Methods in Molecular Biology™

VOLUME 157

Mycotoxin Protocols

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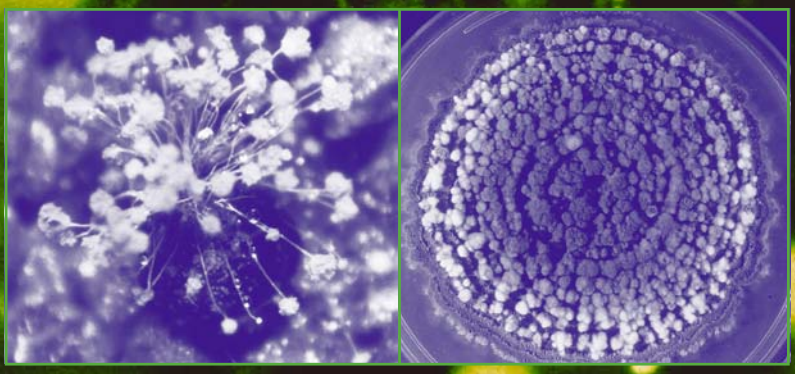

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1

Mycotoxin Method Evaluation

An Introduction

Albert E. Pohland and Mary W. Trucksess

Mycotoxins are toxic secondary metabolites produced by molds, i.e., metabolites not essential to the normal functioning of the cells. Molds are ubiquitous in nature and are universally found where environmental conditions are conducive to mold growth. Because molds are present in soil and plant debris, and are spread by wind currents, insects, and rain, they are frequently found in/on foods together with their associated mycotoxins ***(1)***.

The acute toxicity of mycotoxins has resulted in serious human health problems throughout recorded history ***(2)***. It has only been since the early 1960s, when the aflatoxins were found to be carcinogenic, that it was realized that some of these mold metabolites might have significant sub-acute and chronic toxicity for humans. The public health concerns resulting from the finding of mycotoxins, including metabolites in animal tissues resulting from transmission of mycotoxins present in animal feeds, and the observation of both acute and chronic effects in animals has prompted the research effort focusing on analytical methods development. Analysis for mycotoxins is essential to minimize the consumption of contaminated food and feed.

Method development and evaluation for mycotoxins is not a simple task. Determining the concentrations of toxins in grains at the ng/g or parts-per billion levels required for the most important mycotoxins is difficult. The approach generally followed consists of obtaining a relatively large primary sample representing a lot, reducing it in bulk and particle size to a manageable quantity, and finally performing the analysis on a small representative portion. Sampling commodities for mycotoxin contamination follows the U.S. Depart-

From: *Methods in Molecular Biology, vol. 157: Mycotoxin Protocols*
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ment of Agriculture (USDA) recommendations, which require collection of laboratory samples of at least 5–25 kg of nuts, corn, milo, and other grains ***(3)***.

All analytical methods for mycotoxins consist of four steps: sample preparation, extraction, cleanup or isolation, and measurement of the toxins. To prepare a representative test portion for analysis, the laboratory sample is ground and mixed, so that the concentration of toxin in the small final test portion is the same as that in the original laboratory sample collected. The test portion is extracted with various solvents. The extract is filtered and applied to a cleanup column or immunoassay device, or partitioned with appropriate solvents. This partially purified analyte is further separated and determined by liquid chromatography, capillary electrophoresis, or measured after an immunochemical reaction.

Numerous individual methods have been published for mycotoxin analysis. For aflatoxin alone more than 8000 papers have been published; hence a great deal of judgment is required for the selection of the optimum protocol of analysis. The analyst must use authentic toxin standards of known purity and select appropriate methods according to particular needs. The following criteria are considered in selecting a method: number of analyses, time required, location of analysis (laboratory or field), cost of equipment, safety, waste disposal, and the experience required of the analyst ***(4)***. Some of the published methods have been validated in international collaborative studies, and their precision and accuracy estimated for specific commodities according to internationally harmonized rules. AOAC International is one of the organizations that administer collaborative studies following the AOAC-International Union of Pure and Applied Chemistry (IUPAC) harmonized procedure ***(5)***. Such studies typically yield both precision (intralaboratory relative standard deviation, RSD_r) and reproducibility (interlaboratory relative standard deviation, RSD_R) data. By examination of a large body of data generated in such collaborative studies, Horwitz came to the surprising conclusion that one could predict with some confidence the RSD_R to be expected based solely on the concentration of the analyte, and largely independent of the matrix, type of analyte or type of method ***(6)***. The RSD_R, when plotted as a function of the concentration (expressed as a decimal fraction), described a curve, the so-called "Horwitz Horn" (**Fig. 1**) ***(7)***. Thus at 1 μg/g (C, expressed in toxin mass/commodity mass unit (g), 1 ppm $C = 10^{-6}$) the expected precision would be approx 16%; at 1 ng/g (1 ppb) it would be approx 45%, etc. As a consequence, below 1 ng/g, when the RSD_Rs tend to rise above 50%, the results become uninterpretable because of the appearance of excessive numbers of false positive and false negative results. Such data are considered to be "out of statistical control." Analyses can be conducted below 1 ng/g in specific laboratories if extraordinary steps are taken to ensure quality control.

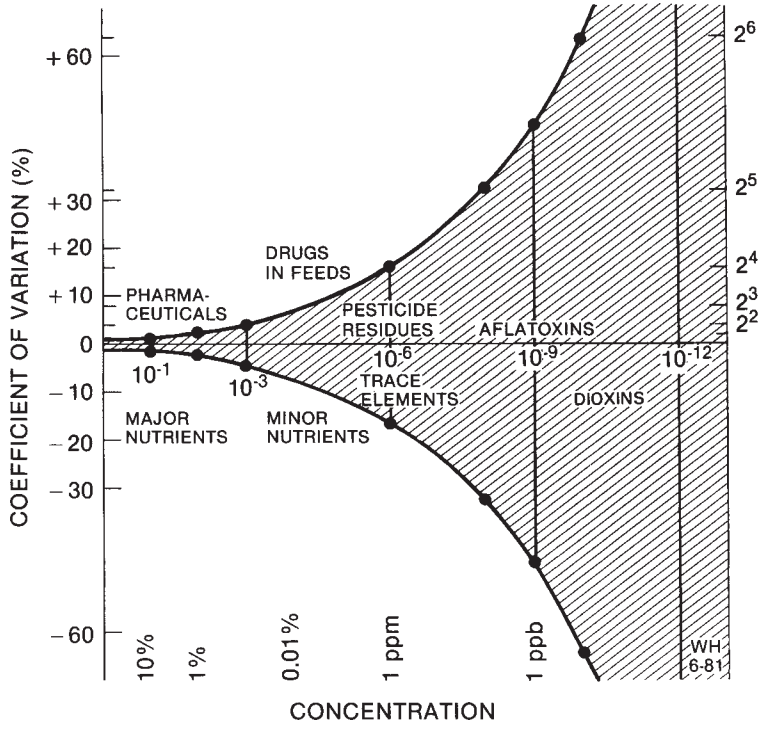

Fig. 1. The "Horwitz Horn.."

The now well-known "Horwitz Equation," $RSD_R = 2C^{-0.1505}$ quantified the relationship between the RSD_R and analyte concentration ***(8)***. C is in terms of a decimal fraction (ranging from pure compound, g/g, C = 1, to ultratrace toxins, ng/kg; $C = 10^{-12}$). Hall and Selinger characterized this relationship as "one of the most intriguing relationships in modern analytical chemistry" ***(9)***. A heuristic derivation of this relationship was recently published based upon the observed change in standard deviation(s) as a function of concentration ***(8)***. It can easily be shown, using this relationship, that the RSD_R will increase by a factor of two for each decrease in analyte concentration by two orders of magnitude.

The Horwitz Equation is extremely useful in evaluating analytical methods, i.e., their fitness for the intended use. A collaborative study is run, and the results are compared with those predicted by the Horwitz Equation. This is done by calculation of the Horwitz Ratio (HORRAT) ***(6)***:

$$\text{HORRAT} = RSD_R \text{ (found)}/RSD_R \text{ (predicted)}$$

Based upon data generated on thousands of samples analyzed in a large number of collaborative studies (each study often included more than 8 collabora-

tor-analysts of international laboratories) over many years, it was concluded that a HORRAT > 2 indicates that the method is not acceptable, i.e., leads to poor results in the hands of experienced analysts in well run laboratories. Values bracketing 1.0 indicate acceptable precision and a method clearly under statistical control. Experience has shown that high HORRAT values usually indicate that (a) the method as written is problematic; (b) the samples distributed to the collaborators were nonhomogeneous; or (c) the laboratories involved had difficulty with the preparation, retention, or stability of standards. Low HORRAT values (<0.5) also have some significance, indicating either (a) lack of independence in the analyses; or (b) the use of advanced technology and strict adherence to a QA program by the laboratories involved. Lack of independence often results from (a) unreported consultation among participants; (b) replication by analysts until results are in agreement; (c) averaging by individual analyst of multiple analyses; or (d) excessive rounding.

The vast majority of published methods have not been validated by interlaboratory collaborative study. However, even with these, one can estimate an approximate HORRAT and come to some defensible conclusions relative to their capabilities.

In **Table 1** are listed some of the mycotoxin methods which have been collaboratively studied and have been approved as "Official Methods" by AOAC International and, as a consequence, are often used by the US Federal and State regulatory laboratories ***(10)***. It gives commodities, levels, statistics, and HORRAT values. The HORRAT were obtained by comparing RSD_R of the particular study to the RSD_R of the corresponding concentration C from **Fig. 1**. In the process of validation, the method is first submitted to collaborative study (test samples with various toxin levels in duplicate are analyzed as unknowns by 8–15 laboratories), and the results are evaluated by a group of expert analysts (the safety officer, the general referee of the topic, the associate referee of the study, the statistician, and the method committee, and the AOACI Official Methods Board, OMB). If the study was properly conducted according to the "harmonized protocol," if the analytical data fulfills the required statistical parameters, and if the reviewers' comments (general referee, the statistician, members of the method committee) have been addressed, the OMB will accept the method as a " First Action AOACI Official Method." After two years of successful use the method is adopted as final action and is published as an "Official Method" in the AOACI Compendium of Official Methods.

The use of a validated (Official) methods or published methods does not automatically ensure that a laboratory will produce acceptable results. It should be clearly understood that such methods are developed for use with a particular commodity (matrix). Should the method be used for other matrices, it is necessary for the analyst to evaluate applicability by conducting recovery studies

Table 1
Some of the AOACI Official Methods for Mycotoxins

Mycotoxin	Method	Commodity	Level ng/g	RSD_r[a] %	RSD_R[b] %	$PRSD_R$[c] %	HORRAT[d]
Aflatoxin B_1	990.32	Corn	11	14.9	45.7	31.5	1.4
	ELISA	Roasted peanuts	6	19.4	52.7	34.5	1.5
Aflatoxins	991.31	Corn	30	16.6	20.1	27.1	0.74
	IAC/LC	Raw peanuts	20	12.8	15.3	28.8	0.53
		Peanut butter	10	11.8	13.6	32.0	0.43
Aflatoxin B_1	993.17	Corn	5	56.6	56.6	35.5	1.6
	SPE/TLC	Raw peanuts	5	21.3	26.4	35.5	0.74
Aflatoxin B_2	993.17	Corn	3	26.8	49.3	38.3	1.3
		Raw peanuts	1.5	38.1	50.3	42.6	1.2
Aflatoxin G_1	993.17	Corn	10	43.6	60.7	32.0	1.9
Aflatoxin G_2	993.17	Corn	3	48.8	2.8	38.3	1.6
Aflatoxin M_1	986.16	Fluid milk	0.2	27.7	57.6	0.48	
	SPE/LC	Fluid milk	0.42	19.2	51.6	0.37	
Aflatoxin M_2	986.16	Fluid milk	0.05	42.1	71.0	0.59	
		Fluid milk	0.13	12.5	61.5	0.20	
Deoxynivalenol	986.18	Wheat	350	30.4	54.0	18.7	2.9
	Column/GC						
Fumonisin B_1	995.15	Corn	500	13.9	7.1	17.8	0.40
Fumonisin B_2	SPE/LC		200	16.3	8.4	20.4	0.41
Fumonisin B_3			100	19.6	10.1	22.6	0.45

continued

Table 1 *(continued)*

Mycotoxin	Method	Commodity	Level ng/g	RSD_r[a] %	RSD_R[b] %	$PRSD_R$[c] %	HORRAT[d]
Ochratoxin A	991.44	Corn	10	20.7	20.1	32.0	0.63
	SPE/LC	Barley	10	7.9	26.5	32.0	0.83
Patulin	995.10 Liquid-liquid Partition/LC	Apple juice	20	18.1	23.5	28.8	0.82
α-Zearalenol	985.18 Liquid-liquid Partition/LC	Corn	100	21.9	22.6	0.97	
Zearalenone	985.18	Corn	500	26.1	17.8	1.5	
Zearalenone	994.01	Corn	800	3.1	16.3	16.5	0.99
	ELISA	Wheat	1000	10.1	15.9	31.9	0.50
		Pig feed	500	16.1	27.2	17.8	1.5

[a]Intralaboratory relative standard deviation.
[b]Interlaboratory relative standard deviation.
[c]Predicted interlaboratory relative standard deviation.
[d]HORRAT = $RSD_R/PRSD_R$.

with emphasis on the acceptability of the results at the level of toxicological concern. The analyst must compare results of his or her studies on the material in question with data of the collaborative studies of published methods. A laboratory quality assurance program should be implemented. Attention should be given to standard operating procedures, sample integrity and traceability, reference material, standard, control charting, and record keeping. The participation in proficiency testing programs is also recommended ***(11)***.

Confirmation of identity of the analyte is necessary when regulatory action is involved or the identity of the analyte is in question. Both chemical derivatization procedures and mass spectrometric analysis are most commonly employed. The chemical derivatization procedures are specific in converting the isolated toxin to a derivative, which exhibits different chromatographic and/or other physical properties from the parent compound. Identity is confirmed when the derivative from the analyte has the same characteristics as the derivative from the analytical standard. The most definitive method for confirming the identity of mycotoxins involves the application of mass spectrometry. The mass spectrum of compounds can be used to identify the analyte and elucidate unknown chemical structures.

In summary, analytical data of high creditability is obtained through the use of properly evaluated methods and the adherence to quality assurance principles by qualified analysts. The data can be used for science-based risk assessments to control mycotoxins in the food and feed supply.

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2

Sampling Techniques

Thomas B. Whitaker

1. Introduction

It is important to be able to detect and quantify the mycotoxin concentration in food and feedstuffs destined for human and animal consumption. In research, regulatory, and quality assurance activities, correct decisions concerning the fate of commercial lots can only be made if mycotoxin test procedures are accurate and precise. However, it is difficult to estimate accurately and precisely the mycotoxin concentration in a large bulk lot because of the large variability associated with the mycotoxin test procedure ***(1–8)***. A mycotoxin test procedure is a complicated process and generally consists of 3 steps: (a) a sample is taken from the lot, (b) the sample is ground in a mill to reduce particle size, and a subsample is removed from the comminuted sample for extraction, and (c) the mycotoxin is extracted from the comminuted subsample and quantified. There have been several reviews published describing accepted procedures for sampling, sample preparation, and analysis for agricultural commodities ***(9–15)***. Even when using accepted procedures, there are errors (the term error will be used to denote variability) associated with each of the above steps of the mycotoxin test procedure. Because of these errors, the true mycotoxin concentration in the lot cannot be determined with 100 percent certainty by measuring the mycotoxin concentration in the sample taken from the lot.

In this chapter we will discuss the different sources of variability that are associated with testing agricultural commodities for mycotoxins. Specifically, we will concentrate on the testing of agricultural commodities for aflatoxin since most published literature is concerned with this mycotoxin. We will show how to reduce the variability of mycotoxin test results and how to design testing programs to determine the mycotoxin level of a contaminated lot as accurately and precisely as resources will permit.

From: *Methods in Molecular Biology, vol. 157: Mycotoxin Protocols*
Edited by: M. W. Trucksess and A. E. Pohland

Table 1
Distribution of Aflatoxin Test Results for Ten 5.4 kg Samples from Each of Six Lots of Shelled Peanuts[a,b]

Lot Number	Sample Test Result (ppb)										Mean (ppb)	SD[c] (ppb)	CV[d] (%)
1	0	0	0	0	2	4	8	14	28	43	10	15	150
2	0	0	0	0	3	13	19	41	43	69	19	24	126
3	0	6	6	8	10	50	60	62	66	130	40	42	105
4	5	12	56	66	70	92	98	132	141	164	84	53	63
5	18	50	53	72	82	108	112	127	182	191	100	56	56
6	29	37	41	71	95	117	168	174	183	197	111	66	59

[a]From Whitaker et al. (1972).
[b]Aflatoxin test results are order by aflatoxin ppb.
[c]SD = Standard Deviation.
[d]CV = Coefficient of Variation = (SD/mean × 100).

2. Variability of Mycotoxin Test Procedures

Assuming accepted test procedures are used to estimate the mycotoxin concentration of a bulk lot, random variation still exists among replicate mycotoxin tests on the same bulk lot. For example, 10 replicated aflatoxin test results from each of 6 contaminated shelled peanut lots are shown in **Table 1** ***(16)***. Each test was made by (a) comminuting a 5.45 kg sample of peanut kernels in a subsampling mill developed by Dickens and Satterwhite ***(17,18),*** (b) extracting aflatoxins from a 280 g subsample with the AOAC Method II (BF method), and (c) quantifying the aflatoxins densitometrically using thin layer chromatography (TLC). The 10 aflatoxin test results from each lot are ranked from low to high to demonstrate several important characteristics about replicated aflatoxin test results taken from the same contaminated lot.

First, the wide range among replicated test results from the same lot reflects the large variability associated with estimating the true mycotoxin content of a bulk lot. In **Table 1**, the variability is described by both the standard deviation and the coefficients of variation (CV). The maximum test result can be four to five times the lot concentration (the average of the 10 test results is the best estimate of the lot concentration). Secondly, the amount of variation among the 10 test results appears to be a function of the lot concentration. As the lot concentration increases, the standard deviation among test results increases, but the standard deviation relative to the lot mean, as measured by the CV, decreases. Thirdly, the distribution of the 10 test results for each lot in **Table 1** are not always symmetrical about the lot concentration. The distributions are positively skewed, meaning that more than half of the sample test

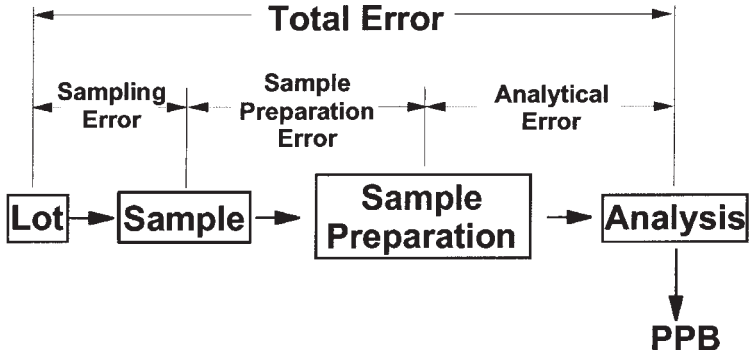

Fig. 1. Types of error associated with mycotoxin testing.

results are below the lot concentration. However, the distribution of sample test results becomes more symmetrical as the lot concentration increases. This skewness can be observed by counting the number of test results above and below the lot concentration in **Table 1**. If a single sample is tested from a contaminated lot, there is more than a 50% chance that the sample test result will be lower than the true lot concentration. The skewness is greater for small sample sizes and the distribution becomes more symmetrical as sample size increases ***(19)***.

The variability shown in **Table 1** is the sum of the variability associated with each step of the mycotoxin test procedure. As shown in **Fig. 1**, the total variability (using variance as the statistical measure of variability) associated with a mycotoxin test procedure is equal to the sum of the sampling, sample preparation, and analytical variances associated with each step of the mycotoxin test procedure.

$$VT = VS + VSS + VA \quad (1)$$

Examples of the magnitude of the variability associated with each step of a mycotoxin test procedure (**Eq. 1**) are given in the sections below.

3. Sampling Variability

There are two important aspects that can affect sampling variability. First is the sample selection procedure, and second is the distribution among contaminated particles within a lot. Generally, using proper sampling equipment and procedures can minimize any effect of sample selection, but only increasing sample size can reduce the effects of the distribution among contaminated particles within a lot on sampling variability. These two aspects affecting sampling variability are discussed below.

3.1. Sample Selection Methods

Procedures used to take a sample from a bulk lot are extremely important. Every individual item in the lot should have an equal chance of being chosen (called random sampling). Biases will be introduced by the sample selection methods if equipment and procedures used to select the sample prohibit or reduce the chances of any item in the lot from being chosen ***(20)***. If the lot has been blended thoroughly from the various material handling operations, then the contaminated particles are probably distributed uniformly throughout the lot. In this situation, it is probably not too important from what location in the lot the sample is drawn. However, if the product is contaminated because of moisture leaks or for other reasons, then the mycotoxin contaminated particles may be located in isolated pockets in the lot ***(21)***. If the sample is drawn from a single location, the contaminated particles may be missed or too many contaminated particles may be collected. Because contaminated particles may not be distributed uniformly throughout the lot, the sample should be an accumulation of small portions taken from many different locations throughout the lot ***(22,23)***. The accumulation of many small incremental portions is called a bulk sample. If the bulk sample is larger than desired, the bulk sample should be blended and subdivided until the desired sample size is achieved. The smallest size sampling unit used before the sample preparation step to estimate the lot mycotoxin concentration is often called the test sample. It is generally more difficult to obtain a representative (lack of bias) sample from a lot at rest (static lot) than from a moving stream of the product (dynamic lot).

3.1.1. Static Lots

Examples of static lots are commodities contained in storage bins, railcars, or many small containers such as sacks. When drawing a sample from a bulk container, a probing pattern should be developed so that product can be collected from different locations in the lot. An example of several probing patterns used by the Agricultural Marketing Service to collect samples from bulk peanut lots is shown in **Fig. 2**. The sampling probe should be long enough to reach the bottom of the container when possible. As a general rule, several hundred grams of sample should be drawn per 1000 kg of commodity.

When sampling a static lot in separate containers such as sacks, the sample should be taken from many containers dispersed throughout the lot. When storing sacks in a storage facility, access lanes should be left in order to gain access to interior sacks. The recommended number of sacks sampled can vary from one-fourth of the sacks in small lots to the square root of the number of sacks for large lots ***(24)***.

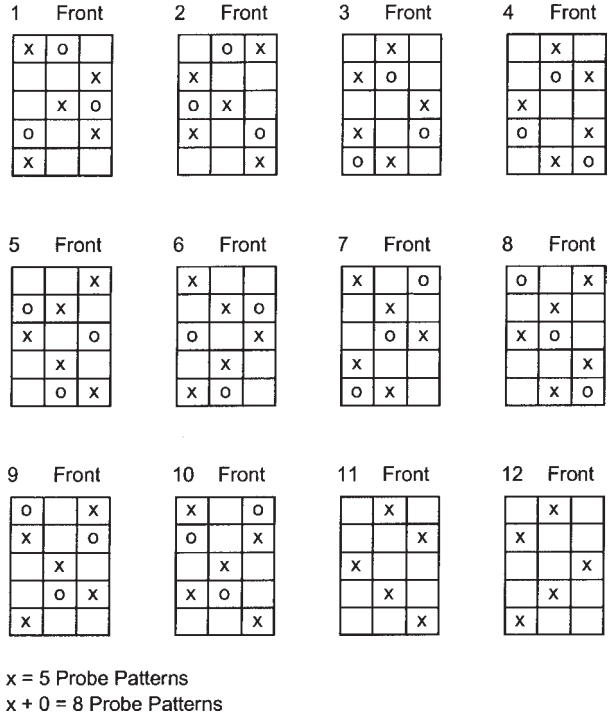

Fig. 2. Example of several five- and eight-probe patterns used by the USDA to sample farmers stock peanuts for grade and support price.

If the lot is in a container where access is limited, the sample should be drawn when the product is either being removed from or being placed into the container. If the accumulated bulk sample is larger than required, the bulk sample should be thoroughly blended and reduced to the required test sample size with a suitable device such as a riffle divider.

3.1.2. Dynamic Lots

True random sampling can be more nearly achieved when selecting a bulk sample from a moving stream as the product is transferred (i.e., conveyor belt) from one location to another. When sampling from a moving stream, small increments of product should be taken along the entire length of the moving stream; composite all the increments of product to obtain a bulk sample; if the bulk sample is larger than required, then blend and subdivide the bulk sample to obtain the desired size test sample.

Automatic sampling equipment such as cross-cut samplers (**Fig. 3**) are commercially available with timers that automatically pass a diverter cup through

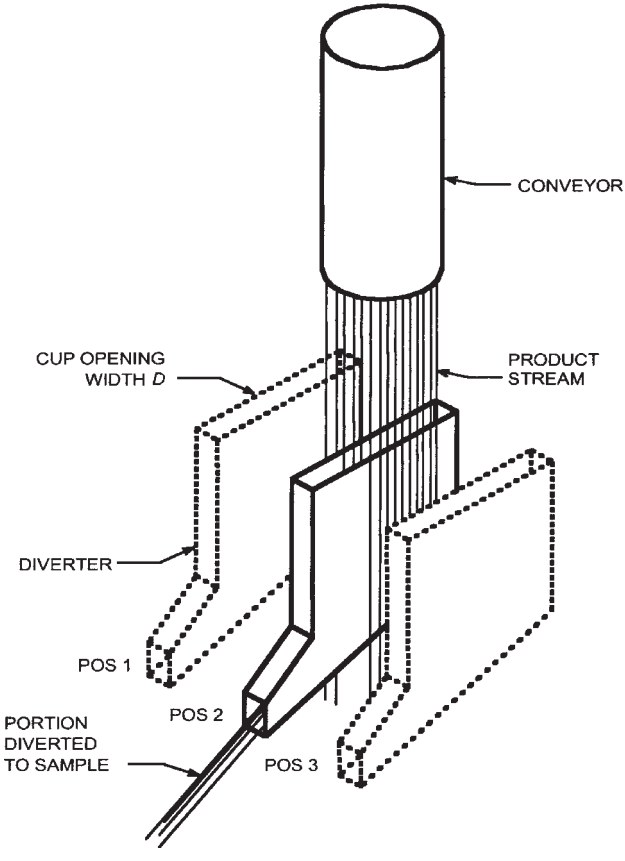

Fig. 3. Automatic cross-cut sampler.

the moving stream at predetermined and uniform intervals. When automatic equipment is not available, a person can be assigned to manually pass a cup though the stream at periodic intervals to collect the bulk sample. Whether using automatic or manual methods, small increments of product should be collected and composited at frequent and uniform intervals throughout the entire time product flows past the sampling point.

Cross-cut samplers should be installed in the following manner: (1) the plane of the opening of the sampling cup should be perpendicular to the direction of flow; (2) the sampling cup should pass through the entire cross sectional area of the stream; and (3) the opening of the sampling cup should be wide enough to accept all items of interest in the lot. As a general rule, the width of the sampling cup opening should be two to three times the largest dimensions of the items in the lot.

The size of the bulk sample, S in kg, taken from a lot by a cross-cut sampler is:

$$S = (D)\,(L) / (T)\,(V) \quad (2)$$

where D is the width of the sampling cup opening in cm, L is the lot size in kg, T is interval or time between cup movement through the stream in seconds, and V is cup velocity in cm/s.

Eq. 2 can also be used to compute other terms of interest such as the time between cuts, T. For example, the required time, T, between cuts of the sampling cup to obtain a 10 kg sample from a 30,000 kg lot where the sampling cup width is 5.08 cm (2 inches), and the cup velocity through the stream 30 cm/s. Solving for T in **Eq. 2**, T = (5.08 cm × 30,000 kg)/(10 kg × 30 cm/s) = 508 s.

If the lot is moving at 1000 kg/min, the entire lot will pass through the sampler in 30 min and only three or four cuts will be made by the cup through the lot. This may be considered too infrequent, in that too much product passes through the sampler between the time the cup cuts through the stream. The interaction among the variables in **Eq. 2** need to be fully understood in terms of the amount of sample accumulated and the frequency of taking the product.

3.2. Contamination Distribution

Studies by researchers on a wide variety of agricultural products (peanuts, cottonseed, shelled corn, and pistachio nuts) indicate that, especially for small sample sizes, the sampling step is usually the largest source of variability associated with the mycotoxin test procedure ***(1–8)***. Accepted sample selection equipment and procedures were used to minimize any effects due to sample selection methods. Sampling error is large because of the extreme distribution among contaminated particles within a lot. Aflatoxin studies on peanuts suggest about 0.1% of the kernels in the lot are contaminated and the concentration on a single kernel may be extremely high. Cucullu et al. ***(25,26)*** reported aflatoxin concentrations in excess of 1,000,000 ng/g (parts per billion, ppb) for individual peanut kernels and 5,000,000 ng/g for cottonseed. Shotwell et al. ***(27)*** reported finding over 400,000 ng/g of aflatoxin in a corn kernel.

Because of this extreme range in aflatoxin concentrations among a few contaminated kernels in a lot, variation among replicated sample test results tends to be large. As an example, the sampling variance associated with testing shelled corn, VSs, was estimated empirically ***(3)*** and is shown in **Eq. 3**.

$$VSs = 3.95M/WSs \quad (3)$$

where M is the aflatoxin concentration in the lot in nanograms of total aflatoxin per g of corn (ng/g) or parts per billion (ppb), WSs is the mass of shelled corn in the sample in kg (kernel count per gram was 3.0). From **Eq. 3** one can see that the sampling variance is a function of the lot concentration M and

sample size WSs. The sampling variance associated with taking a 0.91 kg (2 lb) sample from a lot of shelled corn at 20 ppb is 86.9. The coefficient of variation is 47%.

Researchers have developed equations to describe the sampling variance for several commodities and several mycotoxins ***(1–8)***. The equations are specific for the type of mycotoxin and the type of product studied.

4. Sample Preparation Variability

Once the test sample has been taken from the lot, the sample must be prepared for aflatoxin extraction. Since it is not practical to extract the mycotoxin from a large test sample, the mycotoxin is usually extracted from a much smaller portion of product (subsample) taken from the test sample. If the commodity is a granular product such as shelled corn, it is essential that the entire test sample be comminuted in a suitable mill before a subsample is removed from the test sample ***(9)***. Removing a subsample of whole seed from the test sample before the comminution process would eliminate the benefits associated with the larger size sample of granular product. After the sample has been comminuted, a subsample is removed for mycotoxin extraction. It is assumed that the distribution of contaminated particles in the comminuted sample is similar to the distribution among contaminated kernels found in the lot. As a result, there is also variability among replicated subsamples taken from the same test sample. However, the sample preparation variance is not as large as the sampling variance due to the large number of comminuted particles in the subsample. An example of sample preparation variance for aflatoxin and shelled corn, VSSs, is shown below in **Eq. 4** ***(3)***.

$$VSSs = 0.0125M/WSSs \quad (4)$$

where M is the aflatoxin concentration in the comminuted test sample in ppb, and WSSs is the mass in kg of comminuted shelled corn in the subsample. The variance in **Eq. 4** also reflects a particle size that will pass through a number 20 screen. From **Eq. 4**, it can be seen that the sample preparation variance is also a function of the aflatoxin concentration in the sample and the subsample size. The sample preparation variance associated with a 0.05 kg subsample taken from a sample at 20 ppb is 5.0 and the CV is 11%.

Researchers have developed equations to describe the sample preparation variance for several commodities, type mills, and mycotoxins ***(1–8,28)***. The equations are specific for the type mycotoxin, type mill, and the type product used in the study. The type mill effects the particle size distribution. If the average particle size decreases (number of particles per unit mass increases), then the subsampling variance for a given size subsample decreases.

5. Analytical Variability

Once the subsample is removed from the comminuted test sample, the mycotoxin is extracted. Analytical methods usually involve several steps such as solvent extraction, centrifugation, drying, dilution, and quantification ***(10)***. As a result, there can be considerable variation among replicated analyses on the same subsample extract. The analytical variance, VAbf, associated with AOAC method II extraction and clean-up procedures along with TLC and densitometric quantification techniques to measure aflatoxin in peanuts (BF method) is given by **Eq. 5** ***(1)***.

$$VAbf = 0.064M^{1.93}/Nbf \quad (5)$$

where M is the aflatoxin concentration (ppb) in the subsample, and Nbf is the number of aliquots quantified by TLC methods. For example, at 20 ng/g, the variance and CV associated with the BF method is 20.9 and 22.8%, respectively. Studies on the BF method ***(30)*** indicate that the thin layer chromatography quantification step is the major source of variability in the analytical process associated with analyzing peanuts for aflatoxin.

If extraction and cleanup contribute only a small portion of the total analytical variance, then the immunoassay and high performance liquid chromatography (HPLC) type analytical methods should have lower variances than methods that use TLC quantification techniques. Hagler and Whitaker ***(31)***, and Dorner and Cole ***(32)*** independently measured the analytical variance associated with HPLC type methods to measure aflatoxin in peanuts. Even though Hagler and Dorner used slightly different extraction and cleanup procedures ***(31–33)***, both obtained almost identical results. The relationship between variance and aflatoxin concentration of Hagler's study for HPLC are given below.

$$VAh = 0.0048M^{1.75}/Nh \quad (6)$$

where M is the aflatoxin concentration in the subsample and Nh is the number of aliquots quantified by the HPLC procedure. At 20 ng/g, the variance and CV associated with the HPLC method is 0.9 and 4.8%, respectively. A CV of 4.8% associated with HPLC is much lower than the 22.8% associated with the BF method using TLC quantification techniques.

Immunoassay techniques are a more recent analytical development to measure mycotoxins in agricultural commodities such as peanuts, corn, and cottonseed. Food and feed industries, researchers, and regulatory agencies have studied the variability associated with immunoassay-type analytical methods ***(34,35***; Whitaker, unpublished data, 1991). The variability one might expect using an immunoassay-type analytical method to quantify aflatoxin in peanut products is given below.

$$VAi = 0.013M^{1.57}/Ni \quad (7)$$

where M is the aflatoxin concentration in the subsample and Ni is the number of aliquots quantified by the immunoassay procedure. **Eq. 7** reflects the pooling of variance data from corn, cottonseed, and peanuts. From **Eq. 7**, the variance associated with quantifying the aflatoxin in a subsample at 20 ppb using an immunoassay method is 1.9, and the CV is 7%. The variability associated with immunoassay type methods appears to be less than TLC methods and more than HPLC methods.

All of the analytical variance information described above reflects results from single laboratories and do not reflect among laboratory variances. As a result, some laboratories may have higher or lower variances than those reported in **Eq. 5**, **6**, and **7**. Among laboratory variance is about double the within laboratory variance ***(36)***.

6. Reducing Variability of Test Procedure

The only way to achieve a more precise estimate of the true lot concentration is to reduce the total variability or the variability associated with each step of the mycotoxin test procedure. The sampling variability can be reduced by increasing the size of the sample. The sample preparation variability can be reduced either by increasing the size of the subsample and/or by increasing the degree of comminution (increasing the number of particles per unit mass in the subsample). The analytical variance can be reduced by either increasing the number of aliquots quantified by the analytical method and/or using more precise quantification methods (using HPLC instead of TLC). If the variability associated with one or more of these steps can be reduced, then the total variability associated with a mycotoxin test result can be reduced.

For example, the expected total variance associated with testing a shelled corn lot at 20 ppb when using a 0.91 kg sample, taking a 50 g subsample from a comminuted sample, and using an immunoassay analytical method for quantification can be estimated by summing the variances (equation 1) calculated using **Eqs. 3**, **4**, and **7**.

$$VT = 86.9 + 5 + 1.9 = 93.8 \quad (8)$$

The variance, standard deviation, and CV associated with the total aflatoxin test procedure described above is 93.8, 9.7, and 48.4%, respectively. The sampling, subsampling, and analytical variances account for 92.6, 5.3, and 2.1% of the total testing variance, respectively. The major variance component is sampling which accounts for 92.6% of the total testing variation. It appears that the best use of resources to reduce the total variance of the test procedure would be to increase sample size. Increasing the sample size by a factor of five from 0.91

Table 2
Range of Aflatoxin Estimates for 95% Confidence Limits When Testing a Contaminated Lot of Shelled Corn with 20 ppb Using Different Sample Sizes.

Sample Size (kg)	Standard Deviation[a] (ppb)	Low[b] (ppb)	High[c] (ppb)
1	9.2	2.0	38.0
2	6.8	6.7	33.3
4	5.2	9.8	30.2
8	4.1	12.0	28.0
16	3.4	13.3	26.7
32	3.1	13.9	26.1

[a]Standard deviation reflects sample size shown in table plus a 50 g subsample that will pass a #20 screen and immunoassay analytical method. Sample preparation plus analytical standard deviation = 2.6 and is constant for all sample sizes.
[b]Low = 20 - 1.96(standard deviation)
[c]High = 20 + 1.96(standard deviation)

to 4.54 kg will cut the sampling variance in **Eq. 8** by a factor of five to 17.4. The total variance with the 4.45 kg sample now becomes (**Eq. 9**):

$$VT = 17.4 + 5.0 + 1.9 = 24.3 \quad (9)$$

The variance, standard deviation, and CV associated with the total testing procedure has been reduced to 24.3, 4.9, and 24.6%, respectively.

The range of mycotoxin test results associated with any size sample and subsample, and number of analyses can be estimated from the standard deviation SD (square root of the total variance). Approximately ninety-five percent of all test results will fall between a low of (M – 1.96*SD) and a high of (M + 1.96*SD). The two expressions are only valid for a normal distribution where test results are symmetrical about the mean. The distribution among aflatoxin test results is usually skewed, but will approach a symmetrical distribution as the sample size becomes large. The effect of increasing sample size on the range of test results when testing a contaminated lot of shelled corn that has 20 ppb aflatoxin is shown in **Table 2**. We can see that the range doesn't decrease at a constant rate as sample size increases. For example, doubling sample size has a greater effect on decreasing the range at small sample sizes than at large sample sizes. This characteristic suggests that increasing sample size beyond a certain point may not be the best use of resources and that increasing subsample

size or number of analyses may be a better use of resources in reducing the range of test results once sample size has become significantly large.

As indicated above, there are methods other than increasing sample size to reduce the total variance associated with testing a commodity for a mycotoxin. Different costs are associated with each step of the mycotoxin test procedure, and careful study is required to determine the test procedure that will provide the lowest variance for a given cost. The optimum balance in sample size, degree of comminution, subsample size, number and type of analysis will vary with the costs involved with each step of the testing procedure. In general, the costs of properly designed mycotoxin test procedures will increase as the total variance is reduced.

7. Conclusions

Because of the variability associated with a mycotoxin test procedure, it is difficult to determine with 100% certainty the true concentration of a bulk lot. Even when the sample is correctly selected, there will be variability associated with the mycotoxin test procedure. The variance associated with a mycotoxin test procedure is the sum of sampling, sample preparation, and analytical variances. For small sample sizes, sampling is usually the largest source of variability. The variability associated with a mycotoxin test procedure can be reduced by increasing sample size, the degree of sample comminution, subsample size, and the number of aliquots quantified.

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3

Preparatory Isolation of Mycotoxins from Solid Phase Fungal Cultures

Robert M. Eppley

1. Introduction

Mycotoxins are a large group of secondary fungal metabolites possessing significantly different chemical and physical properties. Because of this diversity, no general procedures can be developed for the isolation and purification of all of the different mycotoxins. The aim of this chapter is to present a procedure that has been successfully applied to the isolation and purification of one mycotoxin group (the fumonisins) which can be adapted to the preparation of many of the other mycotoxins. The fumonisins are water soluble and were missed in many of the early attempts at isolation of the toxic agents produced by *Fusarium verticillioides* (= *F. moniliforme*). These metabolites were not isolated and identified until 1988 ***(1)***. Most of the mycotoxins identified before this time were extracted from the culture media with organic solvents such as chloroform, acetone, ethyl acetate, methanol, and so on ***(2–11)***. Methods using aqueous-organic phase extractants have more versatility and can be made more selective for a particular group of mycotoxins by adjusting the ratio of water to organic solvent.

Various chromatographic techniques have been successfully used in the purification procedures for secondary fungal metabolites; however, open column chromatography and preparatory thin layer chromatography have been the most widely used, separately and in combination ***(2–5)***. These two chromatographic techniques have been very effectively used to isolate and purify multigram quantities of various natural products. More recently, high performance liquid chromatography (HPLC) has become the method of choice for isolation and purification of these metabolites from complex matrices ***(7–11)***.

From: *Methods in Molecular Biology, vol. 157: Mycotoxin Protocols*
Edited by: M. W. Trucksess and A. E. Pohland

The fumonisins have been under intense study since their identification as the probable cause of equine leukoencephalomalacia ***(1)***. The fumonisins also have been shown to be the cause of porcine pulmonary edema ***(12)*** and to cause a number of other toxic effects in laboratory animals ***(13,14)***. The report of carcinogenicity in rats fed at the 50 mg/kg level ***(15)*** set in motion studies to determine the chronic effects of FB_1 in rodents. The following procedure was developed to supply the greater than one kilogram of fumonisin B_1, and gram quantities of fumonisin B_2 and B_3 needed for the toxicity studies.

2. Materials

2.1. Fungal Culture Material

The fungal culture material was prepared by the Fusarium Research Center, Pennsylvania State University (*see* **Note 1**). Briefly, a known fumonisin-producing *Fusarium proliferatum* was inoculated into 500 g yellow corn and 500 mL distilled water, after the mixture was autoclaved in polyethylene bags. The bags were incubated in the dark at 20–22°C for 4 wk.

2.2. Preparatory HPLC System

1. A programmable solvent delivery system capable of producing gradient mixtures of at least two solvents. Pumps capable of delivering 1.0–10 mL/min are usually adequate for gram-quantity purification.
2. A sample loading system. A three-way valve inserted between the elution solvents and the pump to switch between elution solvents and sample solution.
3. Detector is optional, variable wavelength UV, ELSD, and so on. This may require tee to split the eluate flow to protect the detector from overload.
4. Fraction collector, either peak or time actuated.
5. Reverse-phase, 40 × 100 mm or larger columns (Waters, BondaPak, 3 units, 40 × 100 mm, 18–20 µm particle size, 125Å pore size and Rainin Dynamax, 41.4 × 250 mm, 8 µm particle size, 60Å pore size, with guard column).
6. Cyano bonded column (Waters, µBondapak CN, 4 units, 40 × 100 mm, 10 µm particle size, 125Å pore size).
7. Large platform shaker or stirring system.
8. Large (50 cm) Buchner filter and high-flow filter paper (Whatman No. 1).
9. Vacuum evaporator and vacuum pump (Buchi Rotovap).
10. Freeze-dryer equipped with trays.
11. Extraction solvents: Laboratory grade methanol or acetonitrile and distilled water.
12. Filter-aid (Celite, acid washed).
13. 14 qt polyethylene buckets with cap.

2.3. Analytical HPLC System

1. An isocratic or programmable solvent delivery system capable of producing mixtures of at least two solvents. Pumps capable of delivering 0.5–1.0 mL/min are adequate.

2. Fluorescence detector with tunable emission and excitation wavelength control.
3. Sample preparation module and data system are desirable.
4. Reverse-phase analytical column.
5. Analytical solvents: HPLC grade acetonitrile, glacial acetic acid and distilled water.
6. o-Phthaldialdehyde (OPA).
7. 2-Mercaptoethanol.

3. Methods

3.1. Preparation of Reagents

1. Extraction solutions: Mix 3 parts methanol with 1 part water or mix 1 part acetonitrile with 1 part of water.
2. Preparatory LC mobile phases: Methanol/water (3 + 5); (1 + 1); (2 + 1); (4 + 1), and acetonitrile/water (1 + 4); (3 + 7); (4 + 6); (4 + 1).
3. Analytical LC mobile phase: acetonitrile/water/glacial acetic acid (40 + 60 + 1).
4. Dissolve 38.1 g of disodium tetraborate in 1 L of distilled water to give 0.1 *M* solution.
5. OPA Reagent: Dissolve 40 mg OPA in 1mL of methanol, dilute with 5 mL of 0.1 *M* disodium tetraborate solution, and add 50 µL 2-mercaptoethanol. Mix and store in dark.

3.2. Extraction

1. Each 500 g bag of the *Fusarium proliferatum* cultures are extracted with methanol + water (2 L) or acetonitrile + water (2 L) by shaking overnight in covered 14 qt plastic (polyethylene) buckets.
2. The extract is filtered through a large Buchner (filter-aid such as Celite is often needed). Filter cake is washed with 2 L of the extraction solvent. This wash is used for the next extraction or combined with the first extract.
3. The methanol-water filtrate from 3 kg (~10 L) is diluted 1 to 2 with water (volume = 20 L) and pumped onto a preparatory C-18 column. A methanol + water (3 + 5, 1 + 1, and 2 + 1) step-gradient is run and fractions collected. Fumonisin B_1 elutes with approximately (1 + 1) methanol + water. Column is reconditioned with two or three column void volumes of 4 + 1 methanol + water, and/or 4 + 1 acetonitrile + water, and then two or three column void volumes of water.
4. Analyze fractions by an LC-OPA method (*see* **Note 2**).
5. The methanol is removed in a rotary-evaporator from the FB_1-containing fractions and the aqueous FB_1 solution pumped into another type of preparatory C-18 column (Rainin Dynamax, 41.4 × 250 mm, 8 m particle size, 60Å pore size, a guard column is recommended). An acetonitrile + water (1 + 4, 3 + 7, 4 + 6, and 4 +1) step-gradient is run. The FB_1 elutes with acetonitrile + water, 3 + 7. Column is reconditioned with acetonitrile + water, 4 + 1, and then water.
6. Analyze the fractions by the LC-OPA method (*see* **Note 2**).
7. Combine analytically pure fractions and remove acetonitrile in a rotary evaporator.
8. Freeze-dry to remove the water (*see* **Note 3**).
9. Transfer the freeze-dried product to a desiccator.

3.3. Conversion to the Ammonium Salt

1. Remove the acetonitrile (*see* **Subheading 3.2., step 7**) in a rotary-evaporator and add the aqueous solution of fumonisin B_1 to a preparatory cyano-column. Ammonia (1%) in water is used to elute the fumonisin B_1 ammonium salt. The cyano-column is repeated as necessary to separate the fumonisin B_1 from the pigments and other impurities. The fumonisins elute in the void volume. The C-18 column may be used in place of the cyano column (*see* **Note 4**).
2. Freeze-dry the purified ammonium salt solution as above (*see* **Note 5**).

4. Notes

1. The cultured corn (*Fusarium proliferatum*) was prepared by the Fusarium Research Center, Pennsylvania State University, under the direction of Professor Paul Nelson. *F. proliferatum* is another species known to produce fumonisins under laboratory conditions. An advantage of this culture procedure is that it did not turn the corn to mush and thus the filtration of the extracting solutions was more efficient.
2. The fractions are analyzed by the derivatization and LC procedure described in Stack, M. E. and Eppley, R. M. (1992) *J. AOAC, Intl.* **75,** 834–837.
3. The final product is a fluffy, hygroscopic powder. Samples must be stored in a desiccator. The fumonisins will pick up moisture from the air and eventually a hard, glass-like material forms which is very slow to dissolve in water.
4. The ammonia solution is very destructive of the C-18 column and will rapidly destroy it. The cyano column will also be destroyed with time.
5. Generally, the ammonium salt is less electrostatic and somewhat easier to weigh and transfer; however, the ammonia salts of the fumonisins are still very hygroscopic and need to be protected from moisture.

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4

Preparation of Mycotoxin Standards

Stanley Nesheim and Michael E. Stack

1. Introduction

The detection and quantitation of mycotoxins requires pure standards or standards for which the purity and identity are known. Methods for identifying and calibrating standards are necessary. Few commercial sources exist for the mycotoxins discussed in this volume, and for most mycotoxins, specifications of purity are not available. If primary standards are not available commercially, other sources may be investigators who have isolated standard materials which they may be willing to share. As a last resort, it may become necessary to isolate mycotoxins from appropriate fungal cultures. Calibration and purity tests have been developed based on physical and chemical properties such as melting points, visible/ultraviolet, nuclear magnetic, and infrared spectroscopy and mass spectrometry, and various methods of chromatography. Standards are quite expensive, and regardless of the source, the purity and authenticity can be variable. Analysts are responsible for calibrating the standards used in analysis. Procedures have been developed for this purpose ***(1)***. The present protocol outlines the preparation, calibration, purity determination, preparation of solutions, distribution, storage, and uses of quantitative aflatoxin standards, and is intended as a guide for application to other mycotoxins which are used in the protocols in this volume. The amounts needed for most methods of analysis and for fortifying various matrixes for use as laboratory test or control samples, are in the nanogram or microgram ranges. The protocols given are for the aflatoxins B_1, B_2, G_1, and G_2. Mycotoxins standards other than the aflatoxins may be prepared in the same way using the information provided in **Table 1**. For some mycotoxins, such as the fumonisins and deoxynivalenol, UV spectroscopy cannot be used due to the lack of a suitable chromophore in the molecule. For these molecules, use gravimetric

From: *Methods in Molecular Biology, vol. 157: Mycotoxin Protocols*
Edited by: M. W. Trucksess and A. E. Pohland

Table 1
Calibration of Mycotoxins by UV Spectroscopy

Mycotoxin	Solvent	Conc. µg/mL	Molecular Weight	Molar Absorptivity ε	Wavelength,[a] nm	Reference
Aflatoxin B_1	Toluene-acetonitrile (9 + 1)	10	312	19,300	350	3
Aflatoxin B_2	Toluene-acetonitrile (9 + 1)	10	314	21,000	350	3
Aflatoxin G_1	Toluene-acetonitrile (9 + 1)	10	328	16,400	350	3
Aflatoxin G_2	Toluene-acetonitrile (9 + 1)	10	330	18,300	350	3
Aflatoxin M_1	Acetonitrile	10	328	18,900	350	1
Aflatoxin M_2	Acetonitrile	10	330	21,400	350	1
Citrinin	Chloroform	10	250	16,100	332	2
Cyclopiazonic Acid	Methanol	10	336	20,417	284	2
Deoxynivalenol	Ethanol	50	296	4,500	218	2
Moniliformin	Water	50	98	5,600	260	2
Ochratoxin A	Benzene-acetic acid (99 + 1)	40	403	5,600	333	1
Patulin	Ethanol	10	154	14,600	275	1
Sterigmatocystin	Benzene	10	324	15,200	325	1
Zearalenone	Methanol	50	318	6,000	314	1

[a]The wavelength is variable depending on the solvent. The absorbance, A, is the maximum as measured near the indicated wavelength.

methods combined with gas chromatography, liquid chromatography, and/or mass spectrometry.

2. Materials

2.1. Apparatus

1. Spectrophotometer (Beckman, Fullerton, CA).
2. Analytical balance (Mettler, Zurich, Switzerland).
3. Thin layer chromatography (TLC) apparatus, including silica gel 60 TLC plates, 10 µL syringe, TLC developing tank and viewing cabinet with long-wave ultraviolet lamp (VWR Scientific, Philadelphia, PA).
4. Liquid chromatograph with fluorescence detector and 15 cm, 5 µm C_{18} column (Waters Corporation, Milford, MA).
5. Vortex mixer (Fisher Scientific, Pittsburgh, PA).
6. Bottle-top dispenser, adjustable from 1 to 5 mL in 0.1 mL increments (Brinkmann, Westbury, NY).
7. Screw-top, 4 mL, amber, silane-treated vials with polytetrafluoroethylene (PTFE)-lined solid caps (Supelco, Bellefonte, PA).
8. Parafilm (Fisher Scientific, Pittsburgh, PA).

2.2. Reagents

1. Aflatoxins B_1, B_2, G_1, and G_2 (Sigma, St. Louis, MO).
2. Toluene-acetonitrile (9 + 1).
3. Benzene-methanol-acetic acid (90 + 5 + 5).
4. Acetone-chloroform (1 + 9).
5. Ether-methanol-water (96 + 3 + 1).
6. Trifluoroacetic acid-acetic acid-water (2 + 1 + 7).
7. Acetonitrile-methanol-water (1 + 1 + 4).

3. Methods (*see* Note 1)

3.1. Preparation of Aflatoxin Solutions

1. Handle all four aflatoxins, B_1, B_2, G_1, and G_2 in the same manner.
2. Uncap vial containing the mycotoxin, cover with tissue paper, and dry at 50°C for 1 h.
3. Accurately weigh 2.5 mg of aflatoxin onto a foil pan (*see* **Note 2**).
4. Transfer to a 100 mL glass stoppered volumetric flask using toluene-acetonitrile (9 + 1) and fill 2/3 full.
5. Agitate until the crystals are dissolved (*see* **Note 3**).
6. Record the UV spectrum of 4 portions of the aflatoxin solution from 300 to 500 nm against solvent used for the solution in the reference cell (*see* **Note 4**).
7. Calculate the aflatoxin concentration of the solution from the absorbance, A, at the wavelength of maximum absorption close to 350 nm and using the equation: µg aflatoxin/mL = A × MW × 1000/ε (*see* **Table 1**). The concentration will be approx 25 µg/mL.

8. Return aflatoxins to the original volumetric flask.
9. Seal with Parafilm, label, and store in a refrigerator.

3.2. Check of Aflatoxin Purity by Thin Layer Chromatography (see Note 5)

1. Spot 100 ng each of aflatoxins B_1, B_2, G_1, and G_2 on separate spots on three 10 × 20 cm silica gel plates.
2. Develop the first plate with benzene-methanol-acetic acid (90 + 5 + 5).
3. Develop the second with acetone-chloroform (1 + 9).
4. Develop the third with ether-methanol-water (96 + 3 + 1).
5. Dry the developed plates at room temperature under a hood. View the plates under long wave UV light in a chromatogram viewing box. Each aflatoxin should show only one fluorescent spot at the proper R_f for each toxin and no residual spot at the origin. A spot at the origin is indicative of photo-product impurity. The aflatoxins in decreasing order of R_f are B_1, B_2, G_1, and G_2.

3.3. Aflatoxin Purity Check by Liquid Chromatography

1. Transfer 10 µL standard solution 3.1.5 (25 ng/µL) to a 10 mL volumetric flask.
2. Evaporate until dry under a gentle stream of nitrogen at room temperature.
3. Dilute the residue to mark with acetonitrile.
4. Since aflatoxins B_1 and G_1 are not very fluorescent, they must be derivatized to aflatoxins B_{2a} and G_{2a}, respectively. Prepare derivatization solution: mix 10 mL trifluoroacetic acid (reagent grade) with 5 mL glacial acetic acid (reagent grade) and 35 mL water.
5. Place 200 µL of standard solution 3.3.3 in a vial with 700 µL derivatizing solution using a 1000 µL syringe. Close the vial with a cap and mix the solution well. Heat the vial ≥8.5 min in a 65°C water bath (the level of water must be above the level of solution in the vial).
6. Inject 20 µL of solution on liquid chromatograph (LC) with the fluorescence detector set at 360 nm excitation and 440 nm emission. The column is 15 cm C_{18} and the mobile phase is acetonitrile-methanol-water (1 + 1 + 4). Chromatogram should have only one peak (*see* **Note 6**).

