DIETARY SUPPLEMENTS

Determination of Aflatoxin B_1 in Medical Herbs: Interlaboratory Study

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A method was developed for the determination of aflatoxin B_1 in medical herbs (senna pods, botanical name *Cassia angustifolia*; devil's claw, botanical name *Harpagophytum procumbens*; and ginger roots, botanical name *Zingiber officinale*). The method, which was tested in a mini-collaborative study by 4 laboratories, is based on an immunoaffinity cleanup followed by reversed-phase high-performance liquid chromatography separation and fluorescence detection after post-column derivatization. It allows the quantitation of aflatoxin B_1 at levels lower than 2 ng/g. A second extractant (acetone–water) was tested and compared to the proposed methanol–water extractant. Several post-column derivatization options (electrochemically generated bromine, photochemical reaction, and chemical bromination) as well as different integration modes (height versus area) were also investigated. No differences were found depending on the choice of derivatization system or the signal integration mode used. The method was tested for 3 different matrixes: senna pods, ginger root, and devil's claw. Performance characteristics were established from the results of the study and resulted in HorRat values ranging from 0.12 to 0.75 with mean recoveries from 78 to 91% for the extraction with methanol–water and HorRat values ranging from 0.10–1.03 with mean recoveries from 98 to 103% for the extraction with acetone–water. As a result, the method, with all tested variations, was found to be fit-for-purpose for the determination of aflatoxin B_1 in medical herbs at levels of 1 μg/kg and above.

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Aflatoxins are toxic secondary fungal metabolites produced by 3 *Aspergillus* species: *A. flavus, A. parasiticus,* and *A. nomius*. Until now, 18 different types of aflatoxins have been identified. The most frequently occurring types in products of plant origin are aflatoxins B_1, B_2, G_1, and G_2 (AfB_1, AfB_2, AfG_1, and AfG_2). AfB_1 is the most toxic and abundant of this group and is considered by the International Agency for Research on Cancer (IARC) as carcinogenic to humans (1). Acute toxic effects after intake of aflatoxins are called aflatoxicosis. Symptoms of aflatoxicosis are bile duct proliferation, hepatic necrosis, osteoclerosis of bones, childhood cirrhosis, immune suppression, and hepatic veno-oclusive lesions. The risk of suffering aflatoxicosis depends on the level and type of aflatoxins in the diet. These severe symptoms, however, are rarely observed, and the main concern toward consumer safety is related to the subacute and chronic uptake of these carcinogenic mycotoxins. As a result, the summary of the evaluation performed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) has not set a tolerable daily intake figure, but concluded that aflatoxins should be treated as carcinogenic food contaminants, the intake of which should be reduced to levels as low as reasonably achievable (2).

Aflatoxins can be found in various products such as maize, peanuts, Brazil nuts, rice, barley, cotton seeds, pistachio nuts, dried figs, spices, and herbs (3). They are regulated in more than 100 countries worldwide (4), while within the European Community maximum levels for aflatoxins are set for certain

Table 1. Results from duplicate analysis (A and B) obtained with MEP, PCD by KOBRA, and integration by area

	Aflatoxin B_1 found, μg/kg							
	Laboratory 1		Laboratory 2		Laboratory 3		Laboratory 4	
Material	A	B	A	B	A	B	A	B
Senna pods 1 (NC)[a]	1.78	2.03	1.85	1.88	2.83	2.82	1.47	1.66
Senna pods 2 (NC)	13.03	12.81	15.73	15.41	18.61	18.14	11.76	13.82
Ginger root 1 (NC)	<0.4	<0.4	<0.07	<0.07	<[b]	<	<0.06	<0.06
Ginger root 2 (NC)	1.39	1.46	2.37	2.22	2.85	2.96	1.62	2.19
Devil's claw 1 (NC)	<0.4	<0.4	<0.07	<0.07	0.17	0.16	<0.06	<0.06
Devil's claw 1 μg/kg	0.91	1.06	0.89	0.86	0.93	0.86	0.95	0.88
Senna pods recovery, %	72	63	107	105	26	73	92	85
Ginger root recovery, %	82	87	88	97	93	90	95	95
Devil's claw recovery, %	81	64	90	90	83	87	86	81

[a] NC = Naturally contaminated material. The fortification level for the recovery experiments was 2 μg/kg for all matrixes.
[b] The mycotoxin was not detected (below the LOD).

food products at a level of 2 ng/g for AfB_1 and 4 ng/g for total aflatoxins (5). In addition, adequate analytical methods to enforce such regulatory limits for aflatoxins have been developed and validated in collaborative trials for relevant food and feed matrixes (6–8).

As regulatory limits for AfB_1 in medical herbs are currently discussed by the European Pharmacopoeia, this report considers the availability of a validated method of analysis to allow the enforcement of such limits. For medical herbs, a method similar to those currently applied for food and feed has been proposed to determine the content of aflatoxins (9); however, the authors know of no collaboratively validated data to characterize this or any other method for this purpose.

Most of the recent and routinely used chromatographic methods for the determination of aflatoxins have common principles and involve as first step the extraction of the test portion with an aqueous organic solvent, the extractant, containing either methanol, acetonitrile, or acetone. A diluted portion of extract is then purified over an immunoaffinity column to ease the isolation from interfering matrix components. The concentration of AfB_1 is then chromatographically determined. The choice of organic solvent in the extractant, its concentration and the sample-to-extractant-ratio (g sample/mL solvent), differ for most published methods for aflatoxins in general and in particular on the matrix investigated as well as the target level analyzed (10–13).

