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Analysis of Mycotoxins by HPLC with Automated Confirmation by Spectral Library

Application Note

Food Analysis

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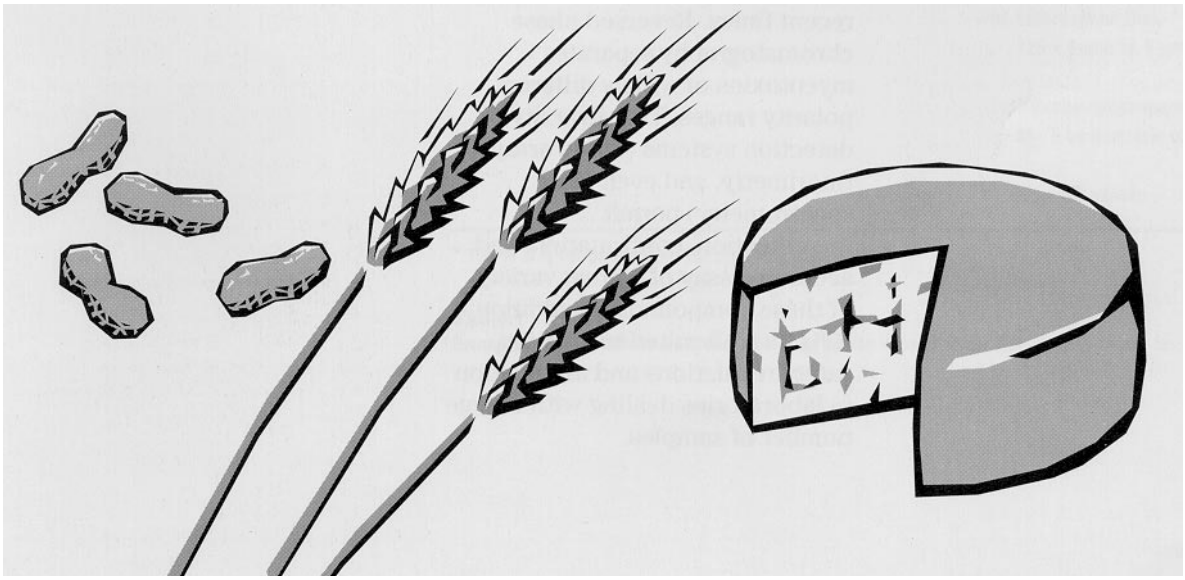
This note describes the sample preparation, chromatographic separation and detection of four different types of mycotoxins in food samples. Deciding which approach to adopt for analyzing these depends on the sample matrix and the type of fungus it has been contaminated with. Various professional organizations have proposed a variety of sample preparation methods–those for aflatoxins, ochratoxin A, patulin and zearaleneone are described here. All HPLC separations have been performed on reversed phase material (normal phase chromatography; a diol column can be used for patulin) and monitored with UV-visible absorbance diode-array detection (DAD) and fluorescence detection (FLD) for aflatoxins, ochratoxin and zearalenones or mass spectrometry (MS) for aflatoxins. Most compounds have been identified and confirmed by UV-visible absorbance spectral library search, purity control and by retention time tagging.



Introduction

Mycotoxins are highly toxic compounds produced by fungi. These toxins can contaminate foodstuffs when storage conditions are favorable to fungal growth. Mycotoxin nomenclature very often results from the fungi where the substance was first detected, for example aflatoxins in Aspergillus flavus strains, ochratoxin in Aspergillus ochraceus, patulin in Penicillium and Aspergillus, zearalenone in Fusarium. Most of these mycotoxins have been identified after cases of poisoning in livestock or the population at large. In 1969 more than 100,000 turkeys died of an unknown condition (so-called Turkey X) that was finally traced to peanuts–a component of their feed contaminated with Aspergillus flavus. During the wartime winter of 1940 in the USSR many people died after eating grain poorly stored and highly contaminated with Fusarium toxins such as zearalenone and fusarin C. A similar case occurred in 1965 in South Africa with ochratoxins found in cereals accumulated unmetabolized in animal kidneys.

Aflatoxins are known to be mutagens, teratogens (causing fetal abnormalities) and carcinogens (particularly in cancer of the liver or kidneys). Ochratoxins cause nephropathies in pigs, are teratogenic, and carcinogenic particularly in the liver and kidneys. Zearalenone shows estrogenic effects in sows and poultry, and affects the liver and kidneys. Patuline is a powerful mutagenic and cytotoxic compound. The intake of these mycotoxins over a long period at very low concentrations may be highly dangerous, yet difficult to combat since the small quantities are difficult to trace.

Currently most mycotoxins are still assayed using thin-layer chromatography (TLC), which permits effective compound separation and characterization. Such assay may be performed with satisfactory sensitivity when the compounds to be detected are fluorescent–a fluorodensitometer reads the plates quantitatively and objectively and has become indispensable to the control laboratory. However due to its higher separation power and shorter analysis times, use of HPLC has expanded rapidly in recent times. Reversed phase chromatography separates mycotoxins of widely different polarity ranges. The diversity of detection systems (diode array, fluorimetry, and even mass spectrometry) permit identification, confirmation and accurate assay of a great variety of these compounds. In addition HPLC is well suited to existing safety regulations and automation in laboratories dealing with a large number of samples.

The complexity in composition of processed foodstuffs makes a fixed routine necessary for analysis:

1. sampling protocol that ensures representative data from any one batch
2. extraction of mycotoxins, using mostly chloroform, acetone, or methanol
3. purification of the extract with clean-up methods
4. concentration of the extract
5. qualitative detection and assay of the mycotoxins.

In this paper we describe the analysis of 4 different types of mycotoxins. First we describe the chemical nature and occurrence of these toxins.

Experimental

Table 1 gives a short overview of analysis conditions for the four different mycotoxins aflatoxins, ochratoxin A, zearalenone and patuline.