3.4. Preparation of Vials of Aflatoxin Dry Film Standards

1. Determine the repeatability of the adjustable dispenser, 0 to 2 mL, by weighing 10 × 1 mL portions of toluene-acetonitrile (9 + 1) into tared flasks, recording the weight of each portion. Calculate the average weight of the portions (about 0.88 g), the standard deviation (about 0.0038 g), and the relative standard deviation (about 0.43%).
2. Carefully dispense approx 1 mL of aflatoxin solution, 25 µg/mL, into clean 4 mL vials (*see* **Note 7**).
3. Evaporate the solvent from the vials to dryness in a nitrogen ventilated oven at 50°C.
4. When dry, randomly select 6 vials and determine the quantity of aflatoxin in each vial by dissolving in 2 mL toluene-acetonitrile (9 + 1) and measuring the UV

spectrum from 300–500 nm of each solution. Calculate the mean, standard deviation, and relative standard deviation. Eliminate any value more than 5% different from the mean and recalculate the mean. Additional vials may be tested, but too much variability indicates faulty dispensing.
5. Cap the vials in a nitrogen atmosphere in a glove box or plastic glove bag.
6. Tighten the cap and wrap a strip of Parafilm around the cap to seal and hold the cap in place.
7. Label each vial with the name of the mycotoxin, amount (mean value from above determination), date prepared, initials of the preparer, and reference number (*see* **Note 8**).
8. Place the standards in a box enclosed in a plastic bag, and store in a refrigerator at 0 to 5°C.

3.5. Preparation of Analytical Standard Solutions

1. To the dry film standard, add a volume of toluene-acetonitrile (9 + 1) calculated to give a concentration of 8 to 10 µg/mL.
2. Vigorously mix the solution on a Vortex mixer for 1 min.
3. Measure the UV spectrum, and calculate the concentration as in **Subheading 3.1., step 6**.
4. Transfer the solution to a new screw cap vial.
5. Prepare the working standard by adding specific amounts of the above solutions of B_1, B_2, G_1, and G_2 to a volumetric flask, and dilute to volume with the appropriate solvent.
6. For TLC determinations, the working standard often contains B_1 and G_1, 0.5 µg/mL, and B_2 and G_2, 0.1 µg/mL dissolved in toluene-acetonitrile (9 + 1). Solutions for LC separation and quantitative standards are prepared in solvents consistent with the mobile phases to be used.
7. Store the solutions in vials sealed with Parafilm in a refrigerator.
8. The vial and solution can be weighed and at some future time the weight can be checked to determine if solvent loss has occurred, which would require recalibration by UV or disposal.
9. Solutions are stable for over one year.

4. Notes

1. The standard solution preparation requires utmost care to avoid calculation, measuring, and dilution errors and contamination or use of the wrong solvents.
2. Weigh using an analytical balance, or for smaller quantities, a microbalance. Aflatoxins must be handled in a glove box, because of their carcinogenicity and their tendency to disperse in the environment as a result of their electrostatic properties.
3. This dissolution may take several hours and require the use of a mechanical shaker for some toxins; an example is aflatoxin B_2 in toluene-acetonitrile (9 + 1).
4. Normal exposure to UV light during a measurement does not result in observable conversion of aflatoxins to photo-products.
5. If from past history or reliability of the source of the standard the purity and high quality can be inferred, it would not be necessary to perform the purity tests.

6. When a mixture of derivatized aflatoxin B_1, B_2, G_1, and G_2 is injected, retention times of G_{2a}, B_{2a}, (derivatives of G_1 and B_1), G_2 and B_2 are 6, 8, 9, and 11 min respectively. G_2 and B_2 do not derivatize but fluoresce adequately for easy detection. For further details see section 994.08, **ref. *1***.
7. Use brown 4 mL silanized vials with Teflon lined screw cap. Aflatoxins are partially absorbed by untreated glass and as dry films are sometimes converted to photo-products by UV light.
8. Use the reference number to refer to the notebook where the data of the standard prepared was recorded, including the source of the aflatoxin, weight, UV data, purity check, date, and the name of the analyst.

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5

Electrospray Mass Spectrometry for Mycotoxin Detection and Purity Analysis

Jon G. Wilkes and Jackson O. Lay, Jr.

1. Introduction

Gas Liquid Chromatography (GLC) has been used to separate a few mycotoxin types: trichothecenes, zearalenone, patulin, and anthraquinones ***(1–9)***. In contrast, most of the known mycotoxins are amenable to high performance liquid chromatographic (HPLC) separation ***(10)***. Accordingly, Frisvad and Thrane developed a general reversed-phase HPLC analysis procedure for simultaneous separation and detection of 182 mycotoxins and other fungal metabolites ***(10)***. This very versatile approach involved the temporal spectroscopic (UV) detection of the chromatographically separated mycotoxins. However, some recently identified mycotoxin classes, (fumonisins and AAL toxins) although amenable to HPLC separation, lack the UV chromophores required for detection using this method. For these toxins, HPLC analysis is most easily accomplished using another approach for the detection step. Electrospray ionization (ES) mass spectrometry (MS) is well suited for mycotoxin analysis, especially for the larger, less volatile toxins not amenable to gas chromatography (GC) and particularly for those like the fumonisins and AAL toxins lacking strong UV chromophores**.** Moreover ES is an ionization method developed in response to the need for direct MS characterization of HPLC separated components.

In HPLC/ES/MS, the chromatographic eluent passes out of a metallic capillary needle maintained at several thousand volts DC (typically 5 KeV) relative to a reference electrode. The electric potential influences both the nebulization of the liquid and the electrostatic charging of the resulting aerosol droplets (electrospray). As the droplets evaporate and shrink, the charge remains with and concentrates on them. Eventually, the force of electrostatic repulsion

From: *Methods in Molecular Biology, vol. 157: Mycotoxin Protocols*
Edited by: M. W. Trucksess and A. E. Pohland

exceeds droplet surface tension. At this point, the Rayleigh limit, a secondary disintegration occurs that produces charged microdroplets. Subsequent evaporation liberates into the gas-phase many large molecules. A significant percentage of these become free ions having the same relative polarity as that of the electrospray needle. These ions, any neutrals, solvent vapor and even ambient air are sampled, typically through a short capillary, into the vacuum system of the mass spectrometer, where the ions can be separated from neutral molecules, steered through ion optics and the mass filter, and impacted onto an electron multiplier.

The ease with which compounds are detected (ionized) in this process varies. Neutral molecules containing electron-rich, basic centers can easily form protonated molecules or ionic adducts of sodium and/or potassium from the solvent, especially at low pH, and so can be detected in the positive ion mode of the mass spectrometer. The HPLC mobile phase can be modified to encourage this, typically using dilute acid, 0.1% formic acid for example. Of course, molecules existing as preformed ions in solution are consistently detectable with the mass spectrometer operating in the mode of corresponding analyte ion polarity. **Table 1** details an evaluation of a number of mycotoxin classes with respect to their suitability for HPLC separation with ES/MS detection. It identifies complementary molecular characteristics associated with facile ES ionization and, where possible, citations of references ***(11–25)*** reporting such an application. Rugged ES/MS systems are a relatively recent instrumental development. Perhaps for this reason, there are relatively few citations of ES/MS methods for many of the mycotoxins which have been the object of major analytical methods development work of the last few decades. (This is indicated in the table by an "X" beyond the final semicolon in each row, indicating an absence of citations.)

Several of the methods in papers cited in **Table 1** demonstrate concentration detection limits (DLs) sufficiently low to allow analysis of mycotoxins in incurred residue samples or in air. (Some results with a conceptually similar instrumental MS method compatible with HPLC sample introduction, atmospheric pressure chemical ionization [APCI], have also been used by several groups for mycotoxin analysis, and are also included in **Table 1**.) Examples include LC/ES/MS of ceftiofur, a β-lactam (DL in milk, 10 ppb) ***(23)***; LC/ES/MS of several other β-lactams (DLs as low as 15 ppb in meats, 40 ppb in plasma) ***(24)***; LC/APCI/MS of sterigmatocystin (4 ppb DL in cheese) ***(25)***; ES/MS/MS of trichothecenes from indoor air (1 pg to 1 ng mass DL) ***(26)***; LC/APCI/MS of zearalenone in food and feed (DLs of 2.5 ppb in extracts and 120 parts per trillion in maize) ***(27)***.

Samples resulting from the biosynthesis and concentration of natural products, especially mycotoxin standards, present difficult challenges for purity

Table 1
Major Compound Classes and HPLC/MS Methods

Mycotoxin Class	Molecular Characteristics; Application and References
AAL toxins	1 amine (basic nitrogen easily protonated in acidic solution, molecule similar to fumonisins, (+) ion ES/MS); X
Aflatoxins	Alcohol –OH, weakly acidic, (–) ion ES/MS?; mercapturic acid metabolites of AFB1, (+) ion ES/MS by Scholl et al., 1997 ***(11)***
Alternariol	Phenol –OH, acidic, (–) ion ES/MS; X
Beauvaricin	3 *N*-methyl L-phenylalanyl residues; (–) ion ES/MS by G. Pócsfalvi et al. ***(12)***
Cephalosporins	Carboxylic acid; (–) ion ES/MS suggested Straub et al., 1993 ***(13)***
Citrinin	Phenol –OH, carboxylic acid, acidic, (–) ion ES/MS; co-chromatograph with ochratoxin A, Frisvad and Thrane, 1987 ***(10)***
Cyclopiazonic acid	β-lactam ring hydrolyses in acid, 2 amine, (+) ion ES/MS;
Fumonisins	1 amine, protonates in acidic solution; ES/MS, Korfmacher et al., 1991 ***(14)***; LC/ES/MS Doerge et al., 1994 ***(15)*** and Plattner, 1995 ***(16)***; LC/ES/MS/MS Josephs, 1996 ***(17)***
Fusaric Acid	Carboxylic acid, (–) ion ES/MS; X
Lolitrems	C31, benzopyrrole substructure; ES/MS by Munday-Finch et al., 1996 ***(18)***
Ochratoxins	Phenol –OH, carboxylic acid, (–) ES/MS; LC/MS by Ominski et al., 1996 ***(19)***, LC/MS/MS, Scott et al., 1998 ***(20)***
Patulin	Hemi-acetal, weakly acidic, but molecule unstable in alkali; (–) ion APCI/MS and (–) ion ES/MS/MS, Rychlik et al., 1998 ***(21)***
Penicillins (β-lactams)	Carboxylic acid, (–) ion ES/MS in alkali, or β-lactam ring hydrolyses in acid, 2 amine, (+) ion ES/MS; ES/MS, W. A. Moats et al., 1998 ***(22)***; LC/ES/MS J. Keever et al., 1998 ***(23)***, LC/ES/MS, V. Hormazabal et al., 1998 ***(24)***
Penicillic acid	Carboxylic acid, (–) ion ES/MS in alkali, or β-lactam ring hydrolyses in acid, 2 amine, (+) ion ES/MS; X
Rugulosin	Phenol –OH, acidic, (–) ion ES/MS?; X
Sterigmatocystin	Phenol –OH, acidic, (–) ion ES/MS?; LC/API/MS, Scudamore et al., 1996 ***(25)***
Tenuazonic acid	Vinyl alcohol, weakly acidic, (–) ion ES/MS?; X
Trichothecenes	Keto and ether linkages; HPLC/ES/MS, Tuomi et al., 1998 ***(26)***
Viriditoxin	Phenol –OH, acidic, (–) ion ES/MS?; X
Zearalenone	Phenol –OH, acidic, (–) ion ES/MS?; (+ & –) ion APCI/MS, Rosenberg et al., 1998 ***(27)***

analysis. Here the problems have to do with method and detector selectivity rather than insensitivity. As noted previously, a major advantage of the use of ES for the detection of HPLC-separated mycotoxins, compared to the more

general HPLC/UV approach, is that some of these compounds would require chemical derivatization for sensitive detection by spectroscopy. Although derivatization is certainly feasible for the analysis of targeted mycotoxins, if an appropriate procedure is either known or can be developed, derivatization is less appropriate for the detection of impurities or metabolites. Whereas metabolite standards may be used to check the efficacy of a derivatization scheme for selected metabolites, the minor components in purity assays are often entirely unexpected prior to the analysis. For this reason, in our laboratory we conduct parallel HPLC/ES/MS experiments for purity determination, even when the target compounds are amenable to HPLC/UV determination.

Purity determination presents other challenges as well, since analytical approaches optimal for ultra-trace determination of a targeted mycotoxin in a specific food matrix may not be well suited for characterization of the purity of a concentrated biosynthesized lot of toxin. Purity analysis problems can include: (1) a lack of certified standards for individual quantification of each constituent; (2) errors arising from any assumptions regarding the molar response factor of the detector; (3) the need to quantify impurities not physically similar to the major component. Traditional chromatographic methods for assessing the contribution of minor constituents to the whole often depend first on the ability to separate all impurities from the major component and then also require that every component possess the same type of UV chromophore, electrophore, fluorophore, or other molecular "handle" appropriate for the chosen detector. They require that the molar response (e.g., the molar extinction coefficient for UV absorption) be known or often simply assumed to be about the same for each sample constituent; a situation that rarely occurs for natural products by traditional HPLC detection methods. For practical and phenomenological reasons, chromatographic resolution in HPLC is always much less than that available by high resolution GC. Consequently, HPLC methods are more prone than are GC methods to errors involving the overestimation of sample purity that arise from the response of impurities that coelute with the main constituent. MS detection can often distinguish contributions from coeluting peaks because of differences in the spectra of individual components. Thus, impurities are less likely to be "lost" under the coeluting peak when their signals can be unambiguously assigned to the minor component.

MS methods may also offer a more uniform molar response, in many cases, than is observed using UV absorption or fluorescence detection. By molar response we refer to a signal proportional to the number of molecules present rather than to the number of carbon atoms or the number of strong UV chromophores. Electrospray/MS is more selective (gives a less uniform molar response factor) than traditional electron ionization (EI) MS. Nevertheless, for the types of HPLC-separated impurities likely to be found in a partially puri-

fied mycotoxin standard, the assumption of a uniform molar sensitivity is often much better for ES/MS than for most other HPLC detectors. The relative molar response factor for several fumonisin-related compounds has been investigated by Musser (***28***). Repetitive collection of HPLC fractions of trace impurities was repeated to produce sufficient material for gravimetric analysis, which showed that the fumonisin B1 (FB1), the isomers FB2 and FB3, FB4, as well as the half-hydrolyzed fumonisins, all gave equivalent molar response factors by ES/MS. Only the fully hydrolyzed FB1 produced a different response, a signal twice that of an equal mass of FB1 (or of the other fumonisins). Thus, the similar-molar-response criterion for purity analysis by ES/MS was fulfilled for these impurities with the highest difference between the parent and one of the other fumonisin related compounds being a factor of two. (A factor of two difference in molar response is relatively small compared to typical multiple order-of-magnitude differences in UV extinction coefficients, for example.) In this instance, the MS response, from protonated molecules, reflected the differences in the proton-accepting nature of these analytes, all of which contain an easily protonated amine moiety.

Other approaches to FB1 characterization have been reported. Fast atom bombardment (FAB)/MS has been used off-line (without chromatography) for purity analysis using fumonisin isolates ***(29)***. Difficulties in producing a practical, flexible system for on-line LC/FAB/MS have precluded the extension of this early FAB/MS work to LC/FAB/MS of fumonisins or other mycotoxins. HPLC using a particle beam (PB) interface with electron impact (EI) ionization is also a possible alternative to HPLC ES/MS. The EI ionization method boasts a more uniform molar response than electrospray, but it and the associated LC interface transport system suffer much greater discrimination from volatility differences among the constituents. PB/MS also shows quantitative anomalies arising from "carrier" effects: greater transport efficiency for a trace component carried through the system with a coeluting major component than would happen in the absence of the major component. Thus, HPLC PB/EI/MS methods, although feasible, have also not been developed for characterizing mycotoxins.

Here we present a reversed phase HPLC separation with ES/MS detection for general analysis of fumonisin mycotoxins. The approach is adaptable for analyzing many other mycotoxins. In addition, we will describe in detail how to use LC/ES/MS data for estimating the purity of fumonisin B1 lots biosynthesized and concentrated for use as standards and for rodent toxicology studies. Using the same fumonisin samples, Wilkes et al. compared results of purity analysis by LC/ES/MS and by LC with evaporative light scattering detection (ELSD) ***(30–32)***. As expected from theoretical considerations, purity assayed by LC/ES/MS tends to establish a lower bound for the true value,

whereas purity assayed by LC/ELSD establishes an upper bound. For very pure samples—those approaching the quality needed for toxicological studies—results by the two assays converge.

2. Materials

2.1. Chemicals, Solvent Mixtures, and Samples

1. HPLC-grade water (purchased or prepared by glass distillation and scrubbed with an organic sieve) (*see* **Note 1**).
2. HPLC-grade organic solvents for HPLC mobile phase, here acetonitrile.
3. Solutions of mycotoxin samples, here fumonisin B1 and impurities (*see* **Note 2**).
4. Mobile phase pH buffers of reagent grade purity, here formic acid (*see* **Note 3**).
5. HPLC Mobile phases A and B (*see* **Note 4**).
6. High purity helium for sparging, degassing mobile phases (*see* **Note 5**).

2.2. Instruments

2.2.1. HPLC System

1. An injector assembly with a sample loop capacity from 1 to 20 µL (*see* **Note 6**).
2. A programmable solvent delivery system capable of producing gradient mixtures of at least two solvents. Depending on the flow constraints of the ES ion source, the pumps should deliver between 0.025 and 1.0 mL/min (*see* **Note 7**).
3. A base-deactivated reversed phase analytical scale HPLC column (*see* **Note 8**).
4. A variable wavelength UV detector. (optional) (*see* **Note 9**.).
5. A post-column flow splitter (optional, for use with a parallel HPLC detector [ELSD in our laboratory] or with larger columns and higher flow rates) (*see* **Note 10**).

2.2.2. ES/MS System

1. A quadrupole mass spectrometer with an ES ion source capable of operating with reasonable sensitivity for liquid flow rates of about 100–200 µL/min and a mass range up to at least *m/z* 800 (*see* **Note 11**).
2. A computerized MS control and data acquisition system. Although full mass scans were used in this study, a capability for scanning multiple, discontinuous, method-defined segments or selected mass-to-charge ranges during a single acquisition is desirable (*see* **Note 12**).

3. Methods

3.1. Sample Preparation and Data Acquisition by LC/EC/MS

1. Degas solvents with helium.
2. Calibrate mass spectrometer, if necessary.
3. Edit and/or load HPLC programs and MS Method files. Define the HPLC gradient. Define the MS acquisition mode, (+) ion, plus retention time and *m/z* interval windows.

4. Equilibrate HPLC system for the starting conditions of the mobile phase composition gradient. When equilibrated go to the next step.
5. Inject 20 µL of sample solution; initiate HPLC program; begin MS data acquisition.
6. After run is completed, reequilibrate column for 10 min under initial conditions.
7. Repeat **steps 5** and **6** until all samples are run.
8. Divert column flow from MS inlet.
9. Flush column for 10 min with 90% methanol and store in 50% methanol.

3.2. Data Manipulation and Purity Calculation for a Representative Sample

1. Define the beginning and ending time of each HPLC/MS peak (based on the appearance of the single ion chromatogram for the *m/z* of the component's protonated molecule, not the total ion chromatogram). With our system peaks can be detected automatically prior to integration (*see* **Note 13**).
2. Integrate the area of each protonated molecule peak (*see* **Note 14**).
3. Total the area of the protonated molecules for all compounds, the main component (here FB1) plus all impurities (*see* **Note 15**).
4. Total the area of the main component only (*see* **Note 16**).
5. Divide the numerical value from **step 4** by that from **step 3**, multiply by 100 to give the percent purity of the FB1.

4. Notes

1. For analyses using a parallel ELSD, one would omit scrubbing distilled water with the organic sieve. The sieve often contaminates the water (and sample blanks) with resins which, although they contain no UV-chromophore and hence are not observed by UV detection, would nevertheless give a strong background signal in an ELSD.
2. In this specific example, we made aqueous solutions of fumonisin B1 at about 1 mg/mL. This high FB_1 concentration allowed the detection of minor components present at levels as low as a few µg/mL.
3. Acid buffers are used when separating acidic mycotoxins. These buffers suppress ionization of acidic mycotoxins, enhancing retention on the reversed phase column so that they can be separated. They also enhance the production of protonated molecules in ES based on the acid-induced shift in the solution-phase equilibrium resulting in enhanced protonation of any basic moieties (e.g., N or O lone pair electrons). We used formic acid as the buffer. Trifluoroacetic acid (TFA) also gives good results both with respect to the HPLC separation and the MS applications but is more toxic than formic acid.
4. For separation of fumonisins we defined mobile phase A as 1% acetonitrile/water with 40 m*M* formic acid and mobile phase B as 90% acetonitrile/water with 40 m*M* formic acid.
5. Helium sparging, or degassing of mobile phases by other methods, is necessary to prevent air bubble formation upon mixing of mobile phases. In solvent delivery systems that use low pressure mixing, these bubbles form after mixing but

upstream of the pumping valves. The valves then can fail to seal, leading to a loss of pressure for one or both strokes of the reciprocal pumping cycle. The problem is less acute for high pressure mixing (2 pump) systems, but high pressure mixing systems are more expensive.

6. We used a 20 µL sample loop on a Rheodyne Model 7125 6-port HPLC valve or an HP 1100 autosampler. Although our mass spectrometer could not program the HPLC or the autosampler, both of these devices could detect start signals (contact closure). Synchronization of the systems was accomplished based on the detection of such contact closure coincident with sample injection.
7. The solvent delivery system pumps should produce a reproducible gradient even at the extremes of Mobile Phase composition (i.e., 2% B or 98% B). We used a Varian model 9012 solvent delivery system, which provides programmable low pressure mixing of up to four different Mobile Phases. Detection of the contact closure initiated the gradient which was programmed directly into the HPLC system.
8. For fumonisins we used Phenomenex UltraCarb ODS 30 columns with a 5 micron particle size. We have used columns from 2 × 250 mm to 3.5 × 150 mm with good results. Appropriate flow rates for these columns range from 0.2 mL/min to 0.6 mL/min. With the larger columns and flow rates, about 80% of the sample (*see* **Subheading 2.2.1., step 3**) is diverted via a split for concurrent analysis by ELSD. (For compounds containing a chromophore we use a UV detector in series with the mass spectrometer, smaller columns, a low flow rate [100 µL/min] and no split.) Our ES/MS system will work with flow rates from 0.001 to 1 mL/min, but the sensitivity falls off rapidly above 100 µL/min.
9. A UV detector inserted post-column but upstream of the ES/MS (in series) is generally useful for troubleshooting the system and for optimizing separations. Of course, it will not detect the fumonisins in this example. It might detect nonfumonisin impurity peaks and so warn the analyst that the selected ion range used for purity analysis (*see* below) may fail to monitor ions from these compounds. For this purpose, we used a Spectra 100 (Spectra-Physics) and typically monitored 254 nm.
10. We built a 4.5:1 mobile phase splitter by passing the column eluent into a zero-dead volume HPLC tee. The capillary flowing out of the tee to the MS was 4.5 times longer than, but had the same internal diameter as, the short one flowing out to the ELSD (or waste). Impedance to flow through a tube is linearly proportional to the inverse of the tube length. Therefore, the majority of the flow followed the path of lesser impedance. The 0.6 mL/min flow out of the HPLC column resulted in only 109 µL/min flowing into the ion source. All of this was done to accommodate an ELSD detector in parallel with the mass spectrometer. In experiments where ELSD was not used, a 2 × 250 mm column and a flow rate of 200 µL min gave excellent results.
11. We used a Finnigan TSQ7000, which has MS/MS capabilities not needed for this work. Whereas the isomeric fumonisins B2 and B3 can be distinguished in the MS/MS mode, in these experiments they were differentiated based on retention times. As noted above, the TSQ7000 ES/MS ion source can accept liquid flows

up to 1 mL/min, but sensitivity decreases drastically for flows much greater than 100 μL/min. For trace level work or detection of targeted mycotoxins splitting is not necessary. Currently available ES/MS systems easily accommodate high flow rates with high sensitivity, and for older systems, many of the smaller columns (1 × 250 and 2 × 250 mm) give excellent separation with flow rates from 50–200 μL/min.

12. Sometimes, when evaluating fairly pure samples, the identities of all impurities may be known or determined experimentally. In this case, impurity ions and retention time interval "windows" can be selected to enhance the analytically relevant signals. One can specify a specialized acquisition method consisting of a sequence of discrete or overlapping windows, each acquiring only the few *m/z* values relevant to the impurity(-ies) expected during a specific time interval. For purity analysis of bulk mycotoxins this sophisticated procedure is often not necessary. Our fumonisin purity determinations were based on full scan ES/MS spectra. Full scan spectral acquisition also has the obvious advantage that it allows detection of unexpected components.

    For analyses of other mycotoxins, the ES/MS system should also be capable of negative ion acquisition. Although we did not use negative ions for our fumonisin work, a number of the references cited in the introduction demonstrated the use of this capability for the analysis of other mycotoxins.
13. Assignment of analyte signals (peaks) should be based on the appearance of the single ion chromatogram for the *m/z* value of the component's protonated molecule, rather than using the total ion chromatogram (TIC). The background signal level of the TIC trace includes background contributions (column bleed, mobile phase ions, clusters, and so on).
14. We generally quantify based only on the signal from the protonated molecule (positive ion mode) even when using full mass scans because this ion is typically the only one detected in the ES mode. (This is verified based on the full scan background subtracted spectra and/or using authentic standards in each case.) With FB1 samples we observed the protonated molecules of: FB1 (*m/z* 722), either of two common fumonisin variants in which one of three hydroxyl groups is missing (FB2 and FB3, *m/z* 706), *N*-methylated FB1 (*m/z* 736), and either of two fumonisins in which one of the two carballylic acid side chains had been hydrolyzed (*m/z* 564). A fumonisin variant (protonated molecule at *m/z* 690) in which two hydroxyl groups are missing is also found in nature, but this component was apparently removed in the preparatory scale HPLC purification, so that it was not a contaminant in our partially purified biosynthetic mycotoxin products. Similarly we also observed no contamination from fully hydrolyzed FB1, the protonated molecule of which would have appeared at *m/z* 406. Other mycotoxins (or dirty samples) may possibly produce an ES/MS spectrum that, in addition to the protonated molecule, also shows significant contributions from fragment ions (or adducts of sodium or potassium). In such cases, the areas of fragment and adduct peaks should be added to that of the protonated molecule to give a number representative of the mass of the substance. Once the total signal has been calculated

for each constituent, the purity calculation proceeds as described previously in **Subheading 3.2., steps 3–5**.

15. If calculating the percent impurity, the instruction would read "total all of the protonated molecule peak areas of whatever *m/z* value except the largest." To calculate the percentage of each impurity, the signals for each minor component are individually compared to the sum of all of the signals.
16. The largest peak with the appropriate *m/z* value, (here *m/z* 722) is the primary component (FB1). Peaks that differ in retention time from the FB1, having the same mass are either isomers or isobars. Peaks with *m/z* values different from the main component, irrespective of retention time, are impurities. The presence of isomeric compounds may be anticipated for assays of other mycotoxins, and can be best resolved using the retention times from authentic standards. For example, FB2 and FB3 are isomers in which each protonated molecule has *m/z* 706. Assaying the purity of an FB2 standard, would require resolution of this problem.

This method has been used to assay fumonisin standards initially thought to be >95% pure. Actual observed purities have ranged from 83% to 98% (molar) pure using this method. For replicate analyses, the standard deviation of the purity measurement has typically been about 0.4%. The "gold standard" fumonisin B1 sample produced by FDA's Center for Food Safety and Applied Nutrition, that has been used for toxicology studies in FDA and elsewhere, was characterized using this method, giving values of 98.0 and 98.4% purity in two different laboratories.

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6

Measurement of Aflatoxins Using Capillary Electrophoresis

Chris M. Maragos

1. Introduction

1.1. The Aflatoxins

The aflatoxins are a group of mycotoxins produced by certain *Aspergillus* species, in particular *Aspergillus flavus* and *Aspergillus parasiticus*. The aflatoxins are extremely potent mutagens, are suspected human carcinogens, and can adversely affect animal health and agricultural productivity. Many countries routinely screen agricultural commodities for the presence of the aflatoxin B_1 (AFB_1), the most potent member of the group. In the United States, the Food and Drug Administration has established a guideline level of 20 ng/g (ppb) total aflatoxins in food destined for human consumption ***(1)***. For breeding cattle, breeding swine, and mature poultry the limit is 100 ppb. For finishing swine and finishing beef cattle the limits are 200 and 300 ppb, respectively. Because of the importance of this group of mycotoxins to human and animal health, all of the common tools of analytical chemistry, including thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), gas chromatography (GC), mass spectrometry, immunoassay, biosensors, and capillary electrophoresis, have been used for their detection. The literature dealing with chromatographic methods for the mycotoxins is extensive and several excellent reviews have been published ***(2–6)***.

The major aflatoxins have a strong UV absorbance with a maximum in the range of 360-365 nm when measured in methanol, with reported extinction coefficients in the range of 21,700 to 27,300 for AFB_1 ***(7)***. The characteristic UV/Vis spectra of the aflatoxins is helpful in confirming the presence of the toxins when they are isolated from complex matrices. Many analytical methods for the aflatoxins, including TLC and HPLC methods rely upon the

From: *Methods in Molecular Biology, vol. 157: Mycotoxin Protocols*
Edited by: M. W. Trucksess and A. E. Pohland

fluorescence of this group of mycotoxins ***(8,9)***. The aflatoxins were given their trivial names in part from their fluorescence characteristics: the AFB toxins exhibit a blue fluorescence whereas the AFG toxins exhibit a blue-green fluorescence on TLC plates when exposed to longwave UV light.

1.2. Capillary Electrophoresis

Although chromatographic separations are based upon the interactions between an analyte and a stationary phase, electrophoretic separations are based upon the behavior of an analyte in the presence of an electrical field. Electrophoretic separations are performed in capillaries, principally because of the high electrical field strengths used and the need to rapidly remove the heat generated. The permutations of capillary electrophoresis (CE) are numerous and range from free zone CE, where separations are performed in simple buffers, to micellar electrokinetic capillary chromatography (MECC), where a pseudostationary phase is used. Capillary chromatography is a hybrid of CE and HPLC where the electrical field is used to draw the buffer through a stationary phase, rather than pumping it through as with HPLC. The application of CE to mycotoxin analysis was recently reviewed ***(10)***.

The simplest and most widely used form of CE is free zone capillary electrophoresis. In free zone CE the composition of the buffers placed at the anode and cathode are identical. With the application of an electrical potential across the capillary the buffer components begin to separate, with the anions moving toward the anode and the cations toward the cathode. In the case where bare fused-silica capillaries are used the application of an electrical potential causes a flow of buffer from the anode toward the cathode. This flow, termed electroosmotic flow (EOF), results from the movement of cations with an accompanying shell of hydration toward the cathode. If the EOF is sufficiently vigorous even compounds having a substantial negative charge can be made to flow toward the cathode. In this manner the components carrying positive charges will elute first, followed by neutral compounds and then negatively charged compounds. The magnitude of the EOF is influenced by a host of factors including the pH, ionic strength, viscosity, and temperature of the solution as well as the magnitude of the applied voltage and the surface characteristics of the capillary wall. Where it is desirable, the surface of the silica capillaries can be treated to reduce, eliminate, or even reverse the EOF.

The major difference between free zone CE and MECC is the use of a buffer containing a pseudostationary phase, usually composed of micelles formed from surfactants such as sodium dodecyl sulfate (SDS) or bile salts such as sodium deoxycholate (NaDC). The components of the sample interact with the micelles and the buffer as they migrate through the capillary. The micelles therefore serve a function analogous to the stationary phase in HPLC. The

major aflatoxins differ from each other in polarity and can therefore be separated by either reversed-phase HPLC or MECC ***(11)***.

1.3. Separation of the Aflatoxins by CE

The aflatoxins G_2, G_1, and B_2 were first separated using CE by Balchunas et al. ***(12)***. The separation was performed using MECC, with fluorescence detection (MECC-LIF). Several papers refining the separation have appeared from the same group ***(11,13,14)***. Cole et al. ***(11)*** formed the micelles for MECC with the bile salt surfactant NaDC rather than SDS. With the use of NaDC buffer the addition of organic modifiers was not necessary to achieve baseline separation of these three aflatoxins. A full examination of the factors affecting separation of aflatoxins, including AFB_1, by MECC-LIF was provided by Cole et al. ***(13)***. AFG_2, AFG_1, AFB_2, and AFB_1 were separated from one another within 30 s. This very rapid separation was achieved by applying 36 kV across a 40 cm capillary (35 cm length to detector, 25 μm id). By using a buffer composed of 50 m*M* SDS, 10 m*M* dibasic sodium phosphate, 6 m*M* sodium borate, and 10% (v/v) acetonitrile the aflatoxins were separated within 5 min when 20 kV was applied. Several samples of spiked corn meal or *Aspergillus flavus* cultures were examined. The limit of detection (LOD) was estimated to be 1 ppm for spiked maize. Whereas the LOD was rather high for spiked maize, the LOD for aflatoxins standards was quite good: ranging from 2.64×10^{-8} *M* (8 ng/mL) for AFB_2 to 4.36×10^{-7} *M* (143 ng/mL) for AFG_1. In part, the difference may be due to the optimization of the system for standards in solution, rather than in purified corn matrix, and the clean-up and concentration steps used.

Recently, MECC-LIF has been applied to the quantitation of aflatoxin B_1 in maize (***15***; **Fig. 1**). AFB_1 was isolated from maize using one of two extraction methods: either a chloroform extraction followed by cleanup on a silica column or a methanol/water extraction followed by cleanup on an affinity column. The electrophoresis buffer contained 50 m*M* NaDC, 10 m*M* dibasic sodium phosphate and 6 m*M* sodium borate (pH 9.1). Baseline separation of AFG_1, AFG_2, AFB_2, and AFB_1 was achieved within 10 min. The order of the four aflatoxins was the same as that reported with reversed-phase HPLC ***(16–18)***. With the silica column cleanup the limit of detection (LOD), defined as the level of AFB_1 required to give a signal to noise ratio of 4, was 0.5 ppb in maize. With the affinity column cleanup the LOD was 1 ppb. This limit of detection compares favorably to the desired range for quantitation of aflatoxins in foods.

2. Materials

1. Sample Grinder: a Stein Mill or equivalent that can be used to attain a particle size able to pass through a number 20 sieve (roughly the consistency of ground coffee).

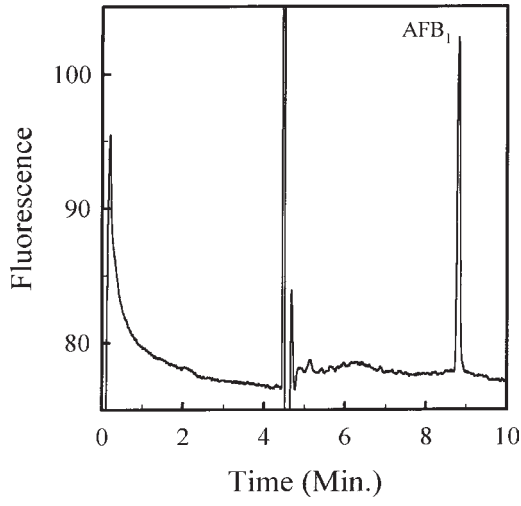

Fig. 1. Electropherogram of a 50 ng/mL solution of AFB_1 in NaDC buffer. The migration time for AFB_1 was 8.8 min. The peak shown at less than 1 min is an artifact from the NaDC buffer. The positive and negative peaks shown at 4.5–5 min arise from the 1% acetonitrile present in the standard.

2. Extraction Solution: 80% methanol/water (v/v), 100 mL per sample.
3. Blender: explosion proof blender made of methanol-resistant materials.
4. Sample Filtering and Dilution: Filter paper, Schleicher & Schuell number 588 (fast), or equivalent. Funnel, vial to collect the filtered extract (volume less than 100 mL), and vial to collect the diluted extract.
5. Aflatoxin Immunoaffinity Columns: AflaTest P columns, Vicam LP (Watertown, MA) (*see* **Note 1**).
6. Nitrogen gas for sample dry-down.
7. CE system:
   a. A capillary electrophoresis unit capable of delivering a voltage of 20 kV or a current of 105 μA, equipped with a fluorescence detector. Preferably a unit with automated sample injection. A Beckman P/ACE 5000 unit has been used previously.
   b. Filters for the fluorescence detector. A 400 nm long-pass filter (Oriel Corp., Stratford, CT) to exclude light from the excitation source and a 400 nm band-pass filter capable of transmission of wavelengths up to 462 nm. Alternatively, a tunable detector with an appropriate monochromator. The emission maximum occurs at 427–440 nm under the described conditions (*see* **Note 2**).
   c. A 19 mW Helium/Cadmium laser with 325 nm output connected as the excitation source (Model 3056, Melles Griot, Carlsbad, CA). For the Beckman P/ACE 5000 unit this involves disconnecting the Argon-ion laser and connecting the He/Cd laser to the fluorescence detector housing. The excitation

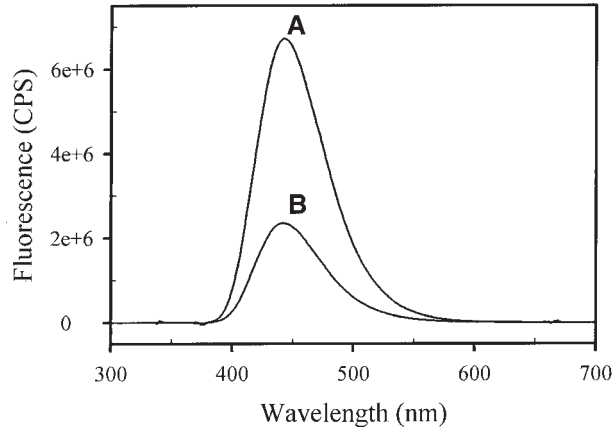

Fig. 2. Fluorescence emission spectra of 1 µg AFB_1/mL in methanol when excited with either light of wavelength 360 nm **(A)** or 325 nm **(B)**. Light from the He/Cd laser excitation source is 325 nm.

maximum for AFB_1 occurs at 360 nm under the described conditions; however the 325 nm source is sufficient to excite the aflatoxins, even though this wavelength does not result in maximum fluorescence (**Fig. 2**).

d. Fused silica capillary: 57 cm total length, 50 cm length to detector, 75 µm inner diameter.
e. Minivials: vials with a capacity of 0.6 mL and which can fit into the CE autosampler (*see* **Note 3**).

8. Electrophoresis buffers:
   To avoid blockage of the capillary, all buffers should be filtered (0.45 µm or smaller) before using them with the CE.
   a. Electrophoresis buffer: 50 m*M* sodium deoxycholate, 6 m*M* sodium borate, and 10 m*M* sodium phosphate, in deionized water, final pH 9.1 (*see* **Note 4**).
   b. Sodium hydroxide, 0.1 *N* in deionized water.
   c. Deionized water: 16 *M*Ω or better.

3. Methods

3.1. Extraction and Isolation of Aflatoxins from Maize

For accurate results it is important that the sample which is ground reflect the composition of the original sample, and that the original sample be obtained using a validated sampling plan. Add 100 mL of extraction solution to 50 g of ground maize and blend for 3 min. Filter the extract, allowing gravity to determine the flow rate. Dilute 10 mL of filtrate with 40 mL of deionized water and filter once more. Quantitatively transfer 10 mL of diluted filtrate, equivalent to 1 g of maize, onto a Vicam Aflatest P column. Wash with 20 mL deionized water

and elute the aflatoxins with 1 mL methanol. Dry the purified extract under a gentle stream of nitrogen and store refrigerated until the day of analysis.

3.2. Separation and Detection by CE-LIF

1. Aflatoxin Standards: Prepare AFB_1 stock solutions in acetonitrile over the range of 0.5 to 100 µg/mL and store at –20°C. Working standards should be prepared fresh daily to minimize the effects from degradation of AFB_1 in the buffer. Prepare the working standards over the range of 5 to 1,000 ng AFB_1/mL by diluting the stock solutions 1:100 (v/v) with electrophoresis buffer.
2. Samples: Prepare the maize samples by dissolving the extract from the immunoaffinity column in 0.8 mL electrophoresis buffer (a level equivalent to 1.25 g maize/mL). Transfer to a minivial for injection.
3. Capillary Electrophoresis: Rinse the capillary with the electrophoresis buffer by applying a pressure of 20 psi for 2 min. The volume of this rinse is equivalent to roughly 10 times the capillary volume. Inject the sample for 5 s at 0.5 psi, approx 30 nL. Transfer the capillary ends to the electrophoresis buffer and apply 20 kV. This should result in a current of approximately 104 µA. The aflatoxins G_2, G_1, B_2, and B_1 will elute within 10 min. After 10 min stop the voltage and rinse the capillary with 0.1 *N* sodium hydroxide for 1.5 min at 20 psi. Rinse the capillary an additional 1.5 min with deionized water. For best results intersperse samples with standards.
4. Data Analysis: The aflatoxins will elute as sharp symmetrical peaks in the order AFG_2, AFG_1, AFB_2, and AFB_1. Determine the peak height of the appropriate aflatoxin in the sample. The peak area, which can be calculated by many software programs designed for CE, can be used in place of the peak height. However, in our hands we found better reproducibility using the height because it is not corrected for mobility, which influences peak area. Calculate the concentration of aflatoxin in the injected sample by comparing the sample to the aflatoxin standard curve (5–1,000 ng/mL). It is important that the aflatoxin concentration be calculated using the appropriate standard curve (i.e., AFG_1 from an AFG_1 standard curve, and so on), due to the differences in fluorescence intensity among the aflatoxins. Calculate the concentration of individual aflatoxins in the maize as follows in **Eq. 1**:

$$[AFB_1]_{maize} \text{ in ng/g} = ([AFB_1]_{sample} \text{ in ng/mL}) \div (1.25 \text{ g equivalents maize/mL}) \quad (1)$$

The total aflatoxin content is obtained by summing the values for the individual aflatoxins.

4. Notes

1. The immunoaffinity cleanup used here is simple and rapid, but is not the only cleanup compatible with the method. Previously we have used the Contaminants Branch (CB) cleanup as well ***(15)***. The CB method, while more laborious, was also slightly more sensitive and gave a slightly cleaner extract for injection. Other

solid phase extraction columns, such as the popular MycoSep columns (Romer Labs, Union, MO) may also be used, provided sufficient sample is purified (i.e., if the equivalent of 1 g maize or greater is purified).

2. The intensity of the fluorescence signal can be increased substantially by removing the bandpass filter in the fluorescence detector. However, more interferences can be expected as the components having emission at wavelengths greater than 462 nm are no longer excluded.
3. For injection of samples the minivials can be purchased from several suppliers. It is just as effective, and considerably less expensive, to remove the caps from 0.6 mL "Eppendorf" style centrifuge tubes and fit these into the standard autosampler vials using adapter springs such as those sold by Beckman-Coulter (Fullerton, CA). Vials with even smaller capacities (so-called microvials, with a capacity of 30 μL) can also be purchased. In our experience the use of microvials has led to a dramatic increase in variability relative to when minivials are used.
4. The buffer composition will influence the fluorescence intensity observed from the aflatoxins and should be rigidly controlled.

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7

Liquid Chromatographic Method for Aflatoxin M_1 in Milk

Hans P. van Egmond and Sylviane Dragacci

1. Introduction

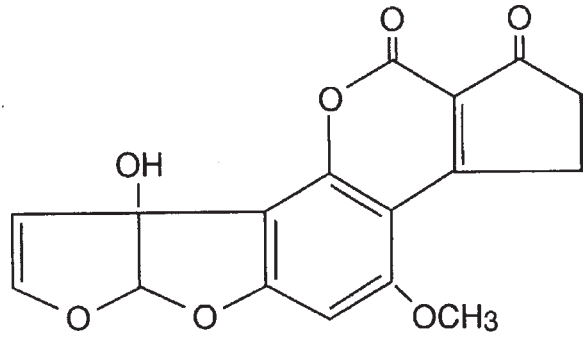
Aflatoxin M_1 is the 4-hydroxy derivative of aflatoxin B_1 (*see* **Fig. 1**). Aflatoxin M_1 appears in milk and milk products as the direct result of the intake of aflatoxin B_1-contaminated feed by dairy cows ***(1)***. The amount excreted as aflatoxin M_1, as a percentage of aflatoxin B_1 in feed, is usually 1–4% ***(2)***, but values as high as 6% have been reported at µg daily intake levels of aflatoxin B_1 ***(3)***. The carry-over of aflatoxin B_1 to milk may vary largely from animal to animal, from day to day, and from one milking to the next.

Most mycotoxins that occur in dairy products are generally present at such low levels of contamination that it is most unlikely that they will lead to adverse toxic effects when consumed by man. There is some concern, however, about aflatoxin M_1. There have been several studies on the toxic effects of aflatoxin M_1 in laboratory animals ***(4)***. In comparison to aflatoxin B_1, relatively little is known about its toxicity. This is mainly because of the difficulty in obtaining sufficient quantities of the pure compound necessary for extensive toxicity testing. Due to this limited supply of pure aflatoxin M_1, most toxicity assays were done for short- and medium-term exposure.

The limited studies carried out to determine the toxicity and carcinogenicity of aflatoxin M_1 tend to come to the same qualitative conclusion: Aflatoxin M_1 has hepatotoxic and carcinogenic properties. Quantitatively considered, the toxicity of aflatoxin M_1 in ducklings and rats seems to be similar or slightly less than that of aflatoxin B_1. The carcinogenicity is probably one to two orders of magnitude less than that of the highly carcinogenic aflatoxin B_1.

The results of various surveys of milk for aflatoxin M_1 presence carried out in various countries since the late 1960s have been summarized and published ***(4,5,6)***. A seasonal trend in milk contamination was noted in a few of these

From: *Methods in Molecular Biology, vol. 157: Mycotoxin Protocols*
Edited by: M. W. Trucksess and A. E. Pohland



aflatoxin M_1

Fig. 1. Chemical structure of aflatoxin M_1.

surveys, with lower aflatoxin M_1 levels in milk in the summer months. This phenomenon was attributed to the fact that the cows are receiving less concentrated feeds in the summer when they are grazing. In almost all surveys carried out in the 1970s positive samples were found with aflatoxin M_1 levels exceeding 0.05 µg/kg. In various studies, samples were reported with levels in the range 0.05-0.5 µg/kg. The establishment of new regulations in the late 1970s and in the 1980s to control the aflatoxin content of dairy rations and the developments in the analytical methodology led to an improvement of the situation ***(4)***. In general, both incidences of positive samples and aflatoxin M_1 levels were lower in the 1980s than in the 1970s. In several countries positive samples could not be found and levels >0.5 µg/kg hardly occurred. In the 1990s the trend towards lower aflatoxin M_1 levels in milk has continued ***(6,7)***, particularly in Europe, where more stringent EU-regulations on aflatoxin B_1 in animal feedstuffs ***(8)*** have led to further decreases in the aflatoxin M_1 concentration. However, isolated elevated values can still be observed on farms accidentally having high concentrations of aflatoxin B_1 in their feed.

To control aflatoxin M_1 in milk and milk products, specific regulations for aflatoxins in feedstuffs for dairy cattle have been established in various countries, and a number of countries have set specific regulations for aflatoxin M_1 in milk and milk products ***(9)***. Among the tolerance levels set for aflatoxin M_1 are values of 0.5 µg/kg milk (as applied in the USA, Mercosur, and some Eastern European countries) and 0.05 µg/kg milk as applied in many European countries. Since January 1, 1999 the latter limit is the official limit in the whole European Union ***(10)***. The rationales for the tolerance levels, set by various countries, seem vague and not based on (published) quantitative risk

assessment. Rationalization is difficult in view of the limited information on the (geno-)toxicity of aflatoxin M_1. In a few cases, some information as to the background of the regulations has been made public. For instance the United States rationale was said to be based on the knowledge of transmission of aflatoxin B_1 in feed to M_1 in milk and on the concept that exposure of (young) individuals to aflatoxin M_1 should be kept at a minimum without jeopardizing the continued supply of milk ***(11)***. In the European Union the new regulation indicates that even if aflatoxin M_1 is regarded as a less dangerous genotoxic carcinogenic substance than aflatoxin B_1, it is necessary to prevent the presence thereof in milk and milk products intended for human consumption and for young children in particular ***(10)***.

The regulatory measures that have been taken worldwide require that reliable validated analytical methodology is available, and over the last decades several methods have become available. Analytical methods for aflatoxin M_1 usually follow the general pattern for mycotoxin assays ***(12)***. A uniform sample is obtained easily with milk, because aflatoxin M_1 is distributed evenly throughout the fluid milk. The initial problem that is encountered in milk analysis is the extraction step. Because milk is a complex natural product, aflatoxin M_1 is not easily extracted and purified for final assay. This may be (partly) due to adsorption of aflatoxin M_1 to casein. A process is needed that separates aflatoxin M_1 from milk easily, efficiently, and economically. Once purified extracts are obtained, several possibilities exist to determine the concentration of aflatoxin M_1. Aflatoxin M_1 is a semipolar component, extractable with solvents such as methanol, acetone, chloroform, or combinations of one or more of these solvents with water. In practice, the choice of solvents depends on the clean-up and determinative steps. In classical methods, chloroform often is used as an extraction solvent, in combination with adsorption chromatography over SiO_2 columns.

The most recent advance in aflatoxin extraction and subsequent clean-up is the use of immunoaffinity (IA) cartridges. These columns are composed of monoclonal antibodies specific for aflatoxin M_1, that are immobilized on Sepharose ® and packed into small cartridges (*see* **Fig. 2**). A milk sample containing aflatoxin M_1 first is loaded on the affinity gel column (A). After washing to remove impurities (B), aflatoxin M_1 is eluted from the column with an organic solvent like pure acetonitrile (C). IA columns remove contaminants efficiently, because the aflatoxin M_1 antibody specifically recognizes aflatoxin M_1, so the column should not adsorb any other materials.

A collaborative study under auspicies of the International Dairy Federation (IDF) was conducted to validate an analysis procedure for aflatoxin M_1 using immunoaffinity in combination with liquid chromatography and fluorescence detection ***(13)***. The study yielded good results, leading to approval of the

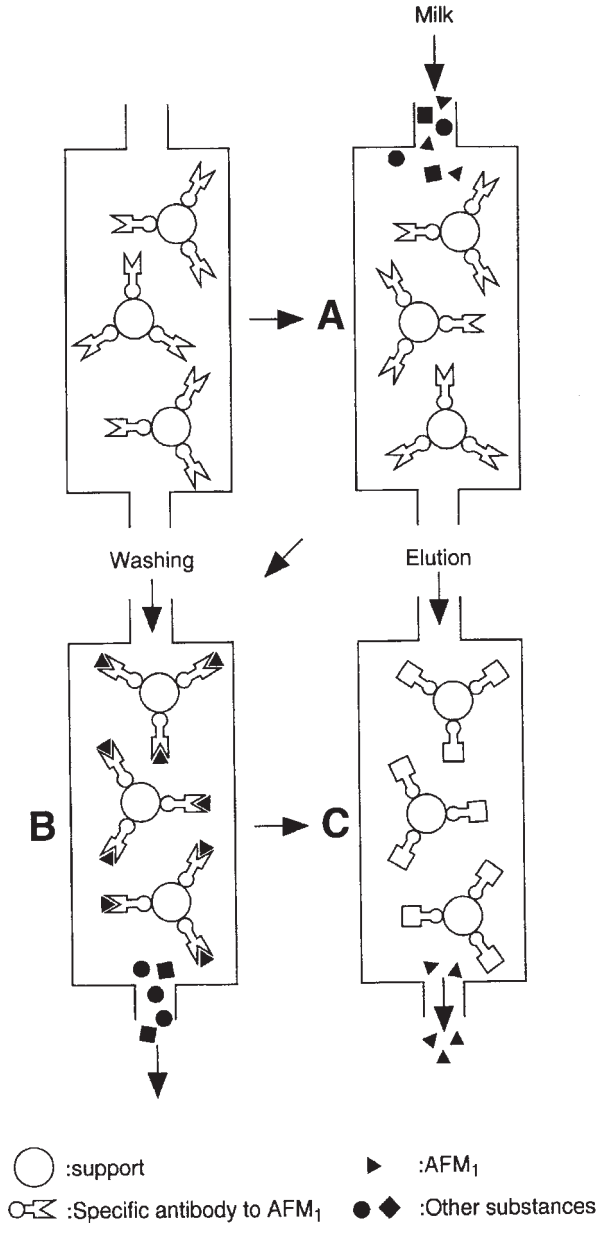


Fig. 2. Schematic diagram for immunoaffinity chromatography for concentration and purification of aflatoxin M_1. Sample containing aflatoxin M_1 is first loaded to the affinity gel column containing antibody against aflatoxin M_1 **(A)**. After washing to remove impurities **(B)**, aflatoxin M_1 is eluted from the column with acetonitrile **(C)**.

Table 1
Method Performance for Determination of Aflatoxin M_1 in Liquid Milk *(16)*

Samples	N	M (µg/L)	SD_r (µg/L)	r (µg/L)	$RSD_{(r)}$ (%)	SD_R (µg/L)	R (µg/L)	$RSD_{(R)}$ (%)	HORRAT
A	12	0.023	0.0040	0.0113	17	0.0061	0.0173	27	0.33
B	12	0.046	0.0056	0.0158	12	0.0104	0.0293	23	0.31
C	12	0.103	0.0077	0.0217	8	0.0220	0.0622	21	0.33

A: batch with presumptive value of 0.027 µg AFM_1/L; B: batch with presumptive value of 0.055 µg AFM_1/L; C: batch with presumptive value of 0.121 µg AFM_1/L; N: number of laboratories; M: overall mean; SD_r (SD_R): standard deviation for repeatability (for reproducibility); r (R): repeatability (reproducibility) value; RSD_r (RSD_R): relative standard deviation for repeatability (reproducibility); HORRAT: value calculated as the ratio of the RSD_R resulting from the trial to the predicted RSD_R. A HORRAT value of 1 indicates an RSD_R value corresponding exactly to the Horwitz equation and HORRAT values bracketing a value of 1 or smaller indicate acceptable precision ***(17)***.

method as an official IDF standard. Unfortunately the study was not carried out according to the harmonized IUPAC/AOAC guidelines for collaborative studies ***(14)***, which prevented it from worldwide acceptance. A new collaborative study was undertaken in 1998 according to the revised harmonized guidelines ***(15)*** to validate the immunoaffinity column clean-up followed by liquid chromatography for determination of aflatoxin M_1 in liquid milk. This method, described in standard operating protocol format in the following sections, was collaboratively studied for liquid milk in 1998 in a collaborative study under the auspicies of the Standards, Measurements and Testing (SMT) Program of the European Commission. The collaborative study protocol was also approved by AOAC International, and the study report is separately published ***(16)***. In **Table 1** the study performance characteristics are summarized.

The method has completed in the approval process of both the SMT Program and AOAC International. The method is approved by the SMT Program and may be used in the EU for official purposes. The method is approved by AOAC International, and it has been adopted for first action in "Official Methods of Analysis" of AOAC International. The protocol presented hereafter describes a method for determining the aflatoxin M_1 concentration in milk, where the aflatoxin M_1 content is expressed as micrograms per liter for liquid milk and micrograms per kg for dry milk. This method can also be applied to skimmed milk and low fat milk. The quantification limit is 0.005 µg/L in liquid milk. The lowest validated level for dry milk is 0.08 µg/kg.

