 280 nm equipped with full spectrum scanning and absorbance ratioing capacity, a Model 730 data module (Waters Assoc.) and a Model 720 system controller (Waters Assoc.). The HPLC columns were a 10-μm CN (An-

alytichem) 15 cm × 4.0 mm I.D. and a radial compression module (Waters Assoc.) equipped with a 5-μm C_{18} cartridge (10 cm × 8.0 mm I.D.).

HPLC

Mobile phase. The HPLC mobile phase used was a mixture of hexane–1-propanol–conc. acetic acid (95:3:2, v/v/v). The mobile phase was filtered through a 0.45-μm filter and degassed prior to use.

Mycotoxin standards. All mycotoxin standards were purchased in various concentrations from Sigma and stored at −4°C. The sterigmatocystin (5.0 mg) was diluted to a final concentration of 1 ng/μl with chloroform, while zearalenone (10 mg) was diluted with chloroform to a final concentration of 5 ng/μl. The patulin (10 mg) and penicillic acid (10 mg) were diluted with chloroform to final concentrations of 15 ng/μl and 30 ng/μl, respectively. All of the diluted standards were stored at −4°C until use, and replaced when a deterioration of the HPLC peak shape was seen. The purity of each standard was confirmed by TLC, HPLC and UV spectrophotometry. The UV specdtra of each individual toxin were compared with the reference spectra obtained from I.U.P.A.C. sources[8].

Sample preparation. A 50-g amount of cocoa beans were placed in a 500-ml Erlenmeyer flask which contained 250 ml of acetonitrile–10% orthophosphoric acid (95:5, v/v) and placed on an orbital shaker at 250 rpm for 30 min. The resulting solution was filtered through Whatman 2v or equivalent. After the filtration was complete, 25 ml of the filtrate was withdrawn and placed into a 125-ml separatory funnel. This solution was extracted with two, 25-ml portions of hexane and the hexane was discarded. The resulting fat free solution was further extracted with two 15-ml portions of chloroform. The chloroform layers were combined and dried with sodium sulfate. The dried extracts were placed into a 100-ml round bottom flask and the solvent was removed under a vacuum at 35°C on a rotary evaporator. The resulting residue was allowed to cool to room temperature and 5.0 ml of chloroform were pipetted into the flask and swirled to dissolve the residue; this solution was then placed on a bonded phase silica column for further manipulations.

Sample cleanup. A 2.8-ml capacity silica Bond-elut column (Analytichem) was conditioned with 3.0 ml of chloroform. A 1-ml aliquot of the above residue in chloroform, corresponding to 1.0 g sample, was placed on the extraction column. The fraction containing the toxins was eluted with a 5-ml portion of 1-butanol–chloroform (91.9, v/v) into a 5-ml capacity conical vial and evaporated to dryness using low heat and a nitrogen stream. After cooling to room temperature, it was reconstituted with 200 μl of mobile phase and vortexed before the analysis step.

TABLE I

CONFIRMATORY TECHNIQUES[1,10]

Mycotoxin	*Technique*
Ochratoxin A	Methyl ester derivative
Ochratoxin A	Post-column reaction with ammonia to form derivative
Penicillic acid	Methyl ester derivative
Zearalenone	Electrochemical detection

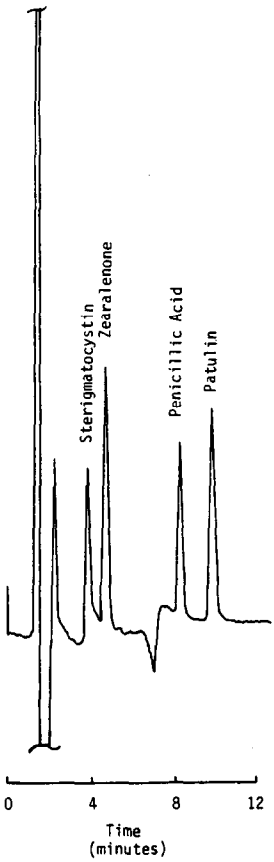

Fig. 2. Chromatogram of mycotoxin standards. Column, cyano; mobile phase, hexane–1-propanol–acetic acid (95:3:2, v/v/v); initial flow-rate, 2 ml/min; detection, UV at 245 nm.

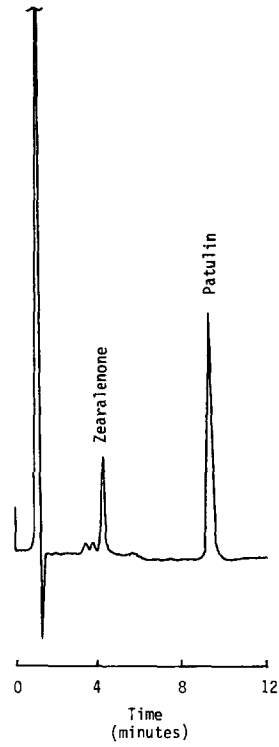

Fig. 3. Chromatogram of mycotoxin standards. Column, cyano; mobile phase, hexane–1-propanol–acetic acid (95:3:2, v/v/v); initial flow-rate, 2 ml/min; detection, UV at 280 nm.

