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Short communication

Immunoaffinity column clean-up prior to thin-layer chromatography for the determination of aflatoxins in various food matrices

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Abstract

A one-dimensional TLC method to determine aflatoxins (B_1, B_2, G_1, G_2) in various food matrices was elaborated which abstains fully on the use of chlorinated solvents. It implements an immunoaffinity clean-up step after extraction with methanol. The aflatoxins were quantified by densitometry. The method has shown to be rapid and efficient. In-house performance characteristics were established. The limit of quantification was found to be significantly lower than current regulatory limits for aflatoxin control outside and within the European Community. The obtained recovery and precision data gave a strong indication, that the method is likely to give satisfactory performance if tested in a future collaborative trial.
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1. Introduction

Aflatoxins are highly toxic metabolites produced by the fungi *Aspergillus flavus* and *Apergillus parasiticus*. They can be found in a wide range of food and feeding stuff and are potentially hazardous to humans and animals. The monitoring of aflatoxins depends on precise and reliable analytical methods. Food products being commonly contaminated with aflatoxins are nuts (e.g. peanuts or pistachios), dried fruits (e.g. figs), grains (e.g. corn) and spices (e.g. paprika). Monitoring of aflatoxins in these products is not only of importance for consumer protection, but also for producers of the raw products prior to cost intensive processing or transport.

Modern aflatoxin determination is commonly based on high-performance liquid chromatography (HPLC) [1–6], while thin layer chromatography (TLC) is still the method of choice where HPLC is not available and the precise determination of aflatoxins is required [7,8]. This holds true for the majority of analysts in developing countries. Those countries are the exporters of food and food products, which are mainly subject to aflatoxin contamination (e.g. figs, spices, pistachios).

Currently available and validated TLC methods for aflatoxin determination still require the use of chlorinated solvents such as dichloromethane or chloroform in the mobile phase, as extraction solvent or for sample clean up [9–11]. Since these solvents are considered to be ecological hazards [12] they are

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constantly being banned in laboratory routine work where possible.

A lot of effort has recently been made to develop, validate and standardise HPLC methods for determination of aflatoxins [13,14]. Nowadays, these methods involve immunoaffinity column (IAC) clean-up procedures, which offer the extraction of aflatoxins from most food matrices with simple aqueous solvent mixtures [15]. Additionally these procedures reduce the amount of work per sample and therefore this clean-up procedure is popular in combination with HPLC. However IAC has only been considered for the application in combination with TLC at higher contamination levels of 10–50 ng/g [16] or more recently for the quantification of aflatoxin M_1 in milk [17] or aflatoxin B_1 in corn [18].

Thus, a validated state-of-the-art TLC method, which is user friendly and easy to perform would be highly desired for those analysts that rely on TLC.

This was achieved by implementation of an already successfully applied IAC clean-up procedure [14] in combination with a modified one-dimensional TLC separation.

2. Experimental

2.1. Sample materials

Paprika powder, peanut butter and pistachios samples have already been previously produced for a collaborative trial [19]. Corn flour samples have been purchased in a local super market.

2.2. Chemicals

Gradient-grade methanol, analytical grade methanol, petrol ether, analytical-grade formic acid, paraffin and silica gel 60 TLC-plates (20 cm×20 cm) were obtained from Merck (Germany). HPLC-grade acetone and *tert*.-butyl-methyl-ether were purchased from Scharlau (Germany). *n*-Hexane for residue analysis, polyoxyethylene sorbitan monolaurate (Tween-20®) and aflatoxins (dry film) were purchased from Sigma–Aldrich (Italy). All filter papers (folded filters V113, glass fibre GF/A and Nylon) were obtained from Whatman (USA). Immunoaffinity columns were from Rhone–Poulenc Diagnostics (UK). PBS buffer tablets were purchased from Sigma–Aldrich (Italy).

2.3. Apparatus

Gas-tight micro-litre syringes were obtained from Hamilton (USA). The TLC sample application device (Linomat), the TLC-scanner (Scanner 3) and the UV-lamp (366 nm) were purchased from Camag (Switzerland). The evaporation block was obtained from Pierce (USA). The high-speed blender (Ultra Turrax) was from Kunkel (Germany).

2.4. Sample preparation

Test portions of 50 g (corn, paprika, peanuts and pistachios) were extracted with 200 ml of methanol–water (8+2, v/v). In the case of peanuts and pistachios, an additional amount of 100 ml of a non-polar solvent (e.g. hexane or petrol ether) was added prior to the extraction. All samples were extracted by high-speed blending for 3 min or in the case of paprika powder and corn flour by shaking for 30 min. Extracts were filtered immediately after extraction through folded filter paper. The details of sample preparation are given in [14].