2. Materials

2.1. Reagents (see Note 1)

1. Immunoaffinity columns. Immunoaffinity columns containing antibodies against aflatoxin M_1 (*see* **Note 2**).
2. Mobile phase. Aqueous solution of acetonitrile at 25% (v + v) for mobile phase: add 1 volume of acetonitrile to 3 volumes of water and degas before use.
3. Chloroform, stabilized with 0.5% to 1.0% of ethanol, by mass (*see* **Note 3**).
4. Aflatoxin M_1 calibrant solution (*see* **Notes 4** and **5**).

2.2. Apparatus (see Note 6)

1. Disposable syringe barrels, to be used as reservoirs (10 mL and 50 mL capacity).
2. Vacuum system.
3. Centrifuge, able to produce a radial acceleration of at least 2000*g*.
4. Volumetric pipets.
5. Hamilton-like microsyringes, of 100 µL, 250 µL, and 500 µL capacity.
6. Glass beakers.
7. Volumetric flasks, of 50 mL capacity.
8. Water bath, able to heat at approx 37°C.
9. Filter paper (Whatman #4, or equivalent).
10. Conical glass tubes and stoppers, of 5 mL and 10 mL capacity.
11. UV Spectrophotometer, able to scan at wavelengths from 200 to 400 nm, with quartz face cells of optical length 1 cm.
12. HPLC equipment:
    a. Pump, suitable for steady optimal flow rate.
    b. Autosampler or injector system, with loop injection of 50 µL to 200 µL.
    c. Reversed phase HPLC conventional analytical column, 250 mm × 4.6 mm (id), with 5 µm packing of octadecyl silicagel plus guard column filled with reversed phase material, or equivalent, or reversed phase HPLC short analytical column, 100 mm × 4.6 mm (id), with 3 µm packing of octadecyl silicagel plus guard column filled with reversed phase material, or equivalent.
    d. Fluorescence detector, able to provide 365 nm excitation and 435 nm emission wavelengths.
    e. Recorder or integrator, or computer-based data processing system.

3. Method (*see* Note 7)

3.1. Preparation of Test Portion

3.1.1. Milk

1. Warm samples before analysis to approx. 37°C in a water-bath and then, very gently stir with a magnetic stirrer to dissolve fat layer (*see* **Note 8**).
2. Spin the liquid milk at 2000*g* at least (but not more than 4000*g*) and discard the upper thin fat layer.
3. Filter through one or more paper-filters. Collect at least 50 mL.

3.1.2. Dry Milk

1. Weigh 10 g of dry milk to the nearest 0.1 g into a 250 mL beaker.
2. Take 50 mL of water warmed to 50°C and add this in small amounts to the dry milk.
3. Mix using a stirring rod, until a homogeneous mixture is obtained.
4. Allow the solution of dry milk so obtained to cool to 20°C and then quantitatively transfer it to a 100 mL volumetric flask using small amounts of water.
5. Make the volume of the dry milk solution up to the mark.
6. Filter enough reconstituted milk through filter paper(s) or centrifuge at 4000*g* for 15 min and collect at least 50 mL (*see* **Note 9**).

3.2. Cleanup

1. Allow the immunoaffinity columns to reach room temperature.
2. Attach the syringe barrel to the top of the immunoaffinity cartridge.
3. Measure 50 mL (V_s) of the prepared test portion into a volumetric flask or use a volumetric pipet.
4. Transfer the test portion into the syringe barrel and allow it to pass through the immunoaffinity column at a slow steady flow rate of about 2–3 mL/min (*see* **Note 10**).
5. Remove the syringe barrel and replace by a clean one.
6. Wash the column with 20 to 50 mL of water at a steady flow rate.
7. After washing, blow the column completely to dryness by using the vacuum system or by pushing air with the help of the syringe.
8. Put another dry clean barrel on the cartridge.
9. Slowly introduce some pure acetonitrile (0.5 to 1.0 mL) into the gel and let it stand in contact with the gel at least 1 min.
10. Then, elute the AFM_1 from the column by passing 3.0 to 3.5 mL of pure acetonitrile for a total volume of 4 mL, by keeping a steady slow flow rate.
11. Collect the eluate in a conical tube.
12. Evaporate the eluate to dryness using a gentle stream of nitrogen.
13. Make up with a volume V_f of at least 200 µL of a 10%-acetonitrile solution to obtain a final extract.

3.3. High Performance Liquid Chromatography (HPLC)

1. Pump the mobile phase at a steady flow rate through the HPLC column (*see* **Note 11**).
2. Check the linearity of the standard injections and the stability of the chromatographic system (*see* **Note 12**).
3. Inject properly the required volume (i.e., 50 µL) (*see* **Note 13**).
4. Inject in sequence a suitable volume V_i of AFM_1 standard solutions containing from 0.05 ng to 1 ng.
5. Prepare a calibration graph by plotting the peak area or peak height against the mass of injected AFM_1.

6. Inject a suitable volume V_i of the final extract into the HPLC through the injection loop or with the help of an autosampler.
7. Using the same conditions as for the standard solutions, perform the injection of standards and sample extracts according to a specified injection scheme (*see* **Note 14**).
8. Determine the AFM_1 peak area or height in the eluate and calculate the AFM_1 amount W_a for the corresponding test sample from the calibration graph, in ng.
9. If the AFM_1 peak area or height in the eluate is greater than the highest standard solution, dilute the eluate quantitatively with a 10% acetonitrile solution and reinject the diluted extract into the HPLC.

3.4. Calculation and Expression of Results

Calculate the AFM_1 content of the sample, using the following equation (**Eq. 1**):

$$W_m = W_a \times (V_f/V_i) \times (1/V_s) \quad (1)$$

where:

W_m: is the numerical value of the AFM_1 mass per liter (for liquid milk) or mass per kg (for dry milk) of the sample, in ng/mL or µg/L (or in ng/g or µg/kg).

W_a: is the numerical value of the amount of AFM_1 corresponding to the area or height of the AFM_1 peak of the sample extract, in ng.

V_f: is the numerical value of the final volume of the eluted sample, in mL.

V_i: is the numerical value of the volume of the injected sample eluate, in mL.

V_s: is the numerical value of the volume of the prepared sample passing through the immunoaffinity column (i.e., 50 mL for liquid milk or 5 g for dry milk), in mL (or in g).

The results are expressed in µg/L or in µg/kg with 3 decimals.

4. Notes

1. Unless otherwise specified, use only reagents of analytical grade and distilled or demineralized water or of equivalent purity.
2. Any immunoaffinity column meeting above specifications can be used provided they have a maximum capacity of not less than 100 ng of AFM_1 and give a recovery of not less than 80% for AFM_1 when a standard solution containing 4 ng of toxin is applied. These criteria could be checked as below:

   Capacity check: prepare an aqueous solution of 50 mL containing 200 ng of AFM_1. For instance, transfer by means of a microsyringe 200 µL of the AFM_1 stock solution to a 5-mL conical tube. Evaporate to dryness using a gentle stream of nitrogen. Dissolve the residue in 5 mL of mobile phase. Shake vigorously and add this solution to 45 mL of water. After mixing, apply the whole volume to the immunoaffinity column. Wash the column with 20 to 50 mL of water, and elute

AFM_1 with 4 mL of pure acetonitrile. Determine by HPLC the amount of AFM_1 eluted from the column after suitable dilution of the final eluate.

Recovery check: prepare an aqueous solution of 10 mL containing 4 ng of AFM_1. For instance, dilute 400 µL of a 0.010 µg/mL AFM_1 working solution to 10 mL of water. Mix vigorously and apply the whole volume to the immunoaffinity column. Wash the column with 20 to 50 mL of water and elute the toxin with 4 mL of pure acetonitrile. Determine by HPLC the amount of AFM_1 bound to the column after suitable dilution of the final eluate. Calculate the recovery for the AFM_1.

3. Chloroform is harmful and a suspected carcinogen, extreme precautions must be taken when in use. *See* OSHA instructions on appropriate gloves and protection. This method requires the use of solutions of AFM_1.
4. Aflatoxins are carcinogenic to humans. Attention is drawn to the statement made by the International Agency for Research on Cancer (WHO). Decontamination procedures for laboratory wastes were developed and validated by IARC ***(18)***.
5. Prepare a standard solution of AFM_1 in chloroform with a nominal concentration of 10 µg/mL. Determine the concentration of the standard solution by measurement of its absorbance at the wavelength for maximum absorption, close to 365 nm, using $\varepsilon = 1995$ m^2/mol. Store the standard solution in a well-stoppered vial and protected from light (i.e, amber-colored vial) at approx 4°C. (Under these conditions, this solution is stable for about one year.)

   Prepare an AFM_1 stock solution by transferring by means of Hamilton-like microsyringes, 50 µL of the standard solution into a vial. Evaporate the solution to dryness using a gentle nitrogen stream. Dissolve vigorously the residue in 500 µL of pure acetonitrile using a Vortex-like stirrer. The AFM_1 content of this solution will be 1 µg/mL. Store the stock solution in a well-stoppered vial, protected from light (i.e., amber-colored vial) at approx 4°C. (Under these conditions, this solution is stable for about one month.)

   Prepare working standard solutions of AFM_1 as follows: before preparing a working standard solution of AFM_1, allow the stock solution to reach the ambient temperature. Prepare working solutions on the day of use. Use the stock solution to prepare a series of appropriate working standard solutions by dilution in a 10%-acetonitrile solution (when the acetonitrile content of the injected standard solutions or extracts containing AFM_1 exceeds the 10% [v + v] limit, peak broadening could occur). Concentrations of working standard solutions depend on the volume of the injection loop, and must be prepared in order to inject, for instance, from 0.05 ng to 1 ng of AFM_1.
6. The use of nonacid-washed glassware (e.g., vials, tubes, flasks) for aflatoxin aqueous solutions may cause a loss of aflatoxin. A special attention should be taken with new glassware. Before use, soak the glassware in dilute acid (e.g., sulfuric acid, 2 mol/L) for several hours; then, rinse it extensively with distilled water to remove all traces of acid (this can be checked by using pH-paper).
7. Aflatoxins are subject to light degradation. Protect analytical work adequately from the daylight, and keep aflatoxin standard solutions protected from light by using amber vials or aluminium foil.

8. Warming of milk samples is required to avoid separation of refrigerated milk into two layers.
9. In case the dry milk is not completely dissolved, place the beaker in a water-bath of 50°C at least 30 min and mix regularly.
10. Gravity or vacuum system can be used to control the flow rate.
11. Depending on the kind of column used, the acetonitrile-water ratio and the flow rate, the mobile phase may be adjusted to ensure an optimal separation of AFM_1 from other extract components. As a guideline for conventional columns (with a length of 250 mm and an internal diameter of 4.6 mm), the flow rate of about 0.8 mL/min gives optimal results, and for short columns (with a length of 100 mm and an internal diameter of 4.6 mm) the flow rate of about 0.6 mL/min gives optimal results. Check optimal conditions with an AFM_1 standard solution and a spiked milk sample before analysis of test samples.
12. Repeatedly, inject an AFM_1 standard solution (fixed amount) until stable peak areas or heights are obtained. Peak areas or heights corresponding to consecutive injections shall be within ±10%.
13. Either inject the precise volume (i.e., 50 µL) in a loop whose volume is at least double (i.e., 100 µL) or saturate a loop whose volume corresponds to the required volume of injection. Follow the manufacturer's instructions.
14. It is recommended that an AFM_1 standard be injected at least every 10 injections.

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8

Immunochemical Method for Cyclopiazonic Acid

Joe W. Dorner, Victor S. Sobolev, Wanjun Yu, and Fun S. Chu

1. Introduction

Cyclopiazonic acid (CPA) (**Fig. 1**) is a toxic, indole tetramic acid that was originally isolated from *Penicillium cyclopium* Westling ***(1)*** and subsequently reported to be produced by numerous species of *Penicillium* and *Aspergillus* ***(2)***. Among the species of *Aspergillus* that produce CPA is *A. flavus*, which is primarily known as a producer of the aflatoxins and is a frequent contaminant of corn, peanuts, and other commodities. The taxonomy of *Penicillium* species that produce CPA has undergone several revisions, but Pitt et al. ***(3)*** concluded that the correct name for most saprophytic Penicillia that produce CPA is *P. commune* with *P. palitans* as a synonym. This would include the original isolate variously identified as *P. cyclopium* ***(1)***, *P. griseofulvum* ***(4)***, and *P. verrucosum* var. *cyclopium* ***(5)***. Pitt et al. ***(3)*** also classified all molds used in the manufacture of white cheeses that produce CPA as *P. camembertii*. Based primarily on chemotaxonomical features coupled with conidial colors on Czapek yeast autolysate agar, Lund ***(6)*** concluded that *P. palitans* was not just synonymous with *P. commune*, but was actually a distinct species. Despite some confusion with regard to the taxonomy of CPA-producing Penicillia, the fact remains that the various species of *Penicillium* and *Aspergillus* that produce CPA are ubiquitous and abundant in nature and are common contaminants of commodities that go into foods and feeds. Therefore, the potential for the contamination of commodities with CPA is widespread ***(7)***.

Natural occurrence of CPA has been reported in corn ***(8–10)***, peanuts ***(10,11)***, cheese ***(12,13)***, millet ***(14)***, sunflower ***(15)***, and various feeds and feedstuffs ***(16)***. The toxin has also been shown to accumulate in meat and eggs of chickens ***(17,18)*** and the milk of sheep ***(18)*** dosed with CPA.

From: *Methods in Molecular Biology, vol. 157: Mycotoxin Protocols*
Edited by: M. W. Trucksess and A. E. Pohland

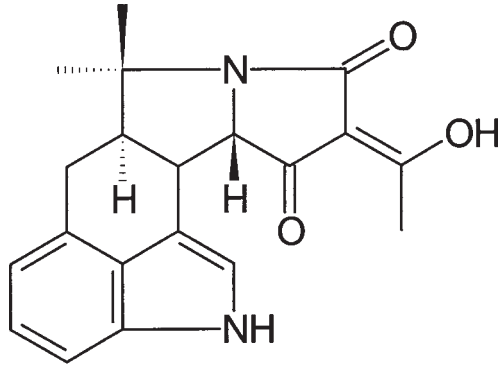

Fig. 1. Chemical structure of cyclopiazonic acid.

Toxicosis resulting from consumption of CPA-contaminated food or feed has not been proven unequivocally, but CPA has been strongly implicated as the causative agent or one of the causative agents in several mycotoxicoses. Whereas the aflatoxins were certainly largely responsible for the outbreak termed turkey "X" disease ***(19)***, Cole ***(20)*** later presented a case of strong circumstantial evidence for the involvement of CPA. This case was strengthened when CPA was detected at a concentration of 31 μg/kg in a sample of groundnut cake that had been saved from the original turkey "X" disease ***(21)***. CPA was strongly implicated in a case of "kodua poisoning" in man ***(14)***. The kodo millet that produced symptoms of giddiness and nausea in two separate instances contained CPA and was heavily infected with CPA-producing strains of *A. flavus* and *A. tamarii*. CPA was also considered to be responsible for the death of quails that consumed feed containing 6000 μg/kg of the toxin ***(22)***.

Many types of methods have been used to detect and quantify CPA in fungal cultures and various agricultural commodities, with thin-layer chromatography (TLC) being the most popular. TLC is typically performed on silica gel plates that have been pretreated with oxalic acid to prevent tailing of CPA ***(23)***. In addition, modification of certain solvent systems with ammonia or acetic acid have been used to prevent tailing ***(24,25)***. CPA can be visualized on TLC plates as a blue-violet spot after spraying with Ehrlich's reagent ***(11)***. TLC has been used to quantify CPA in cheese ***(26)***, corn ***(8,9,27)***, peanuts ***(11,21)***, sunflower ***(15)***, millet ***(14)***, and milk and eggs ***(18)***.

High performance liquid chromatographic (HPLC) methods have also been used to determine CPA in various matrices. Lansden ***(28)*** reported a reversed-phase system for peanuts that used a C_8 or C_{18} column and a mobile phase containing 4-dodecyldiethylenetriamine, zinc acetate, ammonium acetate, 2-propanol, and acetonitrile with UV detection at 284 nm. The detection limit

for pure CPA was 4 ng and recoveries of CPA from spiked peanuts ranged from 72.9% to 85.9%. Goto et al. ***(29)*** used normal-phase HPLC with a silica gel column, mobile phase consisting of ethyl acetate-2-propanol-25% aqueous ammonia (55:20:5, v/v/v), and a SPD-6A spectrophotometer at 284 nm to achieve a detection limit for pure CPA of 0.2 ng. Using an extraction solvent of chloroform-85% phosphoric acid and a silica cleanup cartridge, they reported an 82% recovery of CPA from maize with a lower detection limit of 0.1 μg/g. Urano et al. ***(30)*** described a reversed-phase method using a C_{18} column and a linear gradient of 0–4 m*M* $ZnSO_4$ in methanol-water (85:15, v/v) to quantify CPA in corn and peanuts, with quantitation limits of about 50 and 100 ng/g, respectively. The method was used in a 1990 survey of corn and peanuts which showed extensive contamination of both crops with CPA and aflatoxins ***(10)***. Matsudo and Sasaki ***(31)*** reported a simple HPLC method for analyzing extracts of fungal cultures. The system consisted of a C_{18} column with a mobile phase of 50 m*M* H_3PO_4 plus 1 m*M* $ZnSO_4$-acetonitrile (45:55) and UV detection at 284 nm. Indomethacin was added to sample extracts as an internal standard, and CPA concentrations were calculated on the basis of the ratio of the peak area of CPA to that of the internal standard. The detection limit for CPA was 0.3 ng. Sobolev et al. ***(32)*** recently reported a normal phase ion-pair partition HPLC system that was used to detect CPA simultaneously with other metabolites of various *Aspergillus* species. A silica gel column with a mobile phase of n-heptane-2-propanol-n-butanol-water-tetrabutylammonium hydroxide (2560 + 900 + 230 + 32 + 8, v/v) and a diode array detector allowed separation and detection of at least seven metabolites, including CPA, with a detection limit for CPA of 5 ng/injection.

Several spectrophotometric methods have been used for CPA determination. Rathinavelu and Shanmugasundaram ***(33)*** used TLC to purify extracts, eluted CPA from TLC plates, and added p-dimethylaminobenzaldehyde plus HCl to develop color which was measured with a colorimeter at 560 nm. Variations and improvements to this basic method have been reported by Rao and Husain ***(34)***, Chang-Yen and Bidasee ***(35)***, and Šimůnek et al. ***(36)***, and a detection limit in corn and poultry feed of 80 μg/kg was reported ***(35)***.

The application of capillary electrophoresis to analysis of CPA in milk has been reported recently ***(37)***. The micellar electrokinetic capillary chromatography method involved extraction of milk with basic methanol-water followed by partitioning and Sep-Pak cleanup. The analytical response was linear from 40 ppb to 100 ppm of CPA in milk and recoveries were 78–81% over the range of 20–500 ppb. The minimum quantifiable concentration of CPA in spiked milk samples was 20 ppb.

Enzyme-linked immunosorbent assays (ELISA) have been developed and applied successfully to the analysis of CPA in fungal cultures and cheese ***(38–41)***.

However, it was found that when agricultural commodities were analyzed with an immunoassay, interferences from the sample matrix produced losses in the sensitivity usually associated with these assays ***(42)***. Therefore, an immunoaffinity column containing a monoclonal antibody with high affinity to CPA was developed for the cleanup of sample extracts before ELISA analysis ***(43)***. Use of the immunoaffinity column prior to ELISA analysis improved detection limits for CPA in corn, mixed feed, and peanuts from 100, 300, and 600 ng/g, respectively, to 2.0, 4.4, and 4.7 ng/g, respectively. It was also shown that the immunoaffinity column could be regenerated for reuse at least 10 times by washing with equilibrating buffer and storing in a cold room overnight.

Most published analytical methods for the determination of CPA require extensive, time-consuming cleanup procedures to achieve accurate quantitation by one of the previously described techniques. In many cases, extraction is followed by several liquid-liquid partitioning steps and further cleanup by column, cartridge, or TLC. The purpose of this work was to apply simple immunoaffinity column cleanup to peanut extracts prior to analysis by HPLC.

2. Materials

1. Vertical cutter mixer (VCM) or food processor (e.g. Robot Coupe RSI6Y-1 or Cuisinart DLC-8S).
2. Blender: Waring with 500 mL glass jar.
3. Filter papers: 15 cm coarse fluted filter paper (P8, Fisher Scientific, Atlanta, GA); 4.25 cm glass microfiber filters (GF/C Whatman Ltd., Maidstone, England).
4. Test tubes: borosilicate glass culture tubes with plain end (20 × 150 mm).
5. Flat-bottom boiling flask (1 L).
6. Rotary evaporator (Büchi Rotavapor).
7. CPA-specific immunoaffinity columns (Prepare columns as previously described ***(43)*** by coupling a CPA-specific monoclonal antibody to Sepharose gel and loading a filter tube with 0.2 mL of CPA immunogel in 0.57 mL of slurry. Fill columns with phosphate buffered saline [PBS] containing 0.02% sodium azide and store at 4°C.).
8. Heating block with 9 ports for 4 mL vials.
9. High purity compressed nitrogen.
10. Vortex mixer: Touch-Mixer (Fisher).
11. Vial: 4 mL borosilicate clear autosampler vial with screw cap and PTFE septa (National Scientific, Lawrenceville, GA).
12. HPLC System.
    a. Pump: Model 515 (Waters Chromatography, Milford, MA).
    b. Injector: Model 7125 syringe loading sample injector with 20 µL loop (Rheodyne, Cotati, CA) or Model 712 WISP autosampler (Waters).
    c. Detector: Model SPD-10A diode array with Class-VP chromatography data system (Shimadzu, Kyoto, Japan) or Model 490 E programmable multi wavelength UV detector at 282 nm (Waters).

d. Column: Zorbax Rx-SIL 250 × 4.6 mm id, packed with 5 µ*M* silica gel (MAC-MOD Analytical).

13. Solvents for HPLC: HPLC grade hexane and reagent alcohol (Fisher).
14. Water: Distilled, purified with Milli-Q Water System (Millipore, Burlington, MA).
15. TRIS buffer: 1 g TRIS in 10 mL water.
16. Phosphate buffered saline (PBS): 10 m*M*, pH 7.4 (Sigma, St. Louis, MO).
17. Extraction solvent: 70:30 methanol/1% sodium bicarbonate in water.
18. HPLC Mobile Phase: 500:275:16 hexane/reagent alcohol/TRIS.
19. HPLC Standard: Stock solution: Dissolve 1 mg of CPA in 10 mL of ethyl acetate plus 1% acetic acid; Working standard: Evaporate 20 µL of stock solution to dryness and redissolve in 2 mL of HPLC mobile phase (final concentration of 1 ng/µL).

3. Methods

3.1. Sample Preparation and Extraction

1. Grind peanuts in VCM or food processor to a homogeneous paste (about 6 min).
2. Transfer a 50 g subsample to a Waring blender, add 150 mL of extraction solvent, and blend at high speed for 2 min.
3. Filter extract through fluted filter paper, transfer 15 mL to a test tube, add 2 mL of a saturated solution of sodium bicarbonate, and place in a freezer for at least 30 min.
4. Suction filter contents of test tube through microfiber filter paper into 100 mL boiling flask.
5. Evaporate solvent using a rotary evaporator at 50°C until the volume in the flask is about 4–6 mL.

3.2. Immunoaffinity Column Chromatography

1. Wash column with 10 mL of PBS to remove sodium azide and apply extract from evaporating flask.
2. Rinse flask three times with 2 mL of 1% sodium bicarbonate and add to affinity column.
3. Load column at a flow rate of 1 mL/min and wash with 15 mL of purified water.
4. Wash column with 2 mL of 70:30 water/methanol.
5. Elute CPA with 2 mL of methanol at a flow rate of 0.5 mL/min into vial.
6. Evaporate solvent under stream of nitrogen in heating block.
7. Redissolve eluate in 500 µL of HPLC mobile phase and mix well with vortex mixer.

3.3. Liquid Chromatography

1. Set mobile phase flow rate at 1.2 mL/min or adjust to achieve desired retention time of CPA. Let system equilibrate until steady baseline is achieved.
2. Inject 20 µL of HPLC working standard (contains 20 ng of CPA and is equivalent to 100 ng/g in peanuts) and calibrate data system at 100 ng/g for external standard quantitation, which corresponds to 100% recovery of CPA from peanuts.
3. Inject 20 µL of prepared samples.

Table 1
Method Performance for LC Determination of CPA with Immunoaffinity Column Cleanup

	CPA Added (ng/g)[a]	Recovery (%)	RSDr (%)[b]
Peanuts[c]	10	90.8	7.6
	100	83.7	6.6
Peanut Extract[d]	100	93.8	3.8

[a]Five determinations were made at each spike level.
[b]RSDr = within laboratory coefficient of variation.
[c]50 g of CPA-free ground peanuts. Limit of detection of the method = 2.5 ng/g.
[d]15 mL of filtered CPA-free peanut extract.

3.4. Method Performance

The recovery of CPA from spiked peanuts and peanut extract and the repeatability of the method as measured by the relative standard deviation (RSDr) are presented in **Table 1**. The recovery from spiked peanuts ranged from 83.7–90.8%. The improvement in recovery to 93.8% and the low RSDr associated with spiked peanut extract indicated that the immunoaffinity column cleanup associated with the HPLC system provided excellent recovery of CPA with highly repeatable results.

4. Notes

1. Take safety precautions. Wear protective clothing, gloves, and eye protection. See the Material Safety Data Sheets or equivalent for each reagent. Dispose of waste solvents according to applicable environmental rules and regulations.
2. Addition of saturated sodium bicarbonate to the initial filtrate (*see* **Subheading 3.1.3.**) and freezing causes precipitation of impurities that interfere with complete binding of CPA by the immunoaffinity column. This improved recovery from 55–60% to that shown in **Table 1**.
3. LC analysis of CPA standards was linear (r = 0.9999) over the range of 1–1000 ng of CPA per injection (5–5000 ng/g). However, because the binding capacity of the immunoaffinity column is 4 µg, the upper limit for quantitation is 800 ng/g. For test portions that measure higher, another subsample must be extracted and subjected to the cleanup procedure and LC analysis.
4. The effectiveness of the immunoaffinity column is illustrated by comparing **Fig. 2** (chromatogram of 50 g of peanuts spiked with 100 ng/g of CPA and cleaned up with the immunoaffinity column) with **Fig. 3** (chromatogram of 50 g of peanuts spiked with 100 ng/g of CPA and cleaned up using liquid-liquid partition and solid phase extraction).
5. Use of a diode array detector enables confirmation of the identity of CPA in a sample by comparison of its UV spectrum with that of authentic CPA.

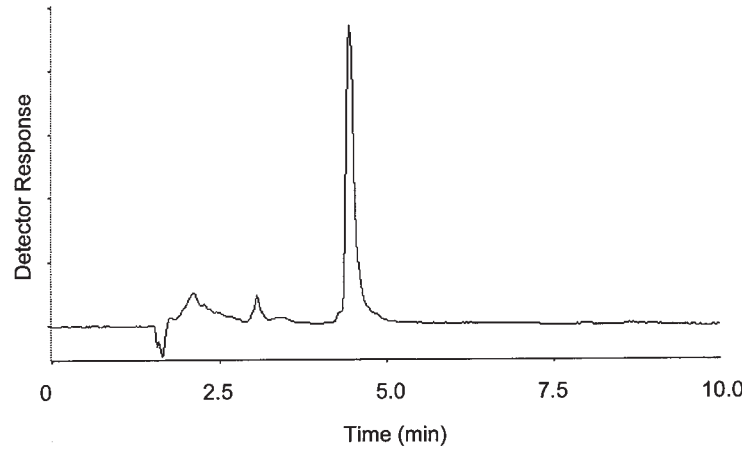

Fig. 2. Chromatogram of an extract of peanuts spiked with 100 ng/g of CPA and cleaned up using immunoaffinity column chromatography.

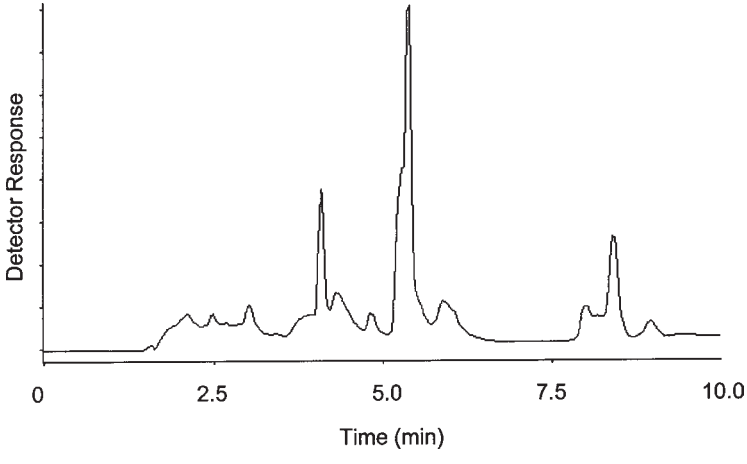

Fig. 3. Chromatogram of an extract of peanuts spiked with 100 ng/g of CPA and cleaned up with liquid-liquid solvent partition followed by solid phase extraction ***(30)***. CPA co-eluted with a major interfering compound at a retention time of 5.4 min. The HPLC system was not optimized for this cleanup procedure.

6. The method was used to analyze peanuts grown under late-season drought stress, conditions that favor contamination of peanuts with CPA and aflatoxins. Results of analyses for CPA using the immunochemical method along with results of analyses for aflatoxins in different seed size categories are shown in **Table 2**.

Table 2
Concentrations of CPA and Aflatoxin in Peanuts Grown Under Late-season Drought Stress

Peanut Seed Size	Weight (g)[a]	CPA (ng/g)[b]	Aflatoxin (ng/g)[a,b]
Jumbo	1390.1	3.0	26.5
Medium	2826.1	44.9	0.0
Number 1	626.0	145.6	122.4
Oil Stock	287.7	170.5	0.6
Damage[c]	46.5	8105.0	7094.3

[a]Values are the means of three replicated, drought-stressed plots (4.0 × 5.5 m).
[b]Values are the total of aflatoxins B_1, B_2, G_1, and G_2.
[c]Damaged seed were hand-picked from each seed size category and combined for one analysis per plot.

Acknowledgment

We thank Milbra A. Schweikert for valuable technical assistance and for help in preparation of the figures for this manuscript.

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9

Immunochemical Method for Ochratoxin A

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1. Introduction

Ochratoxin A (7-L-β-phenylalanylcarbonyl-5-chloro-8-hydroxy-3,4-dihydro-3-R-methylisocoumarin, **Fig. 1**) was first isolated in 1965 from *A. ochraceus* ***(1)***. Meanwhile several *Aspergillus* and *Penicillium* species are known to produce ochratoxin A. The toxin frequently occurs as a contaminant in plant products worldwide, predominantly during storage. Numerous papers have described its occurrence in, for example, cereals, beans, coffee, grapes, beer, and red wine ***(2,3)***. In particular for *Penicillium* spp., cocontamination with ochratoxin A and other *Penicillium* toxins, such as citrinin, is quite common ***(4)***.

Ochratoxin A is a nephrotoxin and a potent renal carcinogen in rodents ***(2)***. It is known to cause mycotoxic porcine nephropathy (MPN) in swine ***(5)***, and is suspected to cause a human disease called "Balkan endemic nephropathy" (BEN) ***(6–10)***. However, no clear proof for the latter hypothesis has been presented so far. The oral acute toxicity (LD_{50}) of ochratoxin ranges from <6 mg/kg bw in female pigs to about 30 mg/kg bw in male rats, with female animals being slightly more sensitive than males ***(2,3)***.

Ochratoxin A binds to blood serum proteins and has a long elimination half-life time in most species ***(11)***. Therefore a carry-over of ochratoxin A via the food chain is possible, the most important source being porcine tissues (kidneys) and products containing porcine blood or serum.

Measurement of ochratoxin A serum levels in humans can be used to estimate the continuous daily intake of ochratoxin A via foods. Breitholtz et al. ***(12)*** made an estimate based on animal data ***(11)***, which gave the following relationship: Ochratoxin A intake (ng/kg bw/day) = 1.34 × ochratoxin A plasma

From: *Methods in Molecular Biology, vol. 157: Mycotoxin Protocols*
Edited by: M. W. Trucksess and A. E. Pohland

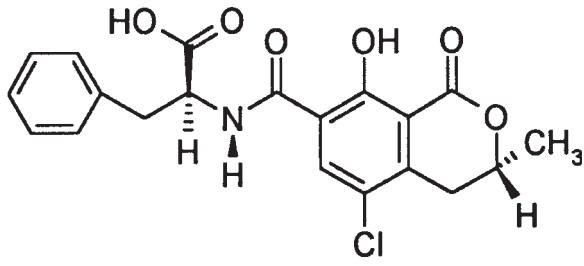

Fig. 1. Structural formula for ochratoxin A.

concentration (ng/mL). However, there are several uncertainties in this estimate, since elimination kinetics and bioavailability of ochratoxin A are not exactly known in humans ***(12)***. Nevertheless, since published data on ochratoxin A in human blood serum are fairly abundant ***(13–16)***, this can be used to compare the overall ochratoxin A intake between countries. As a matter of fact most studies found that 80–100% of all blood samples were ochratoxin positive, with marked regional differences in the mean toxin levels (from <0.5 ng/mL up to 10 ng/mL).

An estimate of the (provisional) tolerable daily intake (TDI) for ochratoxin A made in 1990 by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) resulted in a figure of 112 ng/kg bw per week, corresponding to 16 ng/kg bw per day ***(17)***. This value was based on nephrotoxicity and did not address carcinogenicity. Other calculations of the TDI for ochratoxin A came to figures ranging from 0.2 to 5.0 ng/kg bw per day ***(18)***. According to Scott et al. ***(15)***, the provisional TDI of ochratoxin A in Canada has been set at 3.7 ng/kg bw per day.

Only a few countries have set regulations for ochratoxin A in foods so far ***(19)***. Ochratoxin A in cereals is regulated in Austria, France (5 µg/kg), and in Switzerland (2 µg/kg). Denmark uses the ochratoxin A content in visibly damaged pig kidneys to decide over condemnation of the carcass (10–25 µg/kg). Within the European Union, tolerances for ochratoxin A in the range of 3–5 µg/kg have been under discussion for several years now.

Analytical methods for ochratoxin A are predominantly high-performance liquid chromatography (HPLC) techniques with fluorescence detection ***(20)***, in recent years increasingly employing immunoaffinity chromatography techniques as the extract cleanup method ***(21)***. Several enzyme immunoassay techniques for ochratoxin A, mostly designed as microtiter plate assays, have also been described ***(22,23)***. This chapter gives details about the production of polyclonal rabbit antibodies against ochratoxin A and the development of a

competitive direct enzyme immunoassay for ochratoxin A. This approach uses a mixed anhydride reaction ***(24)*** for immunogen synthesis, and an activated ester method ***(25)*** for production of the labeled antigen. The application of this assay for the determination of ochratoxin A in cereals and in porcine blood serum is described.

2. Materials

2.1. Antigens and Labeled Antigens

1. Ochratoxin A (Sigma-Aldrich Vertriebs GmbH, Deisenhofen, Germany).
2. Human serum albumin (HSA), molecular mass 6.8×10^4 (Sigma-Aldrich) (*see* **Note 1**).
3. Horseradish peroxidase (HRP), enzyme immunoassay grade, molecular mass 4×10^4, (Boehringer Mannheim, Mannheim, Germany).
4. Dimethylformamide (DMF).
5. Isobutyl chloroformate.
6. Tri-n-butylamine.
7. N-hydroxysuccinimide.
8. N,N'-dicyclohexylcarbodiimide.
9. 10 m*M* potassium phosphate, 100 m*M* sodium chloride, pH 7.2–7.3 ("phosphate-buffered saline," PBS).

2.2. Immunizations

1. Complete Freund's adjuvant (Sigma-Aldrich).
2. Sterile pyrogen-free water.

2.3. Assays

2.3.1. Antibody Titer Determination

1. 50 m*M* sodium carbonate buffer, pH 9.6
2. Antirabbit IgG from sheep, affinity-purified (Sigma-Aldrich).
3. Wash solution (8.5 g/L sodium chloride, Tween-20 250 µL/L).
4. Ochratoxin A stock solution in methanol, approx 10 µg/mL (*see* **Note 2**). Stored at –18°C. This stock solution is stable for 2–3 mo. From this stock solution, a dilution with a concentration of 1 ng/mL is prepared with 0.13 *M* $NaHCO_3$ for specific titer determination. Store at 4–8°C for 1–2 wk.
5. Enzyme substrate/chromogen solution ***(26)***:
   a. 65 µL hydrogen peroxide (30% solution) in 200 mL 200 m*M* potassium citrate buffer, pH 3.9. Store at room temperature, protected from light.
   b. 50.4 mg 3,3',5,5'-tetramethylbenzidine in 1 mL acetone plus 9 mL methanol. Store at room temperature, protected from light. Mix 0.5 mL of b) with 10 mL of a) shortly before use.
6. 1 *M* sulfuric acid.

2.3.2. Competitive Direct Enzyme Immunoassay

1. 50 m*M* sodium carbonate, pH 9.6.
2. 0.13 *M* sodium hydrogencarbonate, pH 8.3–8.5.
3. Wash solution (8.5 g/L sodium chloride, Tween-20 250 µL/L).
4. Ochratoxin A stock solution in methanol, approximately 10 µg/mL (*see* **Note 2**). Stored at –18°C, this stock solution is stable for 2–3 mo. This stock solution is diluted with 0.13 *M* $NaHCO_3$ for assay to give final standard concentrations of 16.5 pg/mL to 5000 pg/mL. Store at 4–8°C for 1–2 wk.
5. Enzyme substrate/chromogen solution ***(26)***:
   a. 65 µL hydrogen peroxide (30% solution) in 200 mL 200 m*M* potassium citrate, pH 3.9. Store at room temperature, protected from light.
   b. 50.4 mg 3,3',5,5'-tetramethylbenzidine in 1 mL acetone plus 9 mL methanol. Store at room temperature, protected from light. Mix 0.5 mL of b) with 10 mL of a) shortly before use.
6. 1 *M* sulfuric acid.

3. Methods

3.1. Preparation of Antigens

3.1.1. Preparation of HSA-Ochratoxin A Conjugate

1. Dissolve 5.0 mg ochratoxin A in 2.0 mL DMF in a 10 mL glass vial, add a clean stirring magnet, close vial and cool to 0°C in an ice-water bath.
2. Add 3 µL tri-n-butylamine, and cool down to –10 to –15°C by adding ethanol at a temperature of –18°C to the water bath (*see* **Note 3**).
3. Meanwhile, dissolve 7.5 mg HSA with 2 mL distilled water in a 10 mL glass vial, add 1.5 mL DMF, add a clean stirring magnet, close vial, place in the ethanol-water bath, and let cool down to –10 to –15°C.
4. Add 2 µL isobutyl chloroformate to ochratoxin A solution and stir at –10 to –15°C for 5 min.
5. Using a Pasteur pipet, transfer the ochratoxin A solution into the HSA solution.
6. Incubate conjugation mixture for 1 h at –10 to –15°C under constant stirring. Control pH of the mixture and adjust to approx pH 9 by adding 10–20 µL portions of 0.1 *M* NaOH.
7. Incubate mixture for another 3 h, allowing the temperature of the ethanol-water bath to increase to approx 0°C within the first hour, and maintain at 0 ± 2°C for the next 2 h. Control and adjust pH as above.
8. Add 10 mg $NaHCO_3$ to conjugation mixture.
9. Dialyze the mixture at 4–8°C for 3 d against 5 L of PBS, changing the PBS daily.
10. Determine the protein content of the conjugate by the method of Lowry et al. ***(27)***.
11. Check spectrum of conjugate photometrically for the presence of conjugated ochratoxin A at a wavelength range from 200 nm to 500 nm, using spectra of nonconjugated HSA and ochratoxin A, respectively, for comparison. Conjugation ratio (molecules of ochratoxin A per molecule HSA) may be determined

by using the absorbance at >340 nm of the conjugate solution and calculation from ochratoxin A standard curve in PBS recorded at the same wavelength (*see* **Note 4**).

3.1.2. Preparation of HRP-Ochratoxin A Conjugate

1. Dissolve 5 mg ochratoxin A in 0.5 mL DMF.
2. Dissolve 7.1 mg N-hydroxysuccinimide in 0.25 mL DMF and add to ochratoxin A solution.
3. Dissolve 25.5 mg dicyclohexylcarbodiimide in 0.25 mL DMF and add to ochratoxin A solution.
4. Incubate mixture under slow magnetic stirring at ambient temperature protected from light for 16–20 h (*see* **Note 5**).
5. Dissolve 50 mg HRP in 9 mL 0.13 *M* $NaHCO_3$ solution.
6. Add ochratoxin A reaction mixture dropwise to HRP solution under constant stirring.
7. Incubate at room temperature for 2 h (magnetic stirring at slow speed).
8. Dialyze the mixture at 4°C for 3 d against 5 L of PBS, changing the PBS daily.
9. Dilute an aliquot of the conjugate 1:10 with PBS and record UV spectrum of the conjugate from 200 nm to 500 nm (*see* **Note 6**).
10. Store conjugate in small portions of 0.2 to 0.5 mL at –18°C or lyophilize and store at –18°C (*see* **Note 7**).

3.2. Antibody Production

3.2.1. Immunization of Rabbits

1. Adjust the ochratoxin A-HSA conjugate to a protein concentration of 0.6 mg/mL with sterile distilled water, mix for 30 s on a wrist-action shaker and emulsify per animal 0.5 mL of the conjugate with 1.5 mL of Freund's complete adjuvant (*see* **Note 8**).
2. Inject three or four rabbits (female Chinchilla bastard, 16–20-wk-old) each with 2.0 mL of the mixture intradermally at 20 to 30 sites on shaved backs.
3. Collect blood from the *Arteria auricularis magna,* starting 4 wk after primary injection, and continue to collect blood (20–40 mL per collection) every two weeks over a period of 6–10 mo, depending on the total amount of antiserum required.
4. Depending on the results of the antibody titer determinations (*see* **Subheading 3.2.2.**), perform one or two booster injections using the same amount and composition of immunogen. Either subcutaneous or intramuscular injections (2–4 injection sites) may be given (*see* **Note 9**).

3.2.2. Antibody Titer Determination and Sensitivity Check

1. Dissolve antirabbit IgG in 50 m*M* sodium carbonate, pH 9.6, to give a 10 µg/mL solution. Coat microtiter plate using this solution, 100 µL per well, and incubate overnight in a chamber with >90% relative humidity.
2. Remove the antirabbit IgG solution.

3. Wash each plate with 8.5 g/L sodium chloride containing Tween-20 250 µL/L.
4. Individually prepare serial dilutions (2^n–3^n) in 0.13 *M* $NaHCO_3$ solution of antisera of all rabbits immunized, starting at a 1:100 dilution. Prepare at least 8 dilutions. Always compare antisera with those obtained from the collection before to check whether the titer increases or not.
5. Prepare ochratoxin A standard solution in containing 0.13 *M* $NaHCO_3$ solution at a concentration of 1 ng/mL.
6. Prepare HRP-ochratoxin A solution, diluted 1:10,000 with 0.13 *M* $NaHCO_3$ containing 1% Tween-20.
7. To one half of the microtiter plate, add 35 µL per well ochratoxin A standard solution (positive control); to the other half of the plate, add 35 µL per well 0.13 *M* $NaHCO_3$ solution (negative control).
8. Add 35 µL per well of diluted antiochratoxin A antisera to both the toxin-positive and the toxin-negative half of the microtiter plate, resulting in a positive and a negative control well for each individual antiserum dilution.
9. Add 35 µL per well HRP-ochratoxin A conjugate solution to all wells.
10. Incubate for 2 h at room temperature (*see* **Note 10**).
11. Wash each plate with 8.5 g/L sodium chloride containing Tween-20 250 µL/L.
12. Add 100 µL of enzyme substrate/chromogen solution per well, and incubate for 15 min at room temperature.
13. Add 1 *M* sulfuric acid (100 µL per well) and measure absorbance. On the enzyme immunoassay (EIA) microtiter plate reader (AT400, SLT Labinstruments, Austria, or equivalent) set sample wavelength to 450 nm and reference wavelength to 620 nm.
14. Select antisera which give absorbance values for toxin-negative wells of >1.0 units at high dilutions (usually >1:10,000) and a maximum absorbance for the respective toxin-positive well of <50% of the corresponding toxin-negative well (maximum sensitivity).
15. Individually make checkerboard tritations of the selected antisera versus the HRP-ochratoxin A conjugate, with and without addition of ochratoxin A standard solution, to estimate optimum antiserum dilution and conjugate dilution (*see* **Note 11**).
16. Pool antisera of similar quality to provide a large antiserum stock.
17. Mix an aliquot of antiserum pool with the same volume of 70% saturated ammonium sulfate solution and let stand at room temperature for 4 h.
18. Centrifuge mixture (1500*g*, 15 min, 4°C) and remove supernatant.
19. Reconstitute residue (the immunoglobulin fraction) with PBS to the original antiserum volume, add equal volume of 70% saturated ammonium sulfate solution and incubate at room temperature for 5 min.
20. Repeat **steps 18**, **19**, and **18**.
21. Reconstitute residue with PBS to original antiserum volume and dialyze for three days against 5 L PBS, changing the PBS daily.
22. Store the dialyzed antiserum at –18°C (*see* **Note 12**).

23. Perform checkerboard titrations of antiserum coating versus HRP-ochratoxin A conjugate dilution (with and without standard addition) under the conditions of the competitive direct enzyme immunoassay (*see* **Subheading 3.3.2.**) to determine optimum antiserum dilution for microtiter plate coating and for conjugate dilution.

3.3. Assays

3.3.1. Sample Preparation

3.3.1.1. Cereal Samples

1. Grind grain to pass through 1-mm apertures.
2. Add test sample (2 g) and 5 mL 1 *M* HCl in a 40 mL test tube and mix for 5 min on a magnetic stirrer at full speed.
3. Add 10 mL of dichloromethane and mix at full speed for another 15 min.
4. Centrifuge the mixture (1500*g*, 15 min, 4°C).
5. Remove the upper aqueous layer with a Pasteur pipet and transfer the dichloromethane phase into another 40 mL test tube.
6. Add 10 mL 0.13 *M* $NaHCO_3$ solution to the dichloromethane phase and mix at full speed for 15 min.
7. Centrifuge the mixture again.
8. Remove upper aqueous layer for EIA analysis. If necessary (for high ochratoxin A concentrations) make further dilutions in 0.13 *M* $NaHCO_3$ solution.
9. Recovery checks of the sample extraction procedure in a concentration range from 2.5–10 ng/g should be performed on each day of analysis by adding 25–100 µL methanolic ochratoxin A standard solution to 2-gram portions of toxin-negative sample matrix under analysis and allowing the solvent to evaporate for at least 30 min (*see* **Note 13**). If available, an in-house check-sample, naturally contaminated with ochratoxin A, should also be extracted and analyzed on each day of analysis.

3.3.1.2. Porcine Blood Serum Samples

1. Centrifuge 4–5 mL blood sample at 1200–1500*g* for 10–15 min to separate serum.
2. Pipet 2 mL blood serum in a 20 mL test tube.
3. Add 2.5 mL 1 *M* HCl.
4. Add 4 mL dichloromethane.
5. Mix by magnetic stirring at high speed for 5 min.
6. Centrifuge at 1200–1500*g* for 15 min.
7. Remove upper aqueous phase.
8. Add 3 mL 0.13 *M* $NaHCO_3$.
9. Mix for 30 s (wrist-action shaker).
10. Centrifuge at 1200–1500*g* for 15 min.
11. Collect upper aqueous phase and repeat **steps 8–10**.
12. Mix both aqueous phases in a test tube.

13. Add 1 mL 1 *M* HCl.
14. Add 3 mL dichloromethane.
15. Mix for 30 s (wrist-action shaker).
16. Collect lower dichloromethane phase.
17. Evaporate dichloromethane in a rotary evaporator.
18. Redissolve residue with 2 mL 0.13 *M* $NaHCO_3$ for assay.
19. If necessary, dilute further with 0.13 *M* $NaHCO_3$.
20. Routinely check recovery of this procedure in a concentration range of 0.5–5.0 ng/mL by adding 10–20 µL ochratoxin A standard solution to 5 mL blood serum before extraction (*see* **Note 14**).

3.3.2. Competitive Direct Enzyme Immunoassay

1. Dilute ammonium sulfate-purified antiserum against ochratoxin in 50 m*M* sodium carbonate, pH 9.6 (usually a 1:2000 dilution will work). Coat microtiter plates using this solution, 100 µL per well, and incubate overnight in a chamber with >90% relative humidity.
2. Remove the antiserum solution.
3. Wash each plate with 8.5 g/L sodium chloride containing Tween-20 250 µL/L.
4. By dilution of the ochratoxin A stock solution, prepare seven ochratoxin A standard concentrations in 0.13 *M* $NaHCO_3$ solution, covering a concentration range from 5000 pg/mL to 16.5 pg/mL, including a zero standard (blank).
5. Prepare HRP-ochratoxin A solution (usually a 1:100,000 dilution will work) in 0.13 *M* $NaHCO_3$ solution containing 1% Tween-20.
6. Add 50 µL per well of standard or sample extract solution. Perform at least duplicate analyses of all standards and extracts, fourfold determination should be used if possible.
7. Add 50 µL per well HRP-ochratoxin A conjugate solution to all wells.
8. Incubate for 2 h at room temperature (*see* **Note 10**).
9. Wash each plate with 8.5 g/L sodium chloride containing Tween-20 250 µL/L.
10. Add 100 µL of enzyme substrate/chromogen solution per well, and incubate for 15 min at room temperature.
11. Add 1 *M* sulfuric acid (100 µL per well) and measure absorbance. On the EIA microtiter plate reader (AT400, SLT Labinstruments, or equivalent), set sample wavelength to 450 nm and reference wavelength to 620 nm.
12. If available, evaluate results with immunoassay software capable to calculate competitive immunoassays. Otherwise, calculate mean absorbance for each standard concentration and for all sample extracts. Set absorbance of zero standard (B_0) as 100% and transform absolute absorbance of ochratoxin A standards (B) by using the formula $B/B_0 \times 100$ (relative absorbance). On a semilogarithmic paper, plot concentration versus relative absorbance values. Construct calibration curve by point-to-point interpolation of standards (**Fig. 2**) to determine ochratoxin A concentration of sample extracts. Multiply results with the respective sample extract dilution factors to calculate the ochratoxin A concentrations of sample materials. Do not use the high concentration range corresponding to <20%

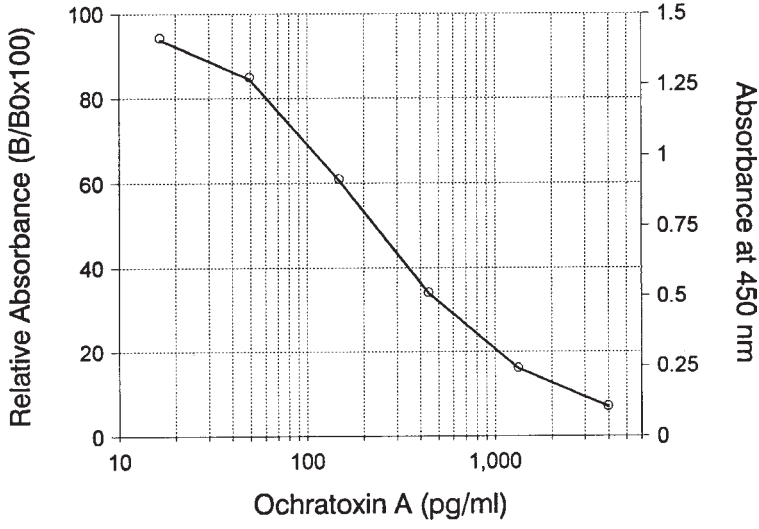

Fig. 2. Standard curve for competitive direct EIA of ochratoxin A with rabbit antiserum and HRP-ochratoxin A conjugate. The left *y*-axis indicates the relative absorbance values ($B/B_0 \times 100$), the right *y*-axis gives the absolute absorbance values (B) measured at 450 nm. Absorbance of the negative control (B_0) was 1.49 units. All standards were performed in quadruplicate. Intraassay coefficients of variation were between 1.5% and 6%. After evaluation of 40 standard curves performed over a period of 2 mo, the 50% inhibition concentration typically was at 250 ± 50 pg/mL, with a standard curve detection limit of 80 ± 30 pg/mL (determined by students T test, one-sided, $n = 4$).

B/B_0 for quantification, but reanalyze using a higher dilution of the extract. Set detection limit of the standard curve to a concentration corresponding to 80% B/B_0; absorbance measurements higher than that should be reported as "not detectable."

13. To check antibody specificity, perform for all available ochratoxin A analogs (for example, ochratoxin B) standard curves under the conditions of the competitive direct enzyme immunoassay, using maximum toxin analogue concentration of at least ten times higher than that routinely used for ochratoxin A. Determine 50% B/B_0 concentrations of the standard curves established for cross-reacting compound and calculate relative cross- reactivities using the formula: "Relative cross-reactivity (%) = 50% B/B_0 concentration of ochratoxin A/50% B/B_0 concentration of test compound × 100". For example, relative cross-reactivity of ochratoxin B was found to be 2%.

4. Notes

1. Instead of HSA, bovine serum albumin (BSA) may also be used with the same reaction protocol.

2. A full UV spectrum should be recorded from 200 to 500 nm to check for impurities. Ochratoxin A in methanol has maximum absorbance at 215 nm and 333 nm, respectively. Concentration should be determined at 333 nm using a molar extinction coefficient of ε =6400 and a molecular mass for ochratoxin A of 403 ***(28)***.
3. This may be a little bit difficult to perform in some labs. It is recommended to put a small (30 × 20 × 5 cm) watertight plastic or metal box on a magnetic stirrer, add ice and a small volume of water, and then add ethanol stored in a –18°C freezer until the required temperature is reached. As the liquid becomes warmer, replace an aliquot with –18°C ethanol from the freezer. In this makeshift waterbath all reaction vials can be placed conveniently.
4. At >340 nm there is no interfering UV absorbance by HSA, i.e., the absorbance measured at this wavelength is only due to ochratoxin A. At the maximum absorbance of ochratoxin A (333 nm) there would be some interference by the 280 nm absorbance maximum of HSA.
5. Precipitates may be visible in the reaction vial, indicating formation of the activated ester intermediate. No attempt to remove these precipitates is required for successful conjugation.
6. Since the absorbance of ochratoxin A interferes with the absorbance maximum of HRP at 403 nm, determination of the conjugation ratio or of the HRP concentration is not possible. However, comparing the spectrum with that of unconjugated HRP and of ochratoxin A solution can be used to qualitatively confirm the success of the conjugation.
7. The HRP-ochratoxin A conjugate is stable, without addition of any additives, at least 5 years when stored at –18°C. Lyophilization may increase shelf-life but also decreases specific activity and is only recommended for longer transport purposes. Repeated (>10 times) thawing and freezing should be avoided. A conjugate predilution of 1:100 in PBS can be stored at 4–8°C for at least 2 wk without loss of specific activity. A typical final working dilution of the conjugate (in 0.13 *M* $NaHCO_3$ solution containing 1% Tween-20) ready for enzyme immunoassay is in the range of 1:100,000 but has to be determined for each conjugate batch prepared.
8. It is essential to prepare a stable emulsion to ensure slow release of the immunogen. It is recommended to mix aqueous phase and oil phase in two syringes of suitable size (5–10 mL) connected by a 40 mm × 0.5 mm id connector with luer fittings on both ends. Mix at least 25 times, starting by as vigorously as possible pressing the aqueous phase into the oil phase. Read also an excellent description of immunogen preparation given by Hurn and Chantler ***(29)***.
9. It is strongly recommended not to follow a predesigned immunization schedule but make booster injections in dependence of the individual immune response. As long as the antibody titer increases, no booster should be given. The first booster may be required any time between weeks 8 and 16 after the primary immunization. Although a terminal bleeding may be taken by cardiac puncture as soon as the antibody titer and the sensitivity are both sufficiently high, it is rec-

Table 1
Recovery of Ochratoxin from Various Cereals at Spiking Levels of 2.5–10 μg/kg

	Ochratoxin A recovery		
Sample type	Mean, %	RSD, %	*n*
Wheat	60	6.3	9
Corn	75	28	14
Barley	69	32	9
Oats	28	6.2	6
Wheat bran	51	18	6

ommended to collect blood samples of 20–40 mL every two weeks. Within 6–8 mo 150–200 mL serum can be harvested, an amount which should be sufficient for most purposes. Stored at –18°C, the antiserum is stable at least 15 yr (probably much longer than that) without any preservatives added.

