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Analysis of Aflatoxins and Ochratoxin A in Cannabis and Cannabis Products by LC–Fluorescence Detection Using Cleanup with Either Multiantibody Immunoaffinity Columns or an Automated System with In-Line Reusable Immunoaffinity Cartridges

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Background: **Evidence of fungal contamination of cannabis plants during drying has raised concerns of potential mycotoxin contamination of leaves and flowers and subsequent contamination of derived edible cannabis products. Methods are, therefore, needed for routine monitoring of cannabis to ensure consumer safety consistent with long-standing controls for mycotoxins such as aflatoxins and ochratoxin A (OTA) in foodstuffs.** ***Objective:*** **To generate preliminary validation data to demonstrate fitness-for-purpose of methods for aflatoxins and OTA in cannabis and cannabis products.** ***Methods:*** **Extraction of solid matrices with acetonitrile–water (75 + 25) and direct analysis of energy drinks after dilution. Extracts were either passed manually though an immunoaffinity column (IAC) containing antibodies to both aflatoxins and OTA or were analyzed sequentially using an automated system with in-line reusable immunoaffinity cartridges for aflatoxins or OTA. In both cases, analysis was by LC with fluorescence detection.** ***Results:*** **Recoveries were in the range of 76–120% with relative SDs from 0.8 to 6.6% for aflatoxins and OTA spiked into cannabis dried leaves and flowers, hemp tea, oils, capsules, cookies, chocolate brownies, and an energy drink.** ***Conclusions:*** **The methods described in this paper are suitable for the cleanup of sample extracts of cannabis and cannabis products.** ***Highlights:*** **Manual and automated methods with IAC cleanup have been shown to be suitable for routine control of aflatoxins and OTA in cannabis and cannabis products.**

In October 2018, Canada formally legalized the cultivation, possession, acquisition, and consumption of cannabis and its byproducts (1). In the United States, medical use of cannabis is now legal in 33 states, while recreational use is only legal in 10 states. Commercial distribution of cannabis is allowed in all jurisdictions in the United States in which cannabis has been legalized (2). In European countries such as the Netherlands, while cannabis use is technically illegal (3), it is tolerated to various degrees, and cannabis and cannabis products can be openly purchased.

Cannabis can be cultivated outdoors, indoors, or in a combination of indoor/outdoor environments in farms that grow, harvest, and process cannabis for commercial distribution (4). Harvesting the plants is done by hand, and leaves are removed and, together with flowers, dried as slowly as possible in the dark, avoiding sunlight and not using artificial heat but ensuring good ventilation and good air circulation (5).

Fresh-cut cannabis plant material has a water activity of 0.95 Aw, but the water activity needs to be below 0.65 Aw to prevent most fungal growth. *Botrytis* spp. are the most commonly found postharvest fungi in cannabis plants and have a water activity threshold of 0.85 Aw. The objective of the drying process is, therefore, to have all plant material below this threshold limit as quickly as possible and certainly within the first 2 days of the drying process (6). The high risk of postharvest fungal growth and subsequent mycotoxin formation in cannabis has been widely recognized (6, 7), although there does not appear to be any published evidence of specific mycotoxins having been identified as contaminants of cannabis. Nevertheless, numerous fungal species have been detected by molecular screening (PCR) in several dispensary-derived cannabis samples. These include the toxigenic *Penicillium* species *P. paxilli*, *P. citrinum*, *P. commune*, *P. chrysogenum*, and *P. corylophilum* as well as *Aspergillus* species including *A. niger*, *A. flavus*, and *A. versicolor* (8).

Cannabis has been cultivated as a medicinal plant for thousands of years. As a result of centuries of breeding and selection, there are now over 700 varieties of cannabis that contain hundreds of bioactive compounds, including cannabinoids and terpenes (9). As a drug, cannabis is usually produced as dried flower buds, resin, or various extracts or oils. These products are very complex matrices with high amounts of waxes, oils, and other components (10). The two most prevalent and commonly known cannabinoids (9) in the cannabis plant are delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD). THC is

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the main psychoactive compound found in cannabis, while CBD is claimed to have medicinal properties. The oil from cannabis is prepared from dried plant material, usually by solvent or gas extraction, for which the composition and quality of the oil will depend on the process employed (11). In Canada, commercial production of cannabis extract is regulated, and only solvent extraction using ethanol or supercritical fluid extraction using carbon dioxide is permitted. CBD oils and capsules are sold for medicinal use and are available as food supplements in some health food outlets. Cannabis extracts, for which olive oil is sometimes used as the extraction solvent, are also incorporated into foodstuffs such as cookies, producing complex matrices presenting an analytical challenge in achieving efficient extraction and good recoveries of target analytes.

There has specifically been concern about the presence of unregulated pesticides or pesticide residues in cannabis (5, 12) above the maximum residue limits that are applied to other agricultural products. The Bureau of Cannabis Control in California, under Title 16 of the California Code of Regulations Section 5715, stipulates laboratory tests to be conducted on cannabis and cannabis products that include tests for pesticides, heavy metals, fungal contamination, and mycotoxins (13). The Cannabis Control Commission of Massachusetts stipulates a limit of 20 µg/kg for unspecified mycotoxins in finished medical marijuana products and marijuana-infused products (14). It seems logical to focus on the same mycotoxins that are of concern in foods resulting from postharvest fungal contamination, and the priority would, therefore, be to focus initially on aflatoxins and ochratoxin A (OTA), with those limits applied in the United States making a good starting point for methods for surveillance of cannabis plant material and the stricter European Union (EU) limits for edible food products. It should be noted that while limits for mycotoxins might be applied to plant materials, there is a risk of toxin concentration during extraction, which is a factor that needs to be taken into account.