Compound class	Matrix	Sample preparation	Chromatographic conditions
Aflatoxins G_2, G_2, B_2, B_1, M_2, M_1	Nuts, spices, animal food, milk, dairy products	Extraction §35LMBG	Hypersil ODS 100 x 2.1-mm id, 3 µm particles *HP 799160D-352* Water-methanol-acetonitrile 63:26:11 as isocratic mixture* Flow 0.3 ml/min at 25°C
Ochratoxin A	Cereals, flour, figs	Extraction §35LMBG Acidify with HCI. Extract with toluene. Clean up SiO_2. Elute toluene-CH_3COOH 9: 1	Lichrospher 100 RP18 125 x 4-mm id, 5 µm particles *HP 799250D-564-3* Water with 2 % acetic acid/acetonitrile, 1 : 1* Flow 1 ml/min at 40°C
Zearalenone	Cereals	Extract with toluene. Sep-pak clean up. Elute toluene-acetone 95: 5.	Hypersil ODS 100 x 2.1-mm id, 3 µm particles *HP 799160D-352* Water-methanol-acetonitrile 5:4:1 as isocratic mixture* Flow 0 : 45 ml/min. at 45°C
Patuline	Apple products	Clean-up on Extrelut Silica gel clean up Elute toluene-ethyl acetate 3: 1.	a)Superspher RP 18 125 x 4-mm id, 4 µm particles *HP 799250D-464,* Water-acetonitrile, 95 % to 5 % gradient Flow 0.6 ml/min at 40° C b) Lichrospher Diol 125 x 4-mm id, 5 µm particles Hexane-isopropanl 95:5 as isocratic mixture Flow 0.6 ml/min

* 100 % B is recommended for cleaning the column.

Table 1. Sample preparation and chromatographic conditions for mycotoxins in foodstuffs

Compound types:

Aflatoxins–are chemical derivatives of difurancoumarin (figure 1). Although a number of different aflatoxin metabolites are known, interest is usually focused on the four main aflatoxins B_1, B_2, G_1, G_2 and the so-called milk toxin M_1.

Aflatoxin B_1 is in the majority of cases the most abundant toxin, the most toxic and the most potent carcinogen. Maximum levels for B1 are usually given for the individual compound, 2 ppb in Germany, 5 ppb in France and 1 ppb in Switzerland, for example. United States legislation regulates the aflatoxin content of a contaminated product as the sum of B_1-plus-B_2-plus-G_1-plus-G_2, which may not exceed 20 ppb. Aflatoxins are most often analyzed in nuts, for example peanuts and pistachios, cereals, figs, bread, meat, eggs, butter, milk, margarine, juices, cottonseed products, and cocoa beans. Considering the complexity of these matrices, sample preparation is the most important step for reliable results.

Since fungal growth–and therefore contamination by aflatoxin–is not homogeneous, normal sampling gives mediocre results. The US Department of Agriculture (USDA) tackled the problem by defining a sampling protocol for peanuts which involves as many as eight assays on four samples of more than
1 kg. Sampling is not specified in European countries. At best, a few hundred grams are taken to determine the mean level of aflatoxins in a batch as large as of a couple of thousand tons of grain.

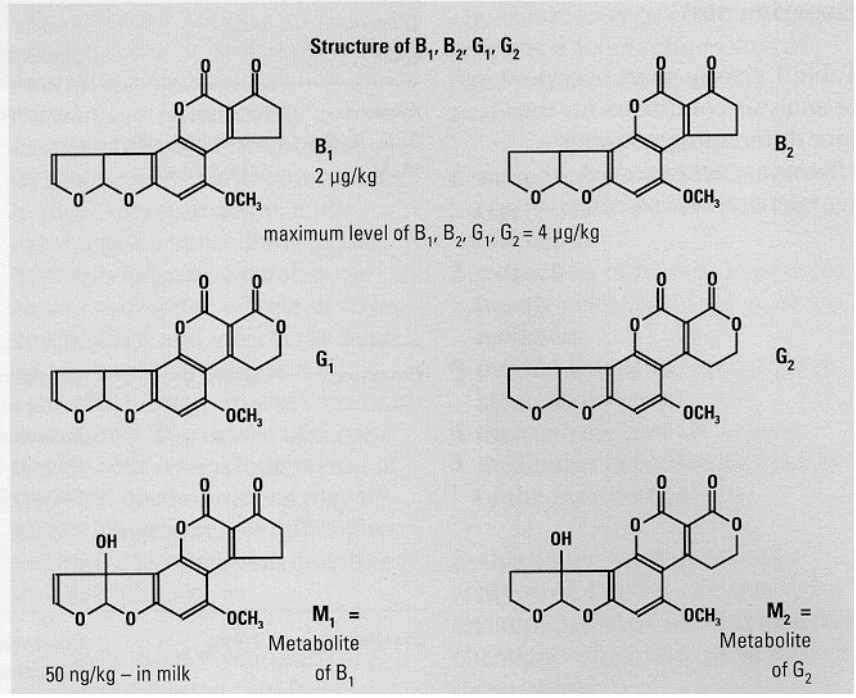


Figure 1. Structure of aflatoxins and their maximum permitted concentrations (given for Germany[1])

Ochratoxins–The mycotoxin ochratoxin A can be produced by different fungi including Aspergillus and Penicillium. Of the ochratoxins A, B, and C, the latter two so far have not been found in naturally contaminated products. Beside nephrotoxicity, ochratoxin A has hepatoxic, teratogenic and carcinogenic properties in the kidneys. Ochratoxin A was found in various foodstuffs. Analysis of more that 900 plant samples show a contamination rate of about 13 %, mostly in barley, oats and wheat. The concentrations of ochratoxin A found varied from 0.1 to 200 µg/kg.[2] A review of results from various countries, covering around 7000 samples, reported that contamination was about 14 %.[3] Ochratoxin A is the primary agent in so-called mycotoxic porcine nephropathy (MPN) a disease prevalent in pigs. The toxicity is a third that of the toxicity of aflatoxin B_1 in rats. The main human intake is assumed to be through the consumption of pork and wholemeal products.