10. If desired, the incubation time can be reduced as much as a few minutes; however, the concentrations of the antiserum and the HRP-ochratoxin A conjugate have to be increased which results in a lower test sensitivity.
11. If only a small quantity of serum (<10 mL) is needed, the antiserum comparison checks may be not necessary. If there is continuous need for serum however, it is preferable to have a larger stock of the same quality. But never pool any antisera unless you are quite sure that you are doing the right thing! Better to repeat some titrations than to inadvertently reduce the quality of the final product. It may be wise to prepare a small test pool of each 100–200 μL of the sera which are supposed to be pooled and check this test pool under the conditions of the enzyme immunoassay.
12. The purified antiserum is stable for at least 10 yr without any preservatives added. However, thawing and freezing the same vial more than a hundred times may have a deleterious effect. Antiserum should be stored in portions of about 0.5 mL.
13. Recovery may vary with the type of cereal analyzed, and therefore should be checked carefully. Sample matrix may affect extraction of ochratoxin A, further losses could occur during the liquid-liquid partitioning steps (HCl:dichloromethane:$NaHCO_3$). The data presented in **Table 1** were obtained by one technician within a period of six weeks. It should be acknowledged that another factor determining the repeatability of the method is the skillfulness of the person doing these extractions. For example, in a different series of analyses, another technician obtained recoveries for ochratoxin A from spiked wheat and barley of 67–78% and 84–97%, respectively, with relative standard deviations of 7.7% to 18%. In the same analytical series, relative standard deviations obtained after repeated analysis of naturally contaminated barley (ochratoxin A content: 2–57 ng/g) were 11–22%.

Table 2
Recovery of Ochratoxin from Spiked Porcine Blood Serum

	Ochratoxin A recovery		
Ochratoxin added (ng/mL)	Mean, %	RSD, %	*n*
0.5	71.6	19.8	6
1	67.5	12.1	6
2.5	82.5	14.4	6
5	68.7	10.9	6

14. Recovery data for ochratoxin A from porcine blood serum are listed in **Table 2**. The detection limit for ochratoxin A in porcine blood serum was at 0.05–0.1 ng/mL. It may be difficult to find control sera containing ochratoxin A at less than 0.1 ng/mL.

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10

Solution Fluorometric Method for Deoxynivalenol in Grains

Bruce Malone

Introduction

Deoxynivalenol (3, 7, 15-trihydroxy-12, 13-epoxytrichothec-9-en-8-one, DON, Vomitoxin) (*see* **Fig. 1**) is a member of the toxic group of fungal metabolites known as trichothecenes. DON is most commonly produced by *Fusarium graminearum* (teleomorph = *Giberella zeae*) ***(1)*** which is the fungal species causing *Fusarium* head blight disease (scab) in wheat and pink ear rot in corn. *Fusarium culmorum* also produces this toxic metabolite ***(2)***. DON was first isolated in Japan ***(3)*** from barley infected with *Fusarium* spp. and in the United States from corn infected with *Fusarium* ***(4)*** in northwestern Ohio. Although DON is not as toxic as other trichothecene mycotoxins, it is one of the most common mycotoxin contaminants of grains worldwide ***(5)***. DON contaminated grains, usually wheat, corn, barley, oats, and rye have been reported to cause emesis, feed refusal, and growth depression in animals, especially dogs and swine, consuming the feed, "gushing" in beer made from contaminated malt, and poor baking performance of wheat flour. DON is very stable in commodities during storage and processing and therefore can occur in foods prepared from contaminated grain ***(6)***.

Because contamination of foods and feed with mycotoxins can be hazardous to the health of humans and animals, the US Food and Drug Administration (FDA) established advisory levels for DON on finished wheat products (e.g., flour, bran, and germ) that may potentially be consumed by humans at 1 µg/g. Advisory levels for DON in animal feeds are as follows: (1) 10 µg/g DON on grains and grain byproduct destined for ruminating beef and feedlot cattle older than 4 mo and for chickens with the added recommendation that these ingredients not exceed 50% of the diet of cattle or chickens; (2) 5 µg/g DON on

From: *Methods in Molecular Biology, vol. 157: Mycotoxin Protocols*
Edited by: M. W. Trucksess and A. E. Pohland

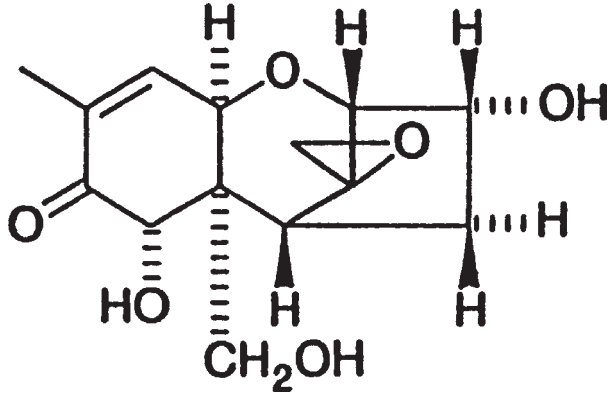

Fig. 1. Structure of Deoxynivalenol (DON, Vomitoxin).

grains and grain byproducts destined for swine with the added recommendation that these ingredients not exceed 20% of their diet and; (3) 5 µg/g on grains and grain byproducts destined for all other animals with the added recommendation that these ingredients not exceed 40% of their diet.

The incidence of *Fusarium* infestation and thus the degree of DON contamination depends on the weather conditions during growth. The contamination of grains usually occurs when crop flowering is accompanied by cool and wet weather conditions. This widespread occurrence of the disease in these crops and the associated contamination of grain with DON has caused increased awareness and a need for testing grains for this mycotoxin. Current methods of analysis for DON include thin-layer chromatography (TLC) ***(7)***, high performance liquid chromatography (HPLC) ***(8)***, gas chromatography (GC) ***(9)***, and enzyme-linked immunosorbent assay (ELISA) ***(10)***.

This chapter describes the use of a one-step solid phase extraction cleanup column followed by a fluorometric detection method for the determination of DON in grains and grain products. This quantitative method is simple, rapid (28 min for one sample and 2 h for twenty-four samples), accurate compared to HPLC and can quantify DON concentrations in samples between 0.5 and 50 µg/g with no dilutions required.

The method is applicable to the determination of DON in barley, corn, wheat, bran, oats, wheat flour, wheat middlings, malted barley and is approved by the U.S. Department of Agriculture Grain Inspections, Packers and Stockyards Administration.

A brief summary of the method ***(11)*** is as follows:

Samples are ground and extracted with acetonitrile/water (86/14). A portion of the extract is purified by passage through a cleanup column and evaporated

to dryness. A derivatization is performed by heating zirconyl nitrate in the presence of ethylenediamine in methanol. The fluorescent adduct produced ***(12)*** is quantified with a calibrated fluorometer containing a broad wavelength pulsed xenon light source.

2. Materials

2.1. Fluorometric Method

1. Grinding/subsampling mill.
2. Fluorometer equipped with a broad wavelength pulsed xenon lamp, selected source filters (365–380 nm excitation, 450–550 nm emission) silicon detector, and RS-232C output for printer.
3. Blender (Osterizer 14 speed).
4. Blender jars (one-half pint, glass jars suitable for use with blender).
5. Vortex mixer.
6. Evaporation system.
7. Repipetor with 5 mL and 1 mL plastic combotips (Eppendorf Repeator pipet).
8. Pipetor with disposable tips, 1.0–5.0 mL.
9. Glass culture tubes, 15 × 85 mm, borosilicate.
10. Glass cuvets, 12 × 75 mm borosilicate fitted with uniflex safety caps.
11. Filter paper, coarse grade.
12. Plastic funnel.
13. Extraction solution-acetonitrile/water (86/14); add 860 mL of acetonitrile (ACS grade) to 140 mL of water (deionized or distilled).
14. Reagent A-Ethylenediamine in methanol 0.04% (store at room temperature, stability 6 mo).
15. Reagent B-Zirconylnitrate in methanol 3.75% (store at room temperature, stability 6 mo).
16. Calibrators: High (1.225 µg/mL DON in acetonitrile), Low (0.125 µg/mL DON in acetonitrile), Control (0.568 µg/mL DON in acetonitrile), (store calibrators at –5°C, stability 6 mo).
17. Cleanup columns (Romer Labs MycoSep #225/227).

2.2. HPLC Validation Method

1. Grinding/subsampling mill.
2. HPLC system, Shimadzu LC-10A pump at 1.0 mL/min with a Sil-10A autoinjector, SPD-10A UV-Vis detector at 220 nm, SCL-10A system controller.
3. HPLC column-SPHERIS, RP18, 5 µ*M*, 4.6 mm × 10 cm (Perkin Elmer).
4. Mobile phase-water/acetonitrile/methanol (92/4/4).
5. Blender (Osterizer 16 speed).
6. Blender jars (one-half pint, glass jars suitable for use with blender).
7. Filter paper, coarse grade.
8. Plastic funnel.
9. Glass culture tubes, 15 × 85 mm borosilicate.

10. Glass culture tubes, 12 × 75 mm borosilicate fitted with uniflex safety caps.
11. Evaporation system.
12. Repipetor with 5.0 mL plastic combo tips (Eppendorf repeator pipet).
13. Vortex mixer.
14. Deoxynivalenol spiking standard-50 µg/mL DON in methanol (Stored at –5°C, stable 6 mo).
15. Deoxynivalenol calibration standard-2.0 µg/mL DON in mobile phase (stored at –5°C, stable 6 mo).
16. Cleanup columns, MycoSep #225 and #210, (Trilogy Analytical Laboratory, Inc.).
17. Extraction solution-Acetonitrile/water (84/16), Add 840 mL of acetonitrile (ACS grade) to 160 mL of water (deionized or distilled).

3. Methods

3.1. Fluorometric Methods

3.1.1. Test Sample Preparation and Extraction

1. Collect a representative sample of grain and grain products (3 lb wheat or similar sized grain or 5 lb of corn) from a lot.
2. Grind the entire sample through a grinding/subsampling mill (*see* **Fig. 2**) to obtain a representative subsample (*see* **Note 1**). Samples of wheat middlings, bran, flour, and similar fine ground materials can be analyzed without further grinding.
   a. Visually check sample for any rocks, metal, or other foreign objects while pouring commodity into the hopper of the mill.
   b. Open the restrictor lever 1 to 2 notches to the left to allow approx 20% of the ground grain to exit the front chute.
   c. Adjust the grind lever to obtain a fine grind.
   d. Turn the mill motor on and allow the sample to grind through the mill. Collect the test sample from the front chute.
   e. Using a spatula mix the contents of the front container for approx 30 s to insure an even mixture of particles (*see* **Note 2**).
   f. Clean the mill after each use to prevent cross contamination (*see* **Note 3**).
3. Weigh out 25 g of the thoroughly mixed ground test sample into a blender jar (*see* **Note 4**).
4. Add 100 mL of acetonitrile/water (86/14) (*see* **Note 5**). Add 150 mL for wheat middlings and bran (*see* **Note 6**) into the blender jar and blend on high speed for 3 min (*see* **Note 7**).
5. Decant the extraction solution into a glass jar through coarse filter paper (*see* **Note 8**).

3.1.2. Test Sample Extraction and Purification

1. Pipet a 4 mL portion of the filtered extract into a 15 × 85 mm culture tube (use 6 mL portion for malted barley).
2. Insert a cleanup column (*see* **Fig. 3**) into the top of the culture tube. Use a MycoSep #227 column for malted barley (*see* **Note 9**). The rubber flange creates a tight seal with the glass wall of the culture tube. Slowly push the column to the

Fig. 2. Series II Subsampling Mill.

bottom of the culture tube (*see* **Note 10**). As the column is pushed into the tube, the sample extract is forced through the packing material. Allow 40 s for pushing the column to the bottom of the culture tube for malted barley, wheat middlings, and bran. Allow 30 s for all other matrices; *see* **Note 11**).

3. The purified extract located above the column packing is mixed with repeated filling and discharging from the pipetor.
4. Transfer 1.5 mL of the properly mixed purified extract into a 12 × 75 mm cuvet (*see* **Note 12**).
5. Transfer 1.5 mL of the high and low calibrators and control solution into separate 12 × 75 mm cuvets. Allow calibrators and control solutions to equilibrate to room temperature before use. One calibrator and control set is required per set of samples (*see* **Note 13**).
6. Evaporate the sample, calibrators and control to complete dryness in a dry bath at 60–70°C on an evaporation system (*see* **Fig. 4**), or equivalent (*see* **Note 14**).

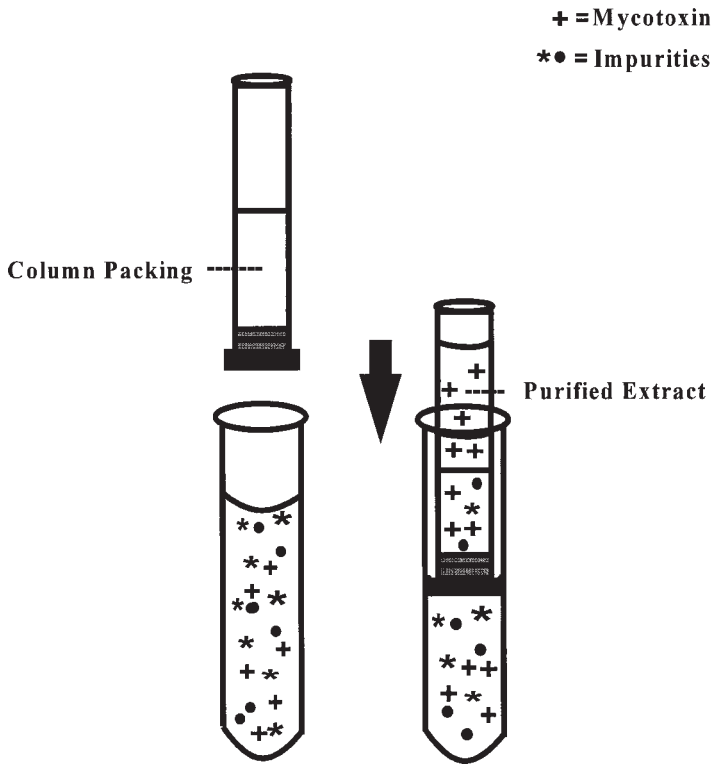

Fig. 3. Cleanup column. This column rapidly purifies extracts by binding maxtrix interferences and allowing the mycotoxin to pass through the packing material into the column cylinder above the matrix.

3.1.3. Fluorescent Derivatization Reaction

1. Add 1.5 mL of reagent A and 50 µL of reagent B to each evaporated sample, calibrator, and control cuvet using an Eppendorf repipetor or equivalent.
2. Cap each tube and vortex for 10 s.
3. Heat the tubes for 10 min at 50°C for 10 min in a dry bath or water bath.
4. Place the cuvets in a rack and cool to room temperature by placing the rack in cool tap water for 30 s.
5. Dry the cuvets completely wiping off all lint and fingerprints before inserting into the RL-100 fluorometer (*see* **Fig. 5**).

3.1.4. Fluorometric Calibration

The fluorometer is calibrated before use with the high and low calibrators (*see* **Note 15**). The appropriate calibration factors must be used depending on the matrix analyzed (*see* **Table 1**). These different factors compensate for dif-

Fig. 4. Solvent Evaporation System.

Fig. 5. Fluorometer.

ferences in matrix fluorescent backgrounds. The fluorometer is internally programmed to perform a simple two point calibration to calculate the unknown sample concentration.

3.1.5. RL100 Fluorometer Operating Procedure

1. Turn the power switch on and the fluorometer will perform a series of diagnostic tests.
2. After the diagnostics are complete, the date and time can be corrected or press continue.

Table 1
DON Fluorometric Calibration Factors

Matrix	Calibrator low	Calibrator high	Control range
Wheat	0.0	5.0	1.6–2.4
Wheat middlings	–0.6	7.4	2.2–3.0
Bran	–0.6	7.4	2.2–3.0
Wheat flour	0.2	5.5	2.0–2.8
Corn	–0.4	5.2	1.5–2.3
Barley	–0.4	4.9	1.3–2.1
Oats	–0.6	4.5	1.2–2.0
Malted barley	–0.5	5.4	1.8–2.6

3. The method screen allows three different options; method number selection, print a current list of methods, or method setup.
4. Push the method set up key to program a new method by selecting the following options:
   a. Method number, input a number 1–30 that is currently not being used.
   b. Time delay between samples, input 3 s.
   c. Result units, select ppm.
   d. Number format, select decimals.
   e. High and low calibrator values, input appropriate values based on the matrix being analyzed (*see* **Table 1**).
5. Select the appropriate method number from the method screen.
6. Place the high calibrator and then the low calibrator in the fluorometer when prompted.
7. When the calibration is complete, analyze the control cuvet as the first sample. The control value should fall within the specified range (*see* **Table 1**) to insure a proper calibration.
8. Insert the sample cuvets into the fluorometer as prompted. The DON results in ppm will be displayed and printed (*see* **Note 16**).

3.1.6. Fluorometric Method Validation Data

1. Cross reactivity data: Other mycotoxins were analyzed with this fluorometric method to determine any cross reactivity with DON. All the type B trichothecenes tested were detectable with varying percentages of cross reactivity (*see* **Table 2**). However aflatoxin B1, zearalenone, and the type A trichothecenes were not detected using this method ***(12)***.
2. Linearity: To examine the procedure for linearity, 5 samples of wheat containing no detectable DON were spiked in triplicate at 0.5, 5, 10, 25, and 50 µg DON /g and analyzed. The results obtained were plotted versus the expected value of the spikes (*see* **Fig. 6**). The results demonstrate that this method is sufficient to determine any significant levels of naturally occurring deoxynivalenol in grains without dilutions.

Table 2
DON Fluorometric Cross Reactivity Data with Other Mycotoxins

Toxin	% Cross reactivity
Deoxynivalenol	100
Nivalenol	100
Fusarenon-X	90
3 Acetyl DON	75
15 Acetyl DON	38
T2 toxin	0
HT-2 toxin	0
Neosolaniol	0
Aflatoxin B1	0
Zearalenone	0

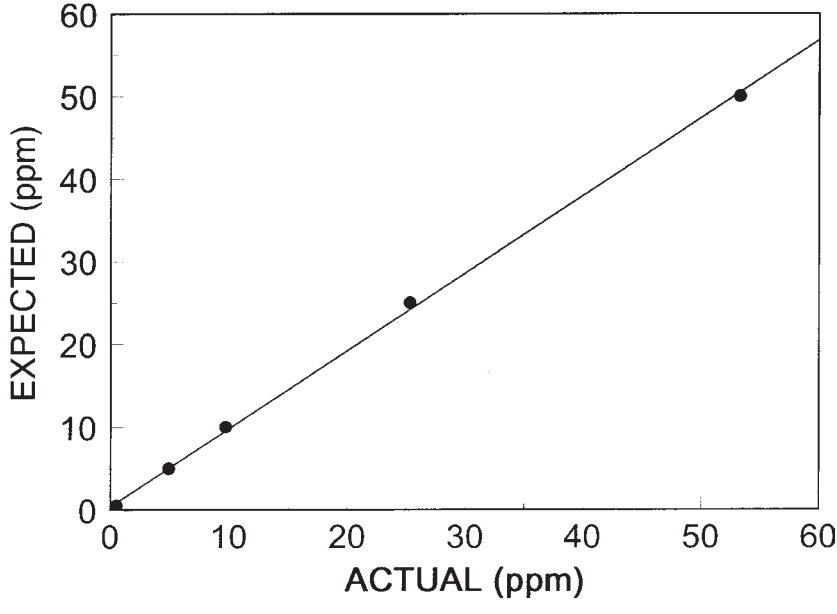

Fig. 6. Linearity of fluorometric measurement of derivatized DON in wheat spiked at 0.5, 5, 10, 25, and 50 µg/g. Reprinted from *The Journal of AOAC International* (1998) **18**, pp. 448–452. Copyright by AOAC International *(11)*.

3. Accuracy and precision studies on spiked matrices of corn, barley, malted barley, and oats: A 25 g sample of finely ground commodity containing no detectable level of DON was spiked at 0.5, 2.5, and 5.0 µg DON/g. Each spiked sample was

Table 3
Accuracy and Precision Data on Spiked Matrices of Corn, Oats, Barley, and Malted Barley[a]

Matrix	*n*	DON spike µg/g	Mean	% Recovery	SD	RSD (%)
Corn	18	0.5	0.45	90.0	0.06	13.3
	18	2.5	2.18	87.2	0.32	14.7
	18	5.0	4.38	87.2	0.36	8.2
Oats	18	0.5	0.60	120.0	0.08	13.3
	18	2.5	2.26	90.4	0.13	5.8
	18	5.0	4.76	95.2	0.33	6.9
Barley	18	0.5	0.52	104.0	0.10	19.2
	18	2.5	2.24	89.6	0.15	6.7
	18	5.0	4.58	91.6	0.50	10.9
Malted barley	18	0.5	0.69	138.0	0.13	18.8
	18	2.5	2.71	108.4	0.40	14.8
	18	5.0	4.98	99.6	0.39	7.8

[a]SD = standard deviation, RSD = relative standard deviation.

Table 4
Accuracy and Precision Data on Spiked Wheat Matrix[a]

Matrix	*n*	DON spike µg/g	Mean	% Recovery	SD	RSD (%)
Wheat	90	0	0.05	—	0.012	—
	90	0.5	0.50	100.0	0.10	20.0
	90	1.0	1.14	114.0	0.20	17.5
	90	2.5	2.66	106.4	0.37	13.9
	90	5.0	5.04	100.8	0.40	7.9

[a]SD = standard deviation, RSD = relative standard deviation.

extracted according to the test method. For each spiked matrix three analysts performed three analyses of each extract using two different fluorometers for a total of 18 results per concentration per matrix. The average relative standard deviation for all matrixes at the 0.5, 2.5, and 5.0 µg/g spiked concentration were 16.2%, 10.5%, and 8.4%, respectively. The average% recovery for all the matrices at the 0.5, 2.5, and 5.0 µg/g spiked concentrations were 113.0%, 93.9%, and 93.5%, respectively (*see* **Table 3**).

4. Accuracy and precision studies on spiked matrices of wheat: Five sets of 25 g portions of finely ground wheat containing no detectable level of DON were spiked at 0.0, 0.5, 1.0, 2.5, and 5.0 µg DON/g. Each spiked sample was extracted according to the test method. Three analysts performed three analyses of each extract using two different fluorometers for a total of 90 results per concentration. The relative standard deviations at 0.5, 1.0, 2.5, and 5.0 µg/g spiked concen-

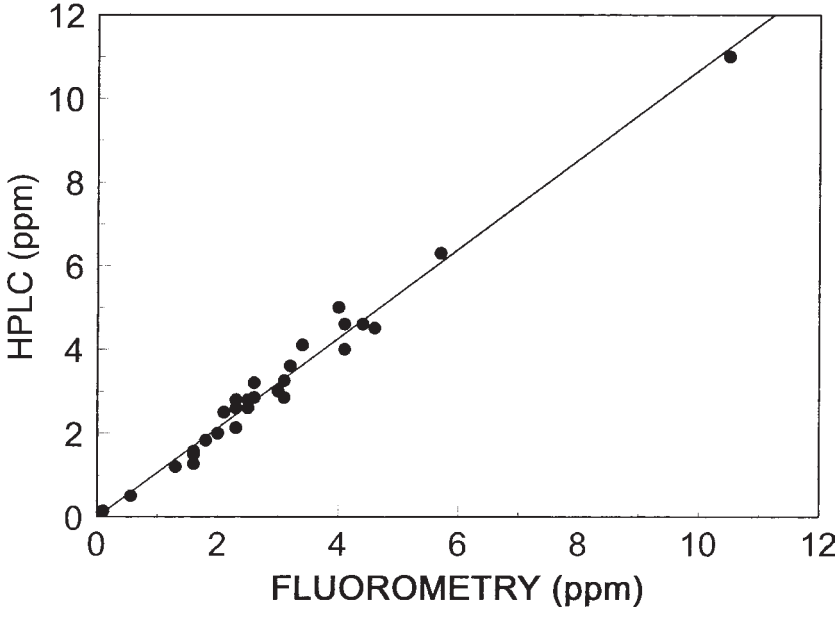

Fig. 7. Fluorometric measurement of DON compared to HPLC analysis in twenty-nine naturally contaminated wheat samples. Correlation coefficient = 0.99. Reprinted from *The Journal of AOAC International* (1998) **18**, pp. 448–452. Copyright by AOAC International *(11)*.

trations were 20.0%, 17.5%, 13.9%, and 7.9% respectively. The % recovery for the 0.5, 1.0, 2.5, and 5.0 µg/g spiked concentrations were 100.0%, 114.0%, 106.4%, and 100.8%, respectively (*see* **Table 4**)

5. HPLC comparison results on naturally contaminated samples: The results of testing 29 samples of naturally contaminated wheat by this fluorometric procedure were similar to those obtained by HPLC analyses (correlation coefficient = 0.99) of the same samples (*see* **Fig. 7**). Correlation coefficients of 0.93, 0.98, 0.99, and 0.99, respectively were obtained when 25 naturally contaminated wheat flour (*see* **Fig. 8**), 21 wheat middlings, 15 corn, and 14 barley samples were analyzed by both fluorometric and HPLC methods.

3.2. HPLC Validation Method

The HPLC method used to validate the fluorometric procedure was that of Trucksess et al. (8) with some modifications.

3.2.1. Test Sample Preparation and Extraction

The test sample preparation and extraction is the same as the fluorometric method (*see* **Note 17**) (*see* **Subheading 3.1.1.**).

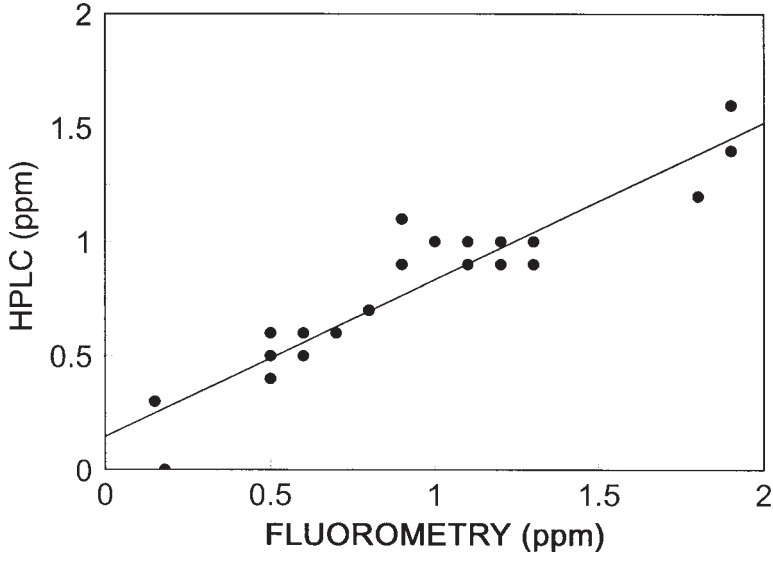

Fig. 8. Fluorometric measurement of DON compared to HPLC analysis in twenty-five naturally contaminated wheat flour samples. Correlation coefficient = 0.93. Reprinted from *The Journal of AOAC International* (1998) **18**, pp. 448–452. Copyright by AOAC International *(11)*.

3.2.2. DON Spike Preparation

1. Pipet 10 mL of acetonitrile/water (84/16) into a 15 × 85 mm glass culture tube using a 10 mL volumetric pipet.
2. Add 100 µL of a 50 µg/mL DON standard in methanol (*see* **Note 18**). Mix well. This spike solution is equivalent to 2.0 ppm DON concentration in a sample.
3. Insert a MycoSep #225 column into the top of the culture tube and push to the bottom of the tube (10 to 20 s time elapse) (*see* **Note 19**).
4. The solution located above the column packing is mixed with repeated filling and discharging from the pipetor.
5. Proceed with **step 4** in **Subheading 3.2.3.**, pipeting 2.0 mL of spiking solution in triplicate (*see* **Note 20**).

3.2.3. Sample Extract Purification

1. Place a 4 mL portion of the filtered extract into a 15 × 85 mm culture tube (use 5 mL portion for wheat middlings and bran) (*see* **Note 21**).
2. Insert a MycoSep #225 column into the top of the culture tube and push to the bottom of the tube (10–20 s time elapse) (*see* **Note 22**).
3. The purified extract located above the column packing is mixed with repeated filling and discharging from the pipetor.

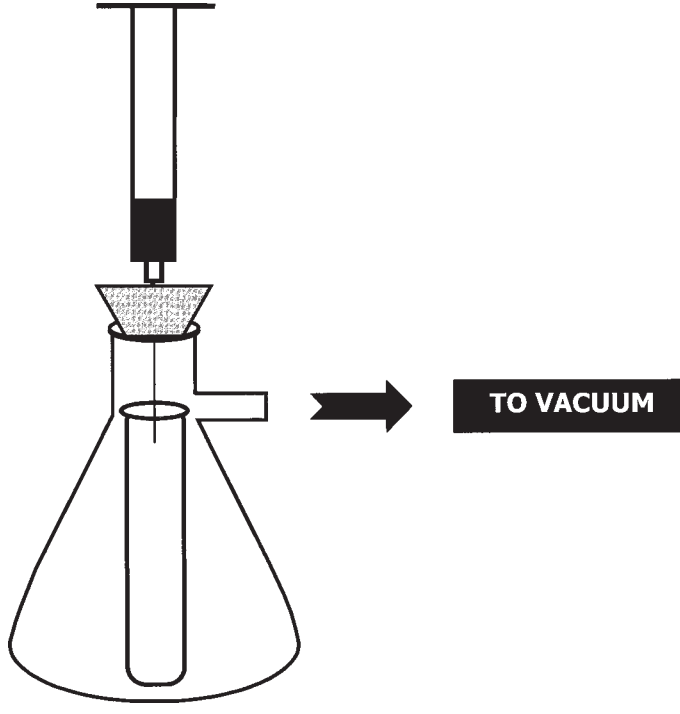

Fig. 9. Illustration of #210 cleanup column and vacuum assembly.

4. Wheat middlings and bran require an additional cleanup using a #210 column as follows. For all other samples, transfer 2.0 mL of the properly mixed purified sample extract into a 12 × 75 mm cuvet and proceed to **step 9**.
5. Attach a #210 column to a vacuum apparatus (*see* **Fig. 9**). Prewash the column by filling the column barrel with acetonitrile/water (84/16) and adjusting the flow rate to approx 1 mL per minute (*see* **Fig. 3**). Discard the wash solution.
6. Place a 15 × 150 mm culture tube inside the side arm flask of the vacuum apparatus for collection of the purified sample extract and rinse solution.
7. Transfer 3.0 mL (*see* **Note 23**) of the purified wheat middlings or bran extract obtained from the MycoSep #225 cleanup into the top of the #210 column.
8. Allow the sample extract to drain completely through the #210 column and add 9 mL of acetonitrile/water (84/16) rinse solution (*see* **Note 24**). Collect both the sample extract and rinse solution.
9. Evaporate all of the collected solution from **step 8** or the 2.0 mL of purified extract in **step 4** to complete dryness in a dry bath at 60–70°C using a solvent evaporation system (*see* **Fig. 4**).
10. Redissolve the tube residue in 500 µL of HPLC mobile phase and vortex for 20–30 s. Inject 125 µL into the HPLC system.

3.2.4. HPLC External Standard Calibration

1. Inject 125 µL of the 2.0 µg/mL DON standard in mobile phase. This standard is equivalent to 2.0 ppm DON in a sample. Use this standard to ensure correct chromatography conditions, determine DON retention time and calculate recovery of the spiked samples.
2. Inject 125 µL of each of the three DON spiked test solutions. Use the average DON peak area counts of the three spikes with a sample equivalent of 2.0 ppm to calibrate the HPLC system.
3. Determine the% recovery of the method using the following calculation (**Eq.1**):

$$\frac{\text{Area count of DON Spike}}{\text{Area count of the DON Standard}} \times 100 = \%\ \text{recovery} \quad (1)$$

   The % recovery of the single column cleanup method (MycoSep #225 column) and the dual column method (MycoSep #225 column plus #210 column) should be approximately 85% and 70% respectively.
4. Calculate the DON concentration of the sample unknowns using the following calculation (**Eq. 2**):

$$\frac{\text{Area count of unknown}}{\text{Average area counts of DON spikes}} \times 2.0\ \mu g/g = \text{concentration of sample } (\mu g/g) \quad (2)$$

4. Notes

1. This mill will simultaneously grind the entire sample of grain and collect a smaller representative subsample to be used for the analysis.
2. This step is critical to achieve the most accurate and precise results. A portion of each kernel must be present in the test sample to ensure that a representative test sample is obtained.
3. A small amount of ground test sample will remain in the mill after the final sample has been ground and a subsample collected. Use one of the following three cleaning procedures to prevent contamination of subsequent samples.
   a. After a sample has been ground and collected and while the unit is still running place an operating vacuum cleaner hose at the bottom of each chute for approx 10–15 s. The hose attachment should be one whose opening just covers the mouth of the chute.
   b. Add the entire sample to the hopper. Collect the first 30–50 g of subsample, turn the power off, and discard. Turn the power on and collect the remaining subsample for analysis.
   c. A small sample, 100–200 g of a known mycotoxin free sample can be ground in between samples.
4. Use of the one-half pint jar for extraction was required to get adequate mixing of the entire test sample. Larger vessels allowed test sample to splash and cling to areas of the vessel beyond the solvent limits and therefore are not extracted.
5. The acetonitrile/water (86/14) is used for the extraction instead of the more popular acetonitrile/water (84/16). The extra 2% acetonitrile eliminates a cloudy

precipitate from forming in some extracts during the derivatization and has no significant difference in extraction efficiency.

6. The 50 mL of additional solvent is necessary for the extraction of wheat middlings and bran due to the excessive adsorption of solvents by these matrices.
7. Alternatively the test sample and extraction solvent can be shaken on a reciprocating shaker for 1.5 h
8. Coffee filters can be used for the sample extract filtration. Only a coarse filtration is required because the MycoSep cleanup column contains an internal porous filter.
9. The MycoSep #227 column provides a higher capacity extract cleanup required to determine the concentration of DON in malted barley.
10. The MycoSep column must be pushed completely to the bottom of the culture tube. This will ensure that an adequate and consistent volume is obtained for the analysis.
11. The rate the sample extract is pushed through the cleanup column is critical. Purifying the sample extract through the MycoSep column faster than 30 s, or 40 s for wheat middlings and bran, can cause interferences to pass through the column, producing a cloudy precipitate during the derivatization.
12. This 12 × 75 mm cuvet must be manufactured from borosilicate glass. Other glass products may exhibit inherent fluorescence and cause erroneous false positive results.
13. One or as many as 50 test samples can be analyzed at one time.
14. The 1.5 mL of purified sample extract and calibrators must be taken to complete dryness to prevent a cloudy precipitate from forming during the derivatization reaction. A dried residue will be observed in the bottom of the sample tubes only. This residue originates from residual packing material washing off the column by the sample extract and will not interfere with the analysis.
15. The low calibrator is equivalent to the fluorescent background of a wheat sample matrix containing no detectable DON. The high calibrator is equivalent to the fluorescence of the wheat matrix background plus 5.0 ppm of deoxynivalenol. These fluorescent equivalents were determined by analyzing wheat extracts that were nondetect for DON and 5.0 ppm deoxynivalenol wheat matrix spikes. The wheat calibration factors were then adjusted to compensate for other matrix differences.
16. The samples should be analyzed immediately after the fluorometric calibration is complete. However, the fluorometric measurement can be delayed up to four hours if the fluorometer is recalibrated with the high and low calibrators immediately before analyzing the samples.
17. Some of the same sample extract (acetonitrile/water 86/14) used for the fluorometric method, if available, can be used for the HPLC validation method. There is no significant difference in the extraction efficiency of acetonitrile/water (86/14) or acetonitrile/water (84/16).
18. The concentration of the DON standard in methanol can be determined by measuring its UV absorbtion at 220 nm and using the following calculation (**Eq. 3**):

$$\mu g/mL\ DON = \frac{\text{Absorbance (220 nm)} \times MW \times 1000}{\varepsilon} \quad (3)$$

MW = molecular weight = 296.1
ε = 6129

19. The column purification time for the HPLC validation method is not as critical as the fluorometric method.
20. The use of spikes for HPLC calibration compensates for recovery loss through the cleanup column.
21. More purified extract is needed for wheat middlings and bran to compensate for the 50 mL additional extraction solvent used for these matrices.
22. The same MycoSep #225 column can be shared between the fluorometric method and the HPLC validation method. After 1.5 mL of the purified extract is removed from the top of the MycoSep #225 column for use with the fluorometric method (*see* **Subheading 3.1.2.**), remove the MycoSep column from the culture tube. Pippet 4.0 mL of additional sample extract back into the culture tube. Place the MycoSep column back into the culture tube and push the column to the bottom of the tube. There will be a sufficient amount of purified extract on top of the MycoSep #225 column to use with the HPLC validation method.
23. 3.0 mL of extract is used for wheat middlings and bran instead of the 2.0 mL used for other matrices in **step 4** in **Subheading 3.2.3.** This compensates for the 150 mL of extraction solvent used instead of 100 mL used for the other matrices.
24. DON will be rinsed off the #210 column with the acetonitrile/water (84/16) solution whereas interferences will adhere to the column packing.

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11

Chromatographic Methods for Trichothecenes

Takumi Yoshizawa

1. Introduction

Trichothecenes are secondary metabolites of *Fusarium*, *Stachybotrys*, *Myrothecium*, *Trichothecium,* and other fungal genera and are classified into two groups, macrocyclic and nonmacrocyclic trichothecenes (*see* Chapter 15 for the former). Although about 100 nonmacrocyclic trichothecenes have been identified, only a few have been detected as naturally occurring contaminants of grains and other agricultural commodities. Deoxynivalenol (DON) and nivalenol (NIV), which are type B trichothecenes, possessing a conjugated 8-carbonyl group, and type A trichothecenes, T-2 toxin (T-2), HT-2 toxin (HT-2), and diacetoxyscirpenol (DAS), which lack the 8-carbonyl group, are the main ones found **(Fig. 1)**.

Fusarium is the most important genus producing the nonmacrocyclic trichothecenes ***(1,2)***. DON and its monoacetates, such as 3-acetyl-DON (3-ADON) and 15-acetyl-DON (15-ADON), are produced by strains of *F. graminearum* and *F. culmorum*, which are divided into two chemotypes in terms of production of these monoacetates: 3-ADON-producing type (type IA) and 15-ADON-producing type (type IB). NIV is produced, together with 4-acetyl-NIV (4-ANIV, fusarenon-X), by strains of *F. graminearum* (type II), *F. crookwellense* and *F. poae*. It is worth noting that *F. graminearum* as an important pathogen of cereals, particularly of wheat and corn, produces either DON or NIV, and its distribution varies by region in the world. T-2 and HT-2 are produced principally by *F. sporotrichioides*, and DAS is produced by *F. poae* and *F. equiseti*.

DON is the most widely distributed *Fusarium* mycotoxin and occurs worldwide in cereals of temperate regions. Representative cereals contaminated with this toxin include wheat, barley, maize, oats, and rye ***(3–5)***. NIV also occurs in

From: *Methods in Molecular Biology, vol. 157: Mycotoxin Protocols*
Edited by: M. W. Trucksess and A. E. Pohland

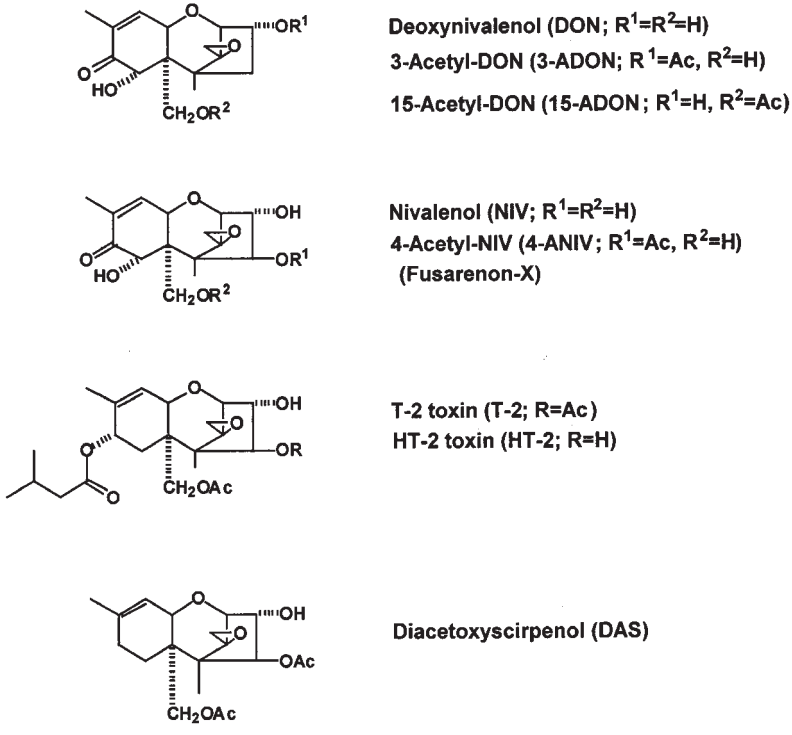

Fig. 1. Chemical structures of major trichothecenes occurring naturally in foods and feeds.

these cereals and has been found extensively in Japan and Korea, and at relatively low levels in samples from Europe, southern Africa and Australia, but it is virtually unknown in grains in North and South America *(3,4)*. Monoacetate derivatives including 3-ADON, 15-ADON, and 4-ANIV also occur as minor concomitants of DON and NIV *(6–8)*. T-2 along with HT-2 has been reported in cereals at relatively low levels *(3–5)*. Trichothecenes are apparently not subject to legal regulation in any country; however, guidelines or advisory levels for DON exist in Austria and USA, and guidelines and recommendations for DON and HT-2 exist in Canada *(9)*.

T-2 and DAS are the most potent for animals of the naturally occurring trichothecenes as food and feed contaminants. Compared with these toxins, NIV is less potent in some systems and DON is the least toxic of the four *(10,11)*. The trichothecenes are toxic for actively dividing cells, such as the intestinal crypt epithelium and the hematopoietic cells. The cytotoxicity has been associated with either impairment of protein synthesis by the binding of

the toxins to the ribosome of eukaryotic cells, or the dysfunction of cellular membrane ***(10,12)***. Suppression of cell-mediated and humoral immunity has been demonstrated in studies with T-2, DON and DAS, and observations include effects such as reduced concentrations of immunoglobulins and depressed phagocytic activity of both macrophages and neutrophils ***(13,14)***. Trichothecene-induced thymic atrophy is associated with cell death through a mechanism of apoptosis ***(15,16)***. The immunosuppressive effect of trichothecenes results in decreased resistance to secondary infection by bacteria, yeasts, and viruses ***(13,14)***. Trichothecenes were not mutagenic, but T-2 had weak clastogenic activity in some assays ***(4)***. There is inadequate evidence in experimental animals for the carcinogenicity of DON and NIV, and trichothecenes are not classifiable as to their carcinogenicity to humans (Group 3) ***(4)***.

Regarding the determination of trichothecenes occurring in foods and feeds, the following steps are included in the analytical procedures: solvent extraction of toxins from samples, column cleanup of extracts, derivatization of toxins in some cases, and separation and quantitation of toxins ***(3,17–21)***. Aqueous acetonitrile and methanol are extraction solvents commonly used for trichothecenes. After column cleanup with charcoal-alumina-Celite, Florisil, silica gel or solid-phase extraction column, trichothecenes are determined by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC), or by gas chromatography with electron-capture detection (GC-ECD) and gas chromatography-mass spectrometry (GC-MS) as various derivatives. **Table 1** summarizes the analytical procedures for trichothecenes, the limit of detection and applicable commodities. There are AOAC-accepted methods for the analysis of DON (***22***).

2. Materials

2.1. Reagents

1. Solvents: LC grade methanol and water for HPLC. Distill all other solvents in glass.
2. Adsorbents for charcoal-alumina-Celite column ***(22)***: Activated charcoal, Darco G-60 (J. T. Baker Chemical Co., Phillipsburg, NJ); neutral alumina, chromatographic grade, 80-200 mesh (No. AX0612, E. M. Science, Cherry Hill, NJ, or equivalent); and diatomaceous earth, acid-washed Celite 545.
3. Solid-phase extraction column: MycoSep No. 225 column (Romer Labs, Inc., Union, MO) packed in a plastic tube (10 × 1 cm id) with a rubber flange on the lower end. In the center of the flange is a porous frit, and above the frit is a one-way valve.
4. Silica gel: Silica gel 60 (0.063–0.200 mm particle size, E. Merck [Darmstadt, Germany], or equivalent).
5. Florisil: Florisil (Wako Pure Chemicals Industries Ltd., Osaka, 60–100 mesh).

Table 1
Summary of Analytical Procedures for Trichothecenes, Detection Limit and Commodities Adopted[a]

Extraction	Cleanup column	Derivatization	Determination	Toxins analyzed	Detection limit	Commodities	Ref.
CH_3CN-H_2O	Solid-phase (84 + 16) (**3.2.1.**)	None extraction column	HPLC (**3.3.**)	DON (UV, 220 nm)	0.5 μg/g[b]	Whole wheat flour, wheat flour, bran	***(29)***
		Zirconyl nitrate-ethylenediamine	Fluorometer	DON	0.5 μg/g[b]	Wheat, wheat flour, wheat middling, barley	(*see* **Ch. 10**)
	Charcoal-alumina-Celite column (**3.2.2.**)	None	TLC $AlCl_3$ spray (**3.4.**)	DON	0.3 μg/g[b]	Wheat	***(22)***
				DON, NIV, T-2, HT-2, DAS and two others	0.3–0.8 μg/g[b]	Wheat, corn	***(30)***
CH_3CN-H_2O (3 + 1)	Florisil column (**3.2.3.**)	TMS (**3.5.1.**)	GC-ECD (**3.6.**) GC-MS (**3.7.**)	DON, NIV, their acetates	10 ng/g	Wheat, barley, corn and their products	***(8,26)***
MeOH-H_2O (7 + 1)	Silica gel column (**3.2.4.**)	TMS (**3.5.1.**) HFB (**3.5.2.**)	GC-ECD (**3.6.**) GC-MS (**3.7.**)	DON, NIV, T-2, HT-2, DAS and two others	20 ng/g 50–200 ng/g	Wheat, wheat flour, barley, corn meal, corn flour, rye flour	***(23,25)***

[a]Numbers in parenthesis indicate sections and sessions of the text, in which individual items are described.
[b]Limit of quantitation.

6. TLC plates: 20 × 10 cm Linear-K High Performance (LHP-K) silica gel plates (No. 4805-710, Whatman Inc., Clifton, NJ, or equivalent). Precoated 20 × 20 cm silica gel 60 plates (No. 5763, E. Merck, or equivalent).
7. Trimethylsilylation reagents: Tri-Sil TBT (Pierce Chemical Co., Rockford, IL), or prepare a mixture of *N*-trimethylsilylimidazole (TSIM) - *N,O*-bis(trimethylsilyl)acetamide (BSA) -trimethylsilylchlorosilane (TMCS) (3 + 3 + 2) (*see* **Note 1**).
8. Heptafluorobutyrylation reagents: Heptafluorobutyric anhydride (HFBA, Pierce Chemical Co., or equivalent). 4-Dimethylaminopyridine (DMAP, Aldrich Chemical Co.) as a catalyst dissolved in toluene-acetonitrile (95 + 5, 2 mg/mL) (*see* **Note 2**).

2.2. Apparatus

1. Densitometer: Shimadzu Model CS-9000 dual wavelength TLC scanner (Shimadzu Ltd., Kyoto, Japan), or equivalent.
2. LC system: Injector (injection volume, 20–100 µL), two pumps, UV detector (variable wavelength, can be set at 220 nm), control and data system. RP-18 column (100 × 3 mm, or equivalent) fitted with RP-18 guard column (15 × 3 mm, or equivalent).
3. GC-ECD system: Varian VISTA 6000, Shimadzu GC-8A (replacement model GC-14B), or equivalent, equipped with split/splitless injector, electron-capture detector (^{63}Ni) and J&W Scientific DB-1701 fused-silica capillary column (15 m × 0.26 mm id, 0.25 µm thickness) ***(23)***, or equivalent.
4. GC-MS system: VG Analytical ZAB-2F interfaced with Varian VISTA 6000 GC, Shimadzu GCMS-QP2000 interfaced with Shimadzu GC-14A (replacement Shimadzu GCMS-QP5000), or equivalent, equipped with J&W DB-5 capillary GC column (30 m × 0.25 mm id, 0.25 µm film) ***(23)***, or equivalent.

3. Methods

3.1. Preparation of Cleanup Columns

3.1.1. Charcoal-Alumina-Celite Column **(22,24)**

1. Place a small ball of glass wool in the bottom of a chromatographic tube (polypropylene, 50 × 10 mm id, equipped with plastic filter disk and reservoir, or equivalent plastic syringe) and add approx 0.1 g of Celite.
2. Thoroughly mix 0.7 g of charcoal, 0.5 g of alumina, and 0.3 g of Celite in a 50 mL beaker with a spatula.
3. Add the mixture to the chromatographic tube and lightly tap the tube to settle the packing.
4. Add a ball of glass wool on the top and apply suction to complete the column packing.
5. Or prepare a mixture of charcoal-alumina-Celite (7 + 5 + 3) in quantities enough for the number of columns needed. Weigh 1.5 g of the mixture for each column.

Keep the mixture from separating by occasional mixing. Columns may be prepared ahead of time and stored in an upright position in a beaker covered with aluminum foil for use as needed *(22)*.

3.1.2. Silica Gel Column **(25)**

1. Add 1 g of anhydrous granular sodium sulfate onto a glass wool plug at the bottom of the glass column (27 × 1.3 cm id) filled with toluene (remove air bubbles).
2. Slowly add 2 g of silica gel 60 as a slurry in toluene and add 1 g of anhydrous sodium sulfate.
3. Drain the toluene to the top of the upper sodium sulfate layer.

3.1.3. Florisil Column **(26)**

1. Add 5 g of anhydrous sodium sulfate onto the glass wool plug at the bottom of the glass column (30 × 2.2 cm id) filled with *n*-hexane.
2. Slowly add 10 g of Florisil as a slurry in *n*-hexane and add 5 g of anhydrous sodium sulfate.
3. Drain the *n*-hexane to the top of the upper sodium sulfate layer.

3.2. Extraction and Cleanup

3.2.1. Solid-phase Extraction Column Cleanup **(27–29)**

1. Place 25 g of ground test sample in a blender jar or 250 mL Erlenmeyer flask and add 100 mL (200 mL for bran) of acetonitrile-water (84 + 16) (*see* **Note 3**).
2. Blend for 3 min at high speed in the blender or shake at high speed for 30 min.
3. Decant the extraction solvent into a glass container through filter paper.
4. Place an 8 mL portion of the extract into a 15 mL polypropylene centrifuge tube.
5. Holding the MycoSep cleanup column in one hand and the centrifuge tube containing the extract in the other, slowly push the cleanup column (rubber flange end) into the tube, creating a tight seal between the rubber flange and the wall of the tube (*see* **Note 4**).
6. Collect approx 2.2 mL (approx 4.2 mL for bran) of purified extract in the column reservoir.
7. Quantitatively transfer 2 mL (4 mL for bran, sample equivalent to 0.5 g each) of purified extract from the top of the column to a 4 mL vial. Evaporate to dryness on a steam bath under a stream of nitrogen. Do not overheat the residue.

3.2.2. Charcoal-Alumina-Celite Column Cleanup **(22,24,30)**

1. Attach charcoal-alumina-Celite cleanup column (*see* **Subheading 3.1.1.**) to vacuum apparatus and place a beaker in the chamber to collect the eluted solvent.
2. Transfer 20 mL of filtrate (*see* **Subheading 3.2.1.**, sample equivalent to 5 g) to the column (2–3 mL/min flow rate).
3. As the solution reaches the top of the packed bed, rinse the cylinder with 10 mL of acetonitrile-water (84 + 16).

4. Add the rinse to the column and continue aspiration until flow stops. Do not let the column go dry between addition of extract and addition of wash solution.
5. Remove the beaker and evaporate the solvent under a stream of nitrogen on a steam bath until approx 3 mL of the solution remains. Do not evaporate to dryness.
6. Transfer the concentrated extract to a 2 dram vial. Wash the beaker with three 1 mL portions of acetonitrile and combine the washes in the vial.
7. Evaporate to dryness under a stream of nitrogen on a steam bath.

3.2.3. Florisil Column Cleanup **(8,26)**

1. Place 20 g of ground test sample in a 300 mL separatory funnel or Erlenmeyer flask along with 160 mL of acetonitrile-water (3 + 1) and shake for 30 min on an automatic shaker (*see* **Note 3**).
2. Filter through filter paper and transfer 80 mL of filtrate to a 300 mL separatory funnel.
3. Add 40 mL of *n*-hexane and shake for 10 s. Discard the upper layer.
4. Add 40 mL of ethanol to the aqueous acetonitrile layer and evaporate to dryness on a rotary evaporator at water bath temperature of 45°C.
5. Dissolve the sample extract in 2.0 mL of methanol with sonication for 2 min and transfer to a Florisil column (*see* **Subheading 3.1.3.**).
6. Drain to the top of the upper sodium sulfate layer and wash with 100 mL of *n*-hexane
7. Elute DON and NIV with 100 mL of chloroform-methanol (9 + 1), flow rate 10 mL/min) into a 300 mL round-bottom flask and evaporate to dryness.
8. Dissolve the residue in 2.0 mL of methanol with sonication. This solution contains 5 g of sample equivalent per mL (*see* **Note 5**).