For the analysis of mycotoxins in a diverse range of matrices, immunoaffinity columns (IACs) have become the preferred method for sample cleanup, as the high specificity of antibodies enables complex matrices to be handled without interference from coextractives (15). When there is a desire to monitor more than one mycotoxin simultaneously, then multimycotoxin IACs are employed (16, 17) or two IACs can be used in tandem (18). When large numbers of samples need to be handled, there are automated systems employing reusable immunoaffinity cartridges (19). Official methods for mycotoxins that have been published by AOAC International (20) or have been established as European Committee for Standardization standards (21) are almost universally based on the use of IACs. Thus, for the analysis of cannabis and cannabis products, it is logical to follow the path of existing, well-established mycotoxin methods in terms of employing an IAC cleanup prior to LC analysis.

In this paper, we describe two IAC-based methods and report preliminary method performance data for the determination of both aflatoxins and OTA spiked into a range of cannabis and cannabis products. One method uses a multimycotoxin IAC for simultaneous analysis of both toxins, and a second, similar method uses a fully automated system with a reuseable immunoaffinity cartridge with sequential LC analysis of aflatoxins and OTA.

Experimental

Caution: This method involves the use of hazardous materials and does not address all the safety problems associated with their use. It is the responsibility of the user of this protocol to establish appropriate safety and health practices and determine the applicability of regulatory limitations before use. The International Agency for Research on Cancer (IARC) has classified aflatoxins as human carcinogens (Group 1) and OTA as a possible human carcinogen (Group 2B). Protective clothing, gloves, and safety glasses should be worn at all times, and all standard and sample preparation stages should be carried out in a fume cupboard. Disposal of waste solvents should be carried out according to applicable environmental rules and recommendations of the IARC (22).

Standards, Samples, and Chemicals

(**a**) *Standards*.—Aflatoxin solution containing 250 ng/mL (each) aflatoxins B_1, B_2, G_1, and G_2 in methanol was AFLASTANDARD, and OTA solution containing 1000 ng/mL was OCHRASTANDARD (both from R-Biopharm Rhone Ltd, Glasgow, United Kingdom).

(**b**) *Samples*.—(*1*) *Sample A*.—Cannabis dried leaves and flowers with a THC content of 21–23%.

(*2*) *Sample B*.—Cannabis dried leaves and flowers with a THC content of approximately 25%.

(*3*) *Sample C*.—Oil containing 2.75% CBD.

(*4*) *Sample D*.—Oil containing 5% CBD.

(*5*) *Sample E*.—Gelatin capsule weighing 0.5 g containing 1% CBD.

(*6*) *Sample F*.—Gelatin capsule weighing 0.6 g containing 1.66% CBD.

(*7*) *Sample G*.—Energy drink containing 0.02% hemp extract but labeled as being THC-free.

(*8*) *Sample H*.—Hemp tea labeled as containing *Cannabis sativa*.

(*9*) *Sample I*.—Butter cookie containing unspecified amounts of THC.

(*10*) *Sample J*.—Chocolate brownie containing unspecified amounts of THC.

All samples were legally available and purchased from retail sources in the Netherlands, except samples C and E, which were purchased from a health food store in the United Kingdom.

(**c**) *Materials and chemicals*.—Multimycotoxin IACs in wide-body format containing monoclonal antibodies specific to both aflatoxins and OTA were obtained from R-Biopharm Rhone Ltd. The columns had a minimum capacity of 150 ng total aflatoxins and 200 ng OTA. Recoveries were not less than 80% for aflatoxins B_1, B_2, G_1, and G_2 when 5 ng equimolar aflatoxins B_1, B_2, G_1, and G_2 were applied in 10 mL methanol–phosphate-buffered saline (PBS; 10+90) and not less than 85% for OTA when applied as a standard solution in methanol–PBS (10+90) containing 5 ng OTA.

IMMUNOPREP® ONLINE AFLATOXIN immunoaffinity cartridges were obtained from R-Biopharm Rhone Ltd. Cartridges had a recovery of ≥80% for aflatoxins B_1, B_2, G_1, and G_2 and a capacity of >1.0 ng total aflatoxin per cartridge, measured by applying 1 mL 1 ng/mL total aflatoxins solution. IMMUNOPREP ONLINE OCHRATOXIN A immunoaffinity cartridges were obtained from R-Biopharm Rhone Ltd. Cartridges had a recovery of ≥80% for OTA and a capacity of >1.0 ng OTA per cartridge, measured by applying 1 mL 1 ng/mL OTA solution.

Distilled/deionized water (suitable for use with LC, e.g., Milli-Q), LC grade methanol, LC grade acetonitrile, sodium

chloride, ammonium acetate, nitric acid, potassium bromide, and sodium hydroxide were from Fisher Chemical (Loughborough, United Kingdom). Triton X-100 (laboratory grade) and Tween 20 were obtained from Sigma-Aldrich Ltd (Gillingham, United Kingdom). PBS tablets were obtained from R-Biopharm Rhone Ltd. One tablet was dissolved in 100 mL water to give an 8.0 g/L solution of sodium chloride, 0.2 g/L potassium chloride, 1.15 g/L disodium hydrogen phosphate, and 0.2 g/L potassium dihydrogen phosphate with a of pH 7.3±0.2 at 25°C.

(**d**) *Calibration standards for manual IAC containing both aflatoxins and OTA*.—Dispense 1 mL methanol–water (50+50), remove 200 µL and discard, and then add 100 µL AFLASTANDARD (1000 ng/mL total aflatoxins) and 100 µL OCHRASTANDARD (1000 ng/mL OTA) to give 100 ng/mL total aflatoxins and 100 ng/mL OTA (working solution A). Dispense 10 mL methanol–water (50+50), remove 125 µL, and discard. Add 125 µL working solution A to give 1.25 ng/mL total aflatoxins (0.3125 ng/mL each) and 1.25 ng/mL OTA (designated as Cal 5). Dilute this solution 1:1 with methanol–water (50+50) to obtain four further calibration standards as shown in Table 1.