Aflatoxins are commonly separated by standard reversed-phase high-performance liquid chromatography (RP-HPLC) systems in combination with a derivatization of AfB_1 and AfG_1 and fluorescence detection. Derivatization is required for fluorescence detection, because AfB_1 undergoes fluorescence quenching in solvents (10). Although pre- and post-column derivatization (PCD) have been applied in the past (10), precolumn derivatization has several disadvantages compared to PCD, even though it is less demanding in instrumentation. Drawbacks are the need for an additional evaporation step, which makes automatization of the process difficult and the fact that the formed derivatives have a higher polarity. This results in shorter retention times that can lead to an elution in the region of matrix interferences.

PCD techniques have been applied successfully in many analytical methods for aflatoxins. The systems widely used are based on the generation of bromine either by the use of pyridinium hydrobromide perbromide (PBPB; 14) or by electrochemical bromination (so-called KOBRA cell; 15). Post-column photolytic derivatization involving UV radiation (PHRED) has also been used successfully (11) and compared with electrochemical derivatization for different food matrixes (16). Another technique involves iodination, but presents some drawbacks due to the need of daily preparation of the iodine solution, a rather longer reaction tube for heating (70°C), and cooling prior to detection. The use of cyclodextrins has been shown to improve fluorescence of AfB_1 (17), but is costly and has no advantage over other derivatization techniques for routine analysis.

In agreement with the European Pharmacopoeia, it was decided to investigate only the determination of AfB_1 rather than all 4 aflatoxins. Therefore, our aim was to identify the most appropriate method parameters for its determination at a level of 2 ng/g for relevant medical herbs, represented by senna pods, ginger root, and devil's claw. Basic considerations were that the method should be based on an immunoaffinity cleanup and RP-LC with fluorescence detection, which are well established and common procedures and avoid the use of chlorinated solvents. The procedure described by Reif and Metzer (9) served as a candidate method while other parameters as discussed above for extraction and PCD were investigated. For the purpose of the validation study, the

Table 2. Results from duplicate analysis (A and B) obtained with MEP, PCD by KOBRA, and integration by height

	Aflatoxin B_1 found, μg/kg							
	Laboratory 1		Laboratory 2		Laboratory 3		Laboratory 4	
Material	A	B	A	B	A	B	A	B
Senna pods 1 (NC)[a]	1.89	2.14	—[b]	—	—	—	1.57	1.80
Senna pods 2 (NC)	13.84	13.1	—	—	—	—	12.80	14.57
Ginger root 1 (NC)	<0.4	<0.4	—	—	—	—	—	—
Ginger root 2 (NC)	1.38	1.47	—	—	—	—	1.72	2.27
Devil's claw 1 (NC)	<0.4	<0.4	—	—	—	—	—	—
Devil's claw 1 μg/kg	0.86	0.95	—	—	—	—	1.00	0.95
Senna pods recovery, %	79	61	—	—	—	—	97	93
Ginger root recovery, %	78	81	—	—	—	—	101	98
Devil's claw recovery, %	62	61	—	—	—	—	93	86

[a] NC = Naturally contaminated material. The fortification level for the recovery experiments was 2 μg/kg for all matrixes.
[b] — = Not determined.

method of Reif and Metzer (9) was rewritten in a style suitable for collaborative trial purposes and has recently been published for comments in Pharmeuropa (18). The study was performed by 4 laboratories, always conducting the common mandatory extraction and the optional one. In the mandatory extraction procedure (MEP), the test portion is extracted with methanol–water. In the optional extraction procedure (OEP), acetone–water is used as extractant in combination with an adopted immunoaffinity cleanup. Electrochemically generated bromination (KOBRA cell) was the mandatory PCD for all participants. Additionally, photochemical reaction (PHRED) or bromination by PBPB were allowed. This was achieved by switching the PCD and re-injection of the purified HPLC extracts after the mandatory analysis sequence.

Results were reported based on peak height and peak area, in order to evaluate possible differences in the robustness of integration modes. Background for this investigation was that with the availability of modern integration software tools for peak evaluation, signals are very often integrated automatically and reported by area. In the past it has been shown that integration is a crucial element for robust and precise analysis (19). This is especially true for trace analysis, as signal evaluation by peak height can be valuable for small peaks (small signal-to-noise ratio); differences in baseline setting tend to influence peak area more than peak height measurements.

This method can be applied to the determination of AfB_1 in medicinal herbs. The limit of quantitation (LOQ) of the method has been demonstrated to be 2 μg/kg or better, depending on the equipment used.

Materials

General

The obtained results are based on data from different laboratories with different but similar instrumentation and sources of reagents. Only those parameters and requirements that were mandatory are listed.

Laboratories received a common written method protocol, a detailed study protocol, and a total of 6 test samples (2 for each matrix). Each matrix was analyzed on a different day, and 2 calibration curves were made: one prior to the analysis of the sample extracts and one thereafter. For calculation of the contamination levels in samples, the mean of both calibration curves was used.