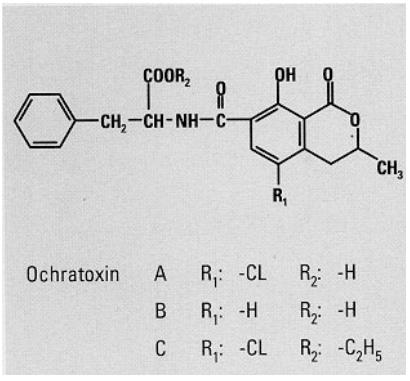


Figure 2. Structure of three common ochratoxins

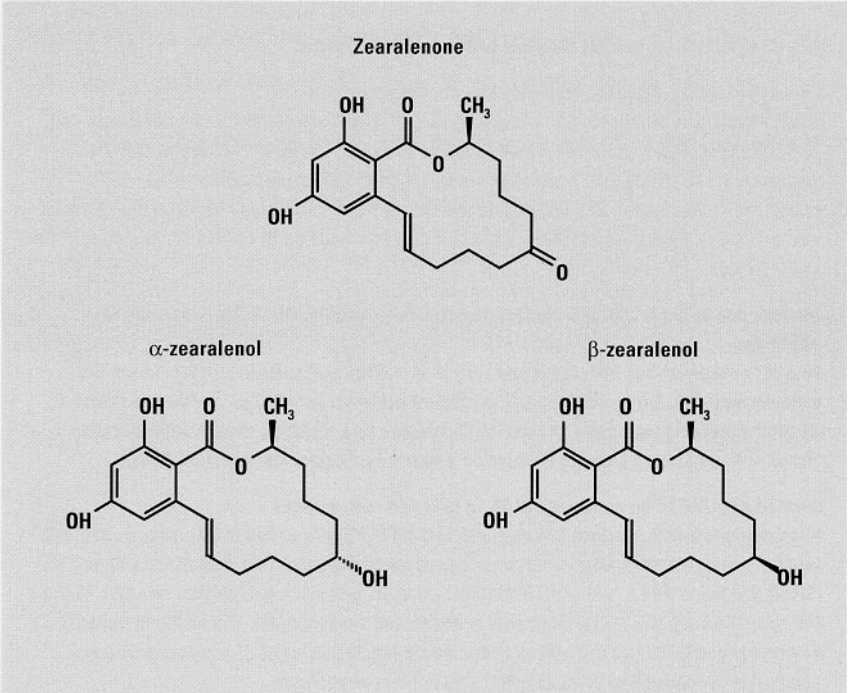


Figure 3. Chemical structures of zearalenone, a–and b-zearalenol and a -zearalanol (Zeranol)

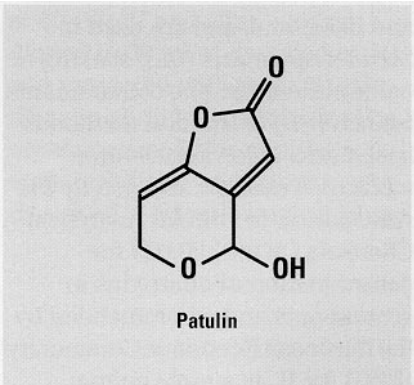


Figure 4. Chemical structure of patulin

Zearalenone–an estrogenic-efficient mycotoxin produced by Fusarium, occurs mainly in a variety of natural products such as corn and other grains. Whereas the acute toxicity of zearalenone is low, its intake is linked to various possible estrogenic disease effects in children.[4] After carcinogenity was determined in rodents, a recent risk assessment resulted in an estimated safe intake of not more than 0.05 µg for each kilogram body weight per day for humans.[12] Use of contaminated animal feed means that these compounds are present in cow's milk as zearalenone and the diastereomer metabolites and b-zearalenol. Another zearalenone derivative is the synthetic a -zearalanol (also known as Zeranol) which is used in some countries for fattening cattle. The recommended limit for zearalanol is 10 µg kg for liver and 2 µg/kg for other meats.[7] Their use as anabolic agents is prohibited in the European Community. investigations of food and animal feedstuffs have shown zearalenone concentrations between 0.001 and 2.0 mg/kg.[5] The highest levels, 1700 mg/kg, were found in silo-corn.[6]

Patulin–unstable in cereals, mainly occurs in fruit, especially apples, is a metabolite of several fungi of Penicillium and Aspergillus. Most of the survey work has been done on apple juices and apple-based products.

Aflatoxin sample preparation

For sample preparation different methods are described in the literature.[8,9,18] Lipid should be eliminated after analyte extraction if lipid content exceeds 5 %. Solvents in which the aflatoxins are insoluble are hexane, petroleum ether, pentane and isooctane, and are used in Soxhlet apparatus (6h), shaking or column clean up. The contaminants branch (CB) extraction method is based on a chloroform-water mixture, a method adopted by the Association of Official Analytical Chemists (AOAC) 20.029 for determination of aflatoxins in ground nuts and recommended by the European Economic Community (EEC) for B_1 in simple animal nutrition foodstuffs.

Whatever extraction method is used the resulting extract still contains, besides the aflatoxins, various impurities (lipids, pigments and so on) requiring an extra clean-up step. Apart from purification by precipitation or by liquid-liquid partition, the most commonly used technique is column adsorption chromatography.

Extraction of aflatoxins with chloroform

CB method (AOAC and EEC) for extraction of aflatoxins

50 g of finely ground sample are mixed with 25 g of diatomaceous earth and moistened with 25 ml of water. This mixture is carefully shaken, diluted in 250 ml of chloroform and shaken vigorously for 30 minutes on a vibration shaker. A 50 ml portion of chloroform extract is collected for purification and assay. The addition of water facilitates chloroform penetration into substrates derived from plants, while the diatomaceous earth retains various substances like pigments.

Method §35 *Lebensmittel und Bedaffspeyenstands Gesetz* **(LMBG)[8] for extraction of aflatoxins**

20 g of finely ground sample are mixed with 20 g of silica gel, particle size 20–45 µm (for example celit 545, Serva, Germany). This mixture is diluted with 200 ml of chloroform and 20 ml of water and vigorously shaken for 30 minutes on a vibration shaker. After filtration, 100 ml are evaporated close to dryness on a rotary evaporator (temperature 40 °C).

Method §35 LMBG[9] for extraction of M_1 in milk and milk powder

50 ml acetone and 5 g sodium chloride and 1 ml 1 N H_3PO_4 are added to 50 g milk, or 10 $_9$ milk powder homogenized in 40 g water, and shaken for 10 minutes. After addition of 100 ml dichloromethane and a further 10 minutes shaking, 25 g of silica gel, particle size 20–45 µm (for example celit 545, Serva, Germany) is added and shaken again. The dichloromethane/ acetone phase is filtered and 100 ml of the filtrate (equivalent to 33.33 g milk or 6.66 g milk powder) is evaporated to dryness at 40 °C in a rotary evaporator.

Purification of aflatoxins

Method according to AOAC/ EEC and §35 LMBG regulations[8]

A glass column (400 x 30mm) is filled in succession with 5 g sodium sulfate, 10 g of silica gel (63–200 µm, dried at 105 °C for lie), and 15 g anhydrous sodium sulfate topped up with some cotton-wool. The extract to be cleaned is added on top and eluted with 15–20 ml chloroform. Then the column is washed with 150 ml hexane and 150 ml diethylether to remove lipids and other interfering compounds from the aflatoxins. The aflatoxins are eluted with 150 ml of a chloroform/methanol (97: 3) mixture. The eluate is dried down and redissolved in a suitable solvent for assay by HPLC (methanol).