(**e**) *Calibration standards for the automated in-line IAC analysis for aflatoxins and OTA*.—Dispense 1000 µL AFLASTANDARD (1000 ng/mL total aflatoxins) into a 10 mL volumetric flask and dilute to volume with methanol to give 100 ng/mL. Dilute 25 µL 100 ng/mL solution to 10 mL with methanol to give 0.25 ng/mL calibration standard. Repeat the above dilutions with OCHRASTANDARD (1000 ng/mL OTA) to produce a separate calibration standard for OTA in methanol containing 0.25 ng/mL OTA. Calibration points ranged from 1.76 to 35 µg/kg depending on the sample matrix, as different gram equivalents were injected for different extracts.

Instruments and Conditions

(**a**) *LC with fluorescence detection after manual IAC cleanup.*—RIDA®CREST LC system operated in a direct injection mode (R-Biopharm AG, Darmstadt, Germany). The system was connected to a postcolumn KOBRA® CELL–K01 (R-Biopharm Rhone Ltd) and to a Shimadzu RF-20A/20Axs fluorescence detector (Shimadzu Benelux, 's-Hertogenbosch, the Netherlands).

(**b**) *LC*.— An Inertsil ODS-3V (150×4.6 mm, 5 µm particle size) analytical column was used for the LC separation (GL Sciences B.V., Eindhoven, the Netherlands). The LC mobile phase A was methanol–water (45+55) containing 350 µL 4 N nitric acid and 119 mg/L potassium bromide (required for postcolumn bromination). The LC mobile phase B was methanol–water (80+20) containing 350 µL 4 N nitric acid and 119 mg/L potassium bromide.

Table 1. Calibration series preparation

Cal No.[a]	Vol. of Cal[a]	Vol. of diluent, mL	Concn of aflatoxins, ng/mL	Concn of OTA, ng/mL
4	5 mL Cal 5	5.0	0.625 total (0.156 each)	0.625
3	5 mL Cal 4	5.0	0.312 total (0.078 each)	0.312
2	2 mL Cal 3	2.0	0.156 total (0.039 each)	0.156
1	2 mL Cal 2	2.0	0.078 total (0.019 each)	0.078

[a] Cal = Calibration standard.

The mobile phase A was isocratic for 14.0 min at a flow rate of 0.8 mL/min and then switched to 35% mobile phase A and 65% mobile phase B at a flow rate of 0.8 mL/min for a further 15.5 min (total run time of 29.5 min) before reconditioning with mobile phase A. The injection volume was 100 µL, and the column temperature was 40°C. The detector had excitation and emission wavelengths of 365 and 442 nm, respectively, for 0–17 min for aflatoxin analysis and then switched to an excitation wavelength of 333 and an emission wavelength of 463 nm for detection of OTA.

(**c**) *LC with fluorescence detection after in-line IAC cleanup*.—Automated analyses were performed on a RIDACREST online solid-phase extraction sample preparation system (R-Biopharm AG). RIDACREST is an HPLC system consisting of two LC pumps, a degasser, an autosampler, an automatic cartridge exchanger, and a high-pressure dispenser (HPD). The system was connected to a postcolumn KOBRA CELL–K01 (R-Biopharm Rhone Ltd) and to a Shimadzu RF-20A/20Axs fluorescence detector (Shimadzu Benelux). For aflatoxin analysis, the fluorescence detector was operated at excitation and emission wavelengths of 362 and 455 nm, and for OTA analysis, it was operated at 335 and 475 nm, respectively.

(**d**) *LC*.—An InertSustain AQ C18 (150×4.6 mm, 3 µm particle size) analytical column (GL Sciences B.V.) was used for the LC separation of aflatoxins and OTA in sequential analysis. The injection volume was 1 mL, and the column temperature was 45°C.

For aflatoxin analysis, the LC mobile phase A was methanol–acetonitrile–water (35+15+50) containing 120 mg/L potassium bromide and 350 µL/L 4 M nitric acid, and mobile phase B was methanol–water–acetic acid (90+5+5). The mobile phase A was isocratic at an initial flowrate of 0.4 mL/min for the first 2.0 min and then switched to a flow rate of 1.2 mL/min for the next 8 min of the analysis. The mobile phase was switched to 100% B for 1 min to clean the analytical column and then back to 100% A to equilibrate the analytical column for 1 min before the next injection.

For OTA analysis, the LC mobile phase was methanol–acetonitrile–water (35+15+50) containing 120 mg/L potassium bromide and 350 µl/L 4 M nitric acid (mobile phase A) and methanol–water–acetic acid (90+5+5; mobile phase B). The mobile phase was isocratic (50% A and 50% B) at an initial flow rate of 0.6 mL/min for the first 1 min and then switched to a flow rate of 1.2 mL/min for the next 9 min of the analysis. The mobile phase was switched to 100% B for 1 min to clean the analytical column and then back to 50% A and 50% B to equilibrate the analytical column for 1 min for the next injection. Although aflatoxins and OTA were analyzed separately, the above programs were selected to enable unattended operation overnight and ease of switching between the analyses for different toxins.

Methods

(**a**) *Sample preparation.*—Grind samples of cannabis leaves, flowers, and hemp tea to a fine powder in a Waring blender (Waring Laboratory Science, Torrington, CT), and for cookies, crumble by hand to a powder. Weigh plant material and tea samples (1.0 g), cookie, CBD oil, and CBD capsules (2.0 g) into a 50 mL centrifuge tube. Add 20 mL of acetonitrile–water (75+25) and mechanically shake for 60 min. Centrifuge at

4000 rpm for 10 min. For manual IAC analysis [*see* (**c**) below], dilute 5 mL supernatant in 75 mL 10% Tween 20 in PBS and stir for 5 min, taking 20 mL for analysis. For automated IAC analysis [*see* (**d**) below] for aflatoxins, dilute seven times with 3% Triton X-100 solution, and for OTA analysis, dilute 10 times with 3% Tween 20 solution, in both cases taking 1 mL extract for analysis on in-line immunoaffinity cartridge [*see* (**d**) below].