Samples

Test materials of senna pod, devil's claw, and ginger root were supplied by Phytolab (Vestenbergsreuth, Germany) and were tested for homogeneity (blank and naturally contaminated) by replicate analysis ($n = 5$) on a 5 g basis. The homogeneity testing was done before the trial by the participants with in-house methods. Coefficients of variation (CVs) ranged from 10.2 to 15.8% for most materials with the exception of devil's claw (CV, 42.8%). The latter result indicated that the material was most likely not homogeneous at 5 g portions. This led to the replacement of the naturally contaminated devil's claw material in the collaborative trial; AfB_1-free (blank) devils claw fortified at 1 ng/g AfB_1 was then used instead, as this was the target working level based on the foreseen legislative limit. Each participant received 6 different test materials (3 naturally contaminated and 3 blank materials), which were split by the participants for duplicate analysis and spiking (Tables 1–6).

Reagents

Use only reagents of recognized analytical grade and water complying with grade 3 of ISO 3696, unless otherwise specified.

(**a**) *PBPB*.—CAS 39416-48-3.

(**b**) *Potassium bromide*.

Table 3. Results from duplicate analysis (A and B) obtained with OEP, PCD by KOBRA, and integration by area

	Aflatoxin B_1 found, μg/kg							
	Laboratory 1		Laboratory 2		Laboratory 3		Laboratory 4	
Material	A	B	A	B	A	B	A	B
Senna pods 1 (NC)[a]	2.30	1.43	1.75	1.83	2.80	3.94	1.77	1.45
Senna pods 2 (NC)	14.42	13.32	18.44	19.52	22.53	22.65	15.83	16.34
Ginger root 1 (NC)	<0.4	<0.4	<0.07	<0.07	<[b]	<	<0.06	<0.06
Ginger root 2 (NC)	2.35	3.58	2.56	2.94	3.08	3.24	2.31	2.80
Devil's claw 1 (NC)	<0.4	<0.4	<0.07	<0.07	0.18	0.18	<0.06	<0.06
Devil's claw 1 μg/kg	0.94	1.06	1.08	1.02	1.09	1.21	1.08	1.02
Senna pods recovery, %	87	102	86	114	102	96	110	83
Ginger root recovery, %	96	93	100	104	112	114	104	104
Devil's claw recovery, %	96	104	102	100	92	96	104	102

[a] NC = Naturally contaminated material. The fortification level for the recovery experiments was 2 μg/kg for all matrixes.
[b] The mycotoxin was not detected (below the LOD).

(**c**) *Acetonitrile.*—HPLC grade.

(**d**) *Methanol.*—HPLC grade.

(**e**) *Acetone.*—Technical grade.

(**f**) *Methanol.*—Technical grade.

(**g**) *Toluene.*—HPLC grade.

(**h**) *Extraction solvent for MEP.*—Methanol–water (7 + 3, v/v). Mix 7 parts per volume methanol with 3 parts per volume water.

(**i**) *Extraction solvent for OEP.*—Acetone–water (85 + 15, v/v). Mix 85 parts per volume methanol with 15 parts per volume water.

(**j**) *Nitric acid, c(HNO$_3$).*—4 M.

(**k**) *Immunoaffinity column.*—The affinity column must contain antibodies raised against AfB_1. The column must have a maximum capacity of not less than 100 ng AfB_1 and must give a recovery of not less than 80% when applied as a standard solution in methanol–water (87.5 + 12.5, v/v) containing 5 ng. For this study, EASI-EXTRACT™ aflatoxin immunoaffinity columns of Rhone-rBiopharm, Glasgow, Scotland, were used.

(**l**) *HPLC mobile phase solvent (A).*—For use with photochemical reactor (PHRED) or with PBPB. Water–acetonitrile–methanol solution (6 + 2 + 3, v/v/v). Degas the solution before use.

(**m**) *HPLC mobile phase solvent (B).*—For use with electrochemically generated bromine: water–acetonitrile–methanol solution (6 + 2 + 3, v/v/v). Add 120 mg potassium bromide and 350 μL nitric acid/L mobile phase. Degas the solution before use.

(**n**) *Post-column reagent.*—Dissolve 50 mg PBPB in 1000 mL water. *Note:* The solution can be used for up to 4 days if stored in a dark place at room temperature.

(**o**) *Toluene–acetonitrile (98 + 2, v/v) mixture.*—Mix 98 parts per volume of toluene with 2 parts per volume acetonitrile.

(**p**) *AfB_1, either in form of crystals or film, or in form of commercially available AfB_1 solution.—Caution*: Adequately, protect the laboratory where the analyses are done from daylight. This can be achieved effectively by using UV absorbing foil on the windows in combination with subdued light (no direct sunlight) or curtains or blinds in combination with artificial light (fluorescent tubes are acceptable). Protect AfB_1 containing solutions from light as much as possible (keep in the dark; use aluminum foil or amber-colored glassware).

(**q**) *$AflB_1$ stock solution for determination of concentration.*—Dissolve AfB_1 in toluene–acetonitrile to give a solution containing 10 μg/mL. To determine the exact concentration of AfB_1 in stock solution, record the absorption curve between a wavelength of 330 and 370 nm in 1 cm quartz glass cells in a spectrometer with toluene–acetonitrile in the reference path. Calculate the AfB_1 mass concentration, C, in μg/mL, as follows:

$$C = \frac{A_{max} \times M_i \times 100}{E_l \times d}$$

where A_{max} is the absorbance determined at the maximum of the absorption curve; M_i is the relative molecular mass of AfB_1, in grams per mol (312 g/mol); E_l is the molar absorptivity of AfB_1 in toluene–acetonitrile (1930 m^2/mol); d is the optical path length of the cell, in cm.