Extraction of aflatoxins from milk, AOAC 26.139

To 25 ml of milk, 10 drops of NH_4OH are added, swirled and diluted by 70 ml acetonitrile. The mixture is shaken for 1 minute and centrifuged for 5 minutes at 1000 rpm. The aqueous alkaline acetonitrile supernatant is transferred and evaporated on a rotary evaporator at 45 °C. The residue is acidified with 15 drops (about 500 µl) of HCl to pH 1.3 and partitioned into methylene chloride on the liquid-liquid extraction column, ChemElut™ (Analytichem, United Kingdom). The dichloromethane is evaporated off by rotary evaporation. After cooling, the residue is redissolved in 1.5 ml of dichloromethane, evaporates and redissolves in 500 µl hexane-dichloromethane (1: 9). Clean-up is performed on a BondElut NH_2 cartridge (Analytichem, United Kingdom) conditioned with hexane-dichloromethane (1: 9).100 µl of the milk extract is transferred to the column; fats are removed with 230 µl hexane-dichloromethane (1: 9) while zearalenone is eluted with 1 ml of methanol. After evaporation of methanol the residue is dissolved in 500 µl of mobile phase.

Extraction of ochratoxins with toluene

According to §35 LMBG Method 15-00-1, AOAC 26.100–26.125

30 ml of 2 M HCl in 50 ml of 0.4 M magnesium chloride solution is added to 20 g of ground and mixed sample. After homogenization, 100 ml toluene is added and shaken vigorously for 60 minutes. The suspension is separated by centrifuge and 50 ml of the toluene supernatant is passed through a preconditioned Sep Pak silica gel column. The column is washed with two 10-ml aliquots of hexane, 10 ml of toluene/acetone (95: 5) and 5 ml of toluene. Ochratoxin A is eluted with two aliquots of 15 ml toluene/acetic acid (9: 1) and dried down at 40 °C. The residue is redissolved in 1 ml of mobile phase and filtered.

Ochratoxin sample preparation

Methylester derivatives of the ochratoxins can also be analyzed. From the extracted sample, 500 µl is evaporated to dryness and redissolved with 1 ml of dichloromethane and 2 ml of 14 % boron trifluoride in methanol. The solution is heated for 15 minutes at 50–60 °C and after cooling diluted in 30 ml of distilled water and extracted with three 10-ml aliquots of dichloromethane. The organic phase is filtered through sodium sulfate, dried down and dissolved in 500 µl of mobile phase.

Zearalenone sample preparation

Thanks to Eppley s technique,[13] zearalenone, aflatoxins and ochratoxin can be simultaneously extracted on Sep-Pak silica cartridges. The sample is added as a toluene extract, washed with toluene and zearalenone is eluted with 10 ml of toluene-acetone (95: 5) mixture.

Patulin sample preparation

Two approaches are documented.[1] Fruit juices can be cleaned on an Extrelut cartridge followed by analyte extraction on a silica gel column with toluene-ethyl acetate (3: 1) before HPLC assay.[14] The analyte can be extracted into ethyl acetate, followed by partition extraction into 1.4 % Na_2CO_3 solution and back into ethyl acetate. After evaporation of the ethyl acetate at 40 °C, the residue is dissolved in methanol-ethyl acetate (9: 1) if it is to be analyzed on a reversed-phase column packing material or in hexane-isopropanol if a diol-phase column packing material is used (details of suitable columns are given in table 1).[18]

Chromatographic separations, peak confirmation and quantification

We used a Hewlett-Packard HP 1090 Series M liquid chromatograph with DR 5 binary solvent-delivery system, variable-volume auto-injector, temperature-controlled column compartment and solvent-preheating device. Mobile phase methanol and acetonitrile were of HPLC reagent quality (Baker, Gross-Gerau, Germany). A diode-array UV-Visible absorbance detector was used together with HPLC^{3D} ChemStation software to automatically quantify the mycotoxins and identify them using spectral libraries. Fluorescent species were detected using an HP 1046A programmable flourescence detector (FLD) under the control of the HPLC^{3D} ChemStation, using lex 265 nm, lem 455 nm for aflatoxins, lex 247 nm, lem 480 nm for ochratoxin A and lex 236 nm, lem 464 nm for zearalenone. Aflatoxins were also determined using mass spectrometry on an HP 5989 MS Engine equipped with negative ion detection and Thermospray options. The electron filament capability was used to provide higher sensitivity. LC eluant passed through a capillary tube and was simultaneously heated to approaching the boiling point. The resulting liquid-vapor was injected into the mass spectrometer where it was ionized and analyzed. The mass spectrometer was controlled and the data were analyzed by the HP 59940A MS ChemStation (HP-UX series).

Results and discussion

Aflatoxin assay by HPLC-DAD and HPLC-FLD

Thin layer chromatography can be replaced by reversed phase HPLC, improving accuracy, and dramatically speeding up the time required to assay, for example B_1 takes three hours by TLC, M_1 four hours. Figure 6 shows a separation of the common aflatoxins M_2 (5 ng), M_1 (10 ng), G_2 (1.5 ng), G_1 (5 ng), B_2 (1.5 ng), B_1 (5 ng) on a reversed phase column (refer to table 1 for conditions).

Flow rate	0.30 ml/min
Mobile phase	Isocratic water–methanol–acetonitrile (63: 26: 11) mixture
Detection:	
Fluorescence	lex 365 m, lem 455 nm
Diode-array	365 nm

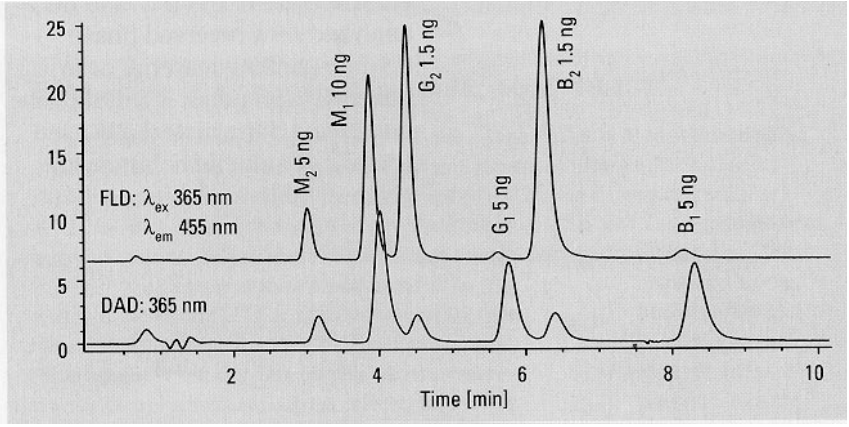


Figure 5. Analysis of the common aflatoxins by fluorescence and diode-array detection

Due to the extreme differences in fluorescence yields for B_2 and B_1 respectively G_2 and G_1 (B_2 FLD yield is about 60 times higher than B_1) it can be useful to run both detectors in series. Diode-array detection in addition gives us the UV-visible absorbance spectra dimension for further identification of the aflatoxins. As an alternative to the isocratic run with subsequent 100 % B wash, a gradient analysis from 35 % B (methanol-acetonitrile, 26: 11) to 55 % B in 10 min and 100 % B in 14 min (at 35 °C) might be used. Peaks become much sharper than under isocratic conditions, with higher signal-to-noise, and less polar compounds in the food extract are eluted in this run.