(**b**) *Sample preparation for energy drink*.—Dispense drink (100 mL) into a glass beaker and place in a sonic bath for 30 min to degas. Weigh sample (1.0 g) into an Eppendorf tube. Spike as required, transfer sample (500 µL) to a beaker, add PBS (9.5 mL), and mix. For manual analysis [*see* (**c**) below], take 3 mL, and for automated analysis [*see* (**d**) below], take 1 mL.

(**c**) *Manual immunoaffinity cleanup*.—Pass diluted supernatant (20 mL) from plant material, cookie, or oil samples or directly pass diluted energy drink (3 mL) through the IAC at 2 mL/min, wash with water (10 mL), pass air through the IAC to remove residual liquid, and elute the toxins with methanol (1 mL). Pass water (1 mL) through the column and combine with the methanol portion, transferring 1 mL to an autosampler vial for LC analysis.

(**d**) *Automated in-line immunoaffinity cleanup*.—The automated in-line immunoaffinity cartridge cleanup system is described in detail elsewhere (19), for which the loading, wash, and elution buffers and dilution solutions were as follows.—(*1*) *Loading buffer A*.—Ammonium acetate (20 mM). Add 1 L water to a flask, followed by ammonium acetate (1.54 g), and adjust the pH to 6.8–7.0 using 1 M sodium hydroxide.

(*2*) *Wash buffer B for aflatoxins*.—Ammonium acetate (20 mM). Add 900 mL water to a flask, followed by acetonitrile (60 mL), methanol (40 mL), and ammonium acetate (1.54 g). Adjust the pH to 8.3–8.5 using 1 M sodium hydroxide.

(*3*) *Wash buffer B for OTA*.—Ammonium acetate (20 mM), sodium tetraborate (25mM), and 0.1% Triton X-100. Add Triton X-100 (1.0 g) to a flask, followed by water (900 mL), methanol (100 mL), ammonium acetate (1.54 g), and sodium tetraborate (9.53 g). Adjust the pH to 8.3–8.5 using concentrated hydrochloric acid.

(*4*) *Elution buffer C for aflatoxins*.—Add 500 mL water to a flask, followed by ammonium acetate (3.85 g), 150 mL acetonitrile, and 350 mL methanol. Adjust the pH to 1.95–2.05 using concentrated nitric acid.

(*5*) *Elution buffer C for OTA*.—Add 330 mL water to a flask, followed by ammonium acetate (1.54 g), 600 mL acetonitrile, and 70 mL acetic acid.

(*6*) *Dilution solution D for aflatoxins*.—Add 100 mL water to a flask plus Triton X-100 (3.0 g) and mix well.

(*7*) *Dilution solution D for OTA*.—Add 100 mL water to a flask plus Tween 20 (3.0 g) and mix well.

(**e**) *Spiking protocol*.—For cannabis plant material and hemp tea, spike 1.0 g powdered sample with 200 µL 100 ng/mL solution of total aflatoxins in methanol and 200 µL 100 ng/mL solution of OTA in methanol to give 20 µg/kg total aflatoxins and 20 µg/kg OTA. Allow the solvent to evaporate and the sample to stand for several hours prior to extraction.

For cookies, CBD oil, and CBD capsules, spike 2.0 g sample with 100 µL 100 ng/mL total aflatoxin solution in methanol and 100 µL 100 ng/mL solution of OTA in methanol to give 5 µg/kg total aflatoxins and 5 µg/kg OTA. Allow the solvent to evaporate and the sample to stand for several hours prior to extraction.

For the energy drink, spike 1.0 g sample with 50 µL 100 ng/mL total aflatoxin solution in methanol and 50 µL 100 ng/mL solution of OTA in methanol to give 5 µg/kg total aflatoxins and 5 µg/kg OTA.

Method Performance

(**a**) *Within-day repeatability and recoveries*.—These were measured by analyzing six replicates of cannabis plant material or either two or six replicates of cannabis products spiked at two different levels. For the automated in-line analysis for all measurements, the injection sequence was designed to ensure that the same immunoaffinity cartridge was utilized for repeat measurements of the same matrix at the same spiking level. For example, one cartridge was used for six replicate analyses of spiked cannabis plant plus a blank and two standards.

(**b**) *LODs*.—LODs were determined by measuring the average noise and assuming the LOD to be equal to 3 times the noise. This was confirmed experimentally by making a spike at the appropriate level and ensuring that a peak could be detected just above the baseline.

Results and Discussion

Method Development

Effective extraction of mycotoxins from complex matrices is an important first step in developing an efficient method. Cannabis plant material has a different composition than the agricultural and food matrices that are conventionally analyzed for mycotoxins. With IAC methods, methanol–water tends to be the preferred extraction solvent, but in this case, a more aggressive acetonitrile–water extraction was employed with subsequent dilution of the extract with Tween 20 or Triton X-100 solution to avoid the damaging effects of acetonitrile on the IAC antibodies, particularly for the reusable immunoaffinity cartridges. Extraction conditions were optimized so that a single procedure could be used for the solid matrices of varied composition, with a different procedure adopted only for the carbonated energy drink.

Initially, the cannabis plant samples and other cannabis products were analyzed using the preliminary method, and it was thereby established that none of the products contained any detectable aflatoxins or OTA. The LODs were slightly different for the two methods and for different matrices, as shown in Table 2, as different gram equivalents were injected in each case.