(**r**) *AfB_1 stock solution for HPLC.*—Prepare a stock solution containing 100 ng/mL AfB_1 by diluting with toluene–acetonitrile. Wrap the flask tightly in aluminium foil and store at <4°C. Before use, do not remove the aluminium foil until the contents have reached room temperature to avoid incorporation of water by condensation. When the solution has to be stored for a longer period (e.g., 1 month), weigh the flask and record any change before and after the solution is to be used.

Table 4. Results from duplicate analysis (A and B) obtained with OEP, PCD by KOBRA, and integration by height

	Aflatoxin B_1 found, μg/kg							
	Laboratory 1		Laboratory 2		Laboratory 3		Laboratory 4	
Material	A	B	A	B	A	B	A	B
Senna pods 1 (NC)[a]	2.83	1.46	1.72	1.84	—[b]	—	1.87	1.56
Senna pods 2 (NC)	14.87	12.96	18.33	19.43	—	—	17.09	17.42
Ginger root 1 (NC)	—	—	—	—	—	—	—	—
Ginger root 2 (NC)	2.31	3.57	2.99	2.59	—	—	2.43	2.94
Devil's claw 1 (NC)	—	—	—	—	—	—	—	—
Devil's claw 1 μg/kg	0.93	0.95	1.09	1.02	—	—	1.14	1.12
Senna pods recovery, %	87	102	87	111	—	—	119	91
Ginger root recovery, %	95	92	102	105	—	—	112	109
Devil's claw recovery, %	93	99	102	100	—	—	115	112

[a] NC = Naturally contaminated material. The fortification level for the recovery experiments was 2 μg/kg for all matrixes.
[b] — = Not determined.

(**s**) *AfB_1 standard solutions for HPLC.*—Use the stock solution (100 ng/mL, *see* instructions for mini-collaborative study) for pipetting the volumes as given in Table 7 into a set of 250 mL calibrated volumetric flasks. Evaporate the chloroform solution just to dryness under a stream of nitrogen at room temperature. To each flask, add 75 mL methanol, let AfB_1 dissolve, dilute to the mark with water, and shake well. *Note*: Methanol and water are subject to volume contraction when mixed.

Apparatus

(**a**) *General*.—All glassware coming into contact with aqueous solutions of AfB_1 must be washed with acid solution before use. Many laboratory washing machines do this as part of the washing program. Otherwise, soak laboratory glassware coming into contact with aqueous solutions of AfB_1 in sulfuric acid (2 M) for several hours, then rinse well (e.g., 3 times) with water to remove all traces of acid. Check the absence of acid with pH paper. *Note*: This treatment is recommended because the use of nonacid-washed glassware may cause losses of AfB_1. In practice, the treatment is necessary for round-bottomed flasks, volumetric flasks, measuring cylinders, vials or tubes used for calibration solutions and final extracts (particularly autosampler vials), and Pasteur pipets, if these are used to transfer calibration solutions or extracts.

(**b**) *Ultrasonic bath.*—Frequency, 35 kHz.

(**c**) *Filter paper.*—24 cm id, prefolded.

(**d**) *Conical flask*.—With screw top or glass stopper.

(**e**) *Glass microfiber filter paper*.—Retention size 1.6 μm or better.

(**f**) *Reservoir.*—50 mL with Luer tip connector for immunoaffinity column.

(**g**) *Vacuum manifold*.

(**h**) *Volumetric glassware*.—5 and 50 mL flasks, with an accuracy of at least 0.5%.

(**i**) *HPLC pump*.—Suitable for flow rate of 1.0 mL/min.

(**j**) *Injection system (manual or automatic).*—Capable of injecting 500 μL by partial or total loop filling (*see* instructions of manufacturer). Smaller volumes are acceptable, provided that enough sensitivity is obtained.

(**k**) *RP-HPLC column*.—LC-18 or ODS-2, which ensures a baseline resolved resolution of the AfB_1 peak from all other peaks. The maximum overlapping of peaks must be <10% (it might be necessary to adjust the mobile phase for a sufficient baseline resolution). A suitable precolumn should be used.

(**l**) *Post-column derivatization system.*—Three derivatization systems have been found appropriate: *(1) System for derivatization with PBPB*.—Second HPLC pulseless pump, zero-dead volume T-piece, reaction tubing minimum 45 cm × 0.5 mm id PTFE (to be used only with mobile phase A). (*2*) *System for derivatization with photochemical reactor (PHRED).*—Reactor unit with one 254 nm low-pressure mercury UV bulb, one polished support plate, knitted reactor coil, length 25 m, 0.25 mm id, nominal void volume 1.25 mL (to be used with mobile phase A (1). (*3*) *System for derivatization with electrochemically generated bromine (KOBRA) cell*.—DC-supply in series with the KOBRA cell, capable of providing a constant current of ca 100 μA, reaction tube Teflon. Dimensions of 120 × 0.25 mm id have been found to be appropriate (to be used only with mobile phase B).

(**m**) *Fluorescence detector*.—With wavelength of $\lambda = 360$ nm excitation filter and a wavelength of $\lambda > 420$ nm cut-off emission filter, or equivalent. Recommended settings for adjustable detectors are 365 nm (excitation wavelength), 435 nm (emission wavelength).