Analysis. Aliquots (10–15 μl) of the sample extract were injected onto the HPLC system and compared with 10–15 μl aliquots of the mixed standard to arrive at the final concentration of the peaks of interest. The analysis was conducted using flow programming. The mobile phase was initially flowing at 2.0 ml/min for 6 min and then increased to 4.0 ml/min for the remainder of the analysis time.

Confirmatory techniques. Any suspect peak was scanned and compared to a UV scan of an authentic standard in order to establish peak identity. Additionally, suspect peaks can also be derivatized or other alternative detection methods used. Table I summarizes some of these techniques.

RESULTS

Figs. 2 and 3 show chromatograms of standards at 245 and 280 nm while Figs. 4 and 5 give example chromatograms at 245 and 280 nm of an artificially spiked sample of cocoa beans.

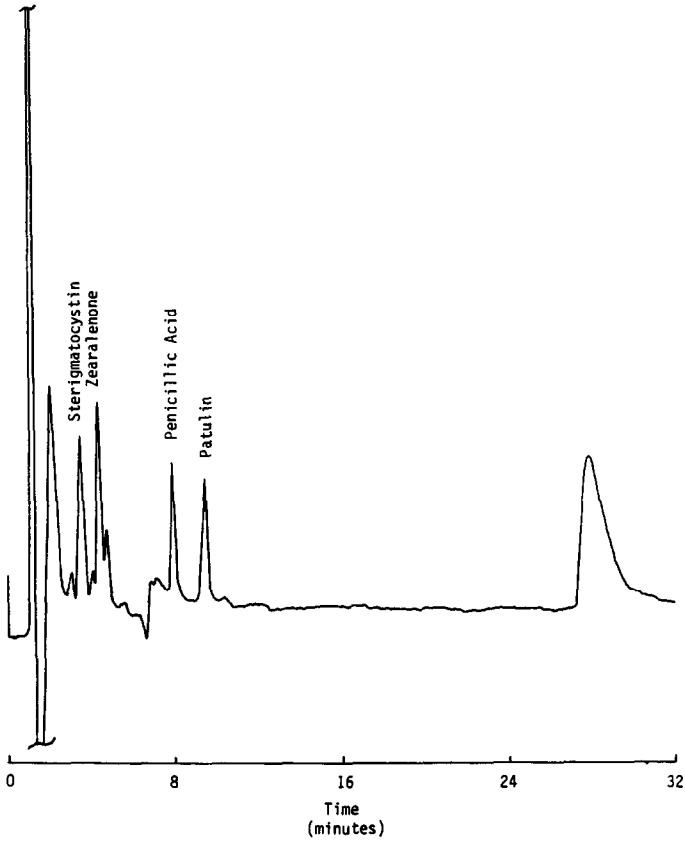

Fig. 4. Chromatogram of spiked cocoa bean extract. Column, cyano; mobile phase, hexane–1-propanol–acetic acid (95:3:2, v/v/v); initial flow-rate, 2 ml/min; detection, UV at 245 nm.

The method that was previously described was evaluated for precision and accuracy. Both standard and sample were injected ten times using the conditions previously described with results shown in Table II.

Various amounts of the four mycotoxins of interest were added to samples of cocoa beans before the initial extraction to evaluate recovery. These data are summarized in Table III.

The standards were injected over a 100-fold range and found to exhibit excellent linearity. Due to the wide variety of mycotoxins evaluated, lower limits of detection range from 1 ng per injection for sterigmatocystin to 40 ng per injection for penicillic acid; zearalenone and patulin had lower detection limits of 15 ng per injection under the actual analysis conditions, (detection limit = $S/N = 2$).

These lower limits then equate to values of 13 ppb*, 200 ppb, 200 ppb and 520

* Throughout this article the American billion (10^9) is meant.