Table 2. LODs for two methods for cannabis plant and different cannabis products

	Manual IAC method		In-line IAC method	
Type of cannabis product	Total aflatoxins, µg/kg	OTA, µg/kg	Total aflatoxins, µg/kg	OTA, µg/kg
Plant material	0.5	0.3	0.2	0.2
CBD oil	0.3	0.2	0.1	0.1
CBD capsules	0.3	0.2	0.1	0.1
Energy drink	0.2	0.1	0.01	0.01
Hemp tea	0.5	0.3	0.2	0.2
Butter cookie	0.3	0.2	0.1	0.1
Chocolate brownie	0.3	0.2	0.1	0.1

The "blank" samples were used for all subsequent experiments to establish recoveries and preliminary repeatability as single-day relative SDs (RSDs). No work was undertaken to examine either heterogeneity of any mycotoxin contamination or the effectiveness of homogenization procedures for plant material. The small sample sizes of 1–2 g were used for convenience and based on an assumption of homogeneity. Spiking was carried out using a small volume of methanol solution containing both aflatoxins and OTA, syringing the solution onto the surface of the matrix, allowing evaporation, and then allowing to stand for several hours to facilitate any potential matrix binding to mimic a naturally contaminated sample. A spiking level of 20 µg/kg for total aflatoxins was used for cannabis plant material and hemp tea as representing the regulatory limit for all foods in the United States and Canada (23), and a similar level was used for OTA. For all other products, such as oils and edible food products, spiking was at 5 µg/kg for total aflatoxins and OTA, being closer to the more demanding EU limits in food products (24, 25).

After extraction, centrifugation to remove fine particulates, and dilution of the supernatant, the manual IAC cleanup procedure was identical to that employed with other extracts (18), and the automated in-line procedure was identical to that employed for aflatoxins (19).

Using the multimycotoxin IAC, the final extract contained both aflatoxins and OTA and was subsequently analyzed in a single LC chromatographic run with aflatoxins eluting first through the KOBRA CELL for postcolumn bromination and the OTA eluting subsequently. The LC solvent was programmed with two different mobile phases to enable good peak shapes and baselines and good separation of the individual aflatoxins and OTA to be achieved in a 40 min run time. Excitation and emission wavelengths were switched during the LC run for signal optimization of aflatoxins and OTA, respectively.

Using the in-line automated procedure, separate aflatoxin and OTA cartridges were used, as multimycotoxin cartridges were not commercially available at the time of this study. Operation of the system involves a series of automated events beginning with conditioning of the reusable immunoaffinity cartridge, loading the sample extract, washing the cartridge, and eluting aflatoxins or OTA from the cartridge directly onto the LC system (19). After 15 injections of sample extract or standards onto the reusable cartridge, the automated cartridge exchanger replaces the used cartridge with a new cartridge, and the sequence of events starts again. The flow of solvents is controlled throughout with an HPD, and the wash solutions are held in reservoirs. Sample extracts are placed in 1.5 mL autosampler vials in the tray ready for analysis. The sequence of events involving sample cleanup is driven through six-way two-position valves, switching from Position 1 to Position 2 and back in order to couple to the different connections. The operation of this system has been described in detail elsewhere, as has method validation for aflatoxin analysis (18). The use of the immunoaffinity cartridges for OTA analysis has not been previously published. Analysis by LC was conducted under different isocratic conditions for aflatoxins and OTA using a KOBRA CELL with postcolumn bromination for the former and using different excitation and emission wavelengths for optimal detection of aflatoxins and OTA.

Method Validation

Typical chromatograms for cannabis plant material, spiked at 20 µg/kg for total aflatoxins and OTA, by manual and automated IAC analysis are shown in Figures 1 and 2. The chromatograms for unspiked material are also shown superimposed: it is evident that there are no detectable peaks above the LODs in the chromatograms at the retention times for either aflatoxins or OTA. The results for spiked samples confirm experience from elsewhere that IAC cleanup of complex biological materials produces extracts that are essentially free of any background matrix interference (15). A similar situation was found to be the case with other matrices such as chocolate brownie, shown in Figures 3 and 4, again with no discernible peaks above the LODs in the unspiked samples. Although peak shapes appear to be poorer for the in-line analysis, it should be noted that the baseline scales are different, showing 0–10 min for in-line analysis compared to 0–40 min for the manual analysis.

Method Performance

A full single-laboratory method validation was not conducted as, rather than establishing detailed method performance data for a single matrix, it was felt to be more important to establish method applicability for a range of different matrix types. In Table 3, the results are given for six replicates measured in a single day for spiked cannabis plant material and analyzed by manual and automated methods. For the manual method, recoveries averaged from 96 to 98% for individual aflatoxins and averaged 88% for OTA, with RSDs from 5.6 to 6.6 and 4.8%, respectively. For the automated IAC method, recoveries averaged from 102 to 107% for individual aflatoxins and averaged 99% for OTA, with RSDs from 1.8 to 2.9 and 2.0%, respectively.

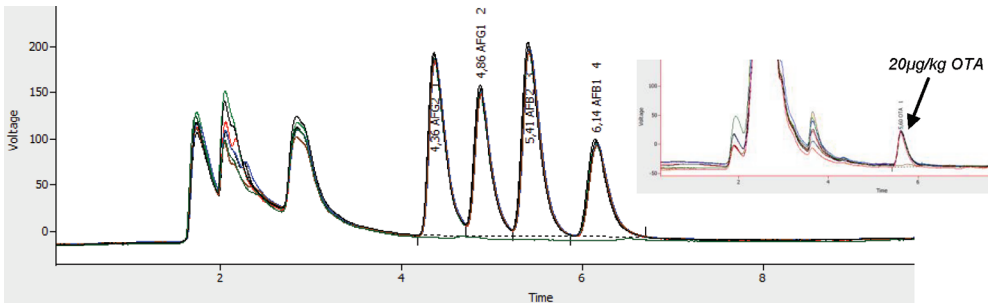

Figure 1. LC chromatogram for in-line IAC analysis of cannabis plant spiked at 20 µg/kg total aflatoxins and 20 µg/kg OTA. Baseline superimposed for blank cannabis plant.