(**n**) *Disposable filter unit (0.45 μm).*—Prior to usage, verify that no aflatoxin losses occur during filtration (recovery

Table 5. Results from duplicate analysis (A and B) obtained with MEP, PCD by PHRED, and integration by area

	Aflatoxin B_1 found, μg/kg							
	Laboratory 1		Laboratory 2		Laboratory 3		Laboratory 4	
Material	A	B	A	B	A	B	A	B
Senna pods 1 (NC)[a]	1.50	2.29	—[b]	—	—	—	1.59	1.83
Senna pods 2 (NC)	13.66	12.97	—	—	—	—	12.86	13.07
Ginger root 1 (NC)	<0.4	<0.4	—	—	—	—	<[c]	<
Ginger root 2 (NC)	1.32	1.40	—	—	—	—	1.66	2.19
Devil's claw 1 (NC)	<0.4	<0.4	—	—	—	—	<	<
Devil's claw 1 μg/kg	0.89	0.66	—	—	—	—	0.99	0.93
Senna pods recovery, %	70	71	—	—	—	—	91	88
Ginger root recovery, %	82	78	—	—	—	—	96	98
Devil's claw recovery, %	37	65	—	—	—	—	99	93

[a] NC = Naturally contaminated material. The fortification level for the recovery experiments was 2 μg/kg for all matrixes.
[b] — = not determined.
[c] The mycotoxin was not detected (below the LOD).

testing). *Note:* There is a possibility that various filter materials can retain AfB_1.

(**o**) *Pipets.*—10 mL capacity.

(**p**) *Analytical balance*.—Capable of weighing to 0.1 mg.

(**q**) *Laboratory balance*.—Capable of weighing to 0.01 g.

(**r**) *Calibrated μL syringe(s) or μL pipet(s)*.—25–200 μL.

METHODS

Extraction of Aflatoxin B_1

Note: Allow the immunoaffinity columns to reach room temperature

(**a**) *MEP*.—Weigh, to the nearest 0.1 g, ca 5 g test portion into a 150 mL conical flask. Add 100 mL extraction solvent. Extract by sonification for 30 min. Filter extract using a prefolded filter paper.

(**b**) *OEP*.—Weigh to the nearest 0.1 g ca 10 g of the test portion into a 150 mL conical flask. Add 100 mL extraction solvent. Extract by sonification for 30 min. Filter the extract using a prefolded filter paper.

Immunoaffinity Cleanup

(**a**) *MEP*.—Pipet 10.0 mL of the clear filtrate into a 150 mL conical flask. Dilute with 70 mL water. Connect immunoaffinity column to vacuum manifold, and attach the reservoir to the immunoaffinity column. Add 40 mL diluted sample extract to the reservoir and pass through the immunoaffinity column at a flow rate of ca 3 mL/min (ca 1 drop/s; gravity). Do not exceed a flow rate of 5 mL/min. Wash the column twice with 10 mL water at a flow rate of maximum 5 mL/min and dry by applying little vacuum for 5–10 s or passing air through the immunoaffinity column by means of a syringe for 10 s.

(**b**) *OEP*.—Pipet 5.0 mL of the clear filtrate into 150 mL conical flask. Dilute with 75 mL water. Connect the immunoaffinity column to the vacuum manifold, and attach the reservoir to the immunoaffinity column. Add 40 mL diluted sample extract to the reservoir and pass through the immunoaffinity column at a flow rate of ca 3 mL/min (ca 1 drop/s; gravity). Do not exceed a flow rate of 5 mL/min. Wash the column twice with 10 mL water at a flow rate of maximum 5 mL/min and dry by applying little vacuum for 5–10 s or passing air through the immunoaffinity column by means of a syringe for 10 s.

(**c**) *Elution of AfB_1 from the immunoaffinity column*.—Apply 0.5 mL methanol on the column and let it pass through by gravity. Collect the eluate in a calibrated volumetric flask of 5 mL. Wait for 1 min and apply a second portion of 0.5 mL methanol. Wait for 1 min and apply a third portion of 0.5 mL methanol. Collect most of the applied elution solvent by pressing air or vacuum through. Fill the flask to the mark with water and shake well. If the solution is clear, it can be used directly for HPLC analysis. If the solution is not clear, pass it through a disposable filter unit prior to HPLC injection.

Note: Methods for loading onto immunoaffinity columns, washing the column, and elution may vary slightly between column manufacturers. Specific instructions supplied with the columns may have to be followed.

(**d**) *HPLC*.—The AfB_1 is separated by isocratic RP-HPLC at ambient temperature with a reversed-phase column and an appropriate mobile phase, respectively. The recommended HPLC operating conditions are flow rate mobile phase, 1 mL/min for columns with 4.6 mm id; injection volume, 500 μL (smaller volumes are acceptable, provided that enough sensitivity is obtained); fluorescence detector settings, described above. AfB_1 should be base-line resolved from

Table 6. Results from duplicate analysis (A and B) obtained with OEP, PCD by PHRED/PBPB[a], and integration by area

	Aflatoxin B_1 found, μg/kg							
	Laboratory 1		Laboratory 2		Laboratory 3		Laboratory 4	
Material	A	B	A	B	A	B	A	B
Senna pods 1 (NC)[b]	1.66	2.35	1.59	1.62	—[c]	—	1.75	1.59
Senna pods 2 (NC)	15.16	13.34	17.96	18.84	—	—	17.10	15.84
Ginger root 1 (NC)	<[d]	<	<	<	—	—	<	<
Ginger root 2 (NC)	2.27	3.41	3.14	2.43	—	—	2.29	2.76
Devil's claw 1 (NC)	<	<	<	<	—	—	<[d]	<[d]
Devil's claw 1 μg/kg	1.02	1.05	1.11	0.88	—	—	1.08	1.02
Senna pods recovery, %	73	97	89	108	—	—	122	92
Ginger root recovery, %	94	96	103	101	—	—	107	107
Devil's claw recovery, %	102	105	110	103	—	—	105	107

[a] PHRED was used by Laboratories 1 and 4, and PBPB by Laboratory 2. The fortification level for the recovery experiments was 2 μg/kg for all matrixes.
[b] NC = Naturally contaminated material.
[c] — = Not determined.
[d] The mycotoxin was not detected (below the LOD).

other peaks. The mobile phase may be adjusted by addition of water, methanol, or acetonitrile for maximum peak resolution and chromatographic performance.