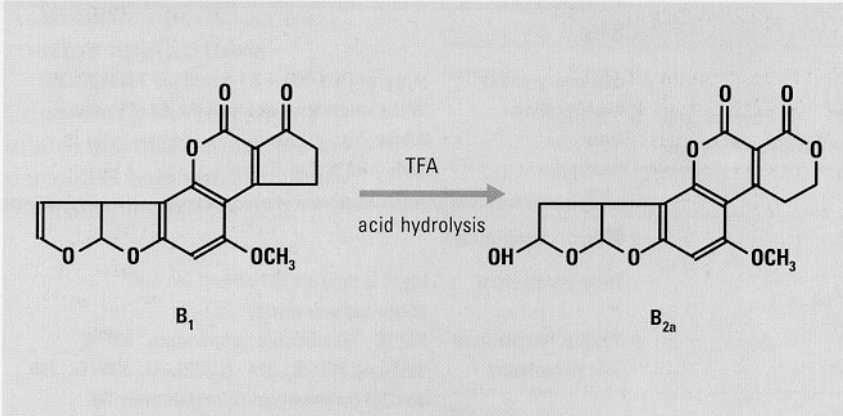


Figure 6. Structure of aflatoxins after hydrolysis with trifluoracetic acid (TFA), from B_1 to B_{2a}

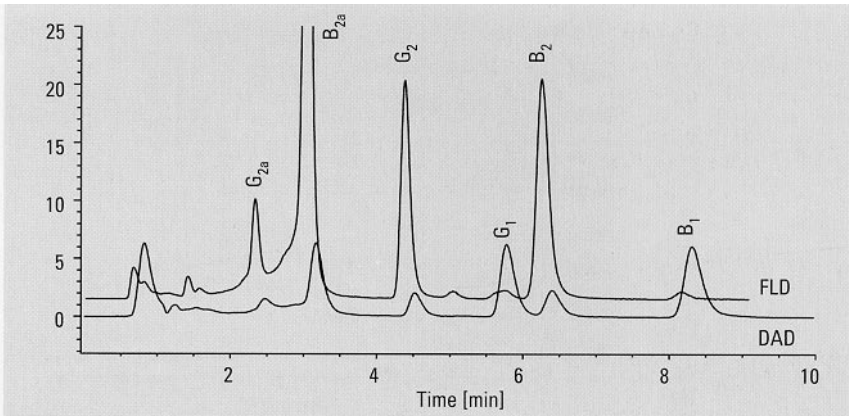


Figure 7. Analysis of the aflatoxins G_2, G_1, B_2, B_1 and G_{2a} and B_{2a} (hemiacetals), with conditions as for figure 1

If fluorescence is used alone it might be desirable to improve the B_1 and G_1 fluorescence yield by hydrating the double bond of the furanic ring (figure 6) with trifluoroacetic acid (TFA) to form the corresponding hemiacetals B_{2a} and G_{2a}. This approach can also be used as a confirmation tool for B_1 and G_1. A separation of the four aflatoxins and the hemiacetals B_{2a} and G_{2a} is shown in figure 7.[17]

Sensitivity of the B_1 can also be improved by formation of an iodine derivative[10] or by modification of the flow cell to a cell filled with fine silica particles.[11]

Aflatoxin assay by LC-MS

For highest sensitivity and selectivity we have investigated the use of mass spectrometry. The aflatoxin standard (not including M_2 and M_1) was diluted fivefold and 1 µl was injected resulting in concentrations of 1 ppm for G_1 and B_1, and 300 ppb for G_2 and B_2 (figure 8). A further dilution of 1: 10 and the 1-µl injection is shown in figure 9. Detection limits for G_1, B_2 and B_1 are less than 50 ppb for this separation. An example of thermospray application is shown in figure 11.

HPLC	
Stationary phase	Hypersil ODS 100 x 2.1 mm, 3 µl 799160D-352
Mobile phase	Water-methanol-acetonitrile 163: 26: 11)
Flow	0.3 ml
Gradient	32 %–60 % B in 10 min
MS	
Tune parameters	Manual tune on 367 adduct ion for polypropylene glycol
Source temperature	250°C Quadropole temperature 120 °C
SIM parameters	SIM ions 312–B_1, 314–B_2, 328–G_1, 330–G_2, 286 and 284 fragments of G_1 respectively G_2
Dwell time	600 msec
Electron multiplier	2500 V On Mode negative
Thermospray stem temperature	95 °C Filament ON

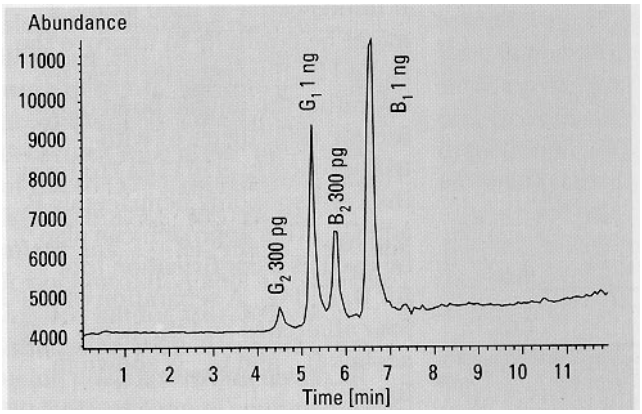


Figure 8. Analysis of G_2, G_1, B_2 and B_1 using HPLC and thermospray mass spectrometry