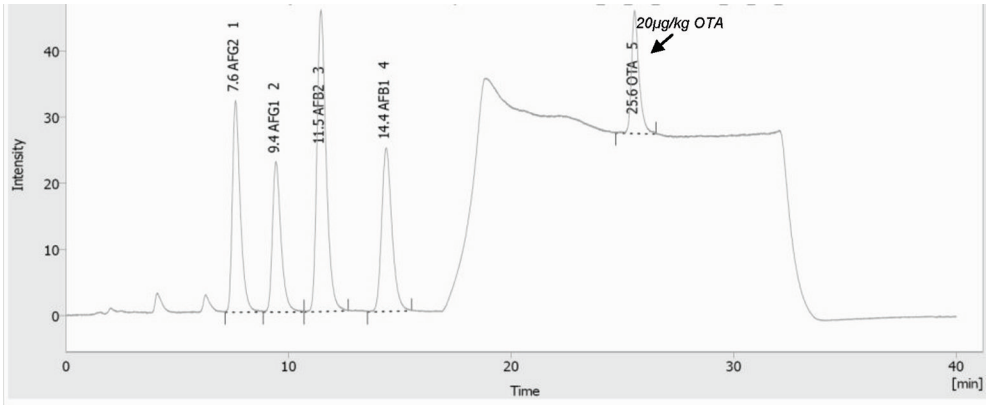

Figure 2. LC chromatogram for manual IAC analysis of cannabis plant material sample spiked at 20 µg/kg total aflatoxins (5 µg/kg each aflatoxin) and 20 µg/kg OTA.

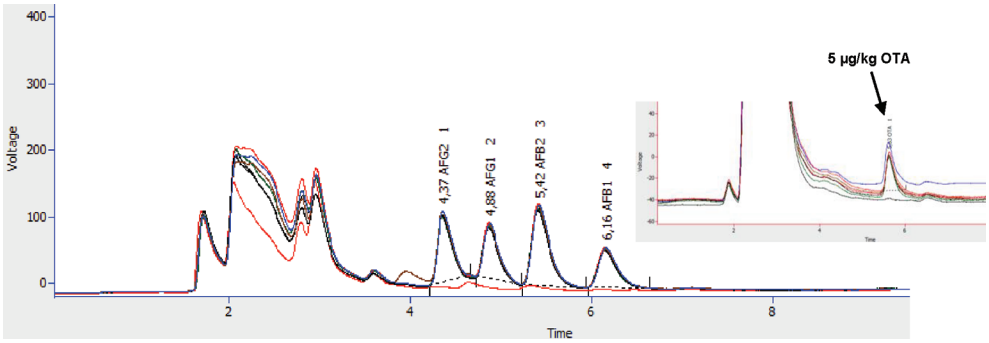

Figure 3. LC chromatograms for in-line IAC analysis of chocolate brownie spiked at 5 µg/kg total aflatoxins and at 5 µg/kg OTA. Baselines superimposed for blank samples.

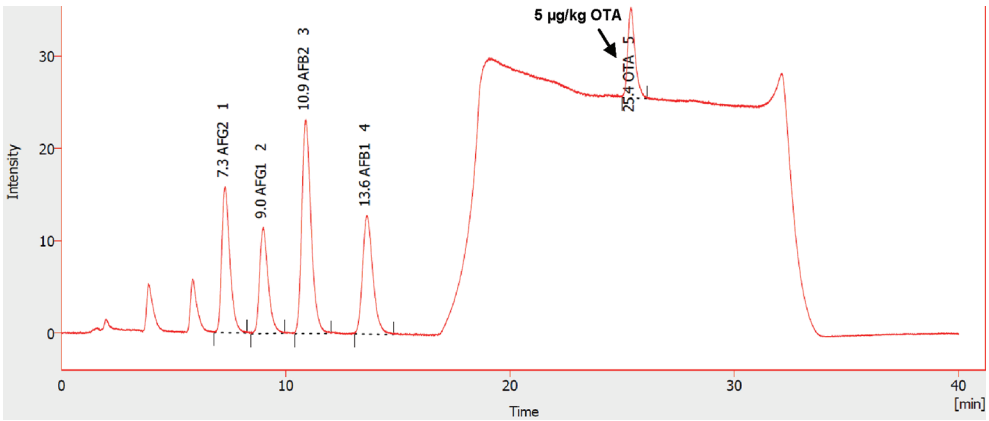

Figure 4. LC chromatogram for manual IAC analysis of chocolate brownie spiked at 5 µg/kg total aflatoxins (1.25 µg/kg each aflatoxin) and 5 µg/kg OTA.

Table 3. Results for single-laboratory, intraday determination of aflatoxins B_1, B_2, G_1, and G_2, total aflatoxins, and OTA in replicates of spiked cannabis plant material