Post-Column Derivatization

(**a**) *PBPB*.—When using PBPB, mount the mixing T-piece and reaction tubing mentioned and then operate using the flow rate of 0.30 mL/min for the post-column reagent.

(**b**) *PHRED*.—When using PHRED, follow the instructions for the installation of the photochemical reactor as supplied by the manufacturer.

(**c**) *KOBRA*.—When using electrochemically generated bromine (KOBRA cell), follow the instructions for the installation.

(**d**) *Calibration curve*.—Prepare the calibration curve using the AfB_1 standard solutions as described in Table 7. These solutions cover the range of 0.2–8 ng/g for AfB_1. Check the plot for linearity. If content of AfB_1 in the test portion is outside the calibration range, the test solution for HPLC analysis may be diluted to an aflatoxin content appropriate for the established calibration curve.

Table 7. Preparation of aflatoxin B_1 standard solutions

Std solution	Amount taken from stock solution, μL	Final concn of B_1 in std solution, ng/mL
1	25	0.01
2	50	0.02
3	125	0.05
4	250	0.10
5	500	0.20
6	750	0.30
7	1000	0.40

Calculations

Plot the data—concentration of AfB_1 (ng/mL; *x*-axis) against the signal (units; *y*-axis)—from the calibration solution experiments into a table and calculate the calibration curve using linear regression. Use the resulting function ($y = ax + b$) to calculate the concentration of aflatoxin in the measured solution. The calibration curve (function) obtained by linear regression for calculation of the concentration of the measured solution is as follows:

$$C_{smp}, \text{ng/mL} = (\text{signal}_{smp}\ [\text{units}] - b)/a$$

where signal_{smp} is the signal of aflatoxin peak obtained from the measured solution [units]. For the calculation of the contamination level Caf1 (in ng/g) use the obtained concentration (C_{smp}) in the following equation:

$$C_{af1} = \frac{C_{smp} \times V_e \times V_{final}}{m_s \times V_{IAC}} \left[\frac{\text{ng} \times \text{mL} \times \text{mL}}{\text{mL} \times \text{g} \times \text{mL}} \right]$$

where m_s is the test portion in g taken for analysis (5 g); V_e is the solvent in mL taken for extraction (100 mL); V_{IAC} is the aliquot in mL taken for immunoaffinity cleanup (5 or 2.5 mL); V_{final} is the final volume in mL achieved after elution from IAC (5 mL); C_{smp} is the concentration in ng/mL of aflatoxin calculated from linear regression.

Table 8. Summary of the LODs, LOQs, and residual CV from the calibration curves[a]

	LOD, μg/kg				LOQ, μg/kg				Residual CV, %			
	KOBRA[b]		Optional[c]		KOBRA		Optional		KOBRA		Optional	
Lab No.	A[d]	h[e]	A	h	A	h	A	h	A	h	A	h
1	0.40	0.29	0.30	0.30	0.60	0.43	0.49	0.44	2.2	1.6	1.8	1.6
2	0.26	0.19	0.60	0.60	0.40	0.29	0.89	0.90	1.4	1.3	3.3	3.3
3	0.55	0.46	—[f]	—	0.83	0.68	—	—	3.6	2.4	—	—
4	0.41	0.40	0.39	0.42	0.63	0.60	0.58	0.62	2.3	2.3	2.1	2.4

[a] Results reflect the mean values of at least 5 calibration curves from each laboratory.
[b] KOBRA = Electrochemical derivatization.
[c] Optional = Photochemical derivatization (Laboratories 1 and 4) and addition of PBPB (Laboratory 2).
[d] A = Integration by area.
[e] h = Integration by height.
[f] — = Integration by height was not determined.

Recovery Experiments (Fortification of Samples)

For recovery experiments, sample materials were fortified by adding aflatoxin solution to 5 g (for MEP) and 10 g (for OEP) material, respectively. The materials were kept for at least 2 h to let the solvent evaporate and were analyzed according to the method description. Recovery was calculated taking into account the subtraction of the naturally contaminated level from the level found, when materials contained traces of AfB_1.

Confirmation of Identity of Aflatoxin B_1

HPLC without post-column derivatization decreases with a factor of 10 or more the fluorescence response of AfB_1 (also for AfG_1). This can be used to confirm the identity of AfB_1 in the test solutions. These test solutions can be stored in the dark at room temperature for at least a week, if desired. In order to confirm the identity of AfB_1 in a test solution, proceed as follows: Using PHRED, switch off the electrical current and re-inject the test solution. Using PBPB, turn off the auxiliary pump with the post-column reagent. Using KOBRA cell, disconnect the HPLC column from the bromination device and connect it directly to the fluorescence detector. Re-inject the relevant test solution. Switching-off the electrical current with the bromination device still in line is not recommended because of the possibility of remaining bromine in the cell membrane of the device.