2.5. Immunoaffinity column clean-up

An aliquot of 20 ml of the filtrate was diluted to 140 ml with phosphate-buffered saline (PBS) solution pH 7.4. This diluted extract was further filtered through glass fibre filters and a micropore filter of 0.45 μm. After conditioning of the immunoaffinity column (IAC) with 10 ml of PBS, 70 ml of the clear glass fibre filtrate was applied onto the IAC at a flow-rate of maximum 3 ml per min. The IAC was first washed with approximately 15 ml of an aqueous washing solution, containing 11 vol% methanol and 0.5% polyoxyethylene–sorbitan monolaurate (Tween-20®) to remove any extract residues from the IAC. This was followed by the addition of 10 ml of water to remove the washing solution. The IAC was dried by pressing air through it for 2 to 3 s with

a syringe and the purified aflatoxins were subsequently eluted with 1.5 ml of methanol into an 2-ml acid washed glass vial. Prior to elution, approximately 50 μl of a methanol–formic acid solution was passed into the glass vial. The eluate was gently mixed and taken to dryness at 40°C under a gentle stream of air. The aflatoxins were re-dissolved in 150 μl of a hexane–acetone–methanol solution (90+5+5, v/v/v). Any irreproducible evaporation was minimised by injecting this solvent into already sealed glass vials (containing the evaporated aflatoxins).

2.6. Thin layer chromatography

Exactly 100 μl of the re-dissolved aflatoxin solution was spotted on a silica-gel 60 TLC plate with a gas-tight microlitre syringe, dried, pre-concentrated with methanol, and finally separated with a mobile phase of *tert*.-butyl-methyl-ether, methanol and water (480+15+5, v/v/v). The spotting scheme used was alternating the samples and the standards (i.e. sample–standard–sample–standard etc.).

2.7. Detection and quantification

The identification and quantification of the fully separated aflatoxins was performed by comparison with aflatoxin standards using densitometry (TLC scanner).

2.8. Determination of the method performance characteristics

The determination of the limit of quantification (LOQ) and the limit of detection (LOD) were performed by analysing nine replicates.

For the determination of the LOQ and LOD using densitometry, a method validation program was applied [20] and both parameters were derived from the 95% confidence interval of the calibration curve (five point calibration composed out of four standards (fortified materials) and a blank). The recovery of the method was derived from the linear slope of a function, in that the added amounts were plotted against the determined concentrations of the analyte.

3. Results and discussion

3.1. Method development

3.1.1. Extraction procedures and immunoaffinity clean-up

Since the sensitivity of TLC is about 100 times less than of fluorescence detection after HPLC, relatively large fractions of the primary extract had to be purified. This was achieved by the extraction with aqueous methanol in addition to an immunoaffinity clean-up [21].

Classical clean-up procedures such as solid-phase extraction have shown to be not always sufficient to eliminate interfering matrix components, which cause problems during TLC separation and aflatoxin identification [22,23].

On the other hand, immunoaffinity columns become more and more popular and have been reported to be re-usable by several authors [24–28]

3.1.2. TLC spotting solvents and application on TLC plates

It has been observed that methanol is inappropriate for the spotting of aflatoxins on silica gel TLC-plates due to its elution strength [29,30].

A solvent mixture of hexane–acetone–methanol (90+5+5, v/v/v) was found to be most suitable concerning spot size, boiling point and recovery from re-dissolving (98–101% for all aflatoxins). Spot diameters were found to be between 2.0 and 2.5 mm if volumes of 100 μl were applied within 2 min.

Additional experiments were carried out to investigate the effect of different spot or band sizes on the densitometric signals. Equal amounts of aflatoxins were sprayed as bands of different sizes from 2 to 8 mm on the TLC-plate. This allowed the investigation of any tendencies or differences that might occur from diverse spot or band dimensions. Aflatoxins were pre-concentrated with methanol to flatten the bands and subsequently developed. No significant difference was observed for corresponding signals of equal amounts, provided that the area scanned recorded the total amount of aflatoxin applied.

3.1.3. TLC plate material and mobile phase

Since the immunoaffinity column clean-up delivered highly purified aflatoxins, a sufficient TLC

separation with a single one-dimensional development was intended. Due to its overall performance, separations were made on silica gel [3,31]. Even though high-performance TLC (HPTLC) offers better results compared to normal TLC [32], the use of ''normal silica gel TLC plates'' was preferred in this study and was found to be sufficient for a full separation of all aflatoxins

Several mobile phases have been tested [8], but only one, based on a mixture of diethyl ether, methanol and water, confirmed to be promising. This mobile phase was further modified with the aim of substituting the highly volatile and peroxide susceptible diethyl ether. In conclusion, a mobile phase composed of *tert.*-butyl-methyl-ether (*t*-BME), methanol and water (480+15+5, v/v/v) was found to deliver the desired results.