3.2.4. Silica Gel Column Cleanup **(25)**

1. Mix 50 g of ground test sample with 150 mL of methanol-water (7 + 3) and blend for 5 min at 60% full speed (*see* **Note 3**).
2. Transfer the mixture to a 250 mL centrifuge tube and centrifuge for 15 min at 200 rpm. Decant the centrifugate and transfer 30 mL of it to a 300 mL beaker.
3. Add 120 mL of 10% aqueous ammonium sulfate and mix. Add 50 mL of diatomaceous earth and stir for 2 min with a magnetic stirrer.
4. Filter through fluted filter paper or decant after standing for 5–10 min.
5. Pipet 10 mL of filtrate and transfer to the top of a Clin-Elut No. 1020 column (20 mL, Analytichem International Inc., or equivalent).
6. Allow 2–4 min for absorption of the aqueous solution onto the hydrophilic matrix after it has drained onto the column.
7. Elute the column with eight 20 mL portions of ethyl acetate, draining each portion to the top of the column after each addition.
8. Collect the combined eluates in a 300 mL pear-shaped flask. Evaporate the solvent on a rotary evaporator in a water bath of approx 30°C.
19. Transfer the extract to a 4 mL vial by rinsing with three 1 mL portions of methylene chloride-methanol (3 + 1). Evaporate carefully to dryness under nitrogen with minimal heating.

10. Dissolve the extract in 0.5 mL of methylene chloride-methanol (3 + 1) using a tube shaker apparatus and transfer to the top of a silica gel column (*see* **Subheading 3.1.2.**) with a pipet.
11. Use an additional 0.2 mL of the same solvent and 0.5 mL of methylene chloride successively to rinse the vial, and add the rinse to the column ***(23)***. Drain to the top of the upper sodium sulfate layer.
12. Wash the column with 15 mL of toluene, approx 2 mL of which is used to rinse the vial, followed by 15 mL of *n*-hexane and discard eluates.
13. Elute DON and NIV with 60 mL of methylene chloride-methanol (9 + 1) into a 250 mL round-bottom flask and evaporate to dryness on a rotary evaporator at a water bath temperature of <40°C.
14. Dissolve the residue in a small volume of methylene chloride-methanol (3 + 1) and transfer to a 4 mL vial with rinsing. Evaporate carefully to dryness under a stream of nitrogen (minimal heating).
15. Dissolve the residue in 1.33 mL of methylene chloride-methanol (3 + 1). This solution contains 0.5 g of sample equivalent per mL ***(25)***.

3.3. RP-HPLC of DON

1. Equilibrate the LC system with methanol-water (15 + 85, solvent A) at a flow rate 0.7 mL/min. Adjust the sensitivity controls of the UV detector (220 nm) to 0.01–0.02 AUF.
2. Dissolve the extract (*see* **Subheading 3.2.1.**) in 200 µL of methanol-water (20 + 80) by mixing for 1 min.
3. Transfer to an 0.4 mL polyethylene microcentrifuge tube, and centrifuge for 6 min at 14,000 rpm.
4. Inject 50 µL (sample equivalent to 0.125 g) onto the LC column (the retention time of DON, approx 15–16 min).
5. At 17 min after injection, immediately pump methanol-water (1 + 1, solvent B) to flush late-eluting impurities from the column.
6. At 25 min, pump solvent A to recondition the column, and at 40 min, inject another extract.
7. Prepare a standard curve for DON each day. The standard curve should be linear in the range 62.5 to 500 ng. The limit of determination of DON is approx 0.5 µg/g (*see* **Note 6**).

3.4. TLC

1. Add 100 µL of methanol to a vial and dissolve the residue (*see* **Subheading 3.2.2.**) by using a vortex mixer approx 1 min (*see* **Note 7**).
2. Spot 5 µL of partially purified extract twice (sample equivalent to 0.5 g) and spot 2, 5, 10 µL of standard solution (25 µg/mL) on a high-performance TLC plate; place spots exactly 1 cm apart and 1.8 cm from the bottom of the plate.
3. Develop the plate in a closed, unequilibrated tank with chloroform-acetone-2-propanol (8 + 1 + 1) to a height of approx 9.0 cm. Remove the plate, and air-dry for 10 min.

4. Spray evenly with aluminum chloride solution (20 g of $AlCl_3 \cdot 6H_2O$ in 100 mL of ethanol-water [1 + 1]). Heat the plate in 120°C convention oven for 7 min. Remove the plate from the oven.
5. Examine under longwave (365 nm) UV light. DON appears as a blue fluorescent spot with Rf = approx 0.78. Compare the spots from the test extract with those from the standard.
6. Scan the spots with a fluorodensitometer (excitation 366 nm, emission filter cut off 400 nm, with electronic integrator) from top to bottom, parallel to the direction of development (*see* **Notes 8** and **9**).

3.5. Derivatization for GC and GC-MS

3.5.1. Trimethylsilylation with Tri-Sil TBT (25)

1. Evaporate 500 µL of the extract (*see* **Subheading 3.2.4.**, sample equivalent to 0.25 g) in a 4 mL vial under a stream of nitrogen to dryness with minimal heating.
2. Add 50 µL of Tri-Sil TBT, firmly cap the vial, mix on a vortex mixer for 1 min, and let stand at room temperature for approx 10 min.
3. Add 500 µL of *n*-hexane, mix, and then add approx 1 mL of phosphate buffer (pH 7.0) or water. Mix for 1 min on a vortex mixer. Let stand to separate the layers.
4. Transfer 160 µL of the top layer to another 4 mL vial containing 1.84 mL of *n*-hexane to give 40 mg sample per mL.
5. Derivatize the standards as for the sample extract, except dilute the final solution to contain 40 ng standard/mL of *n*-hexane (*see* **Note 1**).
6. TMS derivatives of trichothecenes (DON, NIV, T-2, HT-2, and DAS) can be kept at –15°C for several days *(23)*.

3.5.2. Heptafluorobutyrylation with HFBA-DMAP (22)

1. Evaporate 500 µL of the extract (*see* **Subheading 3.2.4.**, sample equivalent to 0.25 g) in a 3 dram vial under a stream of nitrogen to dryness.
2. Transfer 1.0 mL of DMAP catalyst solution (*see* **Subheading 2.1., step 8.**) to the vial and add 50 µL of HFBA. Firmly cap the vial and warm for 20 min on a heating block at 60°C. Let the derivatized reaction mixture cool to room temperature.
3. Add 1.0 mL of 3% aqueous sodium bicarbonate solution to the vial, mix for 2 min on a tube mixer, and let stand to separate the layers.
4. Transfer 100 µL of the organic (upper) layer (represents 0.025 g sample) to a 2 dram vial containing 900 µL of *n*-hexane or an appropriate volume, depending on the concentrations of trichothecenes in the sample.
5. Derivatize the standards as for the sample extract (final concentration, 0.1 ng/µL, *see* **Note 2**).
6. Except for the NIV HFB derivative, the HFB derivatives of other trichothecenes such as DON, T-2, HT-2 and DAS can be kept at -15°C for several days *(23)*.

3.6. GC-ECD

3.6.1. Operating Conditions

Table 2 indicates representative conditions of capillary columns for GC-ECD. If possible, carry out analysis on same day as derivatization. Use a GC equipped with a split/splitless injector operating in the splitless mode; helium carrier gas flow rate 1.9–2.3 mL/min and nitrogen make-up gas flow rate 25.8 mL/min; chart speed 0.5 cm/min; attenuation adjusted to give 10% FSD for 100 pg standard; injection port 250°C; detection port 350°C; oven temperature program (following an initial period of 70°C for 3 min): (a) 30°C/min to 175°C, hold 2 min, 5°C/min to 245°C and hold 20 min (for HFB derivatives) and (b) 30°C/min to 220°C and hold 14 min (for TMS derivatives) ***(23,25)***.

3.6.2. Determination

1. Inject 1-5 µL of the derivatized standards (*see* **Subheadings 3.5.1., step 5** and **3.5.2., step 5**) directly onto the column to obtain the peak response.
2. Construct a standard curve by plotting the amount of derivatized standards vs the detector response for the 100–500 pg range. The detector response (peak area) for 100–500 pg range varies linearly.
3. Inject 2 µL of the sample extract (*see* **Subheading 3.5.1.4.**, sample equivalent to 0.08 mg) into the GC under the same conditions used for preparing the standard curve.
4. Calculate the amounts of trichothecenes in the sample by comparing the peak area of sample with the peak area of derivatized standards.
5. The retention times under the operating conditions listed are 15 and 18 min for DON and NIV TMS ethers, respectively, and 14, 15, 21, 22, and 29 min for NIV, DON, DAS, HT-2 and T-2 HFB derivatives, respectively ***(23)***.

3.7. GC-MS

3.7.1. Operating Conditions

Table 2 indicates representative conditions of capillary columns for GC-MS. Operate in the electron-impact mode at 70 eV and at a mass resolution of 1000–1500. Program the column temperature from 80°C (after 1-min hold) at 50°C/min to 180°C, then at 8°C/min to 250°C (hold) for HFB derivatives ***(23)***. Ions monitored for HFB derivatives: *m/z* 732, 672, and 655 for HT-2; *m/z* 884 for DON; *m/z* 716 and 701 for 4-MAS; *m/z* 656 for 15-MAS; *m/z* 502, 474, and 459 for DAS; *m/z* 501 for T-2, and *m/z* 1096 and 883 for NIV ***(23)***. As for TMS derivatives, program the column temperature from 160°C (after 5-min hold) at 6°C/min to 280°C (hold), and ions monitored: *m/z* 512, 422, and 393 for DON; *m/z* 392 and 377 for 3-ADON; *m/z* 407, 392, and 350 for 15-ADON; *m/z* 379, 349 and 289 for NIV; *m/z* 377 and 320 for 3,15-DADON, and *m/z* 450 and 408 for 4,15-DANIV ***(8)***.

Table 2
Representative Examples of Capillary-column Conditions for Trichothecene Analysis by GC-ECD and GC-MS

Coated phase (film thickness)	Column size (long, id)	Derivatization	Detection	Oven temperature programmed (time hold)	Ref.
J&W DB-5 (0.25 μm)	30 m × 0.25 mm	HFB	MS	80°C (1 min), then 50°C/min to 180°C, and 8°C/min to 250°C (hold)	***(23)***
		TMS, HFB, TFA[a], PFP[a]	MS	80°C to 300°C at 20°C/min; or 80°C to 275°C at 10°C/min	***(37)***
J&W DB-1701 (0.25 μm)	15 m × 0.26 mm	HFB	ECD	70°C (3 min), then 30°C/min to 175°C (2 min) and 5°C/min to 245°C (20 min)	***(23)***
		TMS	ECD	70°C (3 min), then 30°C/min to 220°C (14 min)	***(23)***
Shimadzu HiCap CBP1 (0.25 μm)	50 m × 0.2 mm	TMS	MS	160°C (5 min), then 6°C/min to 280°C (hold)	***(8)***
Noribond OV-1 (0.2 μm)	25 m × 0.32 mm	TMS	ECD	60°C (1 min), then 40°C/min to 180°C, and 5°C/min to 250°C; or 200°C to 280°C at 10°C/min	***(7,33)***
NB-30	25 m × 0.2 mm	TMS	MS	60°C (1 min), then 50°C/min to 200°C, and 5°C/min to 250°C	***(38)***

[a] TFA, trifluoroacetyl ester; PFP, pentafluoropropionyl ester.

4. Notes

1. Mixtures such as pyridine-TSIM-TMCS (90 + 5 + 5) **(32)** and TSIM-TMCS-ethyl acetate (1 + 0.2 + 9) ***(31)*** were used for silylation of trichothecenes. In these cases, portions of the reaction mixtures were directly injected onto the GC column. Note that TSIM causes damage to the capillary column unless removed by washing the reaction mixture with water or phosphate buffer (pH 7.0) ***(18,33)***.
2. Heptafluorobutyrylation is also carried out using heptafluorobutyrylimidazole (HFBI) ***(23)***. Slower derivatization of trichothecenes (1 h at 60°C) is achieved with this reagent than with HFBA-DMAP. After heating, the reaction mixture is washed with phosphate buffer (pH 7.0). Deterioration of HFBI was noted: after storage of the distilled reagent for one month at –8°C, the capillary GC background due to reagent blank showed a marked increase ***(34)***. Instead of DMAP, polystyrene-bound DMAP (Fluka, Buchs, Switzerland) was used as an insoluble catalyst, which has the advantage of no aqueous washing step ***(34)***.
3. The extraction efficiencies of DON and NIV from naturally contaminated or artificially molded grains were examined using different ratios of methanol-water and acetonitrile-water ***(26,30,35,36)***. Acetonitrile-water (3 + 1) and methanol-water (3 + 1) gave the highest recovery of the toxins ***(26,35)***. Less co-extraction of interfering contaminants was observed when the aqueous acetonitrile system was used vs the aqueous methanol system ***(26,36)***.
4. As the column is pushed further into the tube, force the extract through the frit, one-way valve, and packing material, successively. Do not place your finger over the top of the cleanup column reservoir during this step.
5. The cleanup with Florisil is sufficient for quantitative determination of trichothecenes such as DON, NIV and their acetylated derivatives at levels higher than 10 ng/g ***(8,26)***. For determinations of the toxins below this level, however, further cleanup with Sep-pak silica cartridge (Waters Associates Ltd., MA) was required ***(26)***.
6. This method, the solid-phase-extraction/LC method for DON in white flour, whole wheat flour, and bran, was adopted as a peer-verified method by AOAC INTERNATIONAL ***(27,29)***.
7. Sonication recommended.
8. Densitometric results must be confirmed by visual inspection. DON spots from the test extract and the standard should be the same fluorescent color. The response is linear relative to DON concentrations of 10–250 ng. Highly concentrated extracts, containing >100 ng DON/g should be diluted and the TLC redone ***(24)***.
9. Precoated silica gel 60 TLC plates can substitute for HPTLC plates ***(22,30)***. *See* references for developing solvent systems and Rf values of various trichothecenes ***(18,31)***.

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12

Chromatographic Method for the Determination of the Mycotoxin Moniliformin in Corn

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1. Introduction

1.1. Fungal Sources and Natural Occurrence of Moniliformin

Moniliformin is a fungal metabolite structurally characterized as the sodium or potassium salt of 1-hydroxycyclobut-1-ene-3,4-dione (***1***; **Fig. 1**). It was first isolated in 1973 from a corn culture that had been inoculated with *Fusarium proliferatum*, but that had been misidentified as the closely-related species *Fusarium moniliforme*, thus the name moniliformin ***(2)***. The metabolite is produced by at least 30 other *Fusarium* species ***(3–9)***. Of these, several species are particularly important pathogens of cereal grains throughout the world, *F. proliferatum* and *F. subglutinans* being the most important in corn.

Moniliformin has been reported to occur naturally in corn, wheat, rye, triticale, oats, and rice from different parts of the world. Moniliformin (16 to 25 µg/g) was first reported in hand-selected, visibly moldy Transkeian corn ***(10)***. In the US, a sample of corn screenings associated with field outbreak of leukoencephalomalacia was analyzed and found to contain 2.3 µg/g of moniliformin ***(11)***. However, it has been established that fumonisins ***(12)***, and not moniliformin, are the causative agent of the disease. Moniliformin in concentrations varying form 4.2 to 530 µg/g has been reported in *Fusarium*-damaged corn in Poland ***(13,14)***. A survey of samples of food-grade corn and corn products, mainly meal and flour, from different countries revealed that corn from Gambia and South Africa contained the highest concentrations (3.2 and 2.7 µg/g, respectively) of moniliformin, whereas samples of corn meal and corn flour, some of US origin, contained detectable but low concentrations (0.05 to 0.25 µg/g) of moniliformin ***(15)***. In 24 samples of Canadian corn, wheat and rye, moniliformin was only found in 2 corn samples at concentra-

From: *Methods in Molecular Biology, vol. 157: Mycotoxin Protocols*
Edited by: M. W. Trucksess and A. E. Pohland

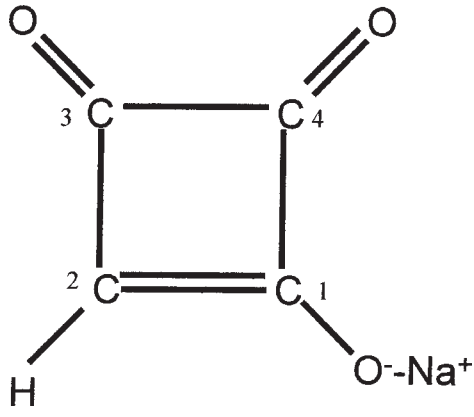

Fig. 1. Structural formula of moniliformin (sodium salt).

tions of 0.06 and 0.2 µg/g ***(16)***. In Poland, wheat and triticale grain naturally infected with *Fusarium* species contained moniliformin in amounts averaging 5.9 and 3.5 µg/g, respectively; healthy-looking kernels of wheat and triticale contained an average of 0.42 and 0.15 µg/g of moniliformin, respectively ***(17)***. Yu et al. ***(18)*** reported that samples of food-grade corn from Chinese areas with Keshan disease (a human cardiomyopathy prevalent in Chinese regions where inhabitants eat home-grown corn infected by *F. subglutinans* and contaminated with moniliformin) and non-Keshan disease did not differ in moniliformin contamination. They contained moniliformin ranging from 0.5 to 1.1 µg/g. Samples of rice from both areas contained less than 0.3 µg/g of moniliformin. Possibly because of lack of suitable analytical methods, moniliformin has not yet been reported in processed products derived from cereal grains contaminated with moniliformin.

1.2. Toxicity of Moniliformin

Moniliformin is a highly toxic metabolite. In addition to its acute toxicity to plants ***(2)***, moniliformin proved to be extremely toxic to various animal species when administered parenterally or by gavage ***(2,19–23)***. The oral LD_{50} of moniliformin in cockerels, chickens, and ducklings are in the range of 3.7 to 5.4 mg/kg body wt ***(2,21,24)***, whereas in rats and mice they are from 42 to 50 mg/kg body wt ***(22,24)***. Clinical signs of moniliformin toxicity observed in most of the affected animals have been generally described as progressive muscular weakness, respiratory distress, cyanosis, coma followed by death. Allen *et al*. ***(20)*** estimated that the LD_{50} of moniliformin upon intravenous injection of 7-wk-old female broiler chickens was 1.38 mg/kg body wt. Dietary exposure of broiler chicks and ducklings to moniliformin and fumonisin B_1,

another mycotoxin produced by *F. proliferatum* and *F. subglutinans*, indicated that moniliformin was more toxic than fumonisin B_1 ***(23,25)***. Toxic effects have been reported in many experimental animals fed diets amended with culture materials containing known amounts of moniliformin ***(6,19–26)***. These toxic effects may have been due to moniliformin and/or other toxic metabolites present in the culture materials. Comparison of cytotoxicity also indicated that moniliformin is more cytotoxic than fumonisin B_1 on cultured chicken primary cells (splenocytes, cardiac and skeletal myocytes) and other cultured mammalian cell lines ***(27,28)***. Moniliformin is a potent cardiotoxic mycotoxin ***(26,29)***. Broiler chickens (3-wk-old) injected with moniliformin (1 mg/kg body wt) died of cardiac failure within 50-min post-injection ***(30)***. Although the acute and long-term toxicity of moniliformin for humans is not yet known, some Chinese scientists suggested that moniliformin may be an etiological factor of Keshan disease ***(31)***.

The predominant molecular mechanism of moniliformin toxicity has been characterized. The toxin selectively inhibits mitochondrial pyruvate and α-ketoglutarate oxidations, thus preventing entrance of pyruvate and α-ketoglutarate into the tricarboxylic acid cycle with consequent reduction of oxidative phosphorylation, i.e., ATP production ***(32)***. Specifically, moniliformin binds to and inhibits pyruvate dehydrogenase ***(33)***. The lack of mutagenicity to *Salmonella typhimurium* ***(34)*** suggests that moniliformin is probably not carcinogenic.

1.3. Analytical Methods for the Determination of Moniliformin

Very few analytical methods have been developed for determining moniliformin in agricultural products. Many investigators used thin-layer chromatography (TLC) with fluorescence quenching ***(21)*** or color reaction with ninhydrin ***(2)***, methylbenzothiazolinonehydrazone (MBTH) ***(3,8,9)*** or 2,4-dinitrophenylhydrazine ***(7,35–39)*** to determine moniliformin, mainly in fungal cultures. A rapid TLC method for moniliformin analysis in corn and poultry feed was described recently ***(40)***. The method involves extraction of the toxin with acetonitrile/water (84/16) followed by one step-cleanup, TLC and fluorescence derivatization of moniliformin with ortho-phenylenediamine. A limit of quantitation of 0.1 μg/g and recovery rates of 85–100% moniliformin added to noncontaminated extracts of corn (0.1–2.0 μg/mL) were reported; but unfortunately, recovery rates of moniliformin added to corn were not presented. Vesonder et al. ***(41)*** described another TLC separation and fluorescence derivatization technique for screening moniliformin in corn. The toxin was extracted with acetonitrile/water (80/20), purified on a weak anion solid phase cleanup column and chromatographed on TLC plates. It was derivatized with 4,5-dichloro-1,2-phenylenediamine. The derivative was more fluorescent than

the derivative of moniliformin and ortho-phenylenediamine. Kamimura et al. ***(35)*** used TLC coupled with densitometry and were able to lower the limit of quantitation of moniliformin in corn, barley, and wheat to 0.05–0.1 µg/g with recovery rates in the range of 71–82%, but at the expense of a lengthy sample preparation and derivatization with 2,4-dinitrophenylhydrazine. Moniliformin was added to the cereals at a concentration of 2.0 µg/g. A gas chromatography-mass spectometry (GC-MS) method with a detection limit of 5 picograms of derivatized standard moniliformin was developed ***(42)***; however the method was not evaluated for determining moniliformin in corn or other cereal grains.

High-performance liquid chromatography (HPLC) is generally preferred over TLC because of its improved sensitivity and resolution. Thiel et al. ***(10)*** used ion-pair reversed phase and ion-exchange chromatography to determine moniliformin in corn samples. The toxin was extracted with distilled water and determined either without any further purification, or after purification on columns packed with diethylaminoethyl (DEAE)-Sephadex. Recovery rates of the procedure were not given and were stated to be low and to vary considerably. The same procedure was used to determine moniliformin in a sample of corn screenings associated with a field outbreak of leukoencephalomalacia in horses in Pennsylvania, USA ***(10)***. The reported limit of quantitation of this procedure was 10 µg/g ***(5)***. An HPLC procedure which uses ion-pairing for the extraction and determination of moniliformin in corn has been described ***(43)***. The procedure was relatively sensitive with a limit of quantitation of 0.1 µg/g and recovery rates in the range of 60–80% at 0.1–1.6 µg/g spiking concentrations. However, the procedure suffered from co-eluting interfering peaks which made interpretation of chromatograms difficult. The procedure, which included a 3-step sample cleanup on an Amberlite IRC-50 column, C_{18} Sep-Pak, and "ChemTube™" is not practical for routine determination of moniliformin in a large number of samples. Scott and Lawrence ***(16)*** developed another HPLC method using ion-pair reversed phase separation for determining moniliformin in corn and wheat. The toxin was extracted with acetonitrile/water (95/5), concentrated by evaporation at approx 50°C and purified successively on C_{18} Sep-Pak and alumina columns. Recovery rates of 74–83% at spiking concentrations of 0.05–1.0 µg/g of corn, with a quantitation limit of approx 0.01 µg/g were reported. However, chromatographic separations were very poor. Thiel ***(44)*** described two HPLC procedures employing ion-exchange and ion-pair reversed phase separation for moniliformin analysis in corn extracts. The detection limit of standard moniliformin was 20 ng and overall recovery rates were on the order of 70%. The procedures depended upon a lengthy extract cleanup (4 h) on DEAE-Sephadex resin which did not eliminate major interfering compounds, and a lyophilization step. Sharman et al. ***(15)*** described a sensitive HPLC method for determining moniliformin in corn, wheat, rye and triticale.

Samples were extracted with acetonitrile/water (95/5). Extracts were concentrated by evaporation at 40°C and purified on a combination of reversed phase and strong-anion exchange disposable columns. Extracts were analyzed by ion-pair reversed phase HPLC with UV detection. Recoveries ranging from 81 to 96% for spiked samples at spiking concentrations of 0.25 and 0.5 µg/g, and a limit of quantitation of 0.05 µg/g moniliformin in corn were reported. Efforts to use this method in our laboratory were not successful. Recoveries of moniliformin extracted with mixtures of acetonitrile and water were very low when extracts were concentrated by evaporation. Recently, Filek and Lindner ***(45)*** reported on an HPLC method which was very sensitive and selective. Moniliformin was derivatized with 4,5-dichloro-1,2-phenylenediamine to yield a fluorescent derivative. The limit of detection (smallest amount of moniliformin derivative which can be qualitatively identified) was 0.5 ng. Moniliformin in corn was extracted with acetonitrile/water (95/5) and purified on a strong-anion exchange disposable column. The procedure detected moniliformin in corn at 0.02 µg/g (limit of determination) with overall recoveries of approx 70% at spiking concentrations in the range of 0.02–0.25 µg/g. Unfortunately, the method requires a time-consuming (2 h) derivatization step. We found the ion-pairing phenomenon very useful in a redesigned analytical procedure for determining moniliformin in corn ***(46)***. Compared with other published HPLC methods employing UV detection, the primary advantage of this procedure is a simple and efficient sample extraction and cleanup resulting in improved recoveries, chromatographic separation, and sensitivity.

2. Materials

2.1. Apparatus

1. Solvent delivery pump capable of delivering 1 ml/min.
2. A sample injector assembly with a 20 µL-sample loop.
3. A UV detector with wavelength and sensitivity set at 229 nm and 0.003 absorbance unit-full scale (AUFS), respectively.
4. Analytical and guard-columns: Ultremex C_{18} reversed phase column (150 × 4.6 mm id., 5 µ*M*, Phenomenex, Torrance, CA) with Partisil 10 SAX guard-column (30 × 4.6 mm id, 10 µ*M*, Phenomenex) or Nova-Pak™ C_{18} reversed phase column (150 mm × 3.9 mm id, 4 µ*M*, Waters Corporation, Milford, MA) with Nova-Pak™ C_{18} reversed phase Sentry guard-column (20 × 3.9 mm id, 4 µ*M*, Waters).
5. A recorder-integrator with chart speed set at 0.85 cm/min or a computer-assisted data collection system.
6. Solid phase extraction (SPE) columns: 1 mL capacity disposable LC-SAX columns containing 100 mg of packing sorbent (Supelco, Inc., Bellefonte, PA 16823-0048).
7. Shaker: wrist-action shaker (Burrell Corp., Pittsburgh, PA).

2.2. Reagents

1. Solvents and reagents: acetonitrile, dichloromethane, methanol, and water (all HPLC grade), 1% and 40% (w/v) tetrabutylammonium hydrogen sulfate, 0.05 *M* potassium dihydrogen phosphate (pH 5.0), 1.1 *M* sodium dihydrogen phosphate monohydrate, and 0.1 *M* ortho-phosphoric acid.
2. Moniliformin standard solution: pure sodium salt of moniliformin (Sigma Chemical Co., St. Louis, MO) dissolved (200 µg/mL) in 0.05 *M* sodium dihydrogen phosphate monohydrate, pH 5.0 and stored at 4°C.

3. Methods

3.1. HPLC

1. LC mobile phase: first, prepare a concentrated solution of ion-pair modifiers by mixing 50 mL of 40% tetrabutylammonium hydrogen sulfate (w/v) with 100 mL of 1.1 *M* potassium dihydrogen phosphate.
2. Next, prepare the mobile phase by diluting 10 mL of concentrated solution of ion-pair modifiers with water to a final volume of 1 liter. Adjust the pH to 6.5 with 5 *N* KOH.
3. Finally, prepare an 8% acetonitrile solution with the diluted solution of ion-pair modifiers and filter through a 47 mm × 0.20 µm nylon membrane.
4. Degas the mobile phase before use. Allow the HPLC system to equilibrate for approx 1 h.

3.2. Working Standard Solution of Moniliformin

1. Prepare an intermediate solution of moniliformin (10 µg/mL) from the standard solution in 0.05 *M* sodium dihydrogen phosphate monohydrate (pH 5).
2. Use fractions of the above solution to prepare different concentrations of moniliformin in the range of 0.01 to 1.0 µg/mL. Store the solutions at 4°C. They are stable for at least 6 mo.

3.3. Sample Preparation

1. Grind corn sample to pass at least US No. 20 sieve and place 10 g of ground corn test sample into a 125 mL-polyethylene sample bottle.
2. Add 50 mL of 1% tetrabutylammonium hydrogen sulfate (TBAHS) prepared in LC grade or double distilled water and shake for 30 min at maximum speed on a wrist-action shaker.
3. Filter the extract by gravity through a Whatman No. 4 filter paper, taking care to retain most of the solid residues in the sample bottle.
4. Add an additional 50 mL of 1% TBAHS to the solid residues in the sample bottle and shake for an additional 30 min.
5. Filter the extract through the same filter paper and combine the 2 extracts.
6. Transfer 25 mL of extract into a 125 mL separatory funnel containing 25 mL of dichloromethane. Mix gently and avoid vigorous shaking.

7. Let phases separate and drain lower phase into a 100 mL container. If an emulsion is formed, centrifuge at 3000*g* for 5 min to allow good phase separation.
8. Repeat the partition with additional 25 mL of dichloromethane, and combine the dichloromethane extracts.
9. Evaporate dichloromethane to 5–10 mL at 50°C in a water bath under a stream of blowing air. Transfer the reduced volume of dichloromethane into a small vial and evaporate to dryness.

3.4. Extract Cleanup

1. Fit a disposable SAX SPE column onto the end of a 10 mL syringe or port of a vacuum manifold.
2. Condition the tube by washing successively with 1 mL of methanol, 1 mL of water and 1 mL of 0.1 *M* ortho-phosphoric acid. Do not allow the tube to dry.
3. Dissolve the extract residue in 1 mL of water (LC grade) and load onto the SPE column.
4. When all the extract has passed through the tube, wash the tube with 1 mL of water and force air through the tube to expel all of the wash solution.
5. Elute the adsorbed moniliformin with 1 mL of 0.05 *M* sodium dihydrogen phosphate monohydrate (pH 5). Filter the eluate through a 0.2 µ*M* nylon membrane and save eluate at 4°C until ready for LC analysis.

3.5. Liquid Chromatography

1. Prepare a standard curve by injecting 20 µL of moniliformin working standards. The retention time of moniliformin is approx 6.2 min. There is no need to prepare the standard curve daily, but injection of a moniliformin standard solution is required for each daily analysis.
2. Inject 20 µL of test solution. Identify peak and determine the quantity of moniliformin by comparing retention time and area with those of reference standard.

3.6. Spiking of Samples and Recovery

1. Prepare moniliformin standard solutions in LC grade water (2.5, 5.0, 25, and 100 µg/mL) and spike ground test sample at concentrations of 0.025, 0.05, 0.25, and 1.0 µg/g with a spiking volume of 0.1 mL for 10 g of test sample.
2. Analyze test samples according to the procedure described above (**Subheadings 3.1.–3.5.**) and calculate percentage recoveries. Use 3 replicates of spiked test samples at each concentration for each run, and repeat the analysis to determine recoveries and daily variation of the analytical procedure.

4. Notes

1. Moniliformin is a toxic substance and should be handled with caution.
2. Water appeared to be the ideal solvent for extracting moniliformin because of the polarity and high solubility of the toxin in water. Thiel ***(44)*** used water (40 mL) to extract moniliformin from spiked ground corn (3 g) and reported a 95% recov-

ery rate for the extraction step. The extraction procedure described here is based on the extraction procedure developed by Shepherd and Gilbert ***(43)*** who used water (150 mL) containing tetra-*n*-butylammonium hydroxide (ion-pairing reagent) to extract moniliformin in ground corn (30 g). The extraction was followed by a cleanup on Amberlite IRC-50 resin and C_{18} Sep-Pak cartridge, before another ion-pairing and final cleanup on a "ChemTube™." Compared to the extraction, ion-pairing and cleanup procedures described by Shepherd and Gilbert ***(43)***, the conditions in our procedure were simplified very much, making the procedure easier and more practical, and reducing analysis-time. Removal of interferences and recoveries were improved. Extraction and ion-pairing of moniliformin were accomplished during a single step by using 1% tetrabutylammonium hydrogen sulfate (TBAHS) in water. No precleanup on Amberlite IRC-50 resin or C_{18} Sep-Pak cartridge was required for successful ion-pairing and subsequent partition of moniliformin. Precleanup on C_{18} Sep-Pak cartridge was omitted in our procedure, because C_{18} Sep-Pak cartridges or columns (Waters, Milford, MA) bound up to 35% of free moniliformin and 100% of paired moniliformin in water solution. Two 50-mL volumes of 1% TBAHS were used to extract moniliformin. In general, the first volume extracted 70–75% of recovered moniliformin.

3. Moniliformin spiked into water or an aqueous extract of corn (0.1 µg/mL) could not be partitioned into dichloromethane, chloroform or ethyl acetate without prior pairing of the toxin with tetrabutylammonium counter-ion. When a moniliformin-free corn sample was extracted with 1% TBAHS, and the extract spiked with moniliformin, approx 100% of moniliformin was partitioned into dichloromethane, 53% into ethyl acetate, and less than 5% into chloroform. Paired moniliformin in aqueous extract was partitioned into two volumes of dichloromethane. Approximately 85% of paired moniliformin was partitioned in the first volume, and 15% in the second volume. Compared to previously published methods, pairing moniliformin with TBAHS, followed directly by partition into dichloromethane before cleanup, was a new step in moniliformin analysis. The described pairing and partition procedure enhanced overall recoveries of moniliformin from spiked corn.
4. Shepherd and Gilbert ***(43)*** indicated that exposure of moniliformin to low pH leads to loss of the toxin. Therefore the stability of moniliformin in 1% TBAHS aqueous extract of corn (pH of approx 2.2) was studied by holding extracts at room temperature for up to 24 h before cleanup and analysis. Results indicated that paired moniliformin was very stable in 1% TBAHS extracts with no loss at all. Additional studies also indicated that the toxin was very stable in dichloromethane after partition, and in water after evaporation of dichloromethane.
5. The cleanup procedure on disposable SAX solid phase extraction columns was adapted from procedures described by other investigators ***(15,45)***. However, the C_{18} SPE column used in combination with SAX SPE column ***(15)*** was omitted in our procedure because of adverse effects of the C_{18} cleanup column on recovery. A 1-mL SAX tube was preferred over the 3-mL tube used by other investigators

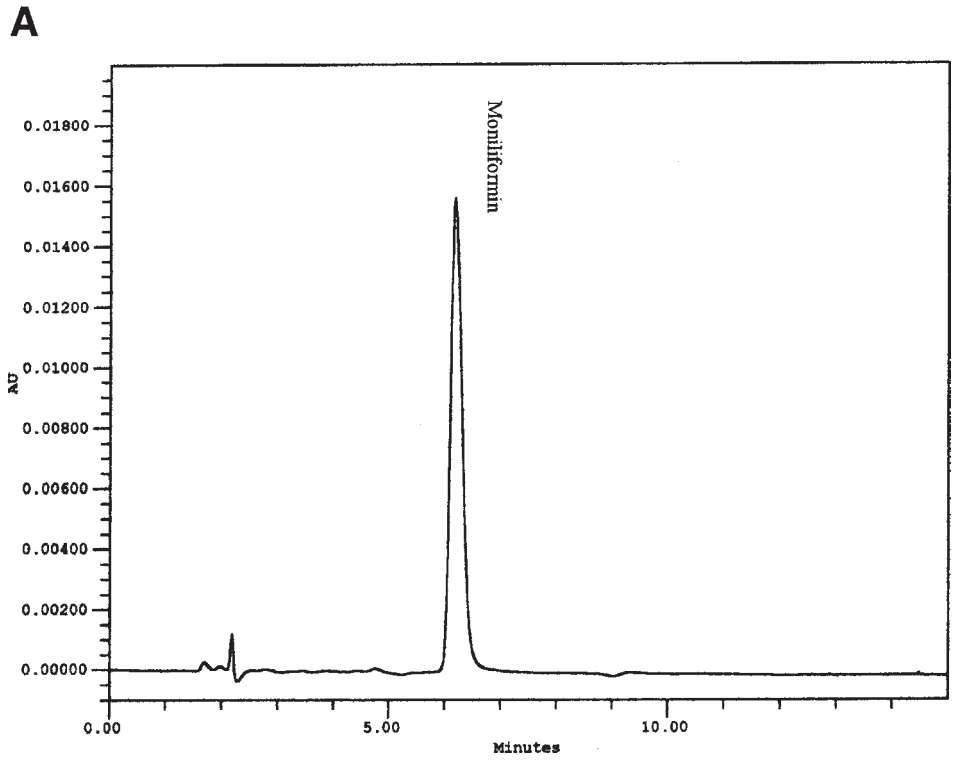

Fig. 2A. Chromatogram of standard moniliformin (20 ng injection).

(15,45). It required lower amounts of solvents and appeared to be more efficient than a 3-mL column at retaining interfering compounds. Sodium phosphate buffer (pH 5) was preferred over solvents used by Sharman et al. ***(15)*** or Filek and Lindner ***(45)*** as the solvent for eluting moniliformin from the SAX column. The buffered water was more efficient at eluting adsorbed moniliformin, and it eluted fewer interferences than other solvents.

6. The extraction and cleanup procedure described above gave excellent chromatograms free of co-extractive interferences. Typical chromatograms of standard moniliformin (20 ng injection), moniliformin-free corn, and naturally contaminated corn (0.2 µg/g) are shown in **Fig. 2A–C**, respectively. In all cases, moniliformin eluted as a very sharp peak without tailing and well separated from other constituents. The limit of detection of pure moniliformin (smallest amount which can be identified) was 0.25 ng (signal-to-noise ratio = 3:1) which is lower than the 1 ng limit reported by Thiel ***(44)*** or 0.5 ng of fluorescent derivative of moniliformin and 4,5-dichloro-1,2-phenylenediamine reported by Filek and Lindner ***(45)***. The chromatographic response was linear ($R^2 = 1.00$) between 0.25 and at least 20 ng moniliformin injected onto the column. The limit of quantitation

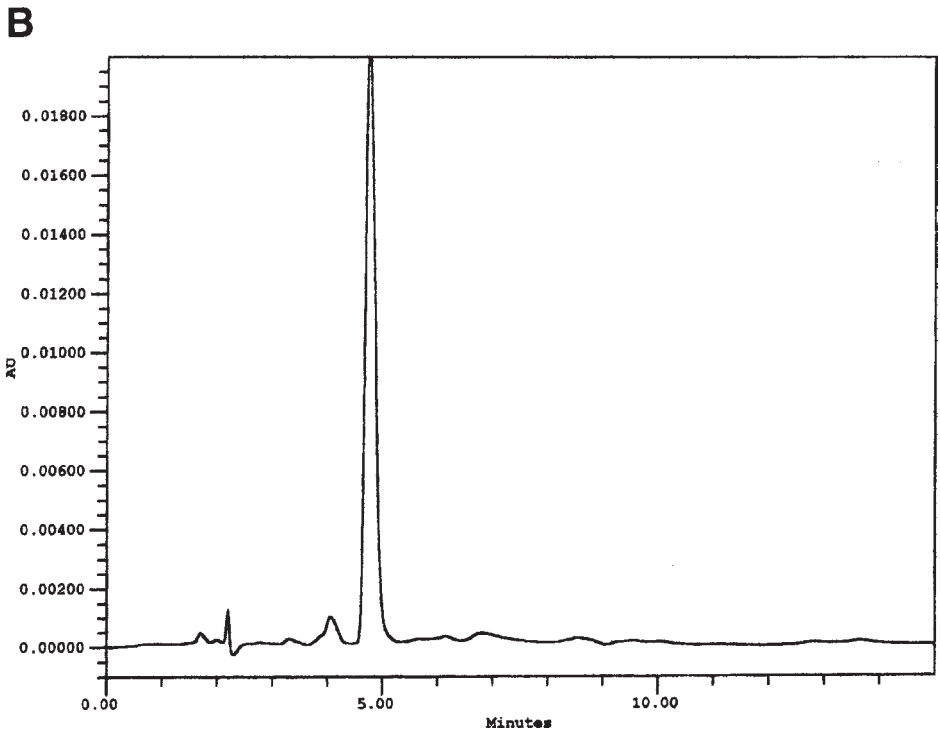

Fig. 2B. Chromatogram of moniliformin-free corn sample; 0.05 g corn equivalent injected; absorbance wavelength, 229 nm; AUFS, 0.003.

of moniliformin spiked into ground corn was 0.025 µg/g corn which is lower than 0.1 µg/g corn reported by Shepherd and Gilbert ***(43)***. It is comparable to 0.01 and 0.02 µg/g corn reported by Scott and Lawrence ***(16)***, and Filek and Lindner ***(45)***, respectively. Chromatograms were better than any previously published. The peak corresponding to moniliformin was sharp and free of interfering compounds. Average recovery rates of moniliformin spiked into ground corn at concentrations in the range of 0.025 to 1 µg/g varied from 96 to 98% (**Table 1**). These recovery rates are higher than 70–80% recovery rates reported by other investigators ***(15,16,43–45)***.

7. Extraction and recovery rates were compared when moniliformin spiked in ground yellow corn at a concentration of 1 µg/g was extracted with water alone followed by ion-pairing with TBAHS, 1% aqueous solution of TBAHS, and aqueous solutions of tetrabutylammonium hydroxide (TBAH) or tetrabutylammonium hydrogen phosphate (TBAHP) (Sigma Chemical Co., St. Louis, MO) with molar concentration equal to that of 1% TBAHS solution. Extraction with 1% TBAHS was the preferred procedure; extracts were very clear and filtered faster than any other extract. TBAHP solution extracted more solids than TBAHS or water alone,

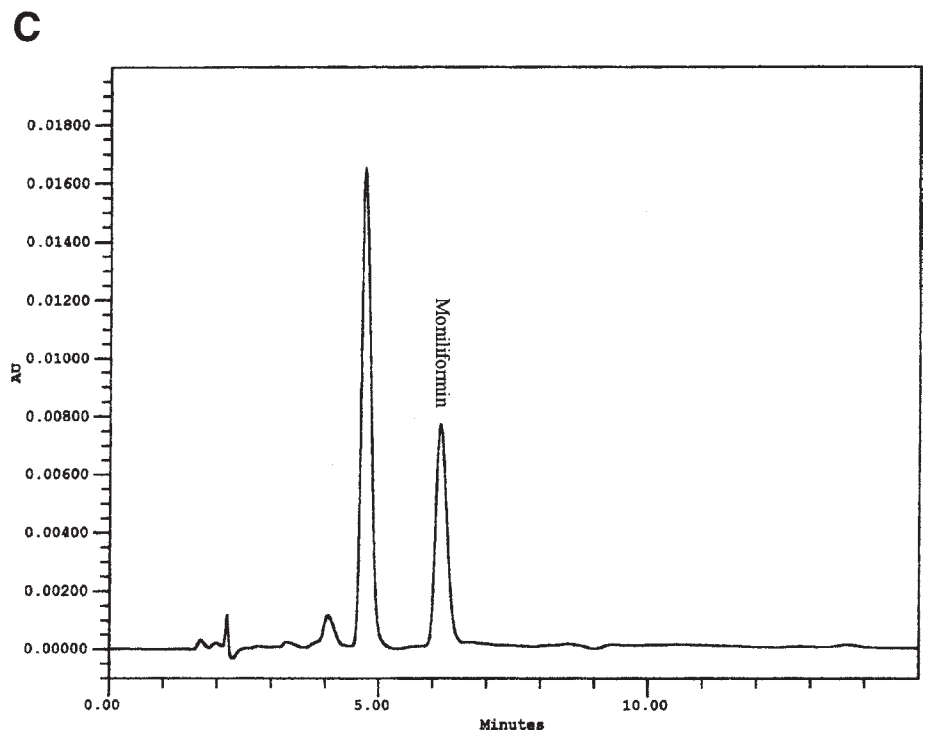

Fig. 2C. Chromatogram of corn sample naturally contaminated with moniliformin (0.2 μg/g); 0.05 g corn equivalent injected; absorbance wavelength, 229 nm; AUFS, 0.003.

Table 1
Recoveries of Moniliformin Added to Ground Corn

Moniliformin added, μg/g	Average, %	Range, %	CV, %
0.025	96.5	86.3–109.9	8.1 (n = 9)
0.050	96.2	83.0–109.1	9.7 (n = 12)
0.250	97.2	88.3–102.2	4.4 (n = 12)
1.0	97.8	95.4–105.7	2.8 (n = 15)

thus making filtration by gravity very difficult. Extracts formed a thick emulsion upon partition with dichloromethane. TBAH solution extracted yellow pigments and a large amount of solids. It is not possible to filter the extracts by simple gravity. Similar average recovery rates, approx 98%, were recorded with TBAHS solution, water alone followed by pairing with TBAHS, and TBAHP solution.

Chromatograms were excellent in all 3 cases. Moniliformin was not detected in spiked samples extracted with TBAH solution. It is possible that the toxin was not stable under the alkaline pH (12.5) of the extraction solvent.

8. Compared to previously published HPLC methods for the determination of moniliformin in corn, this analytical procedure was excellent in terms of efficient and easy test sample extraction and cleanup resulting in improved recovery rates and chromatographic separation. The time required for complete analysis of 15 spiked test samples was approx 8–9 h. The test sample extraction and cleanup procedures described above are suitable for moniliformin analysis in corn, corn meal, and corn flour, but are not as well suitable for corn flakes, corn chips, and extruded corn-based products, because of lower recovery rates.

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13

Liquid Chromatographic Method for Fumonisins in Corn

Gordon S. Shephard

1. Introduction

The fumonisins are an economically important group of mycotoxins that occur worldwide in corn and corn-based products. They are produced by a number of *Fusarium* species, of which *F. moniliforme* and *F. proliferatum* are the most common, as they infect corn crops around the world ***(1)***. Although a number of fumonisins have been identified ***(2)***, the most abundant analogs found in naturally contaminated food and feeds are fumonisin B_1 (FB_1), B_2 (FB_2), and B_3 (FB_3) ***(3)***. Analytical determination of these mycotoxins has shown them to be almost universally present in corn and has heightened concern over the implications that ingestion of these toxins has for animal and human health ***(3)***. Of particular concern, is the reported co-occurrence in corn of fumonisins, which have been shown to have cancer promoting properties ***(4)***, with the known human carcinogen, aflatoxin ***(5–8)***. In animals, fumonisins are the causative agents of the fatal syndromes, leukoencephalomalacia in horses ***(9)*** and pulmonary edema and hydrothorax in pigs ***(10)***. They produce nephrotoxicity ***(11)*** and hepatocarcinoma ***(12)*** in rats and are toxic to turkey poults ***(13)*** and broiler chicks ***(14)***. Fumonisins have been shown to cause developmental toxicity, including fetal deaths and fetal resorptions, in hamsters ***(15)***, mice ***(16)*** and rats ***(17)***. However, experimental evidence suggests that for mice and rats, these effects may be mediated by maternal toxicity ***(16,17)***.

In humans, fumonisin exposure via the ingestion of contaminated homegrown corn as a staple diet has been linked with the high incidence of esophageal cancer in the Transkei region of South Africa ***(18)***. High fumonisin exposure occurs in the high esophageal cancer incidence areas of Linxian and Cixian Counties in China ***(19,20)***, whereas high levels of fumonisin contami-

From: *Methods in Molecular Biology, vol. 157: Mycotoxin Protocols*
Edited by: M. W. Trucksess and A. E. Pohland

nation of polenta, a common food in a region of high esophageal cancer incidence in northern Italy, have also been reported ***(21)***. A recent study has implicated fumonisin contamination of corn as a contributory risk factor for primary liver cancer in Haimen, China ***(6)***. Based on available evidence, the International Agency for Research on Cancer has declared the toxins derived from *F. moniliforme* to be class 2B carcinogens, i.e., possibly carcinogenic to humans ***(22)***.

At the cellular level, fumonisins induce apoptosis and are cytotoxic to a wide range of mammalian cells ***(23–25)***. They are potent inhibitors of *de novo* sphingolipid biosynthesis via the inhibition of the enzyme sphingosine (sphinganine) *N*-acyltransferase (ceramide synthase) in the sphingolipid biosynthetic pathway ***(26)***. This enzyme catalyzes the acylation of sphinganine to dihydroceramide prior to its conversion to ceramide and the subsequent formation of complex sphingolipids. Hence its inhibition leads to an accumulation of the sphingoid base, sphinganine. This elevation of sphinganine, conveniently expressed as an elevation in the sphinganine/sphingosine ratio, has been observed in the cells, serum, and urine of a number of animal species exposed to the fumonisin mycotoxins, including monkeys ***(27)***, ponies ***(28)***, and rats ***(11)***. Although it has been postulated that the fumonisins exert their pathological effects mainly through the disruption of sphingolipid metabolism ***(29)***, their role in carcinogenesis has been shown to also involve changes in growth factor responses ***(30)***, as well as the levels and fatty acid composition of phospholipids, with the consequent possibility of prostaglandin involvement ***(31)***. Studies on the influence of FB_1 on cell cycle progression have shown that FB_1 treatment represses cyclin dependent kinase activity in monkey kidney cells ***(32)*** and have also demonstrated an accumulation of cyclin D1 in rat liver carcinomas caused by chronic fumonisin exposure ***(33)***.

Apart from their impact on human health, the economic importance of fumonisins lies both in direct agricultural losses and in the implications that future governmental regulations may have for international trade. Limits on the levels of fumonisin contamination in animal feeds have been recommended based on the susceptibility of different animal species to fumonisins ***(34)***. Hence, a maximum limit for the most sensitive species, horses, was recommended at 5 μg/g in feed, whereas for beef cattle and poultry, maximum feed levels of 50 μg/g were suggested. Currently, Switzerland is the only country to propose tolerance limits of 1 μg/g for combined FB_1 and FB_2 in corn intended for human consumption ***(35)***. Many samples of field corn, especially from the United States, have been shown to have fumonisin contamination levels well in excess of this level ***(3)***. A recent risk assessment of fumonisins suggested that, based on a tolerable daily intake of 0.8 μg/kg/day, a tolerance level of 0.1 to 0.2 μg/g in corn would be needed to safeguard rural communities consum-

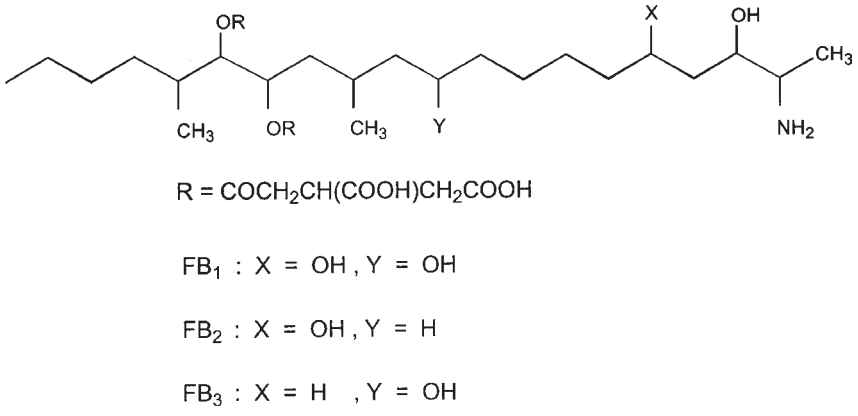

Fig. 1. Chemical structure of FB_1, FB_2, and FB_3.

ing corn as a staple diet ***(36)***. The full implications of fumonisin contamination for human mortality and morbidity have still to be determined. In addition to being statistically linked to esophageal ***(18)*** and liver ***(6)*** cancer, fumonisins have been reported to show immunosuppressive properties ***(37)*** and it has been suggested that, together with other agriculturally important mycotoxins, they contribute significantly to disability adjusted life years lost due to disease in developing countries ***(38)***.

This chapter describes a high-performance liquid chromatographic (HPLC) analytical method, previously validated by international collaborative studies ***(39,40)***, for the determination of FB_1, FB_2, and FB_3 in corn. Although the method has also been widely applied to a range of corn-based products, concern has been expressed over the analytical recoveries achieved from some food matrices such as maize bran flour and mixed baby cereal ***(41)***. As in all quantitative analytical work, analysts should validate the method in their own laboratories with respect to repeatabilities and analytical recoveries of the relevant analytes from the matrix of interest. The fumonisins are diesters of propane-1,2,3-tricarboxylic acid and various 2-amino-12,16-dimethylpolyhydroxyeicosanes in which the hydroxyl groups on C_{14} and C_{15} are esterified with a terminal carboxyl of the acid (**Fig. 1**). The chemical nature of fumonisins makes them amenable to extraction using polar solvents. They are generally extracted from corn using methanol/water ***(39,42,43)*** or acetonitrile/water mixtures (***42,44,45***). Due to the high polarity, water solubility, and zwitterionic nature of fumonisins, these extracts are not readily purified by solvent extraction. Currently used methods involve solid phase extraction, with strong anion exchange providing cleaner extracts than reversed-phase (C_{18}) materials ***(42,46,47)***. Immunoaffinity columns containing antibodies reactive to fumonisin

provide the cleanest extracts, but care must be exercised in their application ***(48,49)***. The purified extracts are analyzed by reversed-phase HPLC. Due to the lack of significant UV absorbing groups, the fumonisins present certain problems as regards their detection after separation by HPLC. A number of recent publications have highlighted the excellent results that can be achieved using mass spectrometry to detect and confirm the presence of the fumonisins ***(50–52)***. Although other detectors, such as evaporative light scattering ***(52)***, can be used to detect underivatized fumonisins, they are generally not sensitive enough for use in the analysis of naturally contaminated corn samples. As mass spectrometry facilities are not always generally available, sensitive detection of fumonisins has more widely been achieved by pre-column derivatization with various fluorogenic reagents, of which *o*-phthaldialdehyde (OPA) has proved to be the most widely used ***(3)***.

2. Materials

1. *HPLC system*: This analytical method for fumonisins requires a standard HPLC system consisting of an isocratic pump capable of a flow rate of 1 mL/min and a suitable injector capable of 10 µL injections. Fumonisins have been separated on a wide range of commercial reversed-phase columns containing C_{18}- or C_8-modified silica packing material of 3 to 5 µm particle size. A fluorescence detector and a suitable data system (electronic integrator or chromatographic package for a personal computer) are required to provide sensitive and specific detection and quantification of fumonisins derivatized with OPA.
2. *Homogenizer*: Fumonisins are extracted from corn using a homogenizer or blender. Although laboratory shakers have been used for extraction, contradictory reports on their efficiency have appeared in the literature ***(43,46)***.
3. *Solid phase extraction (SPE) cartridges*: Sample extracts are generally cleaned-up on SPE columns containing strong anion exchange (SAX) material, e.g., Varian (Harbor City, CA) Bond-Elut 10 mL capacity SAX cartridges containing 500 mg sorbent. For the optimum simultaneous handling of cartridges, the use of a commercial SPE manifold is recommended.
4. *HPLC mobile phase*: The HPLC mobile phase is a mixture of methanol and 0.1 *M* sodium dihydrogen phosphate in water. For most reversed-phase columns, a solvent composition of 75–80% methanol will be required. However, the composition of the mobile phase must be adjusted to optimize chromatographic conditions for individual HPLC columns so as to yield resolution of FB_1 from the initially eluting interferences and to provide adequate separation between the later eluting FB_2 and FB_3. The apparent pH of the mixture is adjusted to 3.35 with *o*-phosphoric acid and filtered through a 0.45 µm membrane filter.
5. *OPA reagent*: OPA reagent for derivatizing the fumonisins is prepared by dissolving 40 mg OPA in 1 mL methanol and diluting with 5 mL 0.1 *M* disodium

tetraborate. While working in a fume hood, 50 µL 2-mercaptoethanol is added and the mixture stored in a capped vial in the dark at room temperature. The reagent should not be kept for longer than a week.

