

Application Note

► Online SPE-LC analysis of aflatoxins – advances in sample preparation

Category	Food
Matrix	Foodstuffs
Method	Online SPE, HPLC
Keywords	Aflatoxins, Coring cell
Analytes	Aflatoxin
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Summary

Sample preparation for instrumental analysis is of crucial importance to the quality of the test results as well as for the speed of the analysis. In liquid chromatography, SPE (solid phase extraction) is a widely used technique for concentrating analytes and for removal of matrix components. SPE has become a popular and indispensable technique, particularly for the analysis of compounds present only in trace amounts (such as antibiotics in feedstuffs).

Introduction

Classical manual SPE sample preparation techniques have two major disadvantages: they are time consuming, entailing high personnel costs, and they offer lower reproducibility in comparison to automated systems. By automating SPE sample preparation one can create independently working offline systems which are independent of the LC analysis. On the other hand, online systems make it possible to couple SPE sample preparation parallel to LC separation. New possibilities in online SPE coupled with LC can be realized with the **KNAUER Smartline Sample Preparation Unit 6300** (Fig. 1). The advantages of this system will be described by using the example of online SPE-LC analysis of aflatoxins.

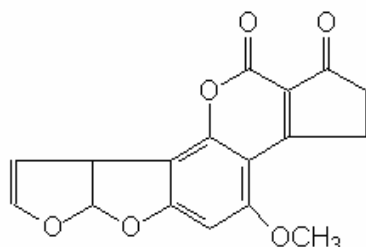
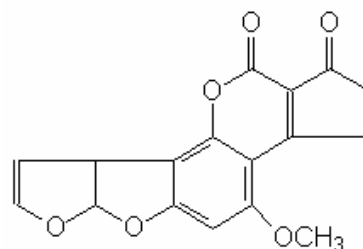
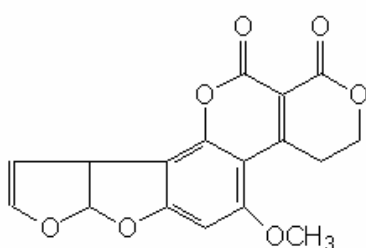
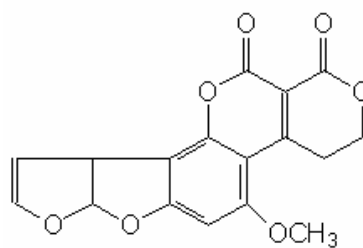


Fig. 1

KNAUER Smartline Sample Preparation Unit 6300

Aflatoxins are known as the most toxic members of the mycotoxin family. While acute toxicity (primarily liver damage) in practice plays a lesser role, chronic toxicity is a particular problem because of the pronounced carcinogenicity of this compound group [1]. The most common aflatoxin B1 (AFB1) is the strongest naturally occurring carcinogen [2]. Food products which pose an increased aflatoxin risk include nuts and seeds (pistachios, peanuts, etc.), dried fruit, spice such as paprika or pepper, as well as corn and other grains [1]. As documented in the *Rapid Alerts* issued by the European Union, aflatoxin-laden food products are encountered often by routine inspections [3].

Chemical Structures

Aflatoxin B₁Aflatoxin B₂Aflatoxin G₁Aflatoxin G₂

Maximum Values

In order to minimize the danger to the consumer posed by aflatoxin-laden foodstuffs, limits have been set by European as well as national German legislation in the lower µg/kg (ppb) range (see Table 1). These limits were set by the European commission regulation on contamination in foodstuffs 466/2001 [4] and are complimented by the German regulation specifying limits on mycotoxin contamination (MHmV) [5] and the German dietary directive [6]. Details concerning sampling and analysis methods have also been fixed [7].

Foodstuff	Maximum level (µg/kg)			Regulation
	B1	B1 + B2 + G1 + G2	M1	
Peanuts, shelled nuts, dried fruits and processed products thereof ¹⁾	2.0	4.0	---	466/2001 [4]
Cereals and related products ¹⁾	2.0	4.0	---	466/2001 [4]
Certain spices (mainly paprika, chili powder, pepper, nutmeg, ginger, turmeric)	5.0	10.0	---	466/2001 [4]
Miscellaneous foodstuffs	2.0	4.0	---	MHmV [5]
Enzymes and enzyme-preparations for production of foodstuffs	---	0.05	---	MHmV [5]
Dietary foodstuffs for infants and small children	0.05	0.05	0.01	DiätV [6]
Supplementary food for infants and small children	0.10	---	---	466/2001 [4]
Infant formula and baby food	---	---	0.025	466/2001 [4]
Dietary foodstuffs for special medical purposes, intended specifically for infants	0.10	---	0.025	466/2001 [4]
Milk	---	---	0.05	466/2001 [4] & MHmV [5]
Milk products	---	---	0.05	MHmV [5]

Table 1.