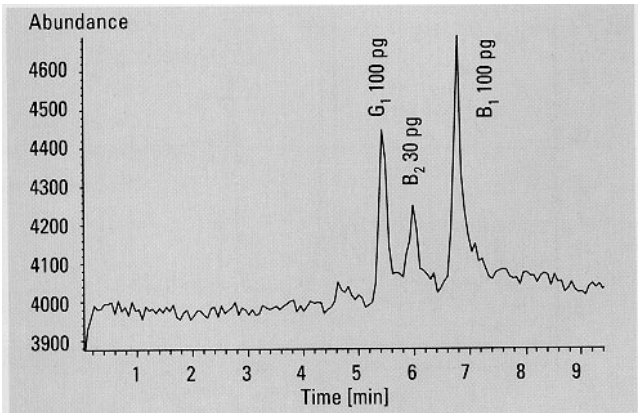


Figure 9. G_1, B_2, and B_1 in the low picogram range at 1-µl injection volume

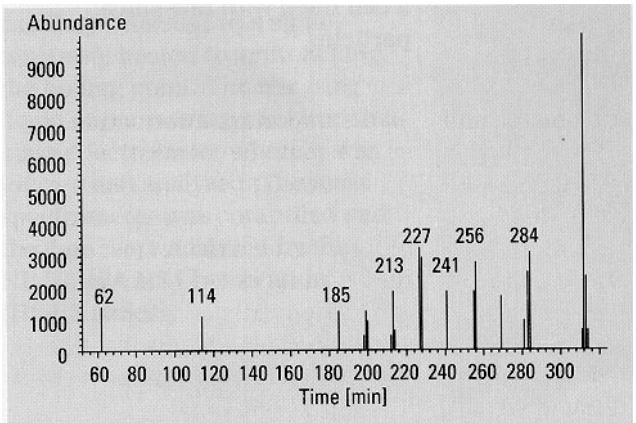


Figure 10. Total ion monitoring spectrum of aflatoxin B_1

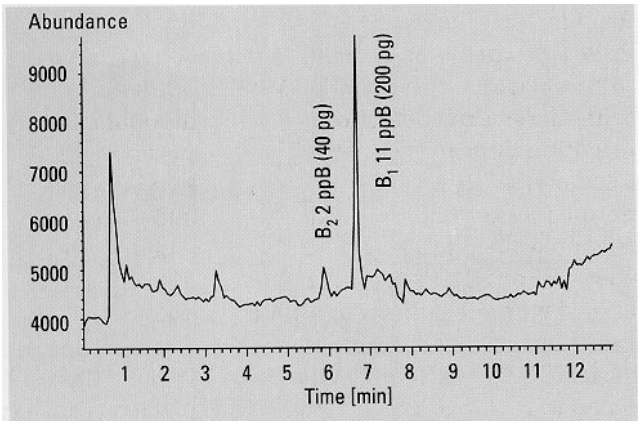


Figure 11. Extract of pistachio nut according to § 35 LMBG with 1-µl injection (see also figure 12 with 2-µl injection volume)

Automatic operation for routine applications

Considering the toxicity of aflatoxins, most countries keep permitted concentrations low, for examples see table 2.

	Aflatoxin B_1	Total aflatoxin	Milk toxin
Germany	2 ppb B_1 20 ppb B_1 animal feed diet and baby food	4 ppb $\Sigma\{B_1, B_2, G_1, G_2\}$ 50 ppt $\Sigma\{B_1, B_2, G_1, G_2\}$	50 ppt M_1 milk
France	5 ppb B_1		
Switzerland	1 ppb B_1 2 ppb B_1 corn, cereals	5 ppb $\Sigma\{B_1, B_2, G_1, G_2\}$ 10 ppt $\Sigma\{B_1, B_2, G_1, G_2\}$ diet and baby food	50 ppt M_1 in milk 20 ppt M_1 baby food 250 ppt M_1 cheese
USA, FDA		20 ppb $\Sigma\{B_1, B_2, G_1, G_2\}$	500 ppt M_1 milk
WHO, FAO	5ppb B_1	10 ppb $\Sigma\{B_1, B_2, G_1, G_2\}$	

Table 2. Limits for Aflatoxins in different countries

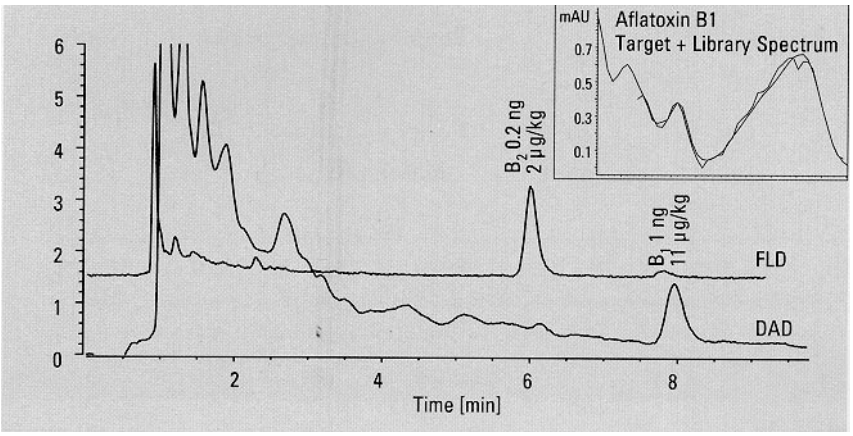


Figure 12. The analysis of a 2-µl injection of pistachio-nut extract (§35 LMBG) detected by FLD and DAD and UV-Spectrum

Sample preparation in the following applications was performed according to §35 LMBG. With diode-array detection, retention time and spectral information can both be incorporated automatically in the report. We created a library of standard mycotoxin spectra tagged with their HPLC retention times. After each run, peak spectra were automatically compared with library spectra, and their purity checked by overlaying several spectra taken in each peak. The customized report prints all this information retention times, chromatogram, library and calibration table, amounts, library search match and purity match factor. The method is fully automatic. Data acquisition and data evaluation including quantification and qualitative identification are performed in one run.

Figure 12 shows a 2-µl injection of pistachio-nut extract detected using FLD and DAD. B_1 is present in less than 1.0 ng (absolute) corresponding to 11 ppb (20 g of material extracted in 500 µl methanol of which 2 µl was injected corresponds to a multiplication factor of 12500 for the ppb value). This is close to the detection limit for fluorescence, while for UV-visible absorbance much lower values are detectable. To determine lower concentrations by FLD, larger injection volumes are needed. Fluorescence has the advantage of high selectivity- and therefore no matrix effects–whereas the diode-array detector shows much higher sensitivity for B_1 and G_1 and can be used for additional confirmation using the automatic library search program. Figure 13 shows the printout of such a search. A report header contains all the important information, such as data file name, the library used, search threshold values, peak purity, while the quantitative report contains the corresponding retention times (from library, calibration table and chromatogram), purity and library match factors and names of the identified compounds.