6. *Fumonisin standards*: Fumonisin standards are prepared in acetonitrile:water (1:1) and stored at 4°C. Standards stored for long periods in methanol undergo slow degradation. Stock solutions of individual fumonisin standards of concentration 250 µg/mL are generally used, from which a working standard is prepared containing 50 µg/mL of each analog.
7. *Additional reagents*: The only additional reagents required are acetic acid and sodium hydroxide (analytical grade).

3. Methods

3.1. Extraction of Fumonisins

1. Finely ground corn products (e.g., corn meal) may be extracted without prior treatment. Corn kernels, corn grits, or other similar food matrices should be ground to a fine flour before extraction.
2. Weigh 25 g of the test sample into a container suitable for centrifuging (e.g., 250 mL polypropylene centrifuge bottle).
3. Add 100 mL extraction solvent (methanol-water, 3:1) and homogenize the contents for 3 min at a speed setting of approx 60% full speed (*see* **Note 1**).
4. Centrifuge the container at 500*g* for 10 min at 4°C.
5. Filter the supernatant through a standard laboratory fluted filter paper (e.g., Whatman No. 4).

3.2. Extract Cleanup

1. Check the apparent pH of the sample extract and adjust to between pH 5.8 and 6.5 with 1 *M* sodium hydroxide (only a few drops should be required) (*see* **Note 2**).
2. Condition the SAX cartridge on the SPE manifold with 5 mL methanol, followed by 5 mL extraction solvent (methanol-water, 3:1) (*see* **Note 3**).
3. Apply 10 mL of the extract to the conditioned cartridge at a flow rate of ≤2 mL/min.
4. Wash the cartridge with 5 mL methanol-water (3:1) and 3 mL methanol. Discard the washings.
5. Place a clean vial (20 mL) below the cartridge and collect the purified fumonisin-containing extract by eluting with 10 mL 1% acetic acid in methanol. It is important to ensure the flow rate does not exceed 1 mL/min (*see* **Note 4**).
6. Dry down the eluate by transferring successive aliquots to a suitable vial (4 mL), where the solvent is evaporated at approx 60°C under a steady flow of nitrogen. Rinse the collection vial with an additional 1 mL methanol and use this to wash down the sides of the 4 mL vial so as to concentrate the residue at the base of the vial. Dry down this additional wash and ensure that all the acetic acid has evaporated. Further small aliquots of methanol may be used to assist the removal of any traces of acetic acid left in the vial.

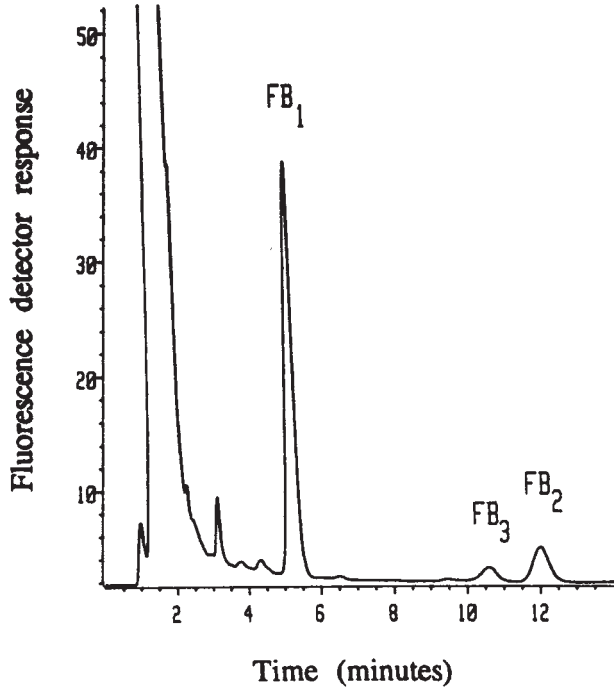

Fig. 2. HPLC chromatogram of the reversed-phase separation of fumonisin-OPA derivatives formed by precolumn derivatization of an extract of naturally contaminated corn.

3.3. Fumonisin Derivatization and HPLC Analysis

1. Prepare the HPLC system for use (*see* **Note 5**).
2. Derivatize standards by mixing 25 µL working standard with 225 µL OPA reagent at the base of a small test tube.
3. Inject 10 µL into the HPLC using a standardized time of 1 to 2 min between the addition of OPA reagent and injection (*see* **Note 6**). This is equivalent to an amount of 50 ng of each fumonisin analog. Fluorometric detector response to the OPA derivatives is generally linear over a wide range. Sample quantification can be achieved by constructing a suitable calibration line from standards of different concentrations. Alternatively, due to the linearity of response, the system can be calibrated using a single point determined in triplicate from the HPLC response to injections of the derivatized working standard as prepared above.
4. After calibration with standards, redissolve the dried test sample residue in 200 µL of methanol.
5. Derivatize the fumonisins by mixing 50 µL of sample with 200 µL of OPA reagent and inject 10 µL, remembering to maintain standardized times between the addition of OPA reagent and the HPLC injection. **Figure 2** shows a HPLC

separation of the fumonisins extracted from a corn test sample. Note the resolution of FB_1 from the earlier eluting impurities and the separation between FB_2 and FB_3.

6. If the fumonisin chromatographic peaks in the sample exceed those of the standard solution, sample extracts should be diluted with methanol and reanalyzed on the HPLC so as to avoid extrapolation beyond the range of calibration.
7. Quantification is achieved using peak areas produced by electronic integration (*see* **Note 7**).

3.4. Calculation

The following equations for the calculation of final fumonisin levels in corn are presented as a guide (*see* **Note 8**).

Assuming a single point calibration, the quantity of each fumonisin analog injected onto the column is given by **Eq. 1**:

$$A\ (ng) = (G/H) \times S \quad (1)$$

where A = ng fumonisin from the sample extract injected into the HPLC

G = fumonisin peak area in the chromatogram from the sample extract

H = fumonisin peak area in the chromatogram of the standard (mean of triplicate determination)

S = amount of fumonisin standard injected (50 ng for standards prepared as above)

Hence the concentration (C) of each fumonisin analog present in the original corn sample is given by **Eq. 2**:

$$C\ (ng/g) = (A \times T \times D)/(I \times W) \quad (2)$$

where A is calculated above

T = total volume of the derivatized solution (250 µL)

D = any dilution factor used in the individual analysis

I = injection volume (10 µL)

W = sample equivalent weight derivatized (0.625 g using quantities above)

The sample equivalent weight is given by **Eq. 3**:

$$W = (M \times V_{sax} \times V_{der})/(V_{ext} \times V_{dis}) \quad (3)$$

where M = mass of the corn test sample extracted (25 g)

V_{sax} = volume of extract applied to the SAX clean-up cartridge (10 mL)

V_{der} = volume derivatized with OPA (50 µL)

V_{ext} = volume of the extraction solvent (100 mL)

V_{dis} = volume of methanol used to redissolve the sample residue (200 µL)

4. Notes

1. It is important to ensure that the speed and placement of the homogenizer probe in the container should be such as to ensure thorough mixing and agitation of the suspension.
2. Retention of fumonisins on the SAX clean-up cartridge is pH-dependent due to the need to ensure full ionization of the fumonisins. If fumonisins are extracted from strongly acidic matrices, this pH adjustment can result in reduced recoveries due to the presence of other ionic compounds which compete for the ion exchange capacity of the SAX column. For the analysis of such matrices, other clean-up mechanisms such as C_{18}-SPE columns or immunoaffinity columns would need to be investigated.
3. At no stage during the clean-up process should the SAX sorbent be allowed to dry out.
4. Faster flow rates lead to reduced recoveries. In cases where a large number of samples are simultaneously purified, it may be easier to allow the SPE cartridges to elute under gravity, although this does lead to increased analysis time.
5. Standard laboratory procedures for the operation and care of the HPLC system and the analytical column should be observed. In particular, the mobile phase is prepared from phosphate buffer and must be filtered through a 0.45 µm membrane filter before use. At completion of the analyses or at the end of the day, buffer must be thoroughly removed from the system before shut down.
6. Fumonisins are detected as fluorescent OPA derivatives which, being unstable, are prepared from solution aliquots immediately prior to each individual HPLC injection. For reproducible results, it is necessary to standardize the time period between OPA reagent addition and the HPLC injection. In this regard, a period of 1–2 min generally yields optimum performance. After injection, the remainder of the derivatized solution is discarded. A wide range of injection volumes can be used (generally 5 to 50 µL) provided allowance is made in the calculation of the final result.
7. Analysts are advised to check for the correct type and positioning of integration markers on each chromatogram.
8. As in all chemical determinations, analysts should check the applicability of the equations to their own individual circumstances.

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14

Enzyme-Linked Immunosorbent Assays of Zearalenone Using Polyclonal, Monoclonal and Recombinant Antibodies

Mi-Gyung Lee, Qiao-Ping Yuan, L. Patrick Hart, and James J. Pestka

1. Introduction

Zearalenone (6-[10-hydroxy-6-oxo-*trans*-1-undecenyl]-β-resorcyclic acid lactone), also known as F-2 toxin, is an estrogenic mycotoxin that is produced by *Fusarium graminearum* ***(1)***. This fungus can infect cereal grains and can elaborate large quantities of zearalenone. The mycotoxin can co-occur with trichothecenes synthesized by *Fusarium* sp. So far, at least 15 naturally produced derivatives of zearalenone including α-zearalenol, α-zearalanol, β-zearalenol, and β-zearalanol have been identified.

The occurrence of zearalenone in agricultural commodities has been reported worldwide. For example, in the 1992–1993 Wisconsin corn crops, the mycotoxin was detected with the distribution of <100 ng/g (33%), 100–500 ng/g (35.2%), 500–1000 ng/g (13.2%), >1000 ng/g (18.7%) ***(2)***. In Canadian corn samples, it has been reported at a 27% incidence level in the years 1978–1981 (detection limit, 30–50 ng/g) and 69% incidence levels in the year 1986–1993 (detection limit, 5–10 ng/g) ***(3)***. In a survey of retail U.S. grain-based food products, zearalenone was detected in 78% of corn meal samples and 57% of popcorn sample (detection limit, 2.5 ng/g) ***(4)***. Zearalenone is stable through processing such as milling, baking, and fermentation.

Zearalenone exerts hyperestrogenism in mammalian reproductive system. The symptoms include hyperemia, edematous swelling of the vulva, mammary gland enlargement, and hypertrophy of the nipples. Also, the mycotoxin is thought to cause infertility and abortions in farm animals. Regarding carcinogenicity, zearalenone has been classified in the category of "limited evidence

From: *Methods in Molecular Biology, vol. 157: Mycotoxin Protocols*
Edited by: M. W. Trucksess and A. E. Pohland

in experimental animals for carcinogenicity" by the International Agency for Research in Cancer ***(5)***.

The daily intake of the highest relative consumption group (1-to-4-yr-old males and females) in Canada for zearalenone and its metabolites was estimated to be 50–100 ng/kg bw/day ***(6,7)***. Regarding exposure assessment of zearalenone, its contribution to the total estrogenic burden from other environmental sources should also be considered. Kuiper-Goodman ***(8)*** estimated the tolerable daily intake (TDI) for zearalenone as 50 ng/kg body wt/d based on the virtually safe dose (VSD), 100 ng/kg body wt/d based on the no-hormonal effect level (NHEL). Maximum tolerated levels of zearalenone have been established in several countries: Austria, 60 ng/g in durum wheat; Brazil, 200 ng/g in maize; France, 200 ng/g in cereals, vegetable oils; Russia, 1000 ng/g in cereals and nuts; Uruguay, 200 ng/g in maize, barley ***(9)***. In the United States, zeranol, a zearalenone derivative is used as a growth-promoting agent for beef cattle and no residual zeranol (detection limit of assay, 20 ng/g) is permitted in uncooked edible tissues of cattle and sheep.

The ability to detect zearalenone and other mycotoxins rapidly is useful in the screening of cereal grains entering the food and feed supply. Antibody-based methods facilitate such rapid assessments. Zearalenone-specific antibodies have been developed for screening of zearalenone in several commodities such as corn, wheat, and barley. Using polyclonal antibodies, zearalenone as low as 1 ng/g corn ***(10)*** and 2.5 ng/g in grain-based food ***(4)*** can be detected by competitive direct enzyme-linked immunosorbent assay (ELISA), and at 1 ng/mL in methanol-water extracts of corn, wheat, and feed by competitive indirect ELISA ***(11)***. With a monoclonal antibody, corn artificially contaminated with zearalenone at 50–500 ng/g can be analyzed by competitive direct ELISA (**Table 1**). In a First Action, AOAC Method ***(12)***, a competitive direct ELISA employing a monoclonal antibody was adopted as applicable to detection of zearalenone in corn, wheat, and pig feed at ≥800 ng/g.

For the direct competitive ELISA, zearalenone-specific antibody is adsorbed on microtiter wells (**Fig. 1A**). A mixture of equal volumes of free zearalenone and zearalenone-enzyme conjugate is placed into each well. Thus, free zearalenone and zearalenone-enzyme conjugate compete for the binding site of antibodies in coated wells. The unbound competitors are removed by washing. The amount of bound zearalenone-peroxidase conjugate is determined after the color is developed with enzyme substrate. The amount of free zearalenone is inversely related to color development.

For the indirect competitive ELISA, zearalenone-carrier conjugate is adsorbed onto the microtiter plate (**Fig. 1B**) wells. Sample extract containing free zearalenone is added to each well and immediately followed by adding of the zearalenone-specific antibody (primary antibody) of the same volume.

Table 1
Recovery of Zearalenone from Spiked Corn by Competitive Direct Monoclonal ELISA[e]

Zearalenone added, µg/kg[b]	Sample	Recovery[a] µg/kg	Recovery[a] %	Interwell CV,[c,d] %
50	1	54.5 ± 9	109	3.8
50	2	49.4 ± 5	99	2.4
50	3	54.5 ± 11	109	5.2
250	1	292 ± 13	117	1.7
250	2	267 ± 20	107	2.9
250	3	279 ± 29	112	4.2
500	1	579 ± 43	116	4.2
500	2	553 ± 34	111	3.6
500	3	606 ± 22	121	2

[a]Each sample was spiked separately and then extracted with 70% methanol and assayed in 4 replicates.

[b]Interassay coefficients of variation (n = 3) for 50, 250, and 500 µg/kg were 4.5, 3.7, and 3.7%, respectively. Mean interassay coefficient of variation was 4%.

[c]Coefficient of variation. (based on absorbance).

[d]Mean interwell CV was 3.3%.

[e]Adapted from **ref. *14***.

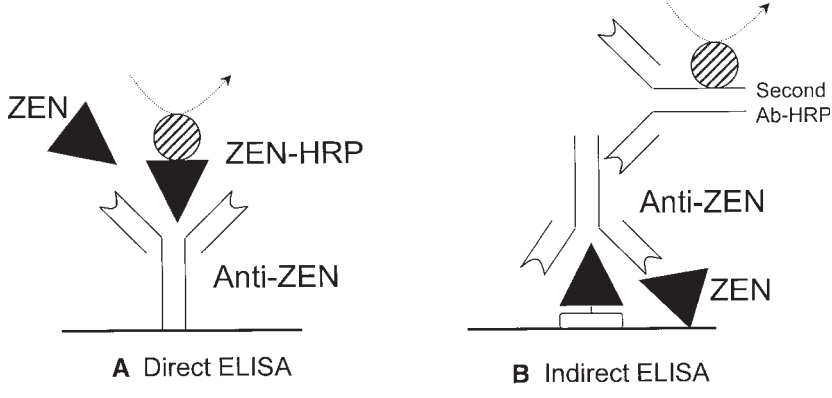

Fig. 1. Detection of zearalenone (ZEA) by direct and indirect ELISA using polyclonal and monoclonal antibodies. HRP refers to the enzyme horseradish peroxidase.

Thus, free zearalenone and conjugated zearalenone compete for the binding sites of the primary antibody. The primary antibody bound with free zearalenone is removed by washing. The primary antibody bound with conjugated zearalenone is detected with an enzyme conjugated second

antibody, which is an antispecies antibody against the primary antibody. The free zearalenone concentration in the sample solution is inversely related to color development.

Both polyclonal and monoclonal antibody-based immunoassays have become important analytical tools in food analysis and environmental monitoring. However, the approach includes inherent disadvantages of the use of animals, tissue culture materials as well as extensive commitment of time, labor, and expense. A recently developed recombinant antibody approach uses a bacterial expression system, instead of mice for the rapid selection of antibodies. Due to the relative ease of bacterial fermentation, cloning and expression of antibody fragments in bacteria, this technology may be an attractive alternative to antibody production by classic animal or hybridoma methods.

ScFv QY1.5 is a specific recombinant antibody for zearalenone that was produced by Yuan et al. ***(13)***. In this approach, mRNA is isolated from an antizearalenone hybridoma cell line (2G3-6E3-2E2) and the first-strand cDNA is synthesized with mRNA template, reverse transcriptase, and primers. The heavy-chain variable (V_H) and kappa light-chain variable (V_L) region genes are isolated and amplified by the polymerase chain reaction using specific primers. The V_H and V_L fragments are joined by a DNA linker encoding peptide $(Gly_4Ser)_3$ to form a scFv DNA fragment. The scFv DNA fragment is cloned into a phagemid (pCANTAB5E) and expressed as a fusion protein with E tag and phage M13 p3 protein through *Escherichia coli* TG1. In the presence of helper phage M13ko7, the scFv fusion protein is displayed on the surfaces of recombinant phages. Using ELISA, high-affinity scFv phages are selected, and the selected scFv phages are infected into *E. coli* HB2151. Soluble E-tagged scFv is secreted into supernatant of the *E. coli* culture. Cross–reactivities of the scFv QY1.5 to α-zearalenol, β-zearalenol and the corresponding diastereomers of zearalanol were in the higher range of 26–82% as compared to a previously described monoclonal antibody, 8–26% (**Table 2**).

For competitive direct assays, antibody to the "E tag" peptide marker of the scFv is adsorbed on microtiter wells to functions as a capture antibody (**Fig. 2A**). Recombinant antibody, scFv QY1.5 specific to zearalenone is attached via the anti-E tag. A mixture of free zearalenone and zearalenone-enzyme conjugate is added, and these compete for the binding site of scFv QY1.5. The amount of zearalenone-enzyme conjugate bound with scFv QY1.5 is determined by measuring absorbance developed after adding of enzyme substrate. The free zearalenone concentration in the sample is inversely related to color development.

For the indirect ELISA using recombinant antibody, microtiter wells are coated with zearalenone-carrier protein conjugate (**Fig. 2B**). Free zearalenone is added into each well and immediately followed by the recombinant antibody

Table 2
Comparison of Cross-Reactivities of scFv Antibody and Monoclonal Antibody to Zearalenone Analogs[b]

	Monoclonal antibody		scFv QY1.5	
	IC_{50} (ng/mL)	Cross-reactivity[a] (%)	IC_{50} (ng/mL)	Cross-reactivity %
Zearalenone	17	100	14	100
α-Zearalenol	66	26	17	82
β-Zearalenol	159	11	54	26
α-Zearalanol	212	8	23	62
β-Zearalanol	175	10	54	26

[a]Cross-reactivity defined as (IC_{50} of zearalenone/IC_{50} of analog) (100%).
[b]Adapted from **ref. *13***.

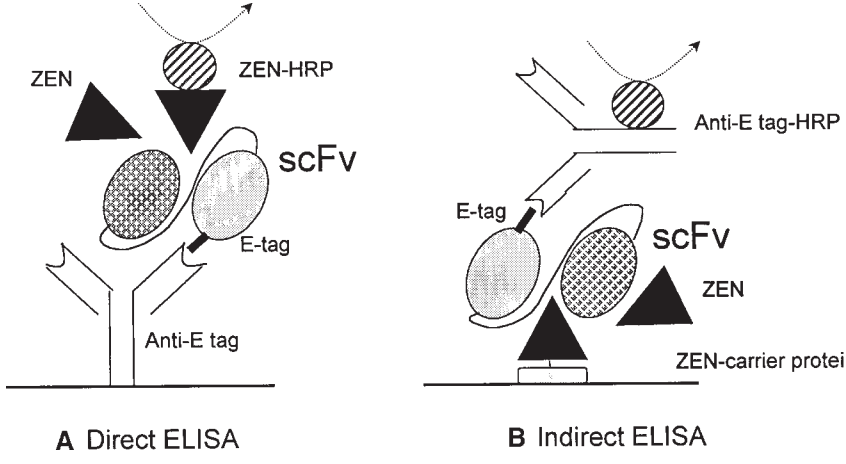

Fig. 2. Detection of zearalenone (ZEA) by direct and indirect ELISA using recombinant single chain antibody variable fragment (scFv). HRP refers to the enzyme horseradish peroxidase.

scFv QY1.5 specific to zearalenone. Free zearalenone and the solid phase conjugated zearalenone compete for the binding site of scFv QY1.5. The scFv (primary antibody) bound with free zearalenone is washed out. Enzyme conjugated anti-E tag is added as a second antibody to bind the primary antibody bound with conjugated zearalenone. The color is developed with enzyme substrate and the absorbance measured is inversely related to the concentration of free zearalenone.

In this chapter, specific steps for the above-described ELISA methods that utilize monoclonal, polyclonal, and recombinant antibodies to zearalenone are described.

2. Materials

1. Microtiter plate (96 well) or strips.
2. Microplate washer and microtiter reader.
3. Forced-air drying oven adjustable to 40°C.
4. Incubators adjustable to 4°C and 37°C.
5. Phosphate-buffered saline (PBS), 0.01 *M*, pH 7.4
6. Zearalenone stock solution of 1 mg/mL in 100% methanol. Stock zearalenone solution should be stored in tightly sealed vial at –20°C. Immediately prior to assay, stock solution is diluted to 0–1000 ng/mL with buffer solution or analyte-free extract. Methanol concentration in standard should be adjusted to the same concentration as the unknown sample solution.
7. Antibodies
   a. Rabbit antibodies to zearalenone were prepared against bovine serum albumin conjugates as described by Warner et al. ***(10)***.
   b. Monoclonal antibodies to zearalenone were prepared as described by Dixon et al. ***(14)***.
   c. Recombinant antibody, scFv QY1.5 supernatant was prepared as described by Yuan et al. ***(13)***.
8. Zearalenone-horseradish peroxidase HRP conjugate prepared as described by Warner et al. ***(10)***.
9. Zearalenone-bovine serum albumin conjugate prepared as described by Liu et al. ***(11)***.
10. Goat antimouse IgG-horseradish peroxidase or goat antirabbit IgG-HRP (Sigma Chemical, St. Louis, MO).
11. Anti-E tag antibody (Phamacia Biotech, Piscataway, NJ).
12. Anti-E tag-HRP conjugate (Pharmacia Biotech).
13. Carbonate-bicarbonate buffer, 0.05 *M*, pH 9.6.
14. PBS-Tween washing solution consisting of Tween-20, 0.02% (v/v), in PBS.
15. Blocking solutions consisting of bovine serum albumin (BSA), 1% (wt/v), in PBS or of 3% (wt/v) nonfat dry milk in PBS; blocking solutions should be prepared fresh daily.
16. Substrate solution: 12.5 mL of citrate buffer, pH 5.3 + 200 µL of 6 mg/mL 3,3',5,5'-tetramethyl benzidine (TMB) substrate in dimethylsulfoxide + 50 µL of 1% H_2O_2 sufficient for one plate. For TMB substrate solution, 1% (v/v) H_2O_2-water is diluted from 30% H_2O_2 stock solution. This should be kept at 4°C in the dark and can be used for one month.
17. Enzyme stopping solution: 10% (v/v) sulfuric acid in distilled water.

3. Methods

3.1. Preparation of Sample Extract

1. Grind corn, wheat, or feed so they can be passed through a No. 20 sieve.
2. Blend the ground sample thoroughly.
3. Place 20 g of test sample into 500 mL glass-stoppered flask.
4. Add 100 mL of 70% (v/v) methanol-water to the flask and close tightly
5. Shake for 30 min on wrist-action shaker.
6. Allow the solution to stand for 10 min at room temperature to facilitate settling.
7. Filter the supernatant through Whatman No. 4 filter paper and collect filtrate.

3.2. Competitive Direct Assay Using Monoclonal and Polyclonal Antibodies

1. Add 100 µL of monoclonal and polyclonal antibody solution diluted to between 0.5 to 10 µg/mL in PBS to each well of a microtiter plate.
2. Incubate the plate overnight in a forced-air drying oven of 40°C.
3. Wash the plate by filling and aspirating four times with 300 µL of 0.02% PBS-Tween washing solution.
4. Block nonspecific binding sites by adding 300 µL of BSA blocking solution (blocking solution) to each well.
5. Incubate the plate for 1 h at 37°C.
6. Wash the plate four times with PBS-Tween.
7. Add the mixture (100 µL) of an equal volume of zearalenone standard or sample solution (*see* **Note 1**) and zearalenone-HRP solution diluted appropriately in BSA blocking solution (e.g., 1:1000 dilution of a 0.5 mg/mL stock) to each well.
8. Incubate the plate for 1 h at 37°C.
9. Wash the plate six times with PBS-Tween.
10. Add 100 µL of TMB substrate solution to each well.
11. Incubate the plate for 30 min at 37°C.
12. Stop enzyme reaction with 100 µL of enzyme stopping solution. The color is changed to yellow.
13. Read the absorbance of each well in a microplate reader at 450 nm. Plot % binding inhibition of control vs log zearalenone standard concentration (**Fig. 3**).

3.3. Competitive Indirect Assay Using Polyclonal or Monoclonal Antibodies

1. Add 100 µL of zearalenone-bovine serum albumin (10 µg/mL) dissolved in carbonate-bicarbonate buffer, pH 9.6 to each well of a microtiter plate.
2. Incubate the plate overnight at 4°C refrigerator.
3. Wash the plate four times with 300 µL of PBS-Tween.

4. Block nonspecific binding sites by adding 300 µL of BSA blocking solution to each well.
5. Incubate the plate for 1 h at 37°C.
6. Wash the plate four times with 300 µL of PBS-Tween.
7. Add 50 µL of zearalenone standard or test sample solution, and then, immediately add 50 µL of monoclonal or polyclonal antibody solution diluted appropriately in BSA blocking solution to each well.
8. Incubate the plate for 1 h at 37°C.
9. Wash the plate six times with PBS-Tween.
10. Add 100 µL of goat antimouse IgG-HRP to the monoclonal antibody coated wells, goat antirabbit IgG-horseradish peroxidase to the polyclonal antibody coated wells diluted appropriately in blocking buffer (e.g., 1:1000–2000 dilution of a 1 mg/mL stock).
11. Incubate the plate for 1 h at 37°C.
12. Wash the plate six times with PBS-Tween.
13. Add 100 µL of TMB substrate solution to each well.
14. Incubate the plate for approx 30 min at 37°C.
15. Add 100 µL of enzyme stopping solution to each well.
16. Read the absorbance of each well at 450 nm. Plot % binding inhibition of control vs log zearalenone concentration (**Fig. 3**).

3.4. Competitive Direct Assay Using Recombinant Antibody

1. Add 100 µL of anti-E tag (10 µg/mL) in PBS to each well of a microtiter plate.
2. Incubate the plate overnight in a forced-air drying oven of 40°C
3. Wash the plate by filling and aspirating it four times with PBS-Tween.
4. Block nonspecific binding sites by adding 300 µL of nonfat dry milk blocking solution to each well.
5. Incubate the plate for 1 h at 37°C.
6. Wash the plate four times with PBS-Tween.
7. Add 100 µL of E-tagged scFv QY1.5 diluted appropriately in nonfat dry milk blocking solution (e.g., 1:2 dilution) to each well.
8. Incubate the plate for 1 h at 37°C.
9. Wash the plate six times with PBS-Tween.
10. Add the mixture (100 µL) of an equal volume of zearalenone-HRP diluted in nonfat dry milk blocking buffer (1:1000) and zearalenone standard or sample solution to the well.
11. Incubate the plate for 1 h at 37°C.
12. Wash the plate 6 times with PBS-Tween.
13. Add 100 µL of TMB substrate solution to each well.
14. Incubate the plate for 30 min at 37°C is developed.
15. Add 100 µL of enzyme stopping solution to each well to stop the enzyme reaction.
16. Read the absorbance of each well at 450 nm using a microtiter plate reader. Plot % binding inhibition of control vs log zearalenone standard concentration (**Fig. 3**).

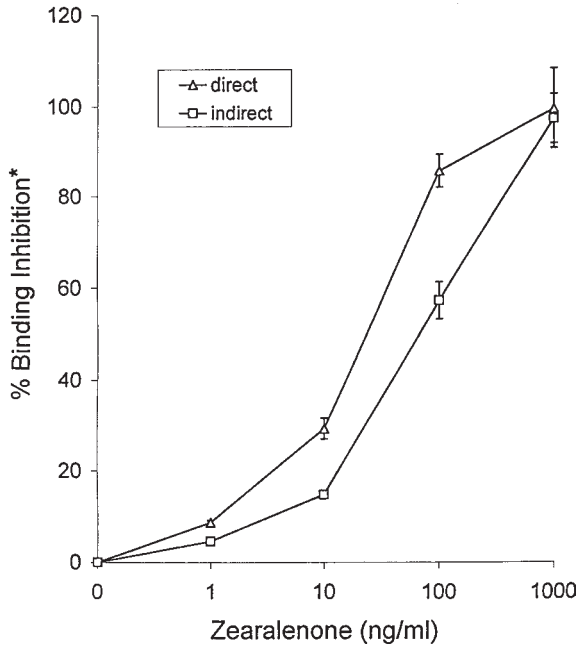

Fig. 3. ELISA standard curves for zearalenone employing recombinant single chain antibody variable fragment (scFv). *Percent inhibition of control activity = $1 - B/B_0 \times 100$, where B_0 is the optical density in a sample known not to contain the analyte (control) and B the optical density of the unknown sample (or standard).

3.5. Competitive Indirect Assay Using Recombinant Antibody

1. Add 100 µL of zearalenone-bovine serum albumin (5 µg/mL) in carbonate-bicarbonate buffer to wells of a microtiter plate.
2. Cover plate with aluminum foil and incubate the plate overnight at 4°C.
3. Wash the plate by filling and aspirating it four times with PBS-Tween.
4. Block nonspecific binding sites by adding 300 µL of nonfat dry milk blocking solution to each well.
5. Incubate the plate for 1 h at 37°C.
6. Wash the plate four times with PBS-Tween.
7. Add 50 µL of zearalenone standard or sample solution, and then immediately add 50 µL of E-tagged scFv diluted appropriately in blocking solution (e.g., 1:15 dilution) to the well.
8. Incubate the plate for 1 h at 37°C.
9. Wash the plate six times with PBS-Tween.
10. Add 100 µL of anti-E tag-HRP diluted in nonfat dry milk blocking solution (e.g., 1:8000) to each well.

11. Incubate the plate for 1 h at 37°C.
12. Wash the plate 6 times with PBS-Tween.
13. Add 100 µL of TMB substrate solution to each well.
14. Incubate the plate for approx 5 min at 37°C.
15. Add 100 µL of enzyme stopping solution to each well.
16. Read the absorbance of each well at 450 nm in a microtiter plate reader. Plot % binding inhibition of control vs log zearalenone concentration (**Fig. 3**).

4. Notes

1. Zearalenone in corn and wheat is usually extracted using 70% (v/v) methanol in water solution. Antibodies, immunoglobulins can be affected by the organic solvents such as acetonitrile, acetone, methanol. The vulnerability of antibodies to concentrations of organic solvent varies and it affects overall performance of the ELISA. During the optimization process, the extraction solvent effect should be evaluated and an appropriate concentration of the organic solvent should be chosen for the assay. The effects of methanol on recombinant antibody scFv QY1.5, which is highly susceptible to the organic solvent, are shown in **Fig. 4**. Sample matrix can also be a factor in determining the sensitivity of ELISA. In sample extracts, there are lipids, pigments, and so on, besides zearalenone.
2. A food matrix such as corn can sometimes alters the ELISA inhibition curve. Thus in the assay of zearalenone in foods, standard curves should be prepared using mycotoxin-free sample extract. When reporting of detection limit of assay, the matrix analyzed should be mentioned (e.g., in 1% methanol-buffer solution, or corn extract diluted to 5% methanol concentration).
3. Using scFv QY1.5, analysis of zearalenone in corn has been successfully applied to in both competitive direct and indirect ELISAs. The detection limits were 4 ng/mL in diluted corn extract (275 ng/g corn) by direct assay and 15 ng/mL (1000 ng/g corn) by indirect assay. Recoveries of zearalenone artificially contaminated at the level of 500–3000 ng/g were 95–113%, and 100–121% by direct and indirect assays, respectively (**Table 3**).
4. The competitive direct assay with scFv QY1.5 can detect zearalenone at 500 ng/g in corn, whereas competitive direct assay with monoclonal antibody noted in earlier section can detect 50 ng/g. Thus, it appears that assay sensitivity by scFv QY1.5 is 10 times lower than that of monoclonal. A major factor in this relatively reduced performance is likely to be destabilization of scFv QY1.5 by methanol. This necessitates more extensive dilution of methanol corn extract (from 70% methanol to 5%), thus lowering sensitivity.
5. Even though the scFv has limitations in sensitivity, it is expected that scFv recombinant antibodies hold promise as alternatives to monoclonal and polyclonal ELISA approaches. It is anticipated that scFvs (i.e., preventing of dissociation of V_H and V_K domains or conformational change of E tag peptide attached to scFv) resistant to organic extract solvent can be developed by strengthening the interactions between V_H and V_K domains by engineering of stronger hydrogen bonds, disulfide bonds, and increasing hydrophobic area or electrostatic interactions.

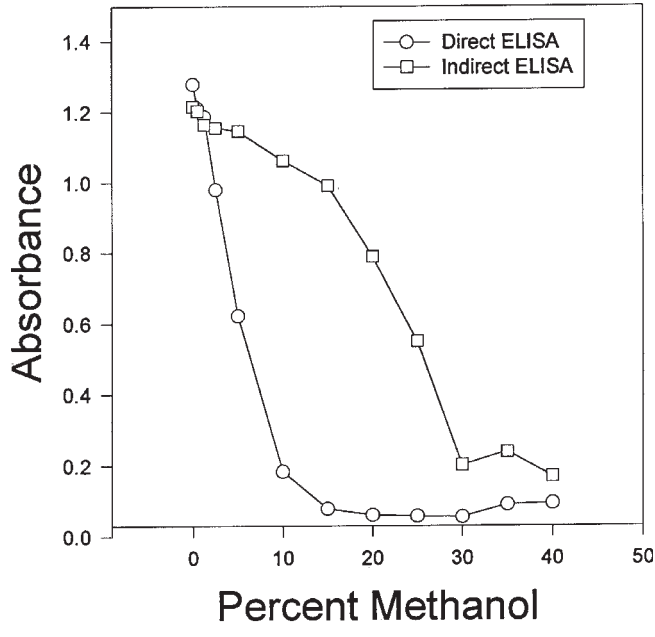

Fig. 4. Effect of methanol on marker ligand binding in ELISAs employing recombinant single chain antibody variable fragment.

Table 3
Recovery of Zearalenone from Spiked Corn by ELISA with scFv QY1.5

Amount added (ng/g)	Percent Recovery			
	Direct		Indirect	
	Mean[a] ± SD	(CV,%)[b]	Mean[a] ± SD	(CV,%)[b]
500	94.6 ± 7.43	(7.8)	120.8 ± 32.0	(26.6)
1,000	112.2 ± 2.06	(1.8)	107.0 ± 1.38	(1.3)
2,000	110.3 ± 9.84	(8.9)	102.0 ± 4.06	(3.9)
3,000	112.6 ± 8.22	(7.3)	100.4 ± 11.7	(11.7)

[a]The assays were repeated two times in total of three runs from spiking to ELISAs.
[b]The average coefficients of variance of interwells ($n = 6$) were in the range of 5.6–7.8% in direct assay, 8.2–27.1% in indirect assay in added zearalenone concentrations.

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15

Chromatographic Method for *Stachybotrys* Toxins

Simon F. Hinkley and Bruce B. Jarvis

1. Introduction

The filamentous fungus *Stachybotrys atra* (also known as *S. chartarum*) has a colorful past. *S. atra* was first described over 150 years ago by Corda in 1837, who isolated the mold from damp wallpaper in a home in Prague. Although *S. atra*-related animal intoxications have no doubt existed for some time, it was not until 1931 that this toxicosis was described, and, not until the late 1930s, was the condition recognized as a mycotoxicosis: stachybotryotoxicosis ***(1)***. Horses are particularly sensitive to this mold which is a common contaminant of damp hay and straw.

Stachybotryotoxicosis in humans is rare and has been reported most commonly in workers who handle moldy straw and hay ***(2)***. However, in 1986, Croft et al. reported an apparent episode of stachybotryotoxicosis in a family living in a Chicago home that was infested heavily with *S. atra* as a result of very damp conditions ***(3)***. Of some notoriety is the report of the strong association of pulmonary hemisiderosis to infant fatalities in Cleveland, Ohio ***(4)***. However, the cause and effect relationship between *S. atra* and this syndrome is controversial ***(5,6)***.

The rare occurrence of *S. atra*-induced toxicosis in humans stems from the specific conditions this fungus requires for significant growth. In damp buildings, high cellulose and fibrous surfaces are favored for growth. For example, water-damaged gypsum board ***(7–9)***, wood fiber board or dust-lined air-conditioning ducting ***(3)***. For the generation of sufficient quantities of the toxins to analyze, we have found that optimum toxin production in the laboratory can be achieved by the use of par-boiled Uncle Ben's rice as a solid culture medium ***(10)***.

Chemical investigation of *S. atra* has provided many highly toxic and novel compounds ***(11)***. In fact, some of the most cytotoxic fungal metabolites ever

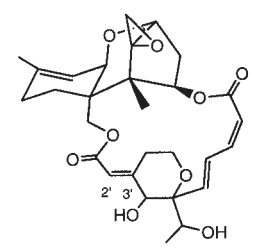
From: *Methods in Molecular Biology, vol. 157: Mycotoxin Protocols*
Edited by: M. W. Trucksess and A. E. Pohland

discovered are products of *S. atra* fermentation ***(12)***, and by no means have all the active constituents been isolated and identified. *S. atra* can produce a diverse array of compounds, and the variation in individual and overall metabolite levels from one isolate to the next is remarkable. Similarly, the toxicity of two isolates of *S. atra* recovered from almost identical environmental conditions, and then grown in the laboratory, may exhibit widely different biological activity profiles ***(13)***.

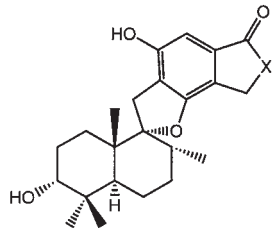
As part of the ongoing research in this laboratory into *S. atra*, we have developed two analytical methods for the identification and quantification of the major bioactive constituents of this species. It is currently accepted that the trichothecene mycotoxins of *S. atra* are responsible for the high toxicity of this fungus (e.g., satratoxins). The components of interest are the series of highly cytotoxic macrocyclic trichothecenes (I) (***12***; **Fig. 1**), and the immunosuppresent phenylspirodrimanes represented by (II), and dialdehydes (III) ***(11)***. Most of the attention on the *Stachybotrys* toxins has been focused on the satratoxins, where LD50s in mice are on the order of 1 mg/kg ***(12)***. However, there are a plethora of other trichothecene toxins present in *S. atra* cultures at much lower levels. Therefore, one method has been designed to quantitate the individual constituents of a crude culture extract, whereas another method has been developed to detect trichothecenes in small environmental samples or in *S. atra* cultures that have only low levels of trichothecenes.

Over a hundred macrocyclic trichothecenes and related trichoverroids have been found as natural products, isolated from a number of fungal genera and from two shrubs of the genus *Baccharis* ***(12,14–17)***. *Stachybotrys atra* produces the macrocyclic trichothecenes: satratoxins F, G, and H ***(12)***, isosatratoxins F, G, and H ***(12–14)***, roridin E ***(12)***, and verrucarins B and J ***(3)***. The trichoverroids, trichoverrols A and B and trichoverrins A and B ***(13,14)***, and roridin L-2 ***(13)*** are also reported isolated from cultures of *S. atra*. Related fungi, *S. cylindrospora* and *Memnoniella echinata* appear not to produce the trichoverroids or macrocyclic trichothecenes, but do produce the simple trichothecenes, trichodermol, and trichodermol acetate (trichodermin) ***(18,19)***.

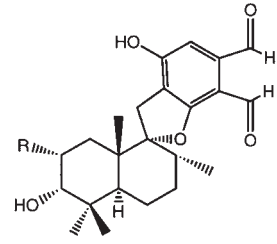
Recently, we have discovered a new series of biologically active molecules which are produced by various isolates of *S. atra*. These compounds, the atranones (IV) ***(20)***, are included in this article even though their complete biological activity spectrum has not been determined, and their role in stachybotryotoxicosis, if any, is not yet clear. Of note is that although they are significantly less cytotoxic than the macrocyclic trichothecenes (unpublished results), they are occasionally expressed at levels considerably higher than those of the trichothecenes. To date, the atranones and macrocyclic trichothecenes have not been detected in the same isolate of *S. atra*. Work is in progress to explain this interesting phenomenon.



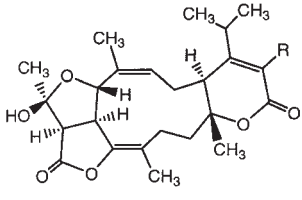
I-a Satratoxin H
I-b Satratoxin G: 2'β,3'β-epoxide



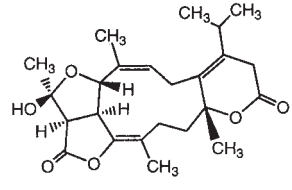
II-a Stachybotrylactone X = O
II-b Stachybotramide X = NCH_2CH_2OH



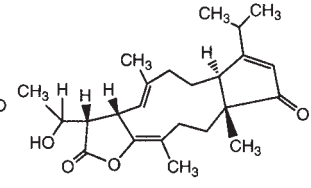
III-a Stachybotrydial R = H
III-b K-76 R = OH



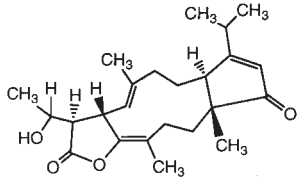
IV-a Atranone A: R = H
IV-b Atranone B: R = OMe



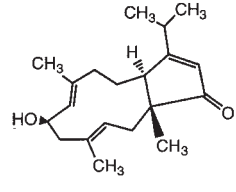
IV-c Atranone C



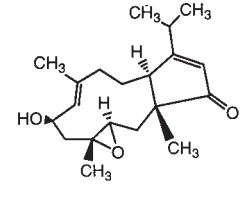
IV-d Atranone D



IV-e Atranone E



IV-f Dolabelladiene A



IV-g Epoxydolabellane A

Fig. 1. Representative examples of mycotoxins produced by *S. atra.*

The macrocyclic trichothecenes, atranones and phenylspirodrimanes are well suited to analysis by high performance liquid chromatography (HPLC). The first analytical method described in **Subheading 3.** utilizes an ultra violet-visible diode array detection (UV/Vis DAD) system. Modern reversed phase chromatography with gradient programmed solvent delivery provides the required separation power needed to analyze the highly complex spectrum of metabolites produced by *S. atra*. The characteristic retention times and UV/Vis spectra allow rapid identification of individual components down to 0.1–1 ng levels.

Reversed phase (RP)-HPLC is a mainstay chemical analysis technique and is well suited for separating and identifying the compounds of interest in this study. Previous analytical analysis of macrocyclic trichothecenes has generally utilized RP-HPLC with ultraviolet/visible detection ***(21,22)*** although studies using gas chromatography-mass spectometry (GC-MS) ***(23)*** and liquid chromatography-mass spectometry (LC-MS) ***(24)*** have also been reported. C-18 Stationary phases are robust, inert and can provide excellent separation for a wide range of constituents in a single chromatographic run. It would appear that normal phase silica and polyethyleneimine silica (PEI) stationary phases would be very suitable as analytical chromatographic stationary phases as they have been used successfully in the large and small scale fractionation of *S. atra* and other crude fungal and plant extracts ***(12,13,20)***. However, only C-18 reverse phase chromatography is able to cleanly separate the individual components and provide adequate separation with a binary solvent system over short total run time. Furthermore, C-18 columns can be reequilibrated post-run much more rapidly than silica or PEI columns and do not require as much maintenance (e.g., guard column replacement).

The macrocyclic trichothecenes are of medium polarity and contain an enone or dienone chromophore that absorbs at ~260 nm (**Fig. 2**). The molar extinction coefficients for most of these compounds is ε~15,000 (**Table 1**), and with UV/Vis detection, it is possible to measure nanogram quantities. Retention times for each peak coupled with the additional information provided from diode array detection is sufficient to characterize individual components.

Atranones (IV) have less characteristic UV absorptions, with only a weak chromophore present as the α,β-unsaturated ketone or lactone group, or just a carbonyl end-absorption (**Fig. 3**). However, relative retention times on C-18 stationary phase coupled with UV/Vis spectra serves clearly to identify these compounds.

The third series of compounds to be quantified are the spirocyclic compounds (II). The UV/Vis spectra for these compounds clearly show that greater sensitivity would be achieved if a shorter wavelength than 260 nm was utilized (**Fig. 4**). However, because the spirocyclic compounds are generally present at a higher level than the trichothecenes, sensitivity is not of great concern, and the 260 nm absorption was routinely used. Shorter wavelength problems such as baseline instability and acid buffer absorption can also be avoided in this way. For very small sample sizes where sensitivity was of premium importance, calibration data at 210 nm are used.

One further series of compounds has been included in our quantitative analysis. These are the spirocyclic aldehydes illustrated by the dialdehydes stachybotrydial III-a and K-76 III-b (**Fig. 1**). Like the spirocyclic series II, a large array of these metabolites are produced by *S. atra*. Rather than isolate

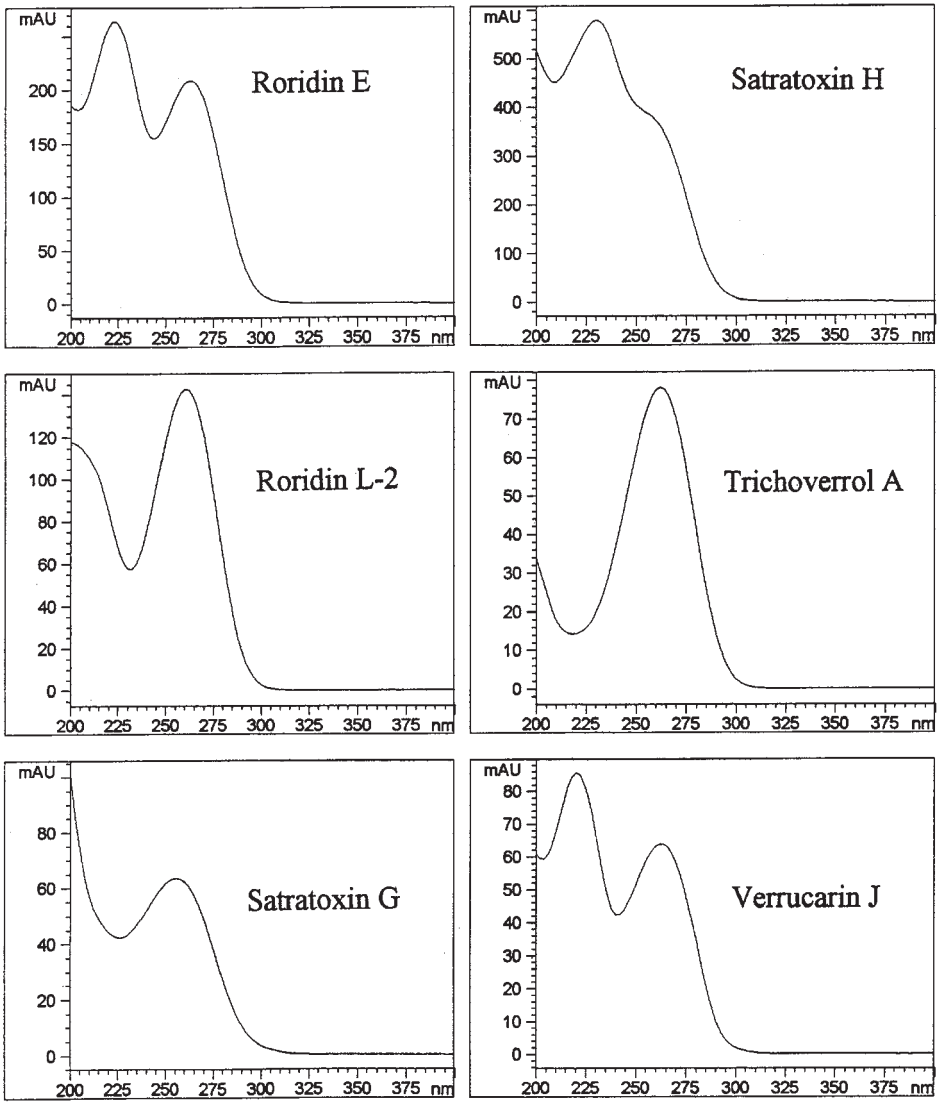


Fig. 2. UV spectra for selected macrocyclic trichothecenes.

and identify each individual component and generate a unique standard curve, we have prepared a standard curve for stachybotrylactone II-a and stachybotrydial III-a only. Other compounds of the related series were identified by DAD UV/Vis spectra, and quantitation approximated using the standard curve for II-a and III-a . This assumption is valid as the molar extinction coefficient at 260 nm for these compounds is very similar. The molecular mass

Table 1
Compounds for HPLC Analysis

	RT[g]	Compound name	UV nm (ε)	Mass/Formula
1	18.1	Dechlorogriseofulvin	322(4100), 290(24500), 253(15100), 235(21400)[b]	318.11 $C_{17}H_{18}O_6$
2	15.0	Epidechlorogriseofulvin	320(7244), 288(28840), 248(21880)[c]	318.11 $C_{17}H_{18}O_6$
3	18.2	Griseofulvin	324(5200), 291(21800), 252(12500), 236(21300)[b]	352.07 $C_{17}H_{17}O_{6Cl}$
4	25.1	Roridin E	263(19900), 223(25100), 195(15800)[a]	514.26 $C_{29}H_{38}O_8$
5	24.2	Isororidin E	262(16000), 223(24000)[a]	514.26 $C_{29}H_{38}O_8$
6	24.0	Epiisororidin E	260, 224 (**Note 20**)	514.26 $C_{29}H_{38}O_8$
7	25.2	Epiroridin E	262, 224 (**Note 20**)	514.26 $C_{29}H_{38}O_8$
8	19.5	Roridin A	263(18600)[a]	532.27 $C_{29}H_{40}O_9$
9	19.1	Isororidin A	262 (**Note 20**)	532.27 $C_{29}H_{40}O_9$
10	15.4	Roridin L-2	259(24650)[e]	530.25 $C_{29}H_{38}O_9$
11	30.8	Roridin H	260(18200), 224(24500), 195(15800)[a]	512.24 $C_{29}H_{36}O_8$
12	17.5	Isosatratoxin F	251 (17700)	542.33 $C_{29}H_{34}O_{10}$
13	15.4	Satratoxin G	256(6500)[a]	544.23 $C_{29}H_{36}O_{10}$
14	19.3	Isosatratoxin G	260 (**Note 1**)	544.23 $C_{29}H_{36}O_{10}$
15	16.5	Satratoxin H	225(14700), 255(10400)[b]	528.24 $C_{29}H_{36}O_9$
16	16.2	Trichoverrin A	260(39800)[d]	532.27 $C_{29}H_{40}O_9$
17	15.9	Trichoverrin B	260(33800)[d]	532.27 $C_{29}H_{40}O_9$
18	10.2	Trichoverrol B	260(33900)[d]	420.21 $C_{23}H_{32}O_7$
19	10.2	Trichoverrol A	260(36300)[d]	420.21 $C_{23}H_{32}O_7$
20	20.2	Verrucarin A	260(17700)[a]	502.22 $C_{27}H_{34}O_9$
21	23.2	Verrucarin B	258.5(23400)[a]	500.20 $C_{27}H_{32}O_9$
22	26.9	Verrucarin J	196(15500), 219(19900), 262(14500)[a]	484.21 $C_{27}H_{32}O_8$
23	22.1	Atranone A	224 (10500)	416.22 $C_{24}H_{32}O_6$
24	24.6	Atranone B	231 (10800)	446.23 $C_{25}H_{34}O_7$

25	22.7	Atranone C	end adsorbtion only	416.22 $C_{24}H_{32}O_6$
26	25.3	Atranone D	231 (14800)	386.25 $C_{24}H_{34}O_4$
27	26.8	Atranone E	226 (12500)	386.25 $C_{24}H_{34}O_4$
28	20.6	Dolabelladiene 6	236 (8620)	302.22 $C_{20}H_{30}O_2$
29	15.1	Epoxydolabellane 7	235 (8870)	318.22 $C_{20}H_{30}O_3$
30	25.3	Stachybotrylactone	218(28000), 268(5300), 309(2600)[f]	386.21 $C_{23}H_{30}O_5$
31	25.9	Stachybotrydial	248, 307, 359 (**Note 20**)	386.21 $C_{23}H_{30}O_5$

[a] EtOH, [b]MeOH from ***(32)***.
[c]$CHCl_3$ ***(19)***.
[d]MeOH ***(33)***.
[e]MeOH ***(34)***.
[f]EtOH ***(35)***.
[g]Retention time in min using the solvent system and columns defined in **Subheading 2.2.1.**

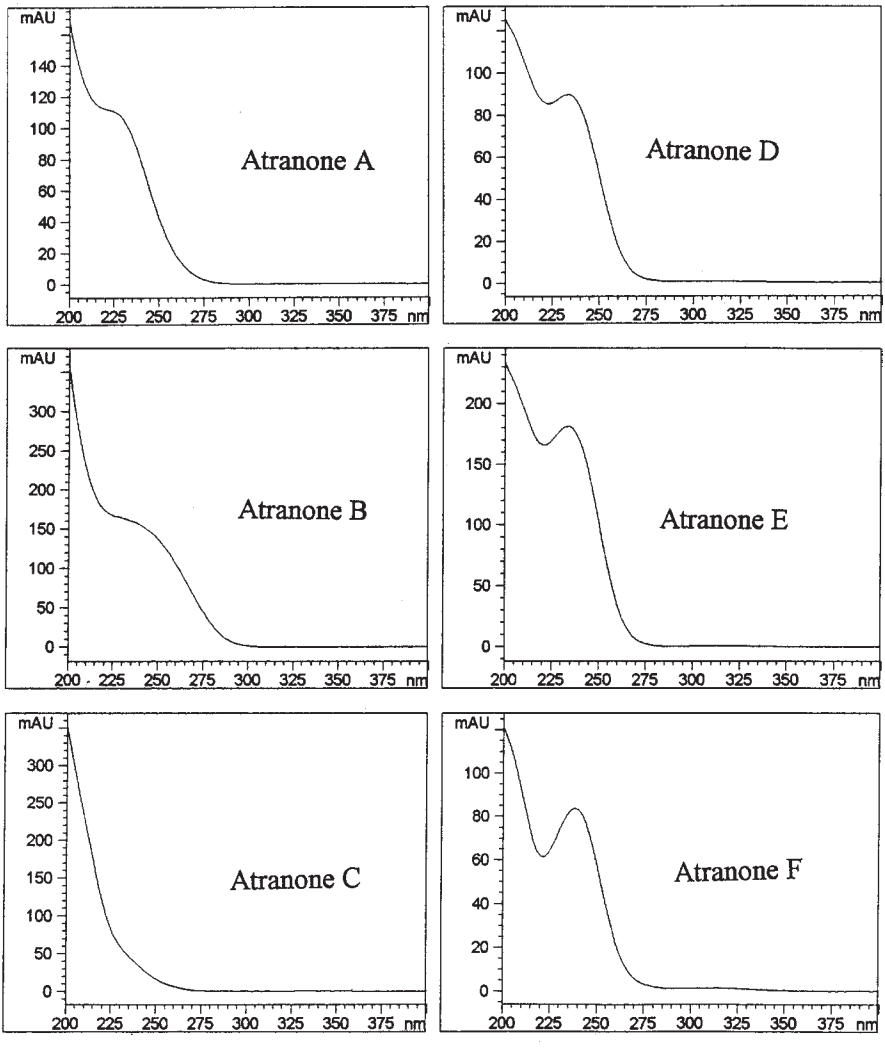


Fig. 3. UV spectra for atranones **A–F**.

for each compound can be determined by high performance liquid chromatography-mass spectrometry (HPLC-MS).