A selection of the maximum levels defined for contaminants in certain foodstuffs.

Analytical Methods

Standardized methods for the analysis of aflatoxins are available, for instance ASU § 64 LFGB-Method L 15.00-2 and L 23.05-2. Special immunoaffinity columns specifically designed for SPE are commercially available for SPE sample preparation (e.g. from r-biopharm, Darmstadt, Germany and Coring System Diagnostix, Gernsheim, Germany). The aflatoxins are isolated from the matrix by selectively binding them with antibodies and are subsequently eluted from the cartridge with organic eluents. The sample extracts produced are then analyzed via LC with fluorescence detection after postcolumn derivatization with bromine or iodine generated by a Coring electrochemical cell (formerly known as CoBrA cell, Coring System Diagnostix, Gernsheim, Germany).

Although these standardized analysis methods offer high selectivity and reproducibility and very low limits of detection for LC, the single-use immunoaffinity columns are relatively costly and the manual processing of SPE is time-consuming, entailing higher personnel costs.

Previous work done in automating the process was focused mainly on automating SPE for use with disposable immunoaffinity (IA) columns (e.g. [9]) as well as on the use of self-made IA materials in connection with automated SPE to analyze aflatoxins [10]. Nevertheless, commercially available multiple-use IA columns are not widely employed in routine analysis. With the **KNAUER Smartline Sample Preparation Unit 6300** it is possible to automate online SPE for aflatoxin analysis with reusable IA columns.

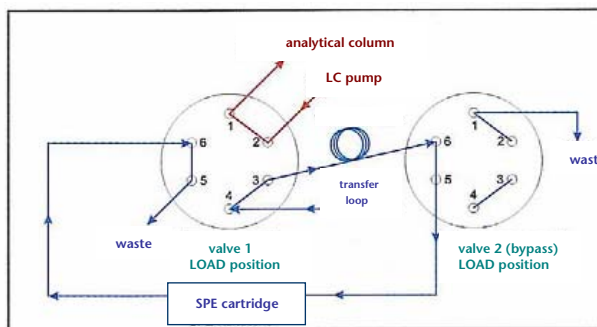
Principle of Online SPE

The **KNAUER Smartline Sample Preparation Unit 6300** can be integrated into practically any HPLC system between the autosampler and the analytical column. The principle of operation is depicted in Fig. 2.

Fig. 2

Step 1 sample introduction

The autosampler injects the sample directly onto the SPE cartridge filled with an appropriate SPE material (in this case, an immunoaffinity material)



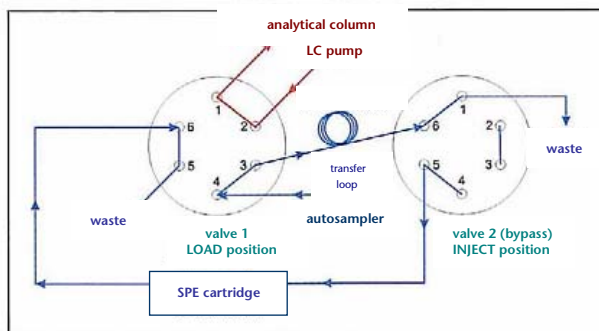
Step 1, 2 and 5: sample introduction, wash, and reconditioning

Step 2 wash

The KNAUER Smartline Sample Preparation Unit 6300 pumps water over the cartridge to remove sample matrix, pushing it to waste.

Step 3 Filling of the transfer loop

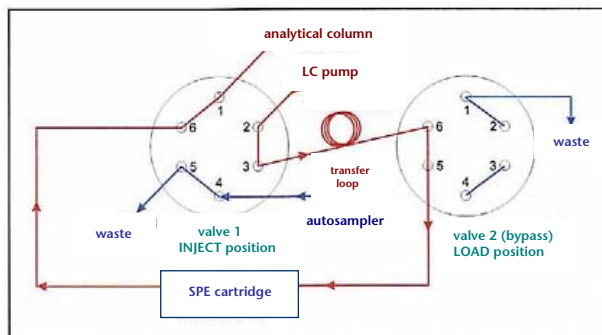
Unique to aflatoxin analysis, a second switching valve is used as a bypass valve to transfer a defined volume (200 µl) of an acetonitrile:water (67:33, v/v) mixture to the transfer loop. This provides for a more effective elution of the analytes.