Cannabis plant material sample A: manual analysis with multimycotoxin IAC							
Added level, µg/kg	Aflatoxin B_1	Aflatoxin B_2	Aflatoxin G_1	Aflatoxin G_2	Added level, µg/kg	Total aflatoxins	OTA
5.0	5.00	5.05	5.13	5.11	20.0	20.29	17.51
5.0	4.97	4.98	5.12	5.07	20.0	20.14	18.50
5.0	4.70	4.77	4.86	4.77	20.0	19.10	17.18
5.0	4.65	4.62	4.74	4.72	20.0	18.73	16.73
5.0	5.07	5.19	5.20	5.12	20.0	20.58	18.91
5.0	4.37	4.32	4.46	4.34	20.0	17.49	17.21
Mean	4.79	4.82	4.92	4.85	—	19.39	17.67
Rec.	96	96	98	97	—	97	88
RSD, %	5.6	6.6	5.8	6.3	—	6.6	4.8
Cannabis plant material sample B: automated analysis with reusable IAC cartridges							
5.0	5.28	5.15	5.33	5.06	20.0	20.82	19.65
5.0	5.45	5.33	5.53	5.23	20.0	21.54	20.04
5.0	5.10	5.05	5.20	5.00	20.0	20.35	19.73
5.0	5.31	5.28	5.44	5.21	20.0	21.24	19.77
5.0	5.17	5.17	5.41	5.11	20.0	20.86	20.27
5.0	5.04	5.07	5.26	5.04	20.0	20.41	19.44
Mean	5.23	5.18	5.36	5.11	20.0	20.87	19.82
Rec.	105	104	107	102	—	104	99
RSD, %	2.9	2.2	2.3	1.8	—	2.2	2.0

In Table 4, the results of duplicate analysis by the manual IAC method are given for two brands of CBD oil, two brands of CBD capsules, energy drink, hemp tea, cookies, and a chocolate brownie. Recoveries ranged from 88 to 117% for individual aflatoxins and from 78 to 103% for OTA. No RSDs were calculated as only two replicates were analyzed.

In Table 5, the results are given for the automated analysis for six replicate samples, each analyzed on single days, for one sample of each of CBD oil, CBD capsule, energy drink, hemp tea, butter cookie, and chocolate brownie. Samples C and F were only analyzed by the manual IAC method. For aflatoxins, recoveries ranged from 95 to 104, 104 to 114, 84 to 90, 108 to 111, 115 to 120, and 95 to 116% for CBD oil, CBD capsule, energy drink, hemp tea, butter cookie, and chocolate brownie, respectively. Although recoveries above 100% can be indicative of matrix effects, there was no evidence from blank chromatograms of any interferences from coextractives. There is also no evidence of carryover using the in-line automated system, as has been demonstrated elsewhere (19). It was noted that the method performance criteria for recoveries of 1–10 µg/kg total aflatoxins are recommended in EU regulations to be in the range of 70–110% (26), for which 110% is exceeded in four instances. For OTA, the recoveries averaged 97, 92, 76, 81, 109, and 111% for CBD oil, CBD capsules, energy drink, hemp tea, butter cookie, and chocolate brownie, respectively. Again, recoveries of 1–10 µg/kg OTA are recommended to be in the range of 70–110% (26), for which the 110% recovery was exceeded for chocolate brownie. Overall, the RSDs averaged 1–4.7% for aflatoxins and 0.8–6.2% for OTA, which were well within the recommended range of <20% for contamination levels of 1–10 µg/kg (26).

Although cannabis and cannabis products have been in use for a long time, it is only recent developments in legalizing medicinal and recreational use that have brought a focus on safety and quality. The drying of cannabis leaves and flowers is a high-risk process in terms of fungal contamination and the potential for mycotoxin formation. Extraction of dried cannabis plant material can produce extracts of variable composition, leading to inconsistencies in edible products, particularly in content of biologically active components (27). Addressing the safety aspect necessitates routine monitoring with at least an initial focus on aflatoxins and OTA. Leghissa et al. (27) propose that it should be mandatory for producers and laboratories to create a standardized set of methods for the analysis of various cannabis edibles to account for matrix effects and possible interferences. It should be noted that, although we have not specifically considered hemp plant material and edible hemp products such as hemp seeds, mycotoxin methods are needed for this matrix (28), and the proposed methods should be equally applicable.

Conclusions

In this paper we address the demand for methods for mycotoxin contaminants in cannabis and cannabis products by proposing two different approaches to determining aflatoxins and OTA using either a multiantibody IAC manual cleanup or an automated system with reusable immunoaffinity cartridges prior to LC–fluorescence detection. Recoveries for both approaches for spiked cannabis plant material and derived products were

Table 4. Results for determinations of aflatoxins B_1, B_2, G_1, and G_2, total aflatoxins, and OTA in duplicate spiked CBD oil, CBD capsules, energy drink, hemp tea, and cookies by manual analysis with multimycotoxin IACs

Sample	Added level, µg/kg	AFB1[a]	AFB2[b]	AFG1[c]	AFG2[d]	Added level, µg/kg	Total aflatoxins	OTA
CBD oil sample C	1.25	1.16	1.17	1.16	1.19	5.0	4.68	4.62
	1.25	1.27	1.24	1.29	1.29	5.0	5.09	4.81
Rec., %	—	97	96	98	99	—	98	94
CBD oil sample D	1.25	1.35	1.32	1.36	1.33	5.0	5.36	4.61
	1.25	1.38	1.36	1.40	1.38	5.0	5.52	4.77
Rec., %	—	109	107	110	108	—	109	94
CBD capsules - E	1.25	1.43	1.41	1.44	1.47	5.0	5.75	5.19
	1.25	1.38	1.40	1.44	1.46	5.0	5.68	5.11
Rec., %	—	113	112	115	117	—	114	103
CBD capsules - F	1.25	1.37	1.29	1.30	1.32	5.0	5.28	5.09
	1.25	1.29	1.23	1.25	1.24	5.0	5.01	4.74
Rec., %	—	106	101	102	102	—	103	98
Energy drink - G	1.25	1.06	1.11	1.13	1.16	5.0	4.46	4.29
	1.25	1.13	1.17	1.21	1.23	5.0	4.74	4.50
Rec., %	—	88	92	94	96	—	92	88
Hemp tea - H	5.0	4.45	4.43	4.54	4.50	20.0	17.92	15.77
	5.0	4.36	4.34	4.42	4.44	20.0	17.56	15.46
Rec., %	—	88	88	90	89	—	89	78
Butter cookie - I	1.25	1.34	1.35	1.36	1.38	5.0	5.43	4.92
	1.25	1.32	1.36	1.36	1.37	5.0	5.41	4.80
Rec., %	—	106	108	109	110	—	108	97
Chocolate brownie- J	1.25	1.28	1.27	1.29	1.27	5.0	5.11	4.58
	1.25	1.37	1.33	1.33	1.30	5.0	5.33	4.71
Rec., %	—	106	104	105	103	—	104	93

[a] AFB1 = Aflatoxin B_1.