Determination of Method Parameters

The limit of detection (LOD) and LOQ were obtained from the 95% confidence interval of the calibration graph, calculated by the Software "Methoden-Validerung in der Analytik" (MVA) by NOVIA, Darmstadt, Germany (20). The calibration points were 50, 100, 200, 300, and 400 pg/mL. The precision parameters (RSD_r and RSD_R, HorRat) were calculated according to the IUPAC/AOAC Harmonized Protocol (21) using an Excel template CLSTD.XLT by Mathieson (22). Generally, HorRat values up to 1.0 indicate a well-performing method, but values up to 2.0 are generally considered as acceptable.

Results and Discussion

The single analytical results obtained in this study are listed in Tables 1–6. The results obtained with the different procedures (mandatory and optional) were compared and discussed in a meeting of the Group of Experts 13B of the European Pharmacopoeia Commission on October 1, 2003. The resulting method performance parameters were compared and evaluated for suitability according to the guideline values for method performance of mycotoxin methods as given in the CEN Technical Report 13505 (23). This report defines minimum performance guideline criteria for characteristics such as repeatability (RSD_r), reproducibility (RSD_R), and recovery for analytical methods in the field of mycotoxin analysis for official food control purposes. Criteria in this report have been selected on the basis of empirical experience of what can be regarded as sufficiently precise and accurate for official use, based on individual experts' opinions as well on interlaboratory studies. According to this report, the recovery of an analytical method for the determination of AfB_1 at levels <1 μg/kg shall not exceed a range of 50–120%. The RSD_r shall not exceed 40%, and the RSD_R shall not be >60%. For AfB_1 levels between 1 and 10 μg/kg and levels >10 μg/kg, these values are 70–110% and 80–110% for recovery, 20 and 15% for RSD_r, and 30 and 20% for RSD_R, respectively. In the following section the conduction of the trial and the statistical performance obtained are discussed.

Extraction Procedures

The sample-to-extractant ratio is an important parameter for several reasons. A higher ratio (more sample per solvent) results in more concentrated extracts. As larger amounts of test portion are taken into analysis, the LOQ is generally decreased. Furthermore, larger sample amounts during

Table 9. Summary of method performance parameters[a]

Parameter	$SP1_{nc}$		$SP2_{nc}$		SP_{rec}		$GR2_{nc}$		GR_{rec}		$DC_{(1\ \mu g/kg)}$		DC_{rec}	
Method	MEP	OEP	MEP	OEP	MEP	OEP	MEP	OEP	MEP	OEP	MEP	OEP	MEP	OEP
Mean, μg/kg	2.0	2.2	14.9	17.9	0.78	0.98	2.1	2.9	0.91	1.03	0.92	1.06	0.83	1.00
RSD_r, %	5.5	24.1	5.1	3.2	22.0	15.3	10.0	17.2	4.2	1.8	5.5	6.3	7.8	3.3
RSD_R, %	27.0	41.4	18.1	21.2	35.2	15.3	30.2	17.2	5.8	7.5	7.6	7.4	10.5	4.6
HoRat	0.7	1.0	0.6	0.7	0.8	0.3	0.8	0.4	0.1	0.2	0.2	0.2	0.2	0.1
Recovery, %	—	—	—	—	78	98	—	—	91	103	92	106	83	100

[a] Method performance parameters calculated for each matrix (senna pods = SP, ginger roots = GR, and devil's claw = DC) and different extraction procedures (mandatory = MEP, optional = OEP) and electrochemical post-column derivatization as performed by all 4 laboratories in the trial study.

extraction reduce the risk that the test portion analyzed would not be representative. This is crucial, as aflatoxins are rather heterogeneously distributed in plant products (24). As a result, test material portions (used for extraction) varied in the range of 25 to 50 g during several validation studies for aflatoxins in various food stuffs (6–8).

Despite these apparent advantages, larger sample-to-extractant ratios have one crucial disadvantage: low recovery rates of the analyte might occur. The reason for such low recovery rates was not subject of this study, but was confirmed in pre-experiments for this study, where senna pods were extracted for AfB_1 and rather low recovery rates <70% were achieved. To maintain sufficiently high recoveries (low sample-to-extractant ratio) and representative sample amounts (larger sample portions), the fact that unfavorably large volumes of extractant might be necessary must also be considered.

Low recoveries were most drastic for methanol-based extractants and were less apparent for aqueous acetone extractants. As a result only portions of 5 g senna pods could be extracted with 100 mL methanol–water (70 + 30) in previous experiments in order to obtain recoveries >70%. When acetone–water (85 + 15) was used, similar recovery rates were obtained even with 10 g senna pods material. An indication about possible types of interaction between matrix and extractant has been reported previously (25).

Another element in the selection of the extractant composition is the fact that immunoaffinity columns are differently sensitive towards organic solvents. Generally, this limits the choice of organic solvents in the extractant to those that are mentioned above. Immunoaffinity columns are fairly robust to methanol. In most cases, solutions of up to 16% methanol can be applied, while aqueous acetone or acetonitrile solutions can harm the antibodies already at concentrations of 5%, resulting mainly in losses of aflatoxins B_2, G_1, and G_2 (26). Therefore, 2 extraction methods were tested: methanol–water (70 + 30) and acetone–water (85 + 15) as they have previously been reported (7, 9, 12). Acetonitrile was not tested as certain matrixes can cause phase separation of the extractant (25). The experiments showed that both selected extractants were suitable. No significant interferences could be observed in the chromatogram for each of the extractants used. The extraction with acetone–water (OEP) also showed sufficient high recovery values in those cases where the sample-to-solvent ratios (more sample material/mL extractant) did not allow a sufficiently complete extraction with methanol–water (decreased recovery).