Step 3: filling of the transfer loop

Step 4 Transfer step

The LC eluent flows over the SPE cartridge after valve switching and pushes along the contents of the transfer loop, transferring the analytes onto the analytical column.



Step 4: transfer step

Step 5 Reconditioning

Parallel to the LC separation, the SPE cartridge is rinsed and reconditioned with water. Afterwards, the cartridge is ready for the next sample.

Materials and Methods:

Sample Preparation

Weigh out 4 g of a ground and homogenized sample into a 30 ml polypropylene centrifuge tube. After addition of 20 ml extraction solution (methanol:water, 70:30, v/v) shake the tube for 30 min and then centrifuge for 5 min at 6000 g. Pipette 5 ml of the sample extract into a 16 ml centrifuge tube and evaporate the methanol fraction under nitrogen at 40°C. The remaining aqueous fraction is brought up to 5 ml with distilled water:methanol (95:5, v/v), membrane-filtered and filled into a standard 2 ml autosampler vial.

Table 2

HPLC Method

analytical column	RP-18, 5µm, 250 x 4 mm
Eluent A	distilled water*
Eluent B	acetonitrile:methanol, 50:50 (v/v)*
Flow rate	0.7 ml/min
Inj. volume	1000 µl
Column temperature	20 °C
HPLC system	KNAUER Smartline LPG (low pressure gradient) system
Online SPE unit	KNAUER Smartline Sample Preparation Unit 6300
SPE cartridge packing material	Immunoaffinity packing material removed from RIDA aflatoxin column (r-biopharm, Darmstadt, Germany)
Postcolumn derivatization unit	Coring electrochemical cell (formerly known as CoBrA cell, Coring System Diagnostix, Gernsheim, Germany)
Detection	Fluorescence (ex. 362 nm, em. 440 nm)
Quantitation	Matrix calibration (spiking with ca. 2 µg/kg aflatoxin B1 and G1 or ca. 0.6 µg/kg aflatoxin B2 and G2)

* each eluent also contains 100 µl HNO₃ (65%) and 119 mg KBr per liter

Results

An LC method based on the classical principle of the ASU § 64 LFGB Method L 15.00-2 [8] was developed for the analysis of aflatoxins involving online immunoaffinity SPE sample preparation using the **KNAUER Smartline Sample Preparation Unit 6300**. The SPE cartridge used is filled with commercially available immunoaffinity packing material. The limited stability of this material against organic eluents was effectively countered by making the eluent milder by the addition of water. By using the eluent described above it was possible to elute all of the analytes completely. Moreover, the antibodies on the immunoaffinity material are not substantially denatured and can therefore be reused several times. This is illustrated in Fig. 3 which compares a chromatogram of a standard solution produced from an immunoaffinity cartridge which had been reused 19 times to a chromatogram produced from a cartridge with 2 uses.

The effectiveness of the elution is depicted in Fig. 4 which shows the chromatogram of an unspiked sample to that of a spiked sample in Fig. 5 (ca. 2 µg/kg B1 and G1; 0.6 µg/kg B2 and G2). The method performance is summarized in Table 2 for a flour sample. The limits of detection reached are in the area of the maximum values set forth by EU and German regulations (2-5 µg/kg AFB1 [4-6]). This method is currently being validated on other regulated foodstuffs including peanuts, pistachios and corn products.

Fig. 3

Stability of the immunoaffinity cartridge after repeated use

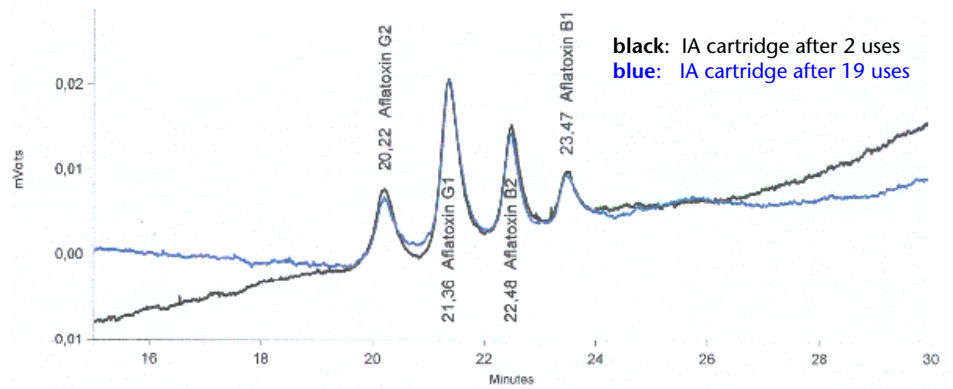


Fig. 4

Chromatogram of a non-spiked sample.