The aflatoxins could be separated in a single run with R_f-values of 0.40 (B_1), 0.35 (B_2), 0.29 (G_1), and 0.25 (G_2) and without any interference from other substances using an unlined and non-equilibrated tank (Fig. 1). This allows the application of several samples and standards on one TLC-plate, while the classical clean-up procedure by solid-phase extraction requires a two dimensional TLC-separation which drastically limits the number of samples that can be applied on a TLC-plate.

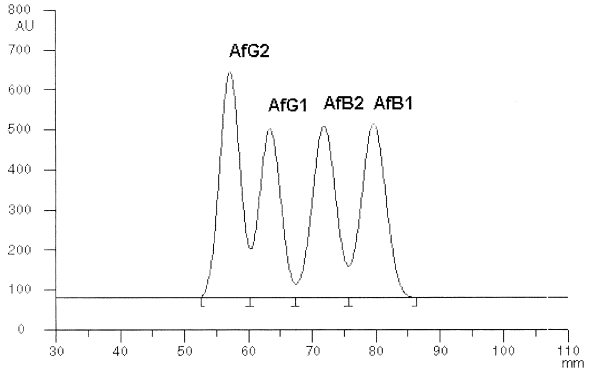

Fig. 1. Chromatogram of the aflatoxins separated by TLC. Separation of aflatoxins extracted from a fortified pistachio sample (4 ng/g each aflatoxin) on a silica gel 60 TLC plate. The start position of the chromatogram was at 20 mm, while the solvent front ended at 170 mm with a run-time of 60 min.

Experiments with a repeatedly used mobile phase resulted in a shift to increased R_f-values. However, the separation of all four aflatoxins was still satisfying. This offers re-use of the mobile phase several times.

3.2. In-house validation of the TLC method

The selected parameters for the in-house validation were mainly taken from the criteria that are laid down by the European Standardisation Committee (CEN) [33].

The LOD and LOQ were calculated from the 95% confidence interval of the calibration curve (chromatographic data), while the recovery was determined from the slope thereof.

The LODs ranged from 0.1 to 0.2 ng/g for all aflatoxins, while the LOQs were found to be from 0.2 to 0.3 ng/g when the calibration was performed with standards. Calibration curves obtained from fortified samples of paprika powder, peanut butter and pistachios (blank materials), that reflect the most difficult matrices in aflatoxin analysis, were spiked at levels of 1–4 ng/g, analysed and the results were plotted against the spiked levels. Table 1 shows the results of the obtained data.

4. Conclusion

The TLC method described here was found to be a simple, robust and efficient option to currently available TLC methods, implementing the postulated aspects of ecology, economy and legislation and can be considered as an alternative to modern HPLC methods. This counts especially in those cases where adequate HPLC equipment is difficult to use or to maintain (e.g. developing countries) and when the contamination level of food with aflatoxins has to be monitored precisely at relatively low levels (2 ng/g and above).

The in-house performance data achieved gives a strong indication that the method is capable of producing acceptable results in a collaborative trial study foreseen in the near future for the analysis of

Table 1
Results from the calibration with fortified samples[a]

Matrix	Analyte	LOD (ng/g)	LOQ (ng/g)	Recovery (%)	Precision (RSD, %)
Peanut butter	AflatoxinB_1	0.6	1.5	85	6.0
	AflatoxinB_2	0.2	0.6	87	2.6
	AflatoxinG_1	0.6	1.7	82	6.7
	AflatoxinG_2	0.4	1.4	78	5.4
Paprika	AflatoxinB_1	0.4	1.2	85	4.9
	AflatoxinB_2	0.3	0.9	87	3.6
	AflatoxinG_1	0.5	1.6	84	6.5
	AflatoxinG_2	0.7	2.2	76	8.9
Pistachios	AflatoxinB_1	0.2	0.5	82	1.9
	AflatoxinB_2	0.1	0.4	87	1.4
	AflatoxinG_1	0.3	0.8	81	3.4
	AflatoxinG_2	0.3	0.8	83	3.3

[a] The values for the LOD, LOQ, recovery and the precision (based on nine replicates) were calculated with software for the validation of analytical methods (MVA). These figures reflect the performance characteristics of the overall analysis method.

aflatoxins at European regulatory limits of 2 ng/g aflatoxin B_1 (respectively 4 ng/g total aflatoxins).

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