The crude extracts prepared by extraction of the rice cultures contain a myriad of components, the majority (by weight) of which are nontoxic. For example, a hexane wash of 10 of organic extract from *S. atra* will remove about 3.5 g of a nontoxic distinctive red oil and concentrate significantly the

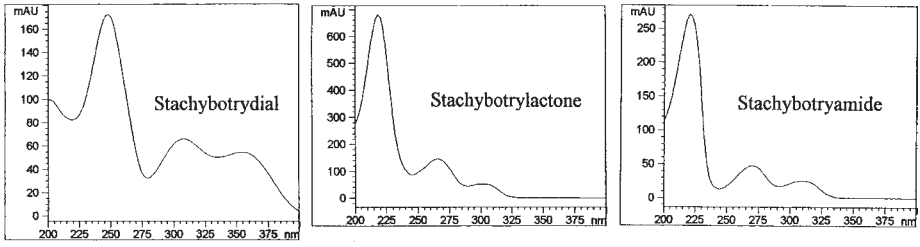


Fig. 4. UV spectra for selected phenylspirodrimanes.

bioactive constituents in the remaining material. Rather than solvent partitioning, we have found that fractionation of the crude extract using PEI silica gel is highly effective. One difficulty with using normal phase chromatography on crude extracts is that the solubility of the crude extract is often very poor in the initial column eluent. This problem has been overcome by adsorbing the crude material onto a portion of PEI stationary phase ***(21)***, which as a dry powder may then be loaded directly onto the column.

A great deal of effort was put into devising a method for the cleanup of *S. atra* crude extracts. Several stationary phases and permutations of solvent systems were attempted, but PEI silica proved to be superior. The advantage of PEI silica over other stationary phases is its remarkable ability to cleanly separate the marocyclic trichothecenes (I) from the bulk extract material. Such a clean separation is also observed for the atranones (IV). These compounds appear in fraction II (dichloromethane eluent, *see* **Subheading 3.3.1.1.**). Furthermore, the spirocyclic series of compounds (II and III) are cleanly separated from the less polar metabolites and are concentrated entirely in fraction III (methanol eluent).

The one disadvantage we have observed in using the PEI system, where the crude extract is adsorbed onto PEI, is that a portion of the more polar trichothecenes, trichoverrols A and B, may irreversibly bind to the PEI during the adsorption stage. This appears to be peculiar to these compounds only. In cases of small sample size, or where this may be a significant concern, the adsorption stage may be excluded. Instead, a portion of the methanol crude extract solution is concentrated to dryness, taken up in the minimum amount of dichloromethane, and applied to a PEI column that was prewashed in dichloromethane (*see* **Subheading 3.3.1.2.**). This column is eluted first with dichloromethane, then methanol, to give only two fractions. With this process the highly nonpolar materials collected previously in Fraction I (hexane eluent) are now combined with the trichothecenes or atranones.

The disadvantage of this method is that the use of dichloromethane for the first eluent combines the highly toxic compounds with the red oily material. The advantages are that without the PEI absorption step, the preparation of samples for HPLC analysis is considerably faster, and all compounds are recovered in high efficiency. However, the combination of the nonpolar lipid fraction in fraction II is detrimental to the HPLC C-18 guard column, and increases the frequency of replacement. For overall performance, the method of direct application (**Subheading 3.3.1.2.**) is the most commonly used in our laboratory.

For the efficient analysis of many samples solid phase extraction (SPE) chromatography has proven very effective. Only small amounts of stationary and mobile phase are required and column elution is rapid. PEI silica is not limited to small scale *S. atra* cleanup, and in fact, the large scale isolation of compounds to be used for reference data and calibration table generation are prepared using a very similar method ***(13,20)***. In brief, a PEI column washed with hexane will elute the macrocyclic trichothecenes (I) or atranones (IV), as increasing amounts of dichloromethane are introduced into the mobile phase. The spirocyclic compounds (II and III) will elute only with the introduction of methanol to the mobile phase ***(25)***.

Toxin analysis of the aqueous extract poses an additional complication in that removal of the extraction solvent by solvent evaporation is tedious. To remove the active organic constituents from this solution a C-18 reversed phase (RP) silica filtration has been developed. Passing the aqueous solution through a plug of RP C-18 traps the organic constituents on the C-18 stationary phase where they may then be eluted with more nonpolar solvents. The power of this method is demonstrated by the large scale filtration of 2.5 liters of aqueous extract using 10 g of RP C-18 stationary phase ***(13)***.

The HPLC method in **Subheading 3.4.** is designed to quantify the major macrocyclic trichothecene metabolites individually. In addition to these known highly toxic compounds, *S. atra* also produces some simple trichothecenes. To further complicate matters, *S. atra* also generates many compounds at very low levels, many of which appear to belong to the macrocyclic trichothecene class of compounds based on our HPLC analysis. In order to determine which isolates, or environmental samples contain trichothecenes, we have developed a sensitive GC-MS method.

Base hydrolysis of a crude sample of *S. atra* extract cleaves the ester linkages at C-15 and C-4 of the macrocyclic trichothecenes and leaves the parent sesquiterpene untouched (**Fig. 5**). This converts all known *Stachybotrys* trichothecenes into either verrucarol (V) or trichodermol. GC-MS analysis of these compounds directly is possible; however, a more distinctive fragmenta-

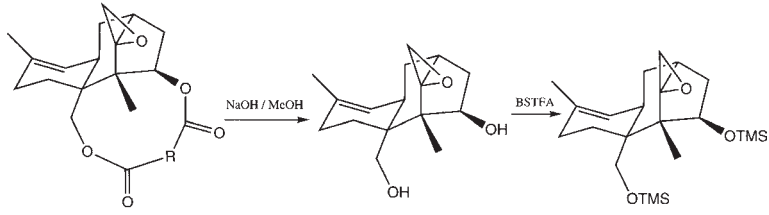


Fig. 5. Preparation of bis-silylated trichoverrol for GC-MS analysis.

tion pattern and greater sensitivity is possible if verrucarol and trichodermol are converted to their more volatile trimethylsilyl ether derivatives.

2. Materials

2.1. General

1. Bis(trimethylsilyl)trifluoroacetamide with trimethylsilyl chloride catalyst (Aldrich, P/N 39465-3).
2. Sep-Pak™ columns (6 mL, P/N 7121-06).
3. Polyethyleneimine silica gel (*see* **Note 1**).
4. Ion exchange resin (Rexyn 300 (H^+-OH^-), Fisher).
5. Rice (Uncle Ben's, Carrol County Foods, P/N 60075).
6. Silica Gel (38–63 µ*M*, Universal Adsorbants P/N 02826).
7. Reversed phase (RP) C-18 silica gel (40 µ*M*, Baker P/N 7025-1).
8. A solution (5% w/v) of NaOH (5 g) dissolved in methanol (100 mL).

2.2 Analysis

2.2.1. HPLC

1. Columns: guard (15 × 1 mm hand packed with Rainin C-18, 8 µ*M*, P/N: PK-201-H), main (150 × 2 mm Phenomenex sphereclone 3 µ*M*, P/N: OOF-4135-BO).
2. HPLC solvent system: Column heater with solvent preheating (40°C) and solvent degassing; Solvents A (water) and B (AcN) each with 0.1% formic acid (88% Fisher) (*see* **Note 2**); Solvent program is a ramp from 25% to 80% B from 0 to 30 min, then ramp to 100% B from 30 to 31 min and maintain 100% until 40 min; Post-run; ramp down to 25% B (over 2 min) then 15 min column reequilibration; Flow rate 0.20 mL/min; Maximum operating pressure 165 Bar (running 25% B).
3. Data collection: Diode Array Detection with only deuterium bulb lit; Chromatograms are recorded at 210, 230, 260, 280, and 300 nm; UV/Vis spectra are saved every 0.5 s from 200 to 400 nm.
4. Injection: Sample filtration through 0.2 mm PTFE filters (Phenomenex, 13 mm); Automated injection with 5 µL sample volume.

2.2.2. GC-MS

1. Resteck column (RTX-5MS, length 30 m, ID 0.25 mm).
2. Method: Column conditions: initial 130°C, ramp to 150°C (over 1 min), ramp to 250°C (over 17.7 min), ramp to 300°C and hold to purge the column; Injector temperature 220°C, transfer line 200°C, source 280°C; Carrier gas He with constant linear velocity of 40 cm/s.
3. Detection: MS was run with full scan mode from 100–420 AMU.
4. Injection by AS9000 autosampler with a split ratio of 25:1 and 1 µL sample size.

3. Methods

3.1. Culture Preparation

1. An Erlenmeyer flask (250-mL) is charged with rice (50 g), water (50 mL, distilled and sterile).
2. Rice is autoclaved for 20–25 min and allowed to cool before *S. atra* spores from an agar slant are introduced.
3. The culture is stoppered with sterile cotton-wool and allowed to sit for 3–5 wk at room temperature with shaking every 1–2 d (*see* **Note 3**).

3.2. Extraction Procedures

3.2.1. Organic Extraction

1. The dark black rice culture is transferred to a small coffee grinder and ground for 1 s (*see* **Note 4**).
2. About 5 grams of coarsely ground rice culture is accurately weighed into an Erlenmeyer flask (50-mL).
3. Chloroform/methanol (1:1, 30 mL) is added and the mixture agitated with ultrasound (1 h).
4. The mixture is allowed to stand overnight (4°C) (*see* **Note 5**).
5. Filter the rice from the mixture using standard vacuum filtration (5.5 cm Buchner funnel and filter paper).
6. Repeat the rice extraction and combine the organic extracts in a 100-mL round-bottom flask.
7. Solvent is removed by rotary evaporation until a constant weight has been achieved (typically 200 mg). The resultant black gum is referred to as the crude extract.
8. The crude extract is transferred to a storage vial using a known volume of methanol and the concentration recorded.

3.2.2. Aqueous Extraction

1. About 5 g of ground rice culture is accurately weighed and introduced into an Erlenmeyer flask (100-mL).
2. Water (40 mL) is added and the mixture is treated to ultrasound (1 h) (*see* **Note 6**).

3. The solution is allowed to stand overnight (4°C).
4. Standard vacuum filtration apparatus is assembled (5.5 cm Buchner funnel and filter paper). The solution is decanted off the rice through the filter paper before tipping the rice into the filtration apparatus (*see* **Note 7**).
5. Return the rice to the Erlenmeyer flask and reextract in an identical fashion. Combine the aqueous extracts.

3.3. Toxin Isolation

3.3.1. Organic Extract

3.3.1.1. PEI Absorption

1. Accurately measure about 1 mL of crude extract in MeOH into a 20-mL vial. This represents ~100 mg of the crude extract.
2. PEI silica (100 mg) is added and the mixture evaporated to dryness using rotary evaporation.
3. Pack PEI (2 g) into a 6-mL filtration column. Wash the PEI stationary phase with hexane (10 mL) and allow the solvent front to drop to the very top of the stationary phase.
4. The crude extract adsorbed onto the PEI is carefully transferred to the top of the column.
5. A small amount of hexane (0.5 mL) is used to wash all of the PEI from the vial onto the top of the column. Ultrasound agitation aides in the removal of the material from the sides of the vial.
6. Weigh three 20-mL vials and label fractions I, II, and III.
7. The column loaded with extract is eluted with the following solvents into the appropriate vials: hexane (15 mL, fraction I), dichloromethane (15 mL, fraction II) and MeOH (15 mL, fraction III).
8. Allow the solvent to evaporate from the vials overnight then reweigh each fraction.

3.3.1.2. Direct Application

1. About 1 mL of crude extract in MeOH is accurately measured into a 20-mL vial. This represents ~100 mg of the crude extract.
2. Evaporate the crude extract to dryness.
3. PEI (2 g) is packed into a 6-mL column. Wash the PEI stationary phase with CH_2Cl_2 (10 mL) and allow the solvent front to drop to the very top of the stationary phase.
4. The crude extract, dissolved in the minimum CH_2Cl_2 (200 µL), is carefully transferred to the top of the column.
5. Use a small amount of CH_2Cl_2 (0.5 mL) to wash all of the extract from the vial onto the top of the column.
6. Weigh and label two 20-mL vials: fractions II and III.
7. The column loaded with extract is eluted first with dichloromethane (15 mL, fraction II), then MeOH (15 mL, fraction III). No vial labeled Fraction I is collected.
8. The solvent is allowed to evaporate from the vials overnight; then each fraction reweighed.

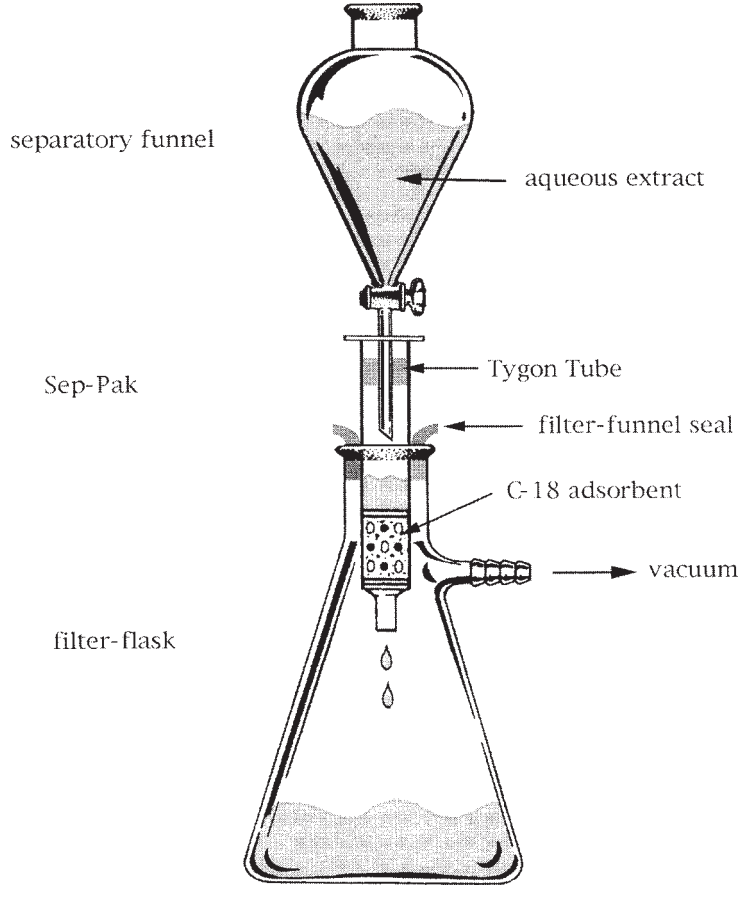


Fig. 6. Method for fractionating aqueous extract.

3.3.2. Aqueous Extract

1. Charge a 6-mL SPE column with RP C-18 silica (40 µm, 2 g).
2. The column is washed with methanol (~10 mL) then water (10 mL).
3. Immediately prior to applying the aqueous extract to the column, filter the extract through two layers of filter paper (*see* **Note 8**).
4. The aqueous extract is passed through the column where the "trapped" compounds are visible as an orange band (10 mm wide) at the top of the column (*see* **Note 9** and **Fig. 6**).
5. Three 20-mL vials are weighed and labeled fractions AQ-I, AQ-II, and AQ-III.
6. Elute the column with the following solvent systems (percentage methanol in water is listed); 20% (20 mL, AQ-I), 70% (20 mL, AQ-II), and 100% (20 mL, AQ-III).
7. Vials are allowed to stand at RT until all solvent has evaporated and then reweighed.

3.4. HPLC Analysis

3.4.1. Specific Operating Conditions

3.4.1.1. Sample Preparation

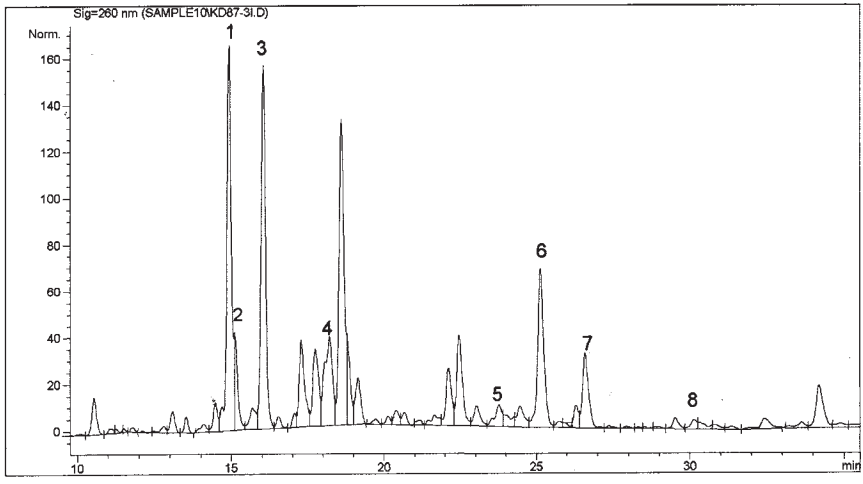
1. The dried column fraction in the glass vial is dissolved in HPLC grade MeOH (1.00 mL) using swirling and ultrasound agitation (*see* **Note 10**).
2. All of the solution is withdrawn into a Lure Lock needle tipped syringe (*see* **Note 11**).
3. Draw the liquid through the needle into the syringe body, remove the needle and replace with a filter (*see* **Note 12**).
4. Pass the solution through the filter into a vial suitable for HPLC analysis.
5. A further aliquot of MeOH (1.00 mL) is washed around the inside of the vial.
6. Repeat **steps 2–4** to give all of the fraction dissolved in 2.00 mL of MeOH in a vial ready for HPLC analysis.
7. Cap and store the vial at 4°C.

3.4.2. Analysis

1. The HPLC column is equilibrated with the starting solvent system for 15 min.
2. A 5 µL injection of the sample is made and the HPLC method started.
3. After the run is completed, the column is returned to initial column conditions and reequilibrated.
4. The chromatogram at 260 nm is integrated between retention times 5 and 35 min.
5. UV/Vis spectra for each peak in the chromatogram are compared to the library spectra for identification. Peak identification is made based on retention time (±5%) and spectral match (>95%).
6. Integration areas for identified peaks are then compared to external calibration curve data and the amount of analyte calculated (in units ng/µL).
7. A report is generated which shows the chromatogram, peak retention time, peak area, peak identification and when relevant, analyte amount (**Fig. 7**).

3.4.3. HPLC Calibration Curve

1. Make a stock solution of the compound to be calibrated by dissolving pure material in MeOH to give a concentration of ~2000 ng/µL (*see* **Note 13**).
2. 25 µL of the above stock solution is dissolved in MeOH (2.00 mL), and the concentration is calculated by recording the UV/Vis spectrum of the compound. If sufficient sample is available (>10 mg), then the concentration is calculated by weighing out the compound.
3. Prepare five calibration solutions in methanol with concentrations ranging from 2 to 200 ng/µL from the solutions made in **steps 1** and **2** above. Vials suitable for HPLC analysis (2-mL) are charged with the calibration solution and sealed. These solutions are stored at 0°C and are suitable for recalibration runs for several months.
4. Three repeats of 5 µL injections from each calibration solution are made using the HPLC run described in **Subheading 2.2.1.** This gives 15 points per calibration curve.



Injection Date : Wed, 19. Aug.1998 File name: C:\HPCHEM\1\DATA\10\KD87-3I.D
Sample Name : KD-87-03 III
Acq Operator : Simon and Kim
Acq. Method : SH21_CAL.M
Analysis Method : C:\HPCHEM\1\METHODS\SH21_CAL.M

#	RT	Peak Area	Compound Name	AMT: ng/μL
1	14.9,	1690	Roridin L-2	56.6
2	15.2	258	Satratoxin G	24.5
3	16.1	1571	Satratoxin H	106.8
4	18.2	386	Isosatratoxin F	82.7
5	23.8	115	Isororidin E	5.5
6	25.1	869	Roridin E	59.2
7	26.6	424	Verrucarin J	18.5
8	30.2	44	Roridin H	0.6

Fig. 7. HPLC chromatogram and quantitative analysis of *S. atra* mycotoxins.

5. Peaks from the calibration runs recorded at 260 nm are integrated and the integration area plotted against the calibration solution's concentration in ng/μL (*see* **Fig. 8** and **Note 14**). Straight line curve fitting is completed and curves with linear regression worse than 0.999 repeated. Diode array detection (DAD) spectra are recorded for each peak and subtracted from the baseline adsorption spectra. A library of UV/Vis spectra of the standard compounds, is generated for use in later analysis (*see* **Figs. 2–4**).

3.5. GC-MS Method

3.5.1. Hydrolysis of Crude Extract

1. A sample of crude extract (~20 mg in methanol) is measured into a centrifuge tube (1.5 mL).

2. Prepare an internal standard solution by accurately measuring about 15 mg of triphenylene and dissolving it in 5 mL of dichloromethane. Store this solution at 0°C.
3. An aliquot of internal standard solution is added (25 µL, 3.9 mg/mL solution in CH_2Cl_2) to the crude extract solution.
4. To the vial is added NaOH/MeOH solution (5% w/v, 250 µL) (*see* **Note 15**).
5. The vial is capped, treated to 60 min ultrasound agitation, then allowed to stand at room temperature for 3 h (*see* **Note 16**).
6. A glass pipet is charged with ion exchange resin (400 mg), contained by tightly packed cotton wool plugs, and washed with MeOH (1 mL) (*see* **Note 17**).
7. Once the hydrolysis is complete, uncap the vial and discharge the contents onto the resin with three washes of MeOH (200 µL each). A centrifuge tube (1.5 mL) collects the eluent. The column is eluted with a further portion of CH_2Cl_2 (200 µL).
8. The eluent is evaporated under a stream of dry nitrogen until all solvent is removed.

3.5.2. Trimethylsilyl Derivatization of Verrucarol

1. The centrifuge tube containing the hydrolyzed extract is charged with silylating reagent (60 µL).
2. The vial is capped, treated to 30 min ultrasound, then stored at room temperature for a further 60 min.

3.5.3. GC-MS Analysis

1. The centrifuge tube contents are diluted with CH_2Cl_2 (anhydrous, 1 mL).
2. Draw the CH_2Cl_2 mixture into a pipet and filtered through a small plug of cotton wool into a vial suitable for GC-MS analysis.
3. The sample is analyzed using the standard method and the resulting total ion-count chromatogram integrated (*see* **Note 18**).
4. Peaks of appropriate retention times are compared to library MS data and identified (*see* **Note 19**).

4. Notes

1. PEI silica (without crosslinking) is prepared according to the method of Jarvis ***(21)***.
2. The use of formic acid as mobile phase buffer stems from using HPLC with mass-spectral identification (HPLC-MS). Our attempts at quantitation with MS using atmospheric pressure chemical ionization (APCI), or electrospray chemical ionization (ESI) gave inconsistent results, most likely due to the inherent variability of the ionization source ***(26)***. However, for identification of unknowns the MS interface was useful, and formic acid gave superior ionization characteristics than other common acidic mobile phase additives.
3. If the flask is covered with a less permeable stopper (e.g., aluminum foil) then the growth is less uniform and slower. Rice inoculated with *S. atra* takes on a distinctive black appearance after 1–2 wk. The white rice grains become totally covered by the black heavily sporulating fungus after 2–4 wk. Great care must be taken in handling this material as skin contact with the spores can result in blis-

tering, especially when particles contact the face. All manipulations should be carried out in a chemical fume hood, particularly the grinding of the rice culture.

4. Rice cultures are coarsely ground prior to extraction. Although this would seem unnecessary as the fungus grows on the surface of the rice and the rice grains do not clump together (in the case of *S. atra*), grinding does provide a more homogeneous sample and toxin recovery is higher in ground rather than unground samples. Rice that is ground too finely will prove very difficult to filter, especially with the aqueous extraction procedure (**Subheading 3.2.2.**).
5. This step increases toxin recovery by up to 20%.
6. A typical organic solvent extraction proves highly effective for the isolation of the bioactive constituents from *S. atra*. However, it has been observed that the principal toxic components, the macrocyclic trichothecenes, are exported to the surface of the spores and may be extracted into water ***(13,27)***. This is unusual considering the generally hydrophobic nature of trichothecenes. Such aqueous solubility of the toxins may further their distribution in the natural environment and increase their human health risk. In order to investigate this phenomenon further, we have also developed an aqueous extraction method for *S. atra* rice cultures.
7. Tipping the rice directly onto the filter paper may result in a rice pad clogging the paper and slowing the filtration considerably.
8. This step is important as it removes fine particles which block the C-18 column. This step must be completed immediately before application of the extract to the C-18 column as fungal growth in the aqueous extract can occur in as little as 8 h (at 4°C).
9. Filtration of a relatively large volume of aqueous extract through the C-18 stationary phase can be time consuming. To load the extract onto the column, use the vacuum filtration apparatus shown in **Fig. 6**. Fraction collection was achieved by using filter flasks (50-mL) and quantitatively transferring the flasks contents to the vial (20-mL) where the solvent can be evaporated. With the following method, the compounds of interest (**Table 1**) are concentrated in fraction AQ-II.
10. The fractions collected were allowed to evaporate at room temperature overnight. If immediate analysis is required, rotary evaporation of the vial's contents speeds the process with no detrimental effects. Alternatively, the solvent can be removed by applying a stream of dry nitrogen. The HPLC method employed reversed phase C-18 column chromatography with AcN and water as mobile phase with added formic acid. It was found that all the compounds of interest in this study exhibited poor solubility in AcN and so all samples, calibration and otherwise, are prepared as methanol solutions. Methanol is employed as the injection solvent with sample size injection of 5 µL to obtain good peak shape, separation and repeatable retention times. If larger sample volumes must be injected, then the samples are diluted to a minimum of 50% water content; however, precipitates may form.
11. As no internal standard is used, it is critical that the solvent volume added to the samples to be analyzed is measured accurately. Furthermore, all of the solution

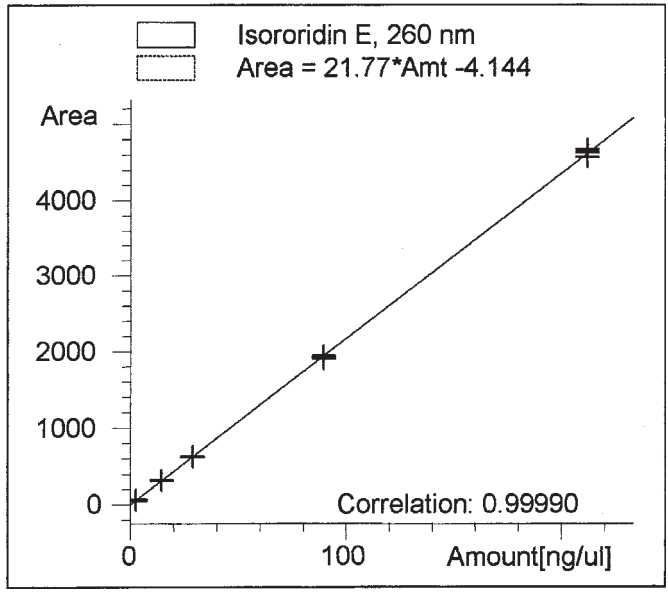


Fig. 8. Calibration curve for isororidin E.

added to the vial must be removed and filtered into a vial suitable for HPLC analysis without any solvent loss.

12. Care must be taken to expunge all of the solvent through the filter into the vial.
13. Quantitative analysis is completed by generating external standard curves for individual components. **Table 1** lists the key compounds that are treated to quantitative analysis. Standard curves are generated using the identical run conditions employed in the sample analysis as this minimizes differences in peak shape and gives a guide as to expected retention time variations.
14. The wavelength 260 nm is used for analysis as most of the macrocyclic trichothecenes feature a maximum absorption near this frequency (*see* **Table 1**). A linear response over two orders of magnitude is observed (**Fig. 8**) and provides sufficient operating range for analysis of the samples of interest.
15. Crude extract (*see* **Subheading 3.2.1.**) is treated to hydrolysis in basic methanolic solution. After HPLC analysis, the crude extract is generally stored at 4°C in methanol at an accurately determined concentration. This facilitates simpler handling for testing samples for biological activity, measuring out portions of crude extract for repeat or further analysis and allows qualitative TLC analysis to be completed. The mass of extract in **Subheading 3.5.1.** is calculated from the known methanol concentration of each crude extract solution.
16. Hydrolysis of the macrocyclic trichothecenes to verrucarol is found to be complete after 1 h. A longer reaction time up to 6 h did not adversely affect analysis. However, if the hydrolysis reaction is allowed to continue for an

extended period (48 h) or heated (60°C), then a decrease in the amount of verrucarol was recorded ***(8)***.

17. It is possible to partially purify the hydrolyzed material by trituration with ethyl acetate ***(14)***; however, we have found that the hydrolyzed material may be silylated directly ***(27)***. Silylation is found to proceed smoothly at room temperature, but extended reaction times or heating (60°C) can result in the degradation of the *bis*-silylated product. Sensitivity can be enhanced by substituting a partition step of water-dichloromethane for **steps 6** and **7**. The methanol solution in **step 5** is poured into 10 mL of water, and the mixture is extracted with 3 × 3 mL of dichloromethane. The organic extracts are combined, dried (anhydrous sodium sulfate), and the procedure from **step 8** onwards is continued. When this procedure is followed, the atranone-producing isolates of *S. atra* can clearly be seen to yield trichodermol but no, or only trace quantities of, verrucarol.
18. The lack of separation procedures required in this method coupled with the high sensitivity of GC-MS analysis allows very small samples to be investigated. Verrucarol is easily detected to the 1 ng levels with a 25:1 split ratio and ion monitoring from 50 to 400 mass units. Much greater sensitivity is clearly available if splitless operation and selective ion monitoring are introduced. Several methods have been reported for the quantification of simple trichothecenes in feed stock and grains ***(28)***. Several techniques have been utilized for their quantification including TL-HPLC ***(29)*** and GC ***(30,31)***.
19. The internal standard triphenylene has retention time 18.6 min and *bis*-TMS-verrucarol 15.1 min. The internal standard and verrucarol derivatives are readily identified by their retention times and fragmentation patterns.
20. The lambda-max for these compounds were determined from UV/Vis spectra obtained by HPLC-DAD and the molar extinction coefficients have not been recorded. The values recorded are therefore in a solution of AcN/H_2O/0.1% formic acid. The proportion of AcN is dependent on the retention time of each compound.

Acknowledgment

We would like to acknowledge support of a contract 98-05 from the Center for Indoor Air Research and EPA grant R-825251. We are particularly grateful for the technical assistance of Ms. Kim Dudley.

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16

Immunochemical Method for Citrinin

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1. Introduction

Citrinin is a toxic metabolite produced by several species of *Aspergillus* and *Penicillium* ***(1)***, and was originally isolated as an experimental antibiotic from fermentation cultures of *Penicillium citrinum* in 1931 *(2)*. Even today, liquid cultures of toxigenic Penicillia, such as *P. citrinum* NRRL-5907 or NRRL-1471, are still the most economical sources for fractional-gram quantities of nearly pure citrinin. A single precipitation from hot ethanol as described ***(2)*** affords crystalline material which is homogeneous when assayed by fluorescence high-performance liquid chromatography (HPLC).

The acute toxicity of citrinin is highly dependent on the animal species, and Hanika and Carlton ***(3)*** cite the following LD_{50} figures: 43 mg/kg for guinea pigs, 57 mg/kg for ducks, 95 mg/kg for chickens, 105 mg/kg for mice, and 134 mg/kg for rabbits. Citrinin and ochratoxin A are often found together. In some cases, the two toxins are produced by the same fungal species, and in other cases they are produced on the same substrate by different storage fungi having similar temperature-moisture requirements ***(4–6)***. In culture studies with *P. verrucosum*, corn and wheat supported the production of citrinin and ochratoxin, whereas rapeseed, soybeans, and peanuts did not ***(7)***. Toxicology studies have shown that citrinin affects the kidney ***(8)*** in test animals. Citrinin, like ochratoxin A, acts as a teratogen as well as a nephrotoxin. Citrinin and ochratoxin A in many cases act synergistically, and have been tested in combination in renal studies ***(9,10)***, in teratogenicity assessments ***(11)***, and in carcinogenicity trials ***(12)***.

Citrinin is detected most often in cereal grains, and has been reported as a natural contaminant in Canadian wheat, oats, and rye ***(13)***, in American corn ***(14,15)***, in wheat flour from Britain ***(16)***, in Swedish barley ***(17)***, and in Danish

From: *Methods in Molecular Biology, vol. 157: Mycotoxin Protocols*
Edited by: M. W. Trucksess and A. E. Pohland

feed grains ***(18)***. Citrinin has also been found in corn, barley, and rice in Egypt ***(19)***. In foodstuffs from Egyptian markets and farms, 8% of corn, 56% of barley, and 39% of rice samples were contaminated with citrinin at levels between 20 and 200 ng/g. Citrinin-producing *Penicillium* species have been isolated from apples in Spain ***(20)***, from stored cereals in Britain ***(21)***, and from naturally-fermented sausages produced in Italy ***(22)***. Citrinin has also been found in both liquid and solid cultures of the filamentous fungi *Monascus purpureus* and *M. ruber* ***(23,24)***; cultures of *Monascus* species are major sources of red pigments that are used as "natural" food additives. Some vegetarian foods colored with *Monascus* pigments have been shown to contain citrinin ***(25)***.

The development of immunoassays for many mycotoxins ***(26)*** has established the enzyme immunoassay (EIA) technique as a convenient alternative to chromatography for assaying these substances in foods. Although EIA methods for mycotoxins such as deoxynivalenol and aflatoxin B_1 have been in use for many years, practical immunoassays for citrinin have only recently been reported ***(27–29)***. The present protocol outlines the preparation of antibodies against citrinin, the use of these antibodies in the direct and indirect EIA formats, and their performance in artificially-contaminated cereal matrix situations.

2. Materials

2.1. Antigens and Labeled Antigens

1. Citrinin (Sigma, St. Louis, MO).
2. Formaldehyde 37% solution, ACS, containing 36.5–38.0% by weight formaldehyde in water with 10–15% methanol (Sigma).
3. Keyhole limpet hemocyanin (KLH), from *Megathura crenulata*, molecular weight $3.0–7.5 \times 10^6$ (Boehringer Mannheim, Mannheim, Germany).
4. Horseradish peroxidase (HRP), enzyme immunoassay grade, molecular weight 4×10^4, (Boehringer Mannheim).
5. Glucose oxidase (GOX), from *Aspergillus niger*, molecular weight 1.86×10^5 (Boehringer Mannheim).
6. 100 m*M* sodium acetate, pH 4.2.
7. 10 m*M* potassium phosphate, 100 m*M* sodium chloride, pH 7.3 ("phosphate-buffered saline," PBS).

2.2. Immunizations

1. Complete Freund's adjuvant (Sigma).
2. 9.0 g/L sodium chloride, sterile.

2.3. Assays

2.3.1. Direct Assay

1. 10 m*M* potassium phosphate, 100 m*M* sodium chloride, pH 7.3 (PBS).
2. 50 m*M* sodium carbonate, pH 9.6.

3. 2% casein (sodium salt) in PBS.
4. 8.5 g/L sodium chloride, Tween-20 250 µL/L.
5. 20 pg/L citrinin in methanol (*see* **Note 1**).
6. Substrate solution 3 m*M* hydrogen peroxide, 1 m*M* 3,3',5,5'-tetramethylbenzidine, 200 m*M* potassium citrate, pH 3.9.
7. 1 *M* sulfuric acid.
8. Antirabbit IgG from sheep, affinity-purified (Sigma).

2.3.2. Indirect Assay

1:5000 HRP-labeled antirabbit IgG from goats (Sigma) in PBS with 1% casein (sodium salt).

3. Methods

3.1. Preparation of Antigens

3.1.1. Preparation of KLH-Citrinin Conjugate

1. Dissolve 6.0 mg KLH in 0.8 mL 100 m*M* sodium acetate, pH 4.2.
2. Dissolve citrinin in methanol to give a 5 mg/mL solution, and add 0.2 mL to the KLH-sodium acetate solution.
3. Without delay, add 320 µL of formaldehyde 37% reagent (*see* **Note 2**).
4. Incubate the mixture for 24 h at 37°C.
5. Dialyze the mixture at 4°C for 3 d against 5 L of PBS, changing the PBS daily. Do not remove precipitates (*see* **Note 3**).

3.1.2. Preparation of HRP-Citrinin Conjugate

1. Dissolve 2.0 mg HRP in 0.8 mL 100 m*M* sodium acetate, pH 4.2.
2. Dissolve citrinin in methanol to give a 5 mg/mL solution, and add 0.2 mL to the HRP-sodium acetate solution.
3. Without delay, add 100 µL of formaldehyde 37% reagent.
4. Incubate the mixture for 24 h at 37°C.
5. Dialyze the mixture at 4°C for 3 d against 5 L of PBS, changing the PBS daily (*see* **Note 4**).

3.1.3. Preparation of GOX-Citrinin Conjugate

1. Dissolve 3.7 mg GOX in 0.8 mL 100 m*M* sodium acetate, pH 4.2.
2. Dissolve citrinin in methanol to give a 5 mg/mL solution, and add 0.2 mL to the GOX-sodium acetate solution.
3. Without delay, add 100 µL of formaldehyde 37% reagent.
4. Incubate the mixture for 72 h at 22°C.
5. Dialyze the mixture at 4°C for 3 d against 5 L of PBS, changing the PBS daily (*see* **Note 5**).

3.2. Immunization of Rabbits

1. After dialysis, adjust the total amount of citrinin-KLH conjugate (*see* **Note 6**) to a final volume of 1.5 mL with sterile 9.0 g/L sodium chloride solution, mix

for 30 s on a wrist-action shaker and emulsify with 4.5 mL of Freund's complete adjuvant.
2. Inject three rabbits (female Chinchilla) each with 2.0 mL of the mixture intradermally at 20 to 30 sites on shaved backs.
3. Collect serum at 13 wk after immunization (*see* **Note 7**).

3.3. Assays

3.3.1. Test Sample Preparation

1. Grind grain to pass through 1-mm apertures (*see* **Note 8**).
2. Stir 2 g with 10 mL of 10% methanol/90% PBS for 30 min.
3. Centrifuge the mixture for 15 min at 1500*g*.
4. Dilute supernate 1:4 with PBS. Further dilutions are made, if necessary, with 2.5% methanol/97.5% PBS prior to assay.

3.3.2. Direct Assay

1. Dissolve antirabbit IgG in 50 m*M* sodium carbonate, pH 9.6, to give a 10 µg/mL solution. Coat microtiter plates using this solution, 100 µL per well, and incubate overnight in a chamber with >90% relative humidity.
2. Remove the antirabbit IgG solution, add 100 µL per well 2% casein (sodium salt) in PBS and incubate for 30 min at ambient temperature.
3. Wash each plate with 8.5 g/L sodium chloride containing Tween-20 250 µL/L.
4. To each well, add 35 µL citrinin standard solution or sample solution, 35 µL of rabbit anticitrinin antiserum (serum from rabbit diluted 1:2000 with PBS), and 35 µL HRP-citrinin conjugate solution, and incubate for 2 h at room temperature (*see* **Note 9**).
5. Wash each plate with 8.5 g/L sodium chloride containing Tween-20 250 µL/L.
6. Add 100 µL of enzyme substrate solution per well, and incubate for 15 min at room temperature.
7. Add 1 *M* sulfuric acid (100 µL per well) and measure the absorbance. On the EIA microtiter plate reader, set sample wavelength to 450 nm and reference wavelength to 620 nm (*see* **Note 10**). Plot relative absorbance vs log citrinin standard concentration (**Fig. 2**).

3.3.3. Indirect Assay

1. Dilute the citrinin-GOX conjugate 1:1000 with sodium carbonate buffer and add 100 µL per well to the microtiter plates; incubate overnight at ambient temperature in a chamber with >90% relative humidity.
2. Remove the citrinin-GOX solution, add 100 µL per well 2% casein (sodium salt) in PBS and incubate for 30 min at ambient temperature.
3. Wash each plate with 8.5 g/L sodium chloride containing Tween-20 250 µL/L.
4. To each well, add 50 µL of rabbit anticitrinin antiserum (serum from rabbit diluted 1:10000 with PBS), then add 50 µL citrinin standard solution or sample solution, and incubate for 1 h at room temperature (*see* **Note 7**).

5. Wash each plate with 8.5 g/L sodium chloride containing Tween-20 250 µL/L.
6. To each well, add 100 µL of goat antirabbit IgG-HRP solution, and incubate for 1 h at room temperature.
7. Wash each plate with 8.5 g/L sodium chloride containing Tween-20 250 µL/L.
8. Add 100 µL of enzyme substrate solution per well, and incubate for 15 min at room temperature.
9. Add 1 *M* sulfuric acid (100 µL per well) and measure the absorbance. On the EIA microtiter plate reader, set sample wavelength to 450 nm and reference wavelength to 620 nm (*see* **Notes 10** and **11**). Plot relative absorbance vs log citrinin standard concentration (**Fig 2**).

4. Notes

1. Prepare citrinin (MW = 250.1) solution in methanol (approx 50 µg/mL) and determine concentration using photometric absorbance measurement at 319 nm, using $\varepsilon = 4700$ ***(30)***. Prepare a citrinin stock solution of 20 µg/mL in methanol and store at +4°C. Prepare citrinin standards for immunoassay by diluting 25 µL stock solution with 975 µL PBS. Prepare further dilutions as required using methanol-PBS (2.5 + 97.5). Methanol solutions of citrinin are stable for several months at +4°C.
2. Formaldehyde was used to successfully conjugate citrinin to the KLH carrier protein by a variant of the condensation procedure originally known as the Mannich reaction ***(31)***. Although citrinin at first appears to offer a choice of several functional groups for chemical reactions, the reactivity of all groups is reduced through a combination of resonance stabilization and hydrogen bonding (**Fig. 1**). This becomes evident when some common coupling methods ***(32)*** are attempted. Unsatisfactory results were obtained in attempts to react the C-6 ketone with aminobenzaldehyde hydrazine or carboxymethoxylamine, or to esterify the C-8 hydroxy group with succinic or glutamic anhydride, or to couple the C-14 carboxylic acid to ε-amino groups of carrier-protein lysine residues via dicyclohexylcarbodiimide. Efforts to diminish resonance stabilization by reduction of the C-6 ketone with lithium aluminum hydride were equally unsuccessful.
3. The appearance of yellow precipitates signifies successful conjugation of citrinin to KLH. This protein is sparingly soluble in buffer solutions even in native form, and tends to precipitate during most common conjugation reactions. Initial trials involving removal of the precipitates by centrifugation showed that almost no protein was left in the supernatant.
4. About 70% of the HRP enzyme activity was retained
5. The incorporation is approx 3 molecules citrinin per molecule GOX. Protein content is determined by the Lowry method ***(33)***, and protein-bound citrinin is estimated using the difference in A_{330} between the conjugate and an equivalent amount of GOX (taking ε_{330} for citrinin in aqueous buffer as 8×10^3).
6. This contained a pale yellow precipitate. Since previous work had shown examples of precipitated KLH coupled to haptens in a potent immunogenic form ***(29)***, the precipitated material was suspended in adjuvant for immunization.

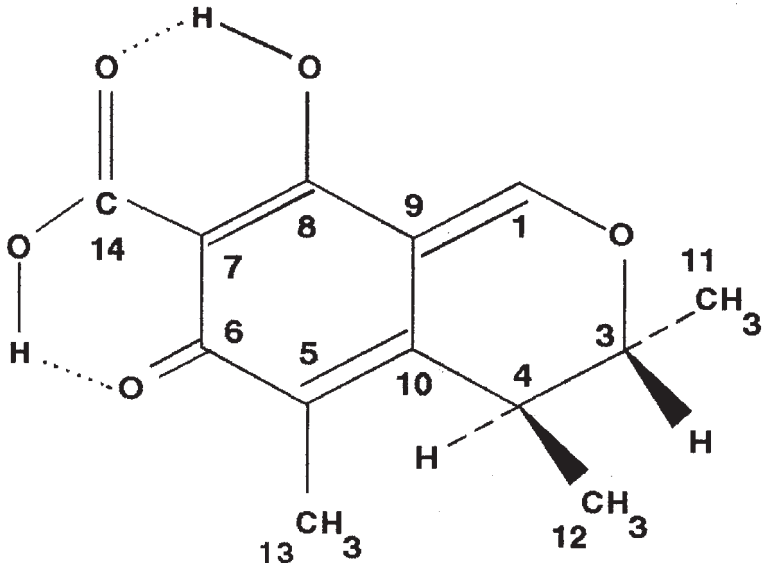

Fig. 1. Structural formula for citrinin showing hydrogen bonding and resonance stabilization.

7. Antibody titer is defined as the serum dilution which gave 0.3 absorbance units in the titer determination described previously ***(24)***. Preimmune sera gave absorbance values of <0.05 absorbance units. Antibodies could be detected in the sera of all three immunized rabbits as early as five weeks after the initial injections. After 13 wk, the titers for sera from 3 rabbits were 1:600,000, 1:400,000, and 1:50,000.
8. An electric coffee mill was used for 3 min with a 30 s on/30 s off duty cycle to minimize sample heating. The resulting particle size was <1 mm.
9. The direct EIA format proved more convenient but less sensitive. To reduce the amount of antiserum required per assay, and to improve the EIA performance, pooled serum from rabbit 1, which showed highest serum titer and strongest binding to citrinin-HRP, was diluted 1:2000 and used for the direct EIA. This enabled the use of 1:400 citrinin-HRP dilutions, and gave working peroxidase concentrations of 2–3 µg/mL. Under these conditions, concentrations of citrinin at the detection limit were 5–10 ng/mL (0.25–0.50 ng/assay), and showed 50% binding inhibition at 40 ng/mL, at a confidence level of 95%. The linear part of the standard curve (**Fig. 2**) was in the range of 8 to 100 ng/mL.
10. For performance of the citrinin EIA in wheat and barley using the direct and indirect formats, *see* **Table 1**.
11. A commercial EIA kit for citrinin, using the indirect assay format with monoclonal antibodies, became available in 1999. The product is called the Ridascreen Fast Citrinin test, and is produced by R-Biopharm GmbH, Darmstadt, Germany.

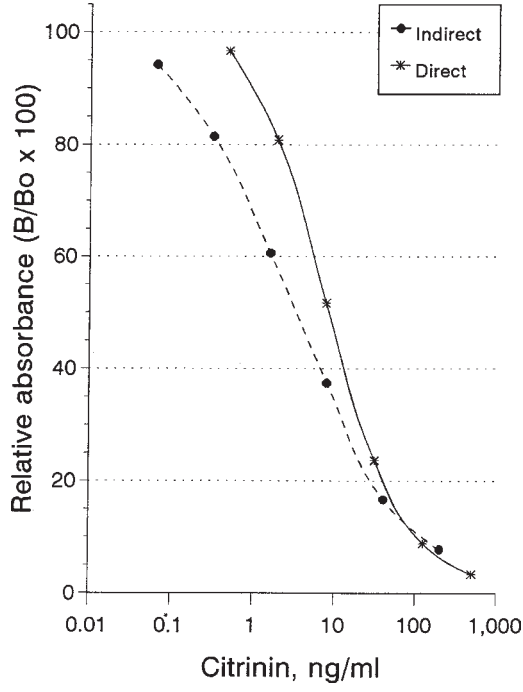

Fig. 2. Standard curves for competitive EIA of citrinin with rabbit antiserum, using the indirect and direct formats. B/Bo = absorbance of test relative to that of the negative control; Bo values = 0.998 (indirect EIA) and 0.984 (direct EIA).

Table 1
Recoveries of Citrinin from Wheat and Barley Using the Indirect and Direct Enzyme Immunoassays

	Added	Found		
Matrix, format	ng/g	Mean % recovery	*n*	CV,%
Wheat, indirect	200	104	6	7.9
	500	93	6	12.0
	1000	89	6	13.0
	2000	94	8	6.9
Barley, indirect	100	105	5	12.4
	500	112	5	11.3
	1000	107	5	4.5
	2000	109	5	10.6
Barley, direct	500	111	5	11.0
	1000	108	5	26.9
	2000	111	5	8.4

Acknowledgment

We thank Margit Straka, Marion Lorber, Diane Smith and Armando Montes for technical assistance.

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17

Solid Phase Extraction Method for Patulin in Apple Juice and Unfiltered Apple Juice

Mary W. Trucksess and Yifeng Tang

1. Introduction

Patulin, 4-hydroxy-4H-furo[3,2c]pyran-2(6H)-one (**Fig. 1**), is a lactone containing secondary metabolite of several species of *Penicillium* and *Aspergillus*. *P. expansum* is the most common mold producing patulin in apples, pears, and cherries. Patulin contamination is primarily associated with areas of decomposing tissue, and can penetrate up to approx 1 cm into the surrounding healthy tissue ***(1)***. The removal of rotten spots and surrounding tissues from apples before processing has been reported to significantly reduce patulin levels in juiced products ***(2)***.

Patulin forms colorless crystals with a molecular weight of 154 Dalton, and melting point of 111°C. It is soluble in water, ethanol, acetone, ethyl acetate, ethyl ether, and chloroform, but insoluble in benzene and petroleum ether and is stable to heat processing at an acid pH. It is gradually destroyed during storage in the presence of sulfites, sulfhydryl groups, and ascorbic acid. Patulin is completely degraded in 15 s in aqueous solution by 10-weight % ozone ***(3)***. Fermentation of apple juice to produce alcoholic beverages results in complete destruction of patulin ***(4)***.

Patulin has a moderate degree of cellular toxicity and has produced local irritation and acute intoxication in humans and laboratory animals experimentally exposed at dosages much higher than would occur as a result of dietary contamination ***(5)***. Evidence of poisoning in animals in the field is indirect and inconclusive. Results of laboratory tests, again using levels higher than those in dietary contamination, for immunosuppression, carcinogenicity and teratogenicity are mixed and their interpretation is controversial ***(6)***. The Joint Food and Agriculture Organization/World Health Organization Expert Committee

From: *Methods in Molecular Biology, vol. 157: Mycotoxin Protocols*
Edited by: M. W. Trucksess and A. E. Pohland

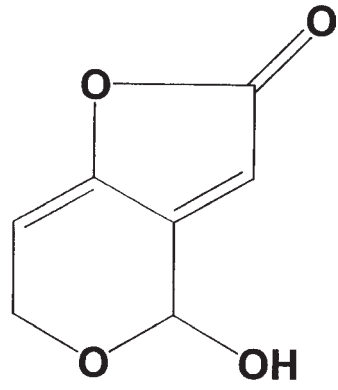

Fig. 1. Chemical structure of patulin.

on Food Additives (JECFA) established a provisional maximum tolerable daily intake (PMTDI) for patulin of 0.4 mg/kg body wt/day. This is based on the calculated no observed effect level (NOEL) and use of a 100-fold safety factor ***(7)***. Many countries regulate patulin in juice at levels ranging from 30–50 µg/L ***(8)***.

Methods of analysis for patulin in apple juice usually consist of multiple liquid-liquid partition steps, concentration, and separation and quantitation by gas chromatography ***(9)***, thin layer chromatography ***(10)*** or liquid chromatography (LC, AOAC 995.10) ***(11)***. Recently, a multifunctional solid phase column ***(12)*** has been used to replace the liquid-liquid partition steps. We developed a method that is applicable to both apple juice and unfiltered apple juice. Immediately after juice is processed from apples it undergoes a sequence of enzymatic changes to produce the color and the aroma. The term "cider" in Britain, and some European countries refers to fermented apple juice that is not covered in this method. In the United States, the terms "apple juice" and "apple cider" are used synonymously for the same product. The only difference between the two for most major retail brands is the label. The term "apple cider" can be used to describe the unfiltered, shelf stable apple juice. The raw juice is nearly always turbid, brown in color. The raw juice can be

pasteurized, filtered, or enzymatically hydrolyzed to a clear juice. Apple juice is prepared commercially by flash heating or by the addition of ascorbic acid; followed by filtration or centrifugation, and pasteurization and packaging. Other procedures are also being used.

The method described in this chapter was published recently ***(13)*** and is simple and rapid; it takes 7 min to extract, isolate, and purify the patulin from apple juice, unfiltered clear apple juice, and unfiltered cloudy apple juice. A commercial hydrophilic-lipophilic, macroporous copolymer, sorbent cartridge is used. The sorbent is a copolymer made from a balanced ratio of two monomers, the lipophilic divinylbenzene and the hydrophilic N-vinylpyrrolidone. The test sample is applied to the cartridge. Patulin is eluted with anhydrous ethyl ether-acetonitrile, separated on a C_{18} reversed-phase liquid chromatography column, and detected with a UV detector set at 276 nm. Recoveries of patulin from apple juice and unfiltered apple juice spiked over the range of 20–100 ng/mL, were 93–104% (*see* **Note 1**).

2. Materials

2.1. Apparatus

1. Centrifuge (e.g., Savant Instruments, Holbrook, NY).
2. Solid phase extraction cartridge (WAT094226, Oasis™ HLB extraction cartridge, 3 cc/60 mg, Waters, Milford, MA).
3. Polypropylene 15 mL solvent reservoir (Alltech, Deerfield, NJ).
4. Extraction cartridge manifold, 12 position, 12 needle tips, with rack for 4 mL vials (Alltech).
5. Vacuum pump, 4 mL vials, 5 mL volumetric pipet, 10 mL centrifuge tubes, general glassware, 500 µL syringe.
6. Heating block with 12 ports for 4 mL vials.