LOD and LOQ from Calibration Curves

The calculated LODs and LOQs as well as the calibration curves residual standard deviations are listed in Table 8. The validation target level was 2 μg/kg AfB_1; thus the reported LOQs must not exceed 2 μg/kg and should be sufficiently below. This was demonstrated in all cases and laboratories reported LOQs ranging from 0.29 to 0.90 μg/kg and LODs ranging from 0.19 to 0.60 μg/kg.

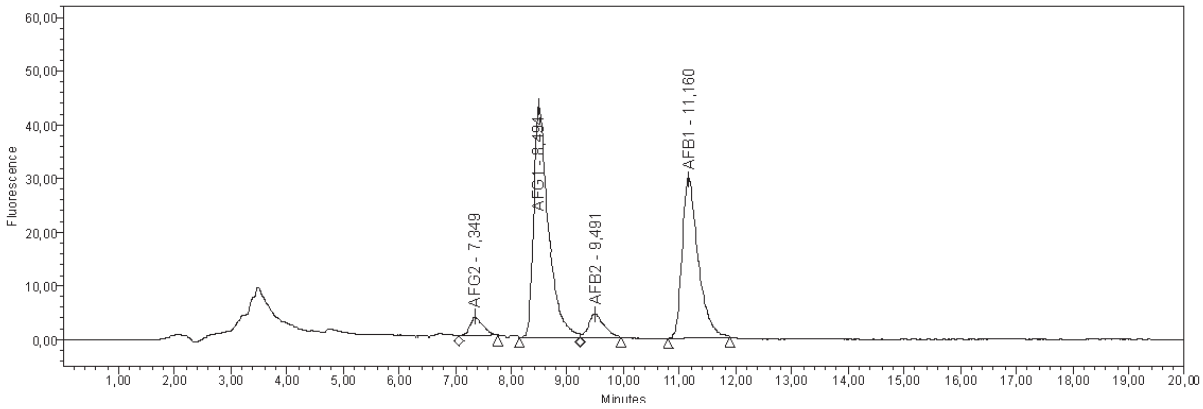

Figure 1. Typical chromatogram of ginger root sample containing aflatoxins G_2, G_1, B_2, and B_1 as 4 well-visible and integrated peaks. The level of aflatoxin B_1 in the sample is estimated as 3 μg/kg.

Method Variability

The summary of performance parameters obtained with these 2 methods is listed in Table 9 for all 3 matrixes. The injection of calibration standards before and after the sample injection sequence did not give any indication of response trends during the sequence of analysis. As a result, the calculation procedure as it was used in this study (mean of 2 calibration curves) does not indicate any advantage compared to calculations with a single calibration curve. It was concluded that the overall recovery for acetone–water extractions (optional extraction) was higher. The between- laboratory variability (RSD_R) and the resulting HorRat values were found to be sufficient for ginger root and devil's claw materials independent of the extractant used, while for senna pods the optional extraction with methanol–water showed higher RSD_R values than those recommended by CEN. It must be noted that these CEN criteria were actually developed for food analysis and due to the complexity of the matrixes analyzed here can be used only as guidelines. However, HorRat values in all cases did not exceed a value of 1, which indicates that a method performs satisfactorily. Remarkable are the impressive precision parameters obtained in the recovery experiments with HorRat values mainly from 0.1 to 0.3.

PCD Techniques

Three out of the 4 laboratories re-injected sample extracts using a second PCD method. This was in 2 cases a PHRED system and in another case derivatization by PBPB. All 4 laboratories tested samples extracted with methanol–water (MEP) with KOBRA PCD, while 3 laboratories also tested samples extracted with acetone–water (OEP) this way. The single analytical results are given in Tables 1–6 sorted by extraction method, PCD, and integration category.

No significant difference in terms of chromatogram purity was observed with any of the derivatization principles, which indicates that the PCD system used is a robust parameter in derivatization of AfB_1. As a result, laboratories might choose any of the PCD systems depending on the available equipment, while maintaining the comparability of results.

Signal Evaluation: Peak Area vs Peak Height

The method was fairly robust regarding the different modes of integration (height vs area). Despite low concentration levels tested, signals seemed to be sufficiently large so that the peak integration mode had no influence on the RSD_r. A typical chromatogram of a ginger root sample containing about 3 μg/kg AfB_1 is shown in Figure 1. The chromatogram was obtained using methanol–water extraction and electrochemical post column derivatization. For all other options, chromatograms looked similar and free of interferences.

Conclusions

A suitable chromatographic method for the determination of aflatoxins in medical herbs (senna pods, devil's claw, and ginger roots) was identified. Different post-column derivatization possibilities such as chemical or electrochemical as well as photochemical derivatization had no influence on the performance of the determination of AfB_1 in medical herbs. The extraction was critical for analysis and a methanol–water extractant was identified as most suitable. The amount of test portion used for extraction in this study was 5 g. This is rather low compared with the amounts used in other methods for aflatoxins. Such low quantities of test portions require a high degree of homogeneity of the whole test sample material prior to analysis.

Despite the limitation that this study was performed with only 4 participants, the proposed method performed satisfactorily in the collaborative trial, showing that the method fulfills the recommended guidelines on method performance according to CEN Technical Report 13505.

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