Report from automatic Library search

***** REPORT *****

Operator Name:	RS	Vial/Inj.No.: 100/1	
Date & Time:	10 Apr 92 4:06 pm		
Data File Name:	FOOD:AFLA-PIST		
Integration File Name:	DATA:AFLA1.I		
Calibration File Name:	FOOD:AFLA-DAD.Q		
Quantitation method:	ESTD	calibrated by Area response	
Sample Info:	PISTATIO NUTS according to $35 LMBG (reduced to 500 µl MeOH)		
Misc. Info:	20g extr. CH2CL2–clean Silicag–HEXANE, ETHER, CHC13		
Method File Name:	AFLA-DAD.M	Wavelength from: 250 to: 400 nm	
Library File Name:	FOOD:AFLATOX.L	Library Threshold: 959	
Reference Spectrum:	8.32 min	Peak Purity Threshold: 950	
Time window from:	5.0 % to: 5.0 %	Smooth Factor: 5	
Dilution Factor: 12.5	Sample Amount: 0.0	Resp.Fact.uncal.peaks: None	

Name	Amount [µg/kg]	Peak-Ret. [min]	Call-Ret. [min]	Lib.-Ret [min]	Purity Match factor	Library	Res.
B1	11.48	A 7.956	7.18	8.18	967	987	6.4
	11.48						

Figure 13. Printout of the report of the analysis in figure 12

A milk sample spiked with 600 ng/l of aflatoxin M_1 was prepared according to § 35 LMBG. 2 µl were injected and detected with FLD and DAD (figure 14). The sub-nanogram amounts of M_1 (0.6 ng) could be detected by DAD and a spectral search for confirmation was performed.

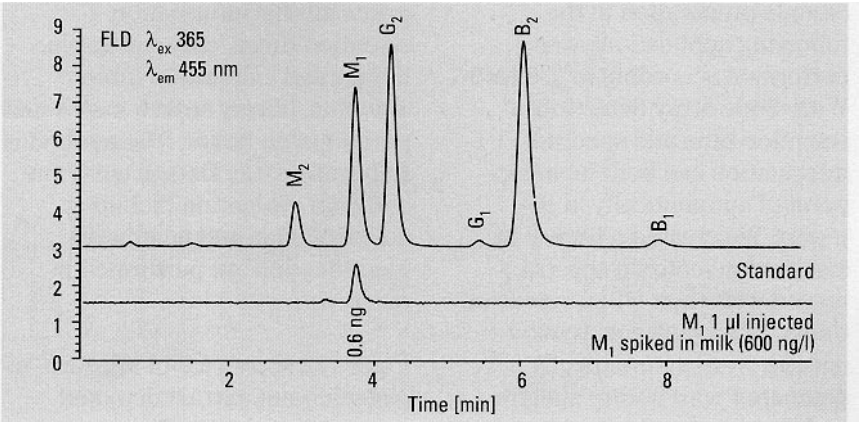


Figure 14. Analysis of spiked milk sample (600 ng/l) using sample preparation § 36 LMBG

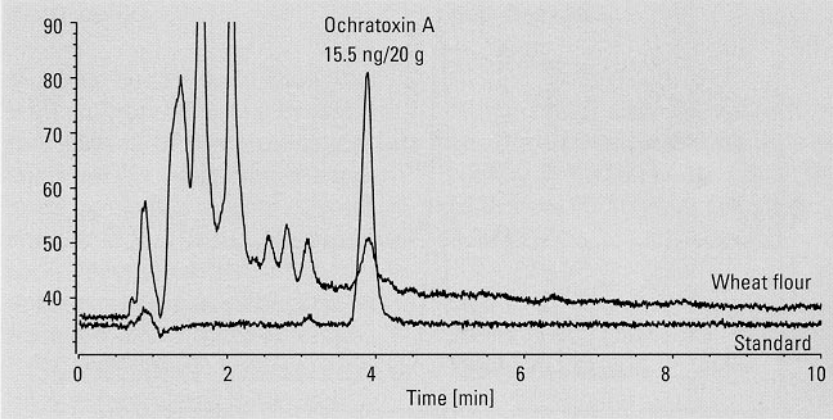


Figure 15. Analysis of ochratoxin A in wheat flour with the corresponding standard

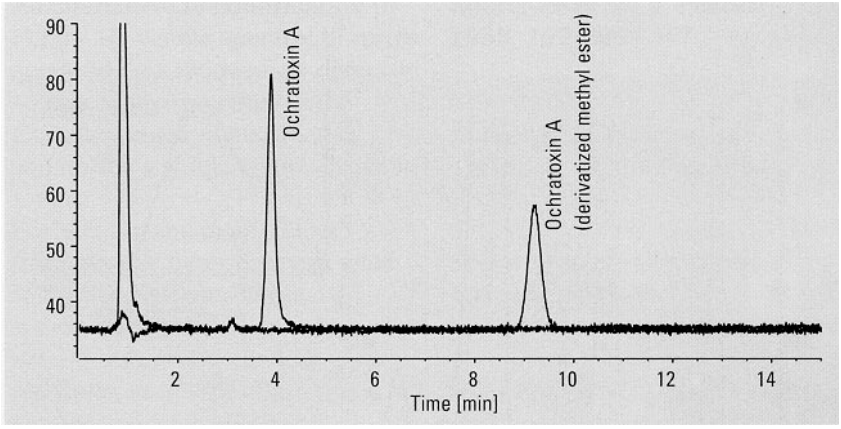


Figure 16. Separation of ochratoxin A and the ochratoxin A methylester derivative (1 ng absolute)