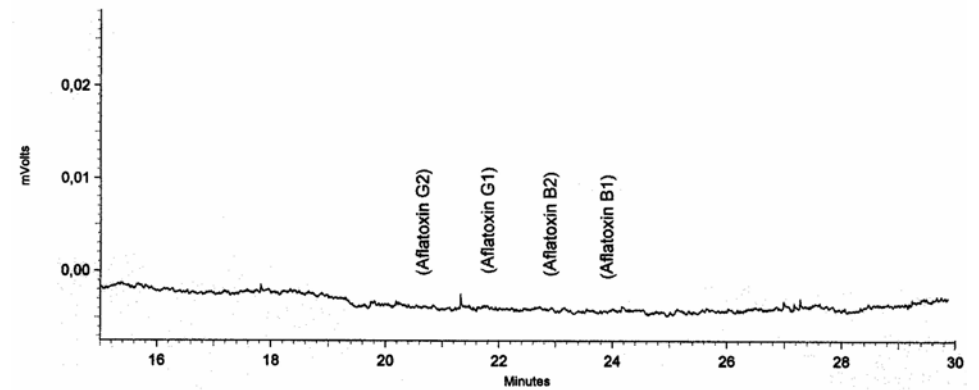
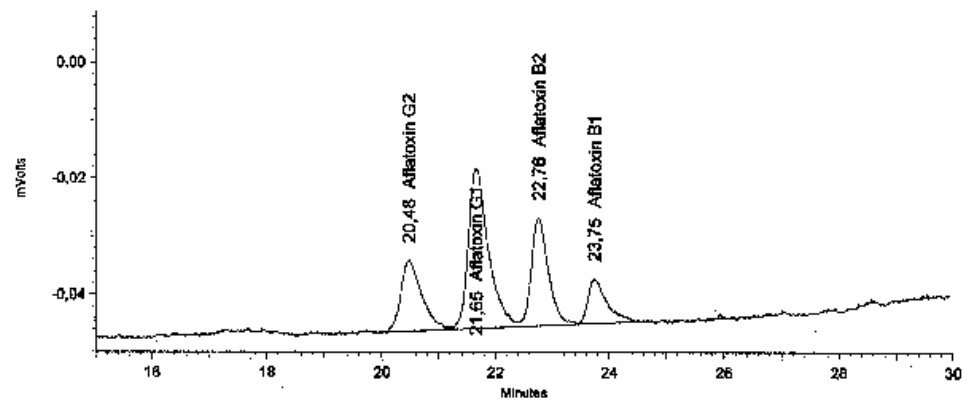


Fig. 5

Chromatogram of sample spiked with AFB1/AFG1 each approx. 2µg/kg AFB2/AFG2 each approx. 0.6 µg/kg).



Method Performance

Table 3

Online SPE-LC analysis of aflatoxins (sample matrix: flour).

Substance	LOD ¹ (µg/kg)	Spiked (µg/kg)	Average recovery ² (%)	%RSD ³
Aflatoxin B ₁	1.93	92.6	92.6	8.8
Aflatoxin B ₂	0.54	96.3	96.3	9.2
Aflatoxin G ₁	2.00	94.0	94.0	6.9
Aflatoxin G ₂	0.63	92.1	92.1	27.1

¹ limit of detection; signal/noise ratio = 5

² n = 3 (repeat measurements)

³ repeat measurements of 4 spiking levels (ca. 1, 2, 3, and 6 µg/kg and 0.3, 0.6, 0.9, and 1.8 µg/kg for aflatoxins B₁ + G₁ and B₂ + G₂, respectively)

Conclusion

The method presented here showed an online SPE procedure based on the example of aflatoxin analysis. The combination of the online SPE sample preparation by means of the **KNAUER Smartline Sample Preparation Unit 6300** with the classical ASU § 64 LFGB offers the following advantages

- cost-effective, simple, and fast sample preparation involving lower personnel costs, thanks to a reusable immunoaffinity column material
- high selectivity by means of antibodies and selective detection (fluorescence detection after postcolumn derivatization)
- good reproducibility thanks to automated sample handling
- low limits of detection (e.g. aflatoxin B₁: 0.2 µg/kg)

The online SPE sample preparation described here can of course also be combined with any LC-MS system available so that the advantages described above can also be enjoyed with MS detection.

This aflatoxin application illustrates only one of the many potential areas of application of the online SPE technique. Other possible application areas include

- antibiotics (sulfonamide, streptomycin, chloramphenicol, etc.)
- pesticides (Carbendazim, Chlorfenvinphos, etc.).

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