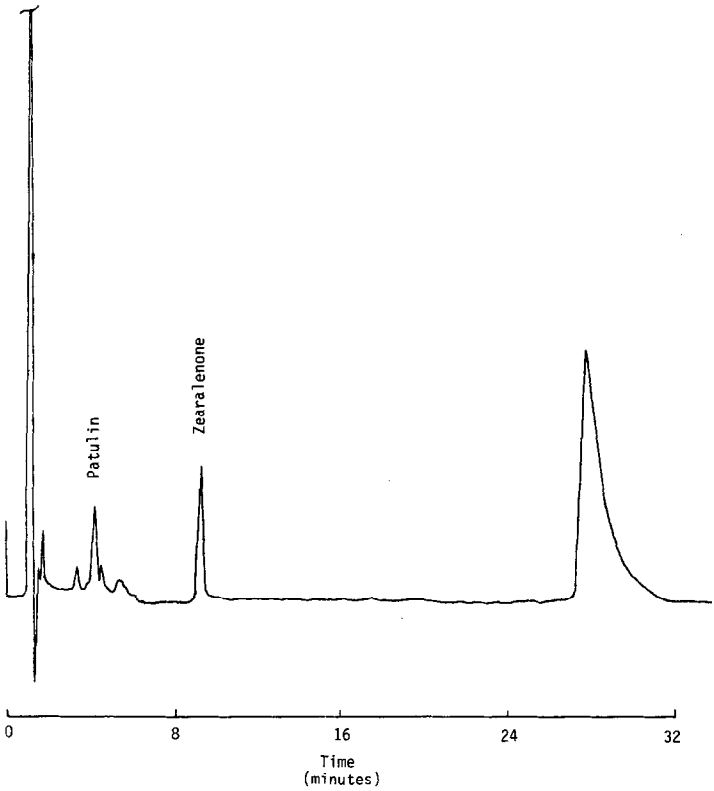

Fig. 5. Chromatogram of spiked cocoa bean extract. Column, cyano; mobile phase, hexane–1-propanol–acetic acid (95:3:2, v/v/v); initial flow-rate, 2 ml/min; detection, UV at 280 nm.

TABLE II

PRECISION DATA (n = 10)

C.V. = Coefficient of variation.

Sample identification	*Concentration (ng/g)*	*C.V. (%)*
Cocoa bean extract (sterigmatocystin)	≈ 50	4.2
Cocoa bean extract (zearalenone)	≈100	1.6
Cocoa bean extract (penicillic acid)	≈100	3.9
Cocoa bean extract (patulin)	≈ 40	2.4
Sterigmatocystin	10	3.4
Zearalenone	50	1.4
Penicillic acid	60	3.7
Patulin	25	2.8

TABLE III

RECOVERY STUDIES FROM ARTIFICIALLY CONTAMINATED COCOA BEANS (n = 2).

	Amount added (ng/g)	*Amount recovered (ng/g)*	*Recovery (%)*
Sterigmatocystin	30	30.15	100.5
	60	64.9	108.3
Zearalenone	150	112	75.0
	300	283	94.2
Penicillic acid	900	909	101
	1800	2050	112
Patulin	450	356	79
	900	795	88.4

ppb for sterigmatocystin, patulin, zearalenone and penicillic acid, respectively, in the matrix.

DISCUSSION

When this assay is combined with the techniques reported earlier[9] for the determination of ochratoxin A in cocoa beans, a multimycotoxin screen of cocoa beans is possible.

Should a researcher not have access to HPLC equipment, the final prepared extract is also suitable for use in TLC studies. The methodology described allows the determination of four mycotoxins via a single cocoa bean extraction.

The method additionally allows the use of other techniques to confirm peak identity. With the use of a detector that has full spectrum scanning capability, a researcher can identify peaks by their characteristic spectra in addition to their retention time when compared to authentic standards.

The choice of 245 and 280 nm was determined after evaluation of UV spectra in the mobile phase used in this study. Several of these toxins exhibit $\lambda^{max.}$ in regions that are outside the UV cut-off of mobile phase used; thus 245 and 280 nm were chosen.

In summary, an accurate precise and time efficient method is now available for use in the screening of cocoa beans for patulin, penicillic acid, sterigmatocystin and zearalenone.

REFERENCES

1 M. J. Shepherd, M. Holmes and J. Gilbert, *J. Chromatogr.*, 354 (1986) 305.
2 W. A. Pons and A. O. Franz, Jr., *J. Assoc. Off. Anal. Chem.*, 60 (1977) 89.
3 P. M. Scott and S. R. Kanhere, *J. Assoc. Off. Anal. Chem.*, 63 (1980) 612.
4 G. M. Ware, C. W. Thorpe and A. E. Pohland, *J. Assoc. Off. Anal. Chem.*, 63 (1980) 637.
5 P. Lepon, *J. Chromatogr.*, 354 (1986) 518.
6 C. A. Linsell, *The Mycotoxins and Human Health Hazards in Mycotoxins in Foodstuffs*, Pergamon Press, London, 1977, p. 1765.

7 L. Stoloff in J. V. Rodricks (Editor), *Mycotoxins and Other Fungal Related Food Problems,* ACS, Washington, DC, 1976, p. 23.
8 A. E. Pohland, P. L. Schuller, P. S. Steyn and H. P. van Egmond, *Pure Appl. Chem.,* 54 (1982) 2219.
9 W. J. Hurst and R. A. Martin, Jr., *J. Chromatogr.,* 265 (1983) 353.
10 D. C. Hunt, L. A. Philip and M. T. Crosby, *Analyst (London),* 104 (1979) 1171.
11 C. P. Gorst-Allman and P. S. Steyn, *J. Chromatogr.,* 175 (1979) 325.
12 M.V. Howell and P. W. Taylor, *J. Assoc. Off. Anal. Chem.,* 64 (1981) 1356.