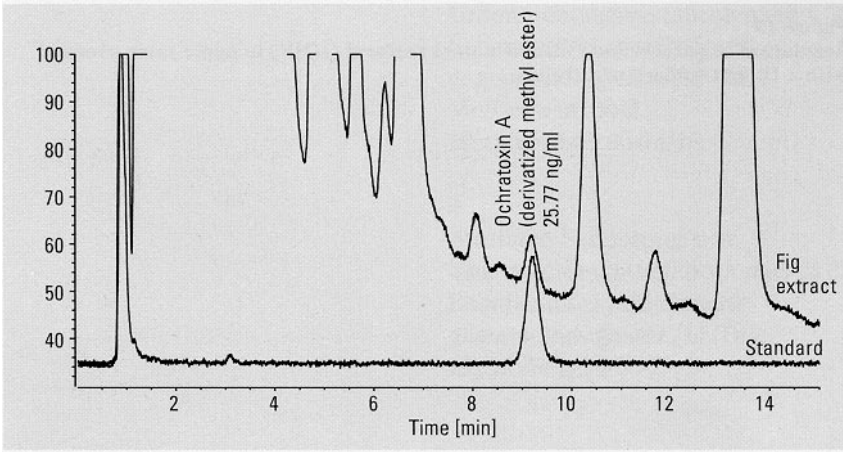


Figure 17. Analysis of a fig extract where ochratoxin A has been derivatized and overlaid with the corresponding methyl ester standard

Ochratoxin A assay by HPLC

Separation was achieved on a reversed phase column (LiChrospher 100 RP 18 125 x 4-mm id, 5 µm particles) with water /2 % acetic acid / acetonitrile (1: 1) and detected at lex 247 nm, lem 480 nm with a fluorescence detector, 20-µl injection volume and 40°C column temperature.

This analysis was confirmed with a derivatization of the mycotoxin to the methyl ester (figure 16).

The analysis also works well in more complicated matrices, for example, figs (figure 17).

Zearalenone assay by HPLC-DAD and HPLC-FLD

Separation was achieved on an Hypersil ODS narrow-bore column (100 x 2.1-mm id, 5-µm particles) using a 50 parts water, 40 parts acetonitrile, 10 parts methanol isocratic mobile phase mixture. DAD detection wavelength was 236 nm with 20 nm bandwidth, fluoresecence detection was at lex 236 nm, lem 464 nm. Figure 18 shows a standard composed of 5 ng a-zearalenol, 2 ng b-zearalenol and 8 ng zearalenone. We recommend the DAD for sensitivity and spectral confirmation, while higher selectivity is given by fluorescence.

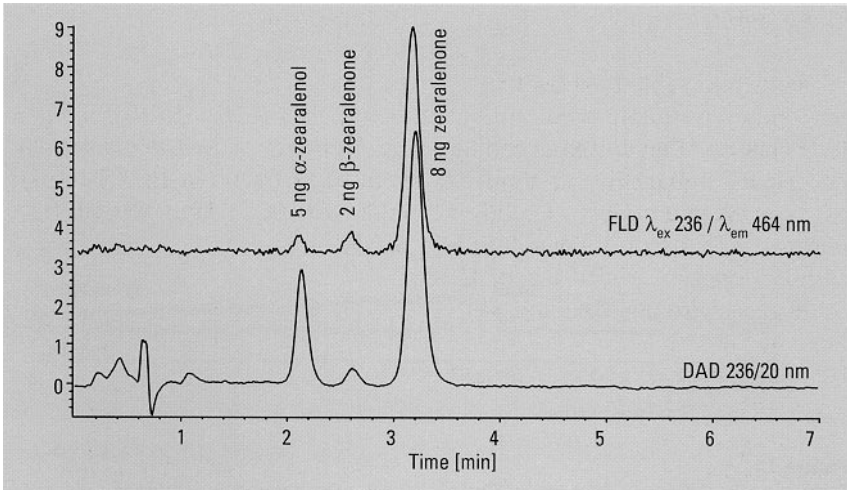


Figure 18. Analysis of zearalenone and its metabolites with FLD and DAD detection

Patulin assay by HPLC-DAD

A major problem for the analysis of patulin in apple products (juice, pies and so on) is the high content of 5-hydroxy methyl furfural (HMF) a compound that elutes close to patulin and absorbs light in the ultraviolet region also. Separation of both HMF and patulin was achieved on a silicagel column and also on a diol column (a reversed phase column based on silica gel with two hydroxyl endings). Figure 18 shows a separation of the compounds in an apple juice sample on a diol column (conditions given in table 1). Patulin was detected at 270 nm with subsequent identification using spectral library search.

With improved reverse phase column materials, separation can be done on Spherisorb RP 18, 5-µm particles using an acetonitrile gradient from 5 % to 100 % at 40 °C.[16]

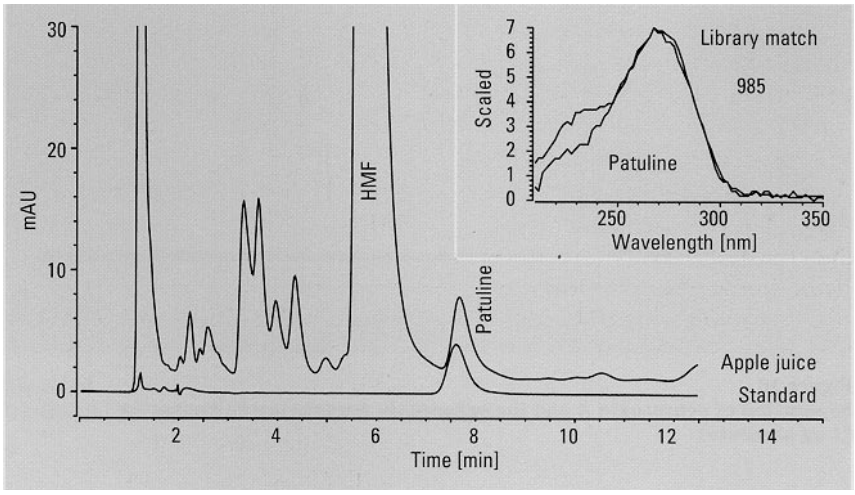


Figure 19. Resolution of patulin and 5-hydro methyl furfural (HMF) in apple juice overlaid with a 10-ng standard of patulin

Conclusion

We have been able to show that with suitable sample preparation four classes of mycotoxins can be successfully quantified at nanogram levels in a variety of solid and liquid foodstuffs. Considering sample complexities, a variety of approaches are possible and we have discussed these at length. HPLC separations were performed on reversed phase materials. Derivatization and subsequent fluorescence detection can improve selectivity for aflatoxins and ochratoxin A, and serve as an additional confirmatory analysis. An alternative to confirmation by FLD—UV-visible spectral libraries acquired on a diode-array detector—can be incorporated in the analytical run and automated, generating a single comprehensive report.

For most of the mycotoxins, fluorescence detection was used for high sensitivity. Mass spectrometry was able to lower detection limits to the low picogram range for aflatoxins, including confirmation via molecular mass.

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