[b] AFB2 = Aflatoxin B_2.

[c] AFG1 = Aflatoxin G_1.

[d] AFG2 = Aflatoxin G_2.

Table 5. Results for determination of aflatoxins B_1, B_2, G_1, and G_2, total aflatoxins, and OTA in replicates of spiked CBD oil, CBD capsules, energy drink, hemp tea, and cookies by automated analysis with reusable IAC cartridges

Added level, µg/kg	Aflatoxin B_1	Aflatoxin B_2	Aflatoxin G_1	Aflatoxin G_2	Added level µg/kg	Total aflatoxins	OTA
CBD oil: sample D							
5.0	5.29	5.15	4.76	4.89	20.0	20.09	19.20
5.0	5.31	5.18	4.79	4.92	20.0	20.20	19.36
5.0	5.38	5.17	4.83	4.95	20.0	20.33	19.01
5.0	5.32	5.13	4.67	4.89	20.0	20.01	19.61
5.0	5.41	5.33	4.89	5.13	20.0	20.36	19.51
5.0	5.25	5.08	4.65	4.86	20.0	19.84	19.32
Mean	5.34	5.20	4.79	4.96	—	20.14	19.33
Rec.	104	102	95	98	—	100	97
RSD, %	1.0	1.2	1.7	2.0	—	1.0	1.1
CBD capsule: sample E							
5.0	5.73	5.65	5.22	5.59	20.0	22.19	18.46
5.0	5.56	5.32	5.03	5.22	20.0	21.13	18.66
5.0	5.59	5.49	5.14	5.51	20.0	21.73	18.42

Table 5. (*continued*)

Added level, µg/kg	Aflatoxin B_1	Aflatoxin B_2	Aflatoxin G_1	Aflatoxin G_2	Added level µg/kg	Total aflatoxins	OTA
5.0	5.80	5.61	5.27	5.59	20.0	22.27	18.32
5.0	5.73	5.61	5.22	5.60	20.0	22.16	18.26
5.0	5.45	5.48	5.07	5.43	20.0	21.43	18.25
Mean	5.68	5.53	5.18	5.50	—	21.81	18.40
Rec.	114	111	104	110	—	109	92
RSD, %	1.8	2.4	1.8	2.9		2.1	0.8
Energy drink: sample G							
1.25	1.04	1.05	1.07	0.98	5.0	4.14	4.07
1.25	1.11	1.111	1.13	1.05	5.0	4.40	3.60
1.25	1.10	1.12	1.14	1.06	5.0	4.42	3.95
1.25	1.10	1.14	1.14	1.08	5.0	4.46	3.60
1.25	1.13	1.15	1.17	1.10	5.0	4.55	4.19
1.25	1.11	1.111	1.13	1.15	5.0	4.50	3.56
Mean	1.095	1.113	1.127	1.05	—	4.41	3.81
Rec.	88	89	90	84	—	88	76
RSD, %	3.0	3.7	3.3	4.7	—	3.3	6.2
Hemp tea: sample H							
5.0	5.51	5.58	5.56	5.60	20.0	22.26	15.64
5.0	5.37	5.45	5.43	5.45	20.0	21.7	16.29
5.0	5.07	5.26	5.25	5.34	20.0	20.92	16.88
5.0	5.43	5.58	5.48	5.62	20.0	22.12	16.86
5.0	5.62	5.69	5.58	5.73	20.0	22.63	15.17
5.0	5.29	5.38	5.25	5.39	20.0	21.31	15.70
Mean	5.40	5.51	5.46	5.55	—	21.82	16.17
Rec.	108	110	109	111	—	109	81
RSD, %	3.9	3.0	2.4	2.8	—	2.9	4.7
Butter cookie: sample I							
1.25	1.53	1.51	1.56	1.47	5.0	6.07	5.41
1.25	1.43	1.45	1.49	1.45	5.0	5.52	5.65
1.25	1.39	1.43	1.46	1.39	5.0	5.67	5.47
1.25	1.49	1.47	1.51	1.47	5.0	5.94	5.27
1.25	1.47	1.46	1.49	1.43	5.0	5.85	5.70
1.25	1.41	1.43	1.48	1.44	5.0	5.76	5.16
Mean	1.45	1.46	1.50	1.44	—	5.80	5.44
Rec.	116	117	120	115	—	116	109
RSD, %	3.4	2.2	2.4	1.9	—	3.4	3.9
Chocolate brownie: sample J							
1.25	1.44	1.41	1.24	1.17	5.0	5.26	5.20
1.25	1.43	1.41	1.20	1.15	5.0	5.19	5.59
1.25	1.53	1.50	1.29	1.23	5.0	5.55	5.63
1.25	1.38	1.39	1.23	1.17	5.0	5.17	5.50
1.25	1.50	1.49	1.28	1.22	5.0	5.49	5.55
1.25	1.44	1.44	1.22	1.15	5.0	5.25	5.73
Mean	1.45	1.44	1.24	1.18	—	5.32	5.54
Rec.	116	115	99	95	—	106	111
RSD, %	3.5	3.3	2.7	3.0	—	3.0	3.0

in the range from 76 to 120%, and RSDs ranged from 0.8 to 6.6%. The elaborated methods described in this paper are suitable for the cleanup of sample extracts of cannabis and various products containing some constituents of cannabis. However, further method validation work is required to provide all information enabling the reliable and accurate testing of cannabis and cannabis products for aflatoxin and OTA contamination.

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