2.2. LC System

1. Programmable solvent delivery system capable of producing gradient mixtures of 2 solvents, 2 pumps capable of delivering 0.1–10 mL/min.
2. Autosampler capable of injecting 10–200 µL.
3. Variable wavelength UV detector capable of monitoring 190–300 nm, set at 276 nm.
4. Computerized data collection system and a printer.
5. LC column: C_{18} reversed-phase, 4.6 × 250 mm, 5 m (MetaSil AQP#0530, Metachem, Torrance, CA).
6. Solvent degassing apparatus.

2.3. Reagents

1. Water, distilled, deionized water purified with a Milli-Q purification system (Waters).
2. HPLC-grade methanol, acetonitrile, and ethyl acetate.

3. Reagent-grade ethyl ether anhydrous, trifluoroacetic acid (TFA), absolute ethanol, high purity compressed nitrogen
4. Patulin (P1639, Sigma Chemical Co., St. Louis, MO); 5-hydroxymethylfurfural (HMF) (Sigma Chemical Co.)
5. Sodium bicarbonate 1%. Dissolve 1 g sodium bicarbonate in 100 mL Milli-Q water.
6. Acetic acid 1%. Add 1 mL acetic acid to 99 mL Milli-Q water
7. Acetic acid solution. Adjust Milli-Q water to pH 4.0 with acetic acid.
8. HMF solution. Dissolve 5 mg in 25 mL ethyl acetate.
9. Patulin standard stock solution. Weigh approx 5 mg patulin into a 25 mL volumetric flask, record weight, and dissolve patulin in ethyl acetate (approx 200 µg patulin/mL). Pipet 500 µL solution into 10 mL volumetric flask and evaporate to dryness in a 60°C water bath with stream of nitrogen. Immediately add absolute ethanol to dissolve residue and dilute to volume (approx 10 µg patulin/mL). Measure UV absorption of patulin standard stock solution at 276 nm. Calculate the concentration as in 974.18C(d) ***(10)*** by using patulin molar absorptivity of 14,600 and molecular weight of 154. Store solutions in freezer.
10. Patulin standard working solutions (*see* **Note 2**). Prepare 0.1, 0.2, 0.5, and 1.0 µg patulin/mL acetic acid solution corresponding to 5, 10, 25, and 50 ng patulin/50 µL injection. Transfer 100 µL patulin standard stock solution (200 µg/mL) into 10 mL volumetric flask. Evaporate just to dryness under stream of nitrogen at room temperature. Immediately dilute to volume with acetic acid solution and mix. Transfer 50, 100, 250, and 500 µL portions to separate 1 mL volumetric flasks and dilute to volume with acetic acid solution. Store patulin working standards solutions in refrigerator at 2–5°C. Make new patulin working standard solutions weekly.
11. HMF-patulin solution. Transfer 100 µL patulin standard stock solution and 100 µL HMF solution to 10 mL volumetric flask, evaporate solvent under stream of nitrogen at room temperature. Dissolve residue and dilute to volume with acetic acid solution.
12. LC mobile phases: Mobile phase A: acetonitrile-0.05% TFA in water (2 + 98); mobile phase B: acetonitrile-water (1 + 1). Degas solvents by application of a vacuum using an appropriately trapped water aspirator connected to a faucet.

3. Methods

3.1. Sample Preparation

1. No preparation is necessary for apple juice and unfiltered clear apple juice.
2. Place 10 mL of unfiltered cloudy apple juice in a 15 mL polypropylene centrifuge tube and centrifuge at 7000 rpm for 10 min.
3. Place the cartridge on the manifold and couple solvent reservoir to the cartridge.
4. Pass 1 mL of methanol through the cartridge followed by 1 mL of water. Do not let cartridge run dry.
5. Pipet 5 mL of test sample into the cartridge reservoir coupled to the cartridge. Let the test sample flow through the cartridge.

6. Wash the cartridge with 1 mL of 1% sodium bicarbonate solution then with 1 mL of 1% acetic acid solution (*see* **Note 3**). Apply a vacuum to the manifold and let the cartridge dry for few seconds (*see* **Note 5**).
7. Place a 4 mL vial under the cartridge and pipet 3 mL of acetonitrile-ethyl ether (2 + 98) into the cartridge reservoir (*see* **Note 4**). Apply a positive pressure on the top of the reservoir until the solvent starts to flow. Let the solvent flow through the column.
8. Evaporate the solvent under a stream of nitrogen at room temperature.
9. Dissolve the residue in 0.25 mL of acetic acid solution and retain for LC determination. Store the test solution in the freezer at –20°C (*see* **Note 6**).

3.2. LC Determination

1. Set the flow rate at 1 mL/min with the mobile phase A. Condition the column for 20 min. Use a step gradient elution: 0–22 min, 100% mobile phase A; 22–30 min, 100% mobile phase B; 30–45 min, mobile phase A.
2. Set the UV detector to 276 nm wavelength and the sensitivity to 0.02 absorbance unit full scale (AUFS) or adjust the detector and integrator system to obtain 50% full scale deflection for 0.5 µg/mL working standard solution.
3. Evaluate the LC column performance by injecting 50 µL of HMF-patulin solution onto the LC column. The HMF and patulin should elute as 2 separate peaks with baseline separation in about 13 and 15 min, respectively. If the HMF and patulin are not completely separated, modify the mobile phases or use a different kind of column. Analysis cannot be performed unless the HMF and patulin are separated (*see* **Notes 7** and **8**).
4. Inject 50 µL of the mobile phase A and each patulin working standard solution. Prepare a standard curve by plotting peak area vs concentration of patulin working standard solutions.
5. Inject 50 µL of the test solution. The patulin concentration in the test solution can be read directly from the plotted graph or calculated from the peak area of the patulin working standard solution.
6. Dilute the test solution and rerun the LC analysis if the peak area of the test solution is outside of the range of standard curve.

3.3. Calculation

1. Calculate the concentration of patulin in test solution (C_t µg/mL) as follows in **Eq. 1**:

$$C_t = [(C_s \times H_t)/H_s] \times F \quad (1)$$

Where:
C_s = concentration of patulin in the working standard solution
H_t = response for the injected test solution
H_s = response for the injected working standard solution
F = dilution factor

2. Calculate the concentration of patulin in the apple juice or unfiltered apple juice (µg/L) as follows in **Eq. 2**:

Table 1
Method Performance for Determination of Patulin

Juice	Patulin added, ng/mL	Recovery[a] %	S[b] %	RSDr[c] %
Apple[d]	20	95	7.8	8.2
	50	93	7.6	8.2
	100	94	3.5	3.7
Unfiltered	20	99	13.3	13.4
apple juice[d]	50	93	1.0	1.1
(clear)	100	95	0.9	0.9
Unfiltered	20	104	4.3	4.1
apple juice[d]	50	93	4.1	4.4
(cloudy)	100	94	3.1	3.3

[a]n = number of analyses at each added patulin level, 8, 4, and 4 for apple juice, clear unfiltered apple juice and cloudy unfiltered apple juice respectively.
[b]S = standard derivation (%).
[c]RSDr = within laboratory coefficient of variation.
[d]Limit of detection of the method = 5 μg/L, signal/noise = 3/1; juice contained patulin at < 5 μg/L.

$$\text{Patulin in apple juice} = (C_t \times 1000)/20 \quad (2)$$

where 20 = the volume ratio of apple juice and test solution because 5 mL apple juice is represented by 0.25 mL test solution (20 = 5 mL /0.25 mL).

4. Notes

1. The performance of the method is shown in **Table 1**.
2. Take safety precautions. Wear protective clothing, gloves, and eye protection. See the Material Safety Data Sheets or equivalent for each reagent. Dispose of waste solvents according to applicable environmental rules and regulations.
3. Patulin is unstable in the basic environment. It is therefore necessary to wash the cartridge with 1% acetic acid immediately after washing with 1% sodium bicarbonate. Recoveries of added patulin at 100 ng/mL were only 50% if the cartridge was prewashed with 3% acetonitrile before washing with 1% sodium bicarbonate in an attempt to obtain a cleaner final extract. Therefore this step was eliminated from the procedure.
4. Ethyl acetate, acetonitrile, methanol, or various combinations of water with acetonitrile and methanol were used to elute patulin from the cartridge. LC chromatograms of the eluates showed baseline elevation and interfering peaks at or near the patulin peak. No baseline separation of the peaks was observed. Chromatograms with the least interfering peaks were obtained by using either 3 mL 2% acetonitrile in anhydrous ethyl ether or 5 mL anhydrous ethyl ether for elution of the patulin. When ethyl ether containing ethanol as preservative was used no clear separation of patulin from interfering peaks was achieved.

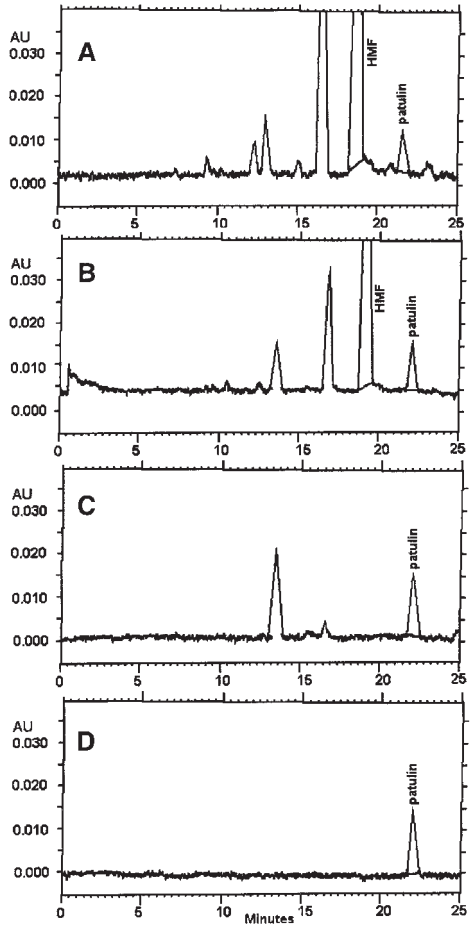

Fig. 2. Liquid chromatograms for **(A)** apple juice (patulin 50 ng/g), **(B)** unfiltered cloudy apple juice (50 ng/g), **(C)** unfiltered clear apple juice (50 ng/mL) and **(D)** patulin standard (50 ng).

5. It is important to dry the column with suction before eluting patulin from the cartridge. Prolonged heating of the eluate during the evaporating step could result in decomposition of patulin. The time required for evaporation is about 1 min. Add 0.2 mL acetonitrile to the residual solvent to aid the evaporation if it is not complete within a short time.
6. Patulin is unstable as a dry film. Immediately dissolve the residue in acetic acid solution to avoid low recoveries. Store the test solution in a freezer if LC analysis is delayed.
7. The amount of acetonitrile in the mobile phase is extremely crucial to achieve separation of patulin from HMF, and an unexpected late eluting peak in some of the apple juice. HMF is well separated from patulin in apple juice, unfiltered clear apple juice and unfiltered cloudy apple juice when 2% acetonitrile was used as mobile phase A (**Fig. 2**).

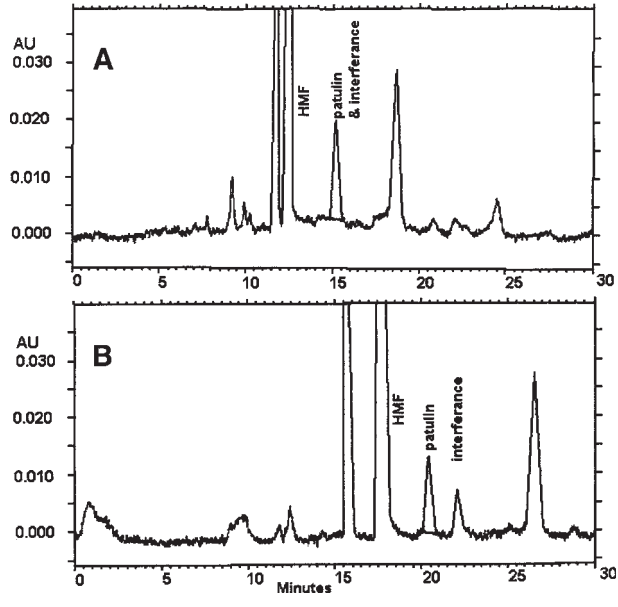

Fig. 3. Liquid chromatograms for apple juice **(A)** using mobile phase A: CH_3CN – 0.05% TFA (4 + 96) and **(B)** using mobile phase A: CH_3CN – 0.05% TFA (2 + 98).

8. When mobile phase A of higher acetonitrile concentration (4% acetonitrile) was used to speed the analysis time we noticed a shift of the interfering peak from a retention time longer than patulin to one overlapping with patulin (**Fig. 3**). The amount of acetonitrile in the mobile phase is extremely crucial to achieve separation of patulin from unexpected interfering peak in some of the apple juice. When using an old column or other types of reverse phase columns the performance of the columns must be properly evaluated.
9. A step gradient with mobile phase B containing 50% acetonitrile was used to elute compounds with retention time much longer than patulin. Otherwise the late eluting peaks sometimes would overlap with the patulin peak in the subsequent injection. The disadvantage is it takes at least 15 min to recondition the column.
10. Follow the published gas chromatography/mass spectrometry method ***(14)*** to confirm the identity of patulin in contaminated juice.

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18

Liquid Chromatographic Method for the Determination of Ergot Alkaloids in Cereal Grains

Gary A. Lombaert

1. Introduction

Ergot is a parasitic infection of cereal grains by the fungus *Claviceps purpurea*. The visible symptom of ergot is the presence of dark purple sclerotia (or "ergot bodies") in place of the cereal kernel. Within these sclerotia the fungus produces ergot alkaloids as secondary metabolites (i.e., they do not contribute directly to the growth of the fungus). The production of ergot alkaloids is affected by many factors including the maturity of the fungus, its host plant, and geographic and prevailing weather conditions. Ergot bodies associated with rye, wheat, triticale, and barley have been found to contain up to 0.45%, 0.31%, 0.75%, and 1.04% of ergot alkaloids ***(1–3)***.

The fungus survives the winter as sclerotia on the ground and infects the new crop especially during periods of cool, wet weather. During dry, warm summers there is usually little ergot infection.

Various fungi occur on different crops and in distinct geographic locations throughout the world, producing different classes of ergot alkaloids. This paper focuses on the North American situation where the infection of cereal crops by *C. purpurea* is most prevalent. The method describes the analysis of the most common and pharmacologically potent ergot alkaloids (**Fig. 1**) produced by *C. purpurea*.

A thorough review of ergot alkaloids, their sources and structures is beyond the scope of this chapter. For more information on these topics the reader is directed to **ref. *4***.

From: *Methods in Molecular Biology, vol. 157: Mycotoxin Protocols*
Edited by: M. W. Trucksess and A. E. Pohland

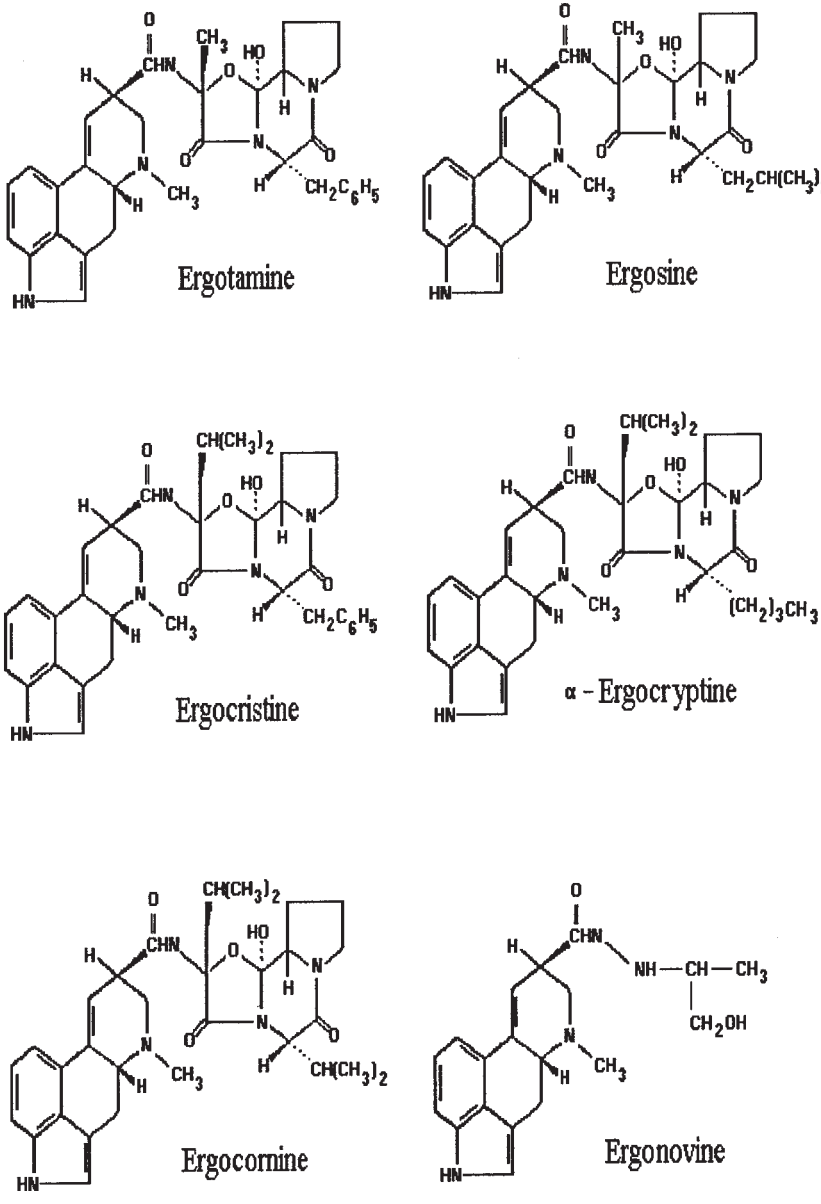

Fig. 1. Ergot alkaloid structures.

1.1. Toxicological Significance

The ergot alkaloids are pharmacologically active and have a very wide range of biological actions. Some have been found useful by the medical profession,

e.g., ergotamine to treat migraine headaches, and ergonovine to stop postpartum uterine bleeding ***(4)***. The inadvertent ingestion of ergot alkaloids, however, can cause serious chronic or acute health conditions.

The symptoms of ergot poisoning are generally classified as either convulsive or gangrenous. Animal feeding studies indicate that cattle are more susceptible than other livestock. In cattle, the usual symptoms of acute poisoning are lameness and gangrene due to constriction of the blood vessels, and, occasionally, convulsions. Chronic ingestion of low levels of the toxins may result in decreased milk production, reduced weight gain, diarrhea, reproductive problems, spontaneous abortion, and heat stress.

Ergotism, or ergot poisoning, is probably the oldest and best known human mycotoxicosis. The symptoms may include vomiting, diarrhea and, in serious cases, gangrene of the extremities and death. Ingestion can also induce hallucinogenic effects. Diseases reported among the Spartans as early as 430 BC certainly resemble ergotism. In the Middle Ages in western and central Europe, ergotism was known as St. Anthony's Fire or Holy Fire. The disease, which was probably due to consumption of bread made from ergot-contaminated rye, reached epidemic proportions and caused thousands of deaths, particularly among the peasants. Speculation persists that ergot-induced hallucinations may have contributed to reports of the supernatural during the fateful Salem witch trials in Massachusetts in 1692. Twentieth century outbreaks of ergotism have been reported in Russia, Ireland, France, India, and Ethiopia.

Research indicates that the ergot alkaloids do not accumulate in the tissues of animals, nor are they transmitted into the milk. Hence any adverse health significance related to ergot alkaloids would be due to direct ingestion from contaminated cereal foods. In developed countries, improved agricultural production, cleaning and inspection procedures have reduced the incidence and levels of ergot-contaminated grain products entering the food chain ***(5)***. Thus the levels of ergot alkaloids in human foods are generally below that considered likely to cause chronic symptoms, and the possibility of acute toxicosis from consumption of processed cereal foods is remote.

1.2. Economic Significance

Ergot contamination can have serious economic impacts on the marketing and utilization of grain, as well as on the rearing of livestock. Although ergot infection does not significantly affect crop yield, its presence does reduce the crop's market value. For grading purposes, the presence of ergot is measured in terms of ergot bodies per unit weight of sample. This measurement, of course, does not provide information on the levels or the particular alkaloids present, information that can be obtained only by chemical analysis employing a method such as presented here.

1.3. Early (and Other) Methods

Infection of crops with *C. purpurea* results in contamination with a mixture of ergot alkaloids. The analysis of this mixture in a crop or a food product can be considered a two step process. The first step is to extract the ergot alkaloids from the sample and isolate them from other components of the sample. Care must be taken to reduce losses of the alkaloids during this step. The second step, involving the separation, identification, and quantitation of the individual alkaloids, is difficult due to their structural complexity and similarities. Fortunately, liquid chromatography provides the analyst with a sophisticated and reliable separation capability.

The present extraction procedure is a modification of that of Scott and Lawrence ***(5)*** and has been used for a multi-year survey of retail cereal products ***(6)***. Other researchers have also modified and improved upon the original extraction procedure ***(7,8)***.

Early research into liquid chromatographic methods for the analysis of ergot alkaloids investigated both reversed phase and normal phase chromatography. By the early 1980s, focus had generally turned to reversed phase applications with either ammonium hydroxide or carbonate ***(5,9)*** as the inorganic modifier. The high pH of these mobile phases, however, slowly dissolves the silica backbone of the column's packing material and, thus, severely reduces the column lifetime. Other researchers ***(10)*** employed highly acidic mobile phases, which also limit column lifetimes due to hydrolysis of the siloxane bond. The liquid chromatographic procedure presented here employs the use of a paired ion mobile phase as first reported by Edlund ***(9)***. This technique extends the column lifetime without sacrificing separation of the ergot alkaloids.

Both ultraviolet and fluorescence detection have been employed with liquid chromatography for routine quantitation of ergot alkaloids. For the critical detection of low (ng/g) levels, the florescence detector is favored. In research laboratories mass spectrometry has been coupled to liquid chromatography to provide unequivocal identification of the alkaloids ***(11–13)***.

The present method is applicable to the routine, quantitative determination of the toxic ergot alkaloids ergonovine (also known as ergometrine), ergosine, ergotamine, ergocornine, α-ergocryptine, and ergocristine (*see* **Fig. 1**) in cereal grains, flours, bran, and breads. In validation studies, samples spiked at levels of 10 ng of ergonovine/g and 40 ng of the other alkaloids/g resulted in mean recoveries of 67 to 79%. The limit of quantitation of the individual alkaloids is about 1 ng/g sample ***(6)***.

The ergot alkaloids are extracted from the sample with an alkaline extraction solvent. After evaporation of the solvent, the ergot alkaloids are taken up

in diethyl ether, extracted into an acidic solution and finally back-extracted into methylene chloride. Following evaporation of the methylene chloride, the ergot alkaloids are dissolved in methanol, diluted with acetonitrile, and injected onto the liquid chromatograph. The ergot alkaloids from the sample are identified and quantitated by comparison to standard solutions chromatographed under identical conditions.

2. Materials

2.1. Liquid Chromatographic System

1. A programmable binary solvent delivery system, capable of reproducible solvent delivery at a flow of 1.0 mL/min.
2. Injection system, capable of reproducible 20 µL injections.
3. Guard column: 40 × 4.6 mm, packed with octadecylsilyl 10 µm or similar reversed phase material.
4. Analytical column: 250 × 4.6 mm, packed with octadecylsilyl 10 µm reversed phase packing material (e.g., Partisil 10, ODS-3; Whatman Chemical Separations Inc., Clifton, NJ; Phenomenex, Inc., Torrance, CA; or similar) operated at 30°C.
5. Variable wavelength fluorescence detector; excitation wavelength 235 nm (use emission cutoff filter KV389), operating range 1 microamp full scale (e.g., Kratos FS970) or emission wavelength 418 nm, gain 10 (e.g., Waters 474).
6. Data handling system (e.g., Varian Vista 402, Waters Millenium, or equivalent).
7. Operating Conditions:

   Flow rate: 1 mL/minute.

   Isocratic operation at 100% mobile phase A (0.014 *M* sodium heptane sulphonate (SHS) + acetonitrile + acetic acid, 60 + 40 + 1). After elution of ergocristine, flush column for 10 min with 50% mobile phase A and 50% mobile phase B (1% acetic acid in acetonitrile). Reequilibrate under initial conditions (100% A) for 10 min prior to subsequent injection. After daily use, wash column with, and store in, pure methanol.

2.2. Laboratory Apparatus

1. Variable volume microliter pipets (10–100 µL and 100–1000 µL) (e.g., Eppendorf Digital or equivalent).
2. Millex HV 0.45 µ*M* HPLC filter cartridges (or equivalent).
3. Horizontal shaker.
4. Ultrasonic bath.
5. Vacuum filtration funnels, with medium porosity sintered glass frits (e.g., Johns Scientific Co., Toronto, CA; or equivalent).
6. Rotary evaporator with water bath controlled at 30ºC.
7. Glass separatory funnels, 125 and 250 mL.
8. Miscellaneous laboratory glassware and supplies.

2.3. Chemicals

1. Hydrochloric acid, 0.5 *N* (*see* **Note 1**).
2. Methanol + acetonitrile (1 + 4). Mix 100 mL HPLC grade methanol with 400 mL HPLC grade acetonitrile.
3. Sodium heptane sulfonate (SHS), 0.014 *M* in 1% aqueous acetic acid. Dissolve 2.8 g 1-heptanesulfonic acid, sodium salt and dilute to 1 liter with 1% acetic acid. Filter through 1.2 µm filter (Millipore RA or equivalent).
4. Acetonitrile + acetic acid (99 + 1, v/v).
5. HPLC Mobile Phases:
   Mobile phase A: 0.014 *M* SHS + acetonitrile + acetic acid (60 + 40 + 1). Mix 600 mL of 0.014 *M* SHS (*see* **step 3**) with 400 mL of acetonitrile + 10 mL acetic acid (99 + 1). Mobile B: Acetonitrile + acetic acid (99 + 1) (*see* **step 4**).

2.4. Standards (see Note 2)

1. Ergonovine (and ergonovine maleate), ergotamine tartrate, ergocornine, α-ergocryptine and ergocristine are available from Sigma-Aldrich Canada Ltd., Mississauga, ON, Canada and from Research Biochemicals International, Natick, MA, USA. Ergosine was a gift of Sandoz Ltd., Switzerland. Dry all standards under vacuum at 70°C overnight before use.
2. Stock standard solutions: accurately weigh 0.5 to 5.0 mg of each dried standard and dissolve separately in 5 mL volumes of methanol, except ergocornine maleate, which is dissolved in methylene chloride. (Resulting concentrations are 100 to 1000 µg/mL) (*see* **Notes 3**, **4**, and **5**)
3. Intermediate Standard Mixture: With variable µL pipet transfer appropriate volumes to a common 10 mL volumetric flask and dilute with methanol + acetonitrile (1 + 4) (*see* **Subheading 2.3., step 2**) to produce intermediate standard mixture of 10 µg ergonovine/mL and 40 µg/mL for each of the other alkaloids.
4. Liquid chromatography standard solutions: dilute 1 mL of intermediate mixture to 100 mL with methanol + acetonitrile (1 + 4) (*see* **Subheading 2.3., step 2**). Concentration is 100 ng ergonovine/mL and 400 ng/mL for each of the other alkaloids. By serial dilutions with methanol + acetonitrile (1 + 4) (*see* **Subheading 2.3., step 2**), prepare three additional LC standard solutions containing 50, 25, and 12.5 ng ergonovine/mL and 200, 100, and 50 ng/mL for each of the other alkaloids.

3. Methods

3.1. Extraction and Cleanup

1. Perform all operations in subdued lighting.
2. Weigh 50 g test sample (*see* **Note 6**) into a 250 mL centrifuge bottle.
3. Add 100 mL methylene chloride, 50 mL ethyl acetate, 10 mL methanol and 2 mL ammonium hydroxide. Seal and shake on a horizontal shaker 10 min.
4. Centrifuge 5 min at 1500*g*.
5. Decant and filter through a sintered glass funnel (*see* **Subheading 2.2., step 5**), under partial vacuum, into a 1 liter boiling flask.

6. Repeat **steps 3–5**.
7. Add 50 mL methylene chloride to the sample remaining in centrifuge bottle; shake contents to break up mass and pour onto funnel.
8. Repeat **step 7** and filter under partial vacuum.
9. Evaporate the combined extracts to dryness on a rotary evaporator.
10. Redissolve the residue with 4 × 10 mL diethyl ether and transfer to 125 mL separatory funnel.
11. Extract the diethyl ether solution by swirling approx 2 min with 60 mL of cold 0.5 *N* HCL.
12. Transfer the acidic extract to a 250 mL separatory funnel.
13. Repeat **steps 11** and **12** (*see* **Note 7**).
14. Wash the acidic extract by shaking vigorously with 100 mL n-hexane for 30 s; discard wash.
15. Add about 10 mL of ammonium hydroxide to bring pH to approx 10.
16. Extract the ergot alkaloids with 3 × 50 mL methylene chloride, shaking vigorously 30 s. Drain and combine extracts into a 250 mL boiling flask.
17. Evaporate combined methylene chloride extracts to dryness on rotary evaporator.
18. Dissolve residue in 1 mL methanol, carefully rinsing flask walls. Place flask in ultrasonic bath 1 min.
19. Add 4 mL acetonitrile, mix, and filter through Millex filter prior to injection onto liquid chromatograph. Sample equivalent in this final extract is 10.0 g/mL.

3.2. Quantitative Determination

1. Inject 20 µL of each liquid chromatography standard solution (*see* **Subheading 2.4., step 4**) (*see* **Fig. 2**) and prepare standard curves of peak area vs concentration for each alkaloid.
2. Inject 20 µL final extract. Identify alkaloid peaks by comparison of retention times to those of standards chromatographed under identical conditions. The pattern of the alkaloid peaks and, generally, their relative abundances aid in their identification (*see* **Note 8**).
3. Calculate the concentration (ng/mL) of each alkaloid in the final extract by comparison of the peak areas to the standard curves.
4. Calculate the concentration (ng/g) of each alkaloid in the original sample by dividing the alkaloid concentration of the final extract by the weight equivalent of sample in the final extract, for example as in **Eq. 1**:

$$\frac{\text{ng alkaloid / mL final extract}}{\text{g sample / mL final extract}} = \frac{\text{ng alkaloid}}{\text{g sample}} \quad (1)$$

4. Notes

1. Store the 0.5 *N* HCl solution in a refrigerator at 2°C. Use of cold solution reduces the formation of emulsions during extraction from the diethyl ether solution.
2. Ergot alkaloids are pharmacologically active and should be handled with caution as toxic materials. For details respecting other chemical hazards, refer to the precautionary notes as described in Chapter 51, Laboratory Safety, of the current

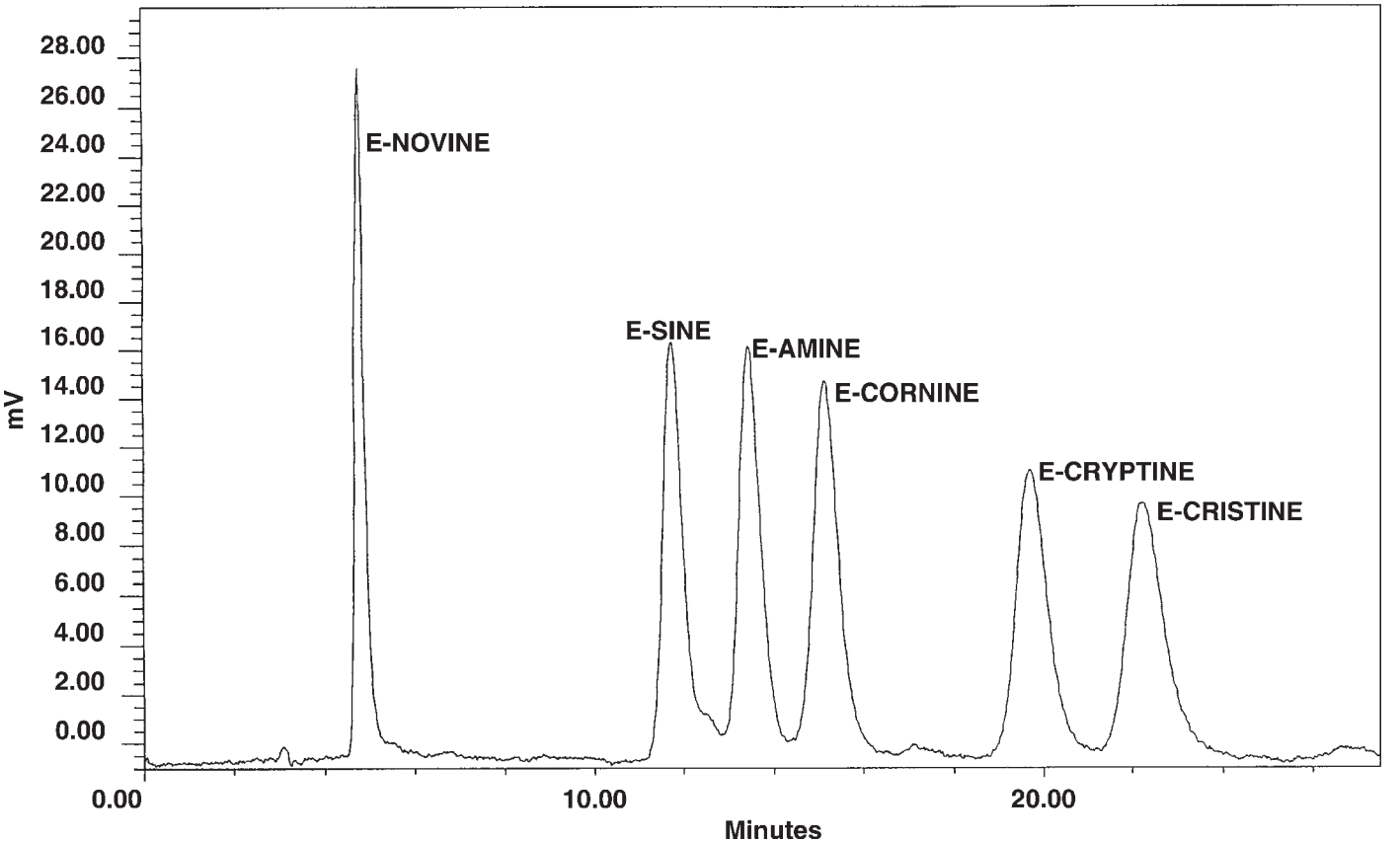

Fig. 2. Chromatogram of ergot alkaloid standard solution. Ergonovine = 25 ng/mL, others = 100 ng/mL; 20 µL injection: ergonovine = 0.5 ng, others = 2.0 ng for conditions, *see* **Subheading 2.4., step 4**.

edition of *Official Methods of Analysis*, AOAC International, Gaithersburg, MD, or other recognized texts respecting laboratory safety.

3. Store all standard solutions in dark at –2ºC. Prepare fresh intermediate and liquid chromatographic solutions weekly, and stock solutions at least monthly.
4. Ergotamine tartrate contains two molecules of ergotamine per molecule of the tartrate. The molecular weight of ergotamine is 581.7, the molecular weight of ergotamine tartrate is 1313.5. The measured weight of ergotamine tartrate must be adjusted by a factor of 0.885 (581.7 × 2 / 1313.5) to accurately reflect the ergotamine component.

   Similarly, ergonovine maleate (also available from Sigma Chemicals) has a molecular weight of 441.5, the molecular weight of ergonovine is 325.4. Therefore, if beginning with ergonovine maleate, the amount weighed should be adjusted by a factor of 0.737 (325.4/441.5) to accurately reflect the ergonovine component.
5. When first applying this method, the analyst is advised to prepare individual liquid chromatography solutions of about 100 ng ergonovine/mL and 400 ng/mL of each of the other alkaloids. Inject 20 µL volumes of these individual solutions to determine retention times and to check for the presence of isomers or other impurities in the standards. Such impurities should represent less than 5% of the total integrated area.
6. Grind whole grain kernels to pass a 2 mm sieve. Cut sliced breads into cubes, weigh a known amount and dry at room temperature 48–72 h; record weight loss and grind to pass a 2 mm sieve. Report analytical results on the fresh weight basis.
7. If an emulsion forms, add 3 mL methanol and swirl gently.
8. The ergonovine peak is occasionally masked by peaks of co-extracted material, especially from rye breads and bran. In some cases, separation and quantitation cannot be readily effected with the chromatographic conditions described.

Acknowledgment

The author expresses his sincere thanks to Mr. P. Pellaers, Ms. V. Roscoe, Dr. G. Rottinghaus, and Dr. P.M. Scott for their thorough and insightful reviews of this manuscript.

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19

Chromatographic Method for *Alternaria* Toxins in Apple Juice

Peter M. Scott and Shriniwas R. Kanhere

1. Introduction

Fungi of the genus *Alternaria* are commonly parasitic on plants and other organic materials. Many are in fact plant pathogens of field crops whereas others infect foodstuffs after harvest ***(1)***. They can grow at low temperatures and so may cause spoilage of fruits and vegetables during refrigerated transport and storage. *Alternaria alternata* is a frequently occurring species of particular interest to mycotoxicologists because it produces a number of mycotoxins, including alternariol (AOH; 3,7,9-trihydroxy-1-methyl-6*H*-dibenzo[*b,d*]pyran-6-one; **Fig. 1**), alternariol monomethyl ether (AME; 3,7-dihydroxy-9-methoxy-1-methyl-6*H*-dibenzo[*b*,*d*]pyran-6-one; **Fig. 1**), altertoxins I, II, and III {[1*S*-(1α, 12aβ, 12bα)] 1,2,11,12,12a,12b-hexahydro-1,4,9,12a-tetrahydroxy-3,10-perylenedione; [7a*R*-(7aα,8aα,8b α,8cα)]-7a,8a,8b,8c,9,10-hexahydro-1,6,8c-trihydroxyperylo[1,2-*b*]oxirene-7,11-dione; and [la*R*-(laα,lbβ,5aα,6aα,6bβ,10aα)]-la,1b,5a,6a,6b,10a-hexahydro-4,9-dihydroxyperylo[1,2- *b*:7,8-*b*']bisoxirene-5,10-dione; respectively}, and L-tenuazonic acid {[5*S*-[5*R**(*R**)]]-3-acetyl-5-(1-methylpropyl)-2,4-pyrrolidinedione} ***(1-5)***. Isolation of AOH and AME was first reported in 1953 ***(2)***. A culture of *A. alternata* on corn flour has been found to be carcinogenic in rats, and culture extracts were mutagenic in various microbial and cell systems ***(6–8)***. *A. alternata* might be one of the etiological factors for human esophageal cancer in Linxian, China ***(8)***. AOH, AME and, in particular, the altertoxins are mutagenic ***(1,7,9–13)***. Although no long term cancer studies of these mycotoxins in laboratory animals have been carried out, there are reports of subcutaneous induction of squamous cell carcinoma in mice by human embryo

From: *Methods in Molecular Biology, vol. 157: Mycotoxin Protocols*
Edited by: M. W. Trucksess and A. E. Pohland

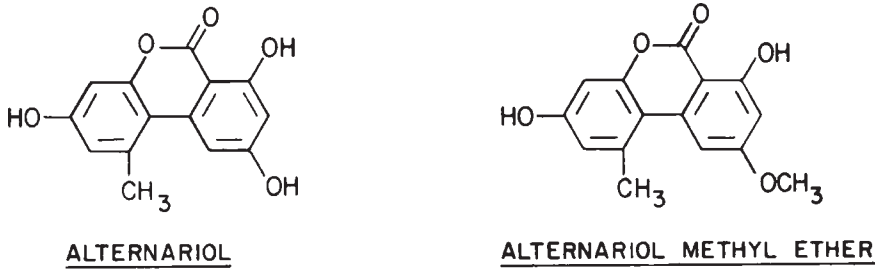

Fig. 1. Structures of alternariol (AOH) and alternariol monomethyl ether (AME).

esophageal tissue treated with AOH and of subcutaneous tumorigenicity with NIH/3T3 cells transformed by AME ***(9,14)***.

The natural occurrence of *Alternaria* toxins in grains ***(1,3,4,15,16),*** sunflower seeds ***(17,18)***, oilseed rape ***(18)***, pecans ***(3)***, and various fruits ***(4,19,20)***, including tomatoes, olives, mandarins, melons, peppers, apples, and raspberries, has been reported. As a result of inoculation experiments, the potential for their occurrence in other fruits (oranges, lemons, and blueberries) has also been demonstrated ***(4)***. The occurrence of AOH in a processed fruit product has only recently been reported—in apple juice ***(21–23)*** and in raspberry drinks ***(20)***. In addition, tenuazonic acid has been found occasionally in tomato products ***(4)*** and AME (mainly traces) has been detected in apple juice ***(22–24)***.

Monitoring of fruit juices for *Alternaria* toxins is necessary to give impetus for further toxicological studies should the level of human exposure from these foods prove to be a concern. Apple juice was chosen as the matrix to be analyzed initially, since interferences in liquid chromatography (LC) for AOH and AME are fewer than in other fruit juices such as citrus and grape juices. As shown by the natural occurrence ***(4)*** and inoculation studies ***(25–28),*** AOH and AME are the main mycotoxins produced in *Alternaria* infected apples and hence would serve as indicators of *Alternaria* contamination of the fruit before processing.

These mycotoxins have been determined by gas chromatography ***(21,29)*** and by LC, mainly with ultraviolet detection ***(24,28,30–38),*** although fluorescence ***(15,31,35,39)*** and mass spectrometry ***(23,40)*** have also been used for detection. A detection limit of 0.05 ng for AOH and AME by fluorescence has been reported ***(35)***. Another very sensitive LC determination procedure for AOH and AME (as well as altertoxins I and II) is electrochemical; 0.05 ng AOH was the detection limit ***(41)***.

A sensitive LC method for AOH and AME in apple juice was developed by Delgado *et al.* ***(24)***, who used two solid phase extraction columns in series for cleanup followed by LC with UV detection at 256 nm. Detection limits were

reported to be 1.6 and 0.7 µg/L apple juice, respectively. A previous LC method for AOH and AME in apple juice had detection limits of 10 and 25 µg/L respectively, by UV detection at 340 nm; extraction was with dichloromethane followed by silica gel column cleanup ***(34)***. With modifications to the cleanup procedure consisting of increased volumes of the wash solvents on both solid phase extraction columns, a change in composition of the acetonitrile-water wash solvent on the C_{18} column, and an increase in volume of the acetonitrile-formic acid (100:1) eluting solvent on the aminopropyl column, the newer method ***(24)*** is described below, together with two further variations (A and B) to the cleanup procedure.

2. Materials

1. Standard solutions of AOH and AME: Weigh crystalline AOH and AME (available from Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178, USA, catalog nos. A 1312 and A 3171, respectively) and dissolve each separately in methanol to make 250 µg/mL stock solutions (*see* **Note 1**). Store stock solutions in a freezer at –12°C (*see* **Note 2**). Evaporate 100 µL AOH stock solution and 100 or 200 µL AME stock solution in a 4-mL screwcap vial under a gentle stream of nitrogen and dissolve in 1 mL methanol to prepare mixed spiking solution containing 2.5 µg AOH/mL and 2.5 or 5 µg AME/mL (*see* **Note 3**). Evaporate aliquot of spiking standard under nitrogen and dissolve in 500 µL of LC mobile phase to give LC standards containing 0.2 µg AOH/mL and 0.2 or 0.4 µg AME/mL. Store spiking and LC standard solutions in a refrigerator (4°C).
2. Pectinase: *Aspergillus niger* solution in 40% glycerol, 445 units/mL (Sigma Chemical Co., catalog no. P4176).
3. Ultrasonic bath: Branson model 1210.
   a. C_{18}: Chromabond, 3-mL, 500 mg, not endcapped (Macherey-Nagel, Neuman Neander Strasse, D-52355 Düren, Germany, catalog no. 730 003) (*see* **Note 4**).
   b. NH_2: Chromabond, 500 mg (Macherey-Nagel, catalog no. 730 033) (*see* **Note 5**).
5. Adaptors: Bond Elut, fits 1, 3, and 6-mL tubes (Varian Sample Preparation Products, 24201 Frampton Avenue, Harbor City, CA 90710, USA, catalog no. 1213 1001).
6. Reservoirs: empty SPE tubes.
7. Stopcocks: Luer (Varian Sample Preparation Products, catalog no. 1213 1005).
8. Reverse phase LC column: Inertsil 5 µ*M* ODS-2, 250 × 4.6 mm (MetaChem Technologies Inc., 3547 Voyager Street, Bldg. 102, Torrance, CA 90503, USA) (*see* **Note 6**).
9. LC mobile phase: Methanol-acetonitrile-1% aqueous *ortho*-phosphoric acid (50:20:30, v/v/v) (*see* **Note 7**).

3. Methods

3.1. C_{18} Cleanup

1. Attach adaptor with reservoir to the top of the C_{18} SPE column and attach the tip of the column to a stopcock inserted into the port on the lid of the vacuum manifold.

2. Condition the C_{18} SPE column with 6 mL methanol followed by 6 mL of water. Adjust the flow rate to about 1 drop/s using the flow control valve of the vacuum manifold and continue with this flow rate for subsequent elutions of this column.
3. Pass a 10-mL test sample of apple juice through the column (*see* **Subheading 3.3.** for cloudy apple juice procedure).
4. Wash the column with 6 mL of distilled, deionized, water followed by 2.5 mL of acetonitrile-water (35:65, v/v). Discard all washings.
5. Elute the toxins with 4 mL acetonitrile-acetic acid (100:1, v/v) into a 50-mL round-bottomed flask or a 4 mL vial.
6. Evaporate the eluate to dryness in a round bottom flask using a rotary evaporator with a water bath temperature of 40°C; or evaporate the eluate in a vial under a stream of N_2 with minimum heating.
7. Dissolve the residue with three 500 µL portions of ethyl acetate (*see also* **Subheading 3.4.**), holding each for 5 min in an ultrasonic bath. Proceed to **Subheading 3.2.**

3.2. Aminopropyl Column

1. Attach the adaptor with reservoir (if required) to the top of the amino SPE column.
2. Condition the column with 6 mL dichloromethane, using gravity flow for this and subsequent elutions from this column.
3. Add the combined extract from **Subheading 3.1.** to the top of the column and wash with 3 mL of acetone followed by 3 mL of acetonitrile (*see also* **Subheading 3.4.**). Discard washings.
4. Elute the toxins with 5 mL acetonitrile-formic acid (100:1, v/v) into a 4-mL vial (capacity 5 mL).
5. Evaporate the eluate carefully to dryness under a stream of nitrogen at 40°C and dissolve residue in 500 µL of LC mobile phase.

3.3. Cleanup Variation A (for Cloudy Apple Juice)

1. In a 15-mL centrifuge tube, mix 10 mL of cloudy apple juice with 25 µL of pectinase on a vortex mixer for 30 s.
2. Heat in a water bath at 40°C for 1 h.
3. Centrifuge at 830 × g for 20 min.
4. Using a Pasteur pipet add the clear supernatant to the conditioned C_{18} SPE column (*see* **Subheading 3.1.**, **steps 1** and **2**).
5. After draining, wash the column with 5 mL of distilled, deionized, water and discard washing.
6. Add 250 µL of acetonitrile-acetic acid (100:1) to the residue in the centrifuge tube with vortex mixing, then place in an ultrasonic bath for 10 min. Add 2.25 mL of distilled, deionized water, vortex mix 30 s, then centrifuge for 10 min.
7. Transfer the clear supernatant to a C_{18} column.
8. Repeat **steps 6** and **7** two more times.
9. Wash the column with 1.5 mL of acetonitrile-water (35:65, v/v). Discard all of the washings.

10. Elute the toxins with 4 mL acetonitrile-acetic acid (100:1, v/v) into a 4-mL vial and evaporate to dryness under nitrogen.
11. Dissolve the residue with three 500 µL portions of ethyl acetate, holding each for 10 min in an ultrasonic bath.
12. Add the ethyl acetate extracts to a conditioned aminopropyl SPE column and carry out the cleanup on this column (*see* **Subheading 3.2.**).

3.4. Cleanup Variation B

Improved recoveries may be obtained by dissolving the residue from **Subheading 3.1.** with 100 µL of methanol plus 400 (or 500) µL of ethyl acetate twice, then 500 µL of ethyl acetate (with ultrasound) (cf. **Subheading 3.1., step 7**). Proceed to **Subheading 3.2.** where the results of the acetone and acetonitrile washes of the aminopropyl column are then omitted.

3.5. Liquid Chromatography (LC)

1. Carry out the determination of AOH and AME by isocratic reverse phase LC on a C_{18} column (*see* **Subheading 2., step 8**) with acetonitrile-methanol-1% aqueous *ortho*-phosphoric acid mobile phase (*see* **Subheading 2., step 9**) at a flow rate of 1 mL/min. The following additional equipment comprised the LC system, but any suitable equivalent apparatus may be substituted:
   a. Pump: Shimadzu model LC-10AD.
   b. Injector: Rheodyne model 7125 with 20 µL loop.
   c. Guard column: Guard-Pak precolumn module (Waters) with Resolve C18 precolumn insert.
   d. In-line degasser (Shodex).
   e. Absorbance detector, 254 nm: Waters model 440 or Thermo Separation Products UV 2000 dual wavelength detector.
   f. Integrator: Varian (Spectra-Physics) 4270.
2. Inject 10 µL of extract solution (20 mL of apple juice equivalent/mL mobile phase) and 10 µL of mixed standard solution of AOH and AME (0.2 µg/mL and 0.2 or 0.4 µg/mL, respectively). The given amounts of AOH and AME injected are for the UV 2000 detector but will depend on the sensitivity of the UV detector. Make two injections of sample extract and compare average peak areas for AOH and AME with those of standards at the same retention time. Typical retention times were 6 and 13 min for AOH and AME, respectively (**Fig. 2**). The standard curve was linear in the range 0.25–12.5 ng AOH or AME injected (0.025–1.25 µg/mL). Detection limits (UV 2000 detector) for standards were 0.05 ng AOH and 0.1 ng AME (S/N 3:1).

3.6. Method Performance

Added at 10 and 20 ng/mL, respectively, percent recoveries of AOH and AME from apple juices averaged 79 ± 14 (n = 9) and 87 ± 16 (n = 13) by the procedure presented here. By the pectinase modification (variation A) (*see*

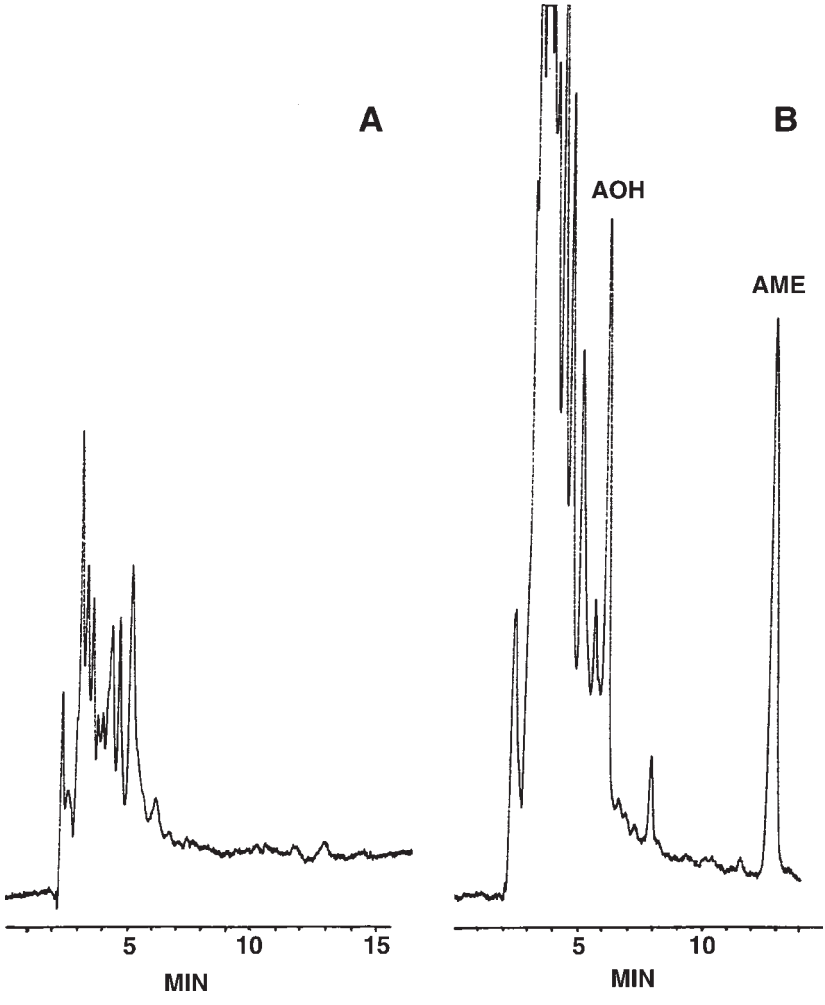

Fig. 2. Chromatograms of apple juice (containing an unconfirmed trace—approx 0.5 ng/mL—of AOH) **(A)** and the same juice spiked with 10 ng AOH/mL and 20 ng AME/mL **(B)**, analysed by method variation B.

Subheading 3.3.), recoveries of AOH and AME added at these concentrations averaged 84% and 76%, respectively ($n = 2$). By the modification described in **Subheading 3.4.** (variation B), recoveries were 87 ± 8 and 84 ± 6% ($n = 5$) when AOH and AME were added together at 10 and 20 ng/mL, respectively, and 94 ± 8 and 102 ± 13% ($n = 12$) in experiments where both toxins were added together at 10 ng/mL. Detection limits for AOH and AME were dependent on the sample but were of the order of 0.5–1 ng/mL.

4. Notes

1. Check the weights of AOH and AME by UV analysis. Prepare dilutions of each at 5 µg/mL in 95% ethanol. For AOH the extinction coefficient at 257 nm is 53700 ***(42)*** and for AME at 259 nm the coefficient of extinction is 47900 ***(43)***.
2. It may be necessary to place the flask containing AME in an ultrasonic bath for 20–30 min to dissolve the AME if it crystallizes out of solution upon storage.
3. This solution can be used to determine by LC the concentration of AME present as an impurity in AOH and vice versa. The standards used here contained 2.5% AME in AOH and 0.1% AOH in AME.
4. Bond Elut 500 mg, 3 mL columns (Varian) also performed satisfactorily, with gravity flow. However, other C_{18} SPE columns may not be suitable.
5. Other aminopropyl columns may not be suitable.
6. The Inertsil column gave a better separation of AOH from an interference compared to two other brands of columns.
7. Mobile phase composition may be varied if not detrimental to resolution. For example, methanol-acetonitrile-1% aqueous *ortho*-phosphoric acid (45:32:23) can also be used.

Acknowledgment

Kind permission of Elsevier Science - NL, Sara Burgerhartstraat 25, 1055 KV Amsterdam, The Netherlands to reproduce the essential content of this method ***(24)*** is acknowledged. Veronica A. Roscoe (Health Protection Branch, Winnipeg, Manitoba) contributed to the **Notes** section and has demonstrated application of the cleanup procedure (including variation A) in her laboratory for a survey of apple juice for AOH and AME ***(23)***.

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