

LC-MS/MS determination of mycotoxins in cannabis and cannabis derived products using different sample preparations

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SUMMARY

The quality control of hemp and cannabis before human consumption e.g., the determination of pesticide residues and mycotoxins in cannabis biomass and its derived products, is mandatory. The U.S. Food & Drug Administration (FDA) and Commission Regulation (EC) No 2023/915 set limit values for mycotoxins in human food and animal feed. Organizations like AOAC are developing method requirements for mycotoxin determination in cannabis matrices. AOAC SMPR® 2021.010 defines aflatoxins B1/B2, aflatoxins G1/G2 and ochratoxin A as analytes of interest and specifies limits of quantification and qualification for cannabis biomass and cannabis derived products. Here, we describe sample preparation methods and an analytical chromatography LC-MS/MS method to detect mycotoxins in different hemp matrixes at ppb level.

INTRODUCTION

Due to the potential healthcare benefits of cannabis and hemp, the market for this plant and its products has increased dramatically in recent years. In several states cannabis is already legal for medicinal and/or recreational use. Quality control before human consumption e.g., the determination of pesticide residues and mycotoxins in cannabis biomass and its derived products, is therefore mandatory. The number of regulated pesticides varies dependent on state/country/region. The U.S. Food & Drug Administration

(FDA) sets a limit value of 20 ppb for mycotoxins in human food and animal feed [1]. The same values are set by Commission Regulation (EC) No 2023/915 [2]. Organizations like AOAC are developing method requirements for mycotoxin determination in cannabis matrices. AOAC SMPR® 2021.010 defines aflatoxins B1/B2, aflatoxins G1/G2 and ochratoxin A as analytes of interest and specifies limits of quantification and qualification for cannabis biomass and cannabis derived products [3].

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Cannabis analysis also includes sample preparation. Four different samples, cannabis/hemp pellets, cannabis/hemp seeds, commercially available hemp flour, and hemp oil were investigated, and four different sample preparation procedures were processed. The following procedures were applied: P1 - solid-liquid extraction/liquid-liquid extraction, P2 - a standard QuEChERS extraction with dispersive cleaning, P3 - extraction with following CrossTOX cleanup and P4 - extraction with following solid phase extraction using immunoaffinity columns. Results are investigated with LC-MS/MS and evaluated in terms of time, costs per sample, solvent consumption, and achievement of limit values. For a direct comparison with fluorescence detection please refer to application note [VFD0192](#). There the same LC method settings and sample preparations were applied. The used CrossTOX and BEKOLut immunoaffinity SPE columns are specific for the desired mycotoxins but are not officially validated for cannabis/hemp matrices.

SAMPLE PREPARATION

Samples of hemp seeds and hemp pellets were shredded before weighing. Commercially hemp flour and oil were used without further pretreatment. Samples were spiked at level L5 (see [Tab.1](#)) for determination of recovery.

Solid-liquid extraction (SLE)/ liquid-liquid extraction (LLE)

Weigh 1 g of sample into a 50 ml falcon tube. Add 5 ml of acetonitrile. Vortex/shake for 10 minutes. Centrifuge for 10 minutes at 3000 rpm. Filter supernatant with 0.2 µm nylon filter. Transfer 500 µl of filtered extract to an autosampler vial and add 500 µl of acetonitrile.

QuEChERS

Weigh 2 g of sample into a 50 ml falcon tube. Add 10 ml of deionized water. Vortex/shake for 10 minutes. Add 10 ml of acetonitrile. Vortex/shake for 10 minutes. Add QuEChERS extraction salts to the falcon tube. Shake for 1 minute. Centrifuge for 5 minutes at 3000 g. Transfer 2 ml of supernatant to the QuEChERS dispersive cleanup tube. Vortex for 30 seconds. Centrifuge for

5 minutes at 3000 g. Filter supernatant with 0.2 µm nylon filter. Transfer extract to an autosampler vial.

(BEKOLut® SALT-Kit-AC, P/N: SK-AC-050; BEKOLut® PSA-Kit-02, P/N: PK-02)

CrossTOX

Weigh 2 g of sample into a 50 ml falcon tube. Add 10 ml of acetonitrile:water 84:16 (v/v). Vortex/shake for 15 minutes. Centrifuge for 5 minutes at 3000 g. Filter a maximum of 3 ml of supernatant through the CrossTOX column. Transfer extract to an autosampler vial.

(LCTech CrossTOX® Clean-up Columns Manual processing, P/N: 17900)

Immunoaffinity chromatography solid phase extraction (IAC SPE)

Weigh 1 g of sample into a 50 ml falcon tube. Add 10 ml of MeOH:ACN:H₂O 25:25:50 (v/v/v). Shake/stir for 15 minutes. Centrifuge for 5 minutes at 3000 g. 3 ml extract are diluted with 20 ml PBS + 2 % Tween20 and are passed through the IAC with a flow rate of 2-3 ml/min. Wash column with 10 ml H₂O. Remaining liquid is removed by applying slight pressure. Elute with 2.5 ml of MeOH:HAc 98:2 (v/v) and 0.5 ml H₂O. For the 1st ml slight pressure/vacuum is applied. For the 2nd and 3rd ml the elution is stopped for 30 seconds after half the volume has passed. Remaining liquid residues are removed by applying slight pressure. The 3 ml of eluted extract are filtered through a 0.2 µm nylon filter. Transfer extract to an autosampler vial. (BEKOLut® IAC Afla/Ochra/ZON/DON/FUM/T2HT2, P/N: 003-AOZDFT)

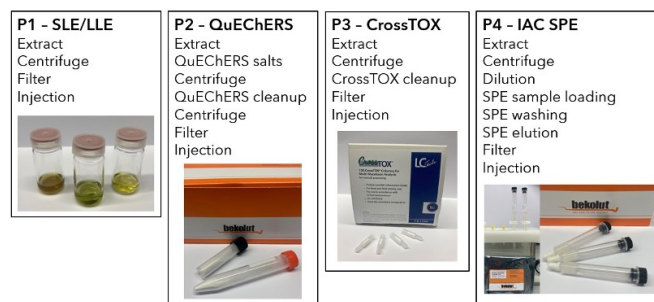


Fig. 1 Simplified overview of sample preparation procedures

RESULTS

Mycotoxins were separated under reversed phase liquid chromatography (LC) conditions. They were detected with mass spectrometry (MS). MRM measurements were conducted using electrospray ionization (ESI) with positive polarity (Fig.2). A 6-point calibration was set up in a range from 0.75-30 ppb for G2/B2, 0.25-10 ppb for G1/B1, 2.50-100 ppb for ZON and 0.50-20 ppb for OTA.

Tab.1 Concentration of calibration levels in ppb (ppb=ng/ml)

Peak	Stock solution [ng/ml]	L6	L5	L4	L3	L2	L1
G2	3000	30	15	6	3	1.5	0.75
G1	1000	10	5	2	1	0.5	0.25
B2	3000	30	15	6	3	1.5	0.75
B1	1000	10	5	2	1	0.5	0.25
ZON	100000	100	50	20	10	5.0	2.50
OTA	10000	20	10	4	2	1.0	0.50

The calculated values for limit of detection (LOD) and limit of quantification (LOQ) for measurements without matrix are below 20 ppb and within the specification of regulations (Tab.2).

For LOD a signal to noise ratio (S/N) of 3 was taken as basis. For the LOQ a ratio of S/N = 10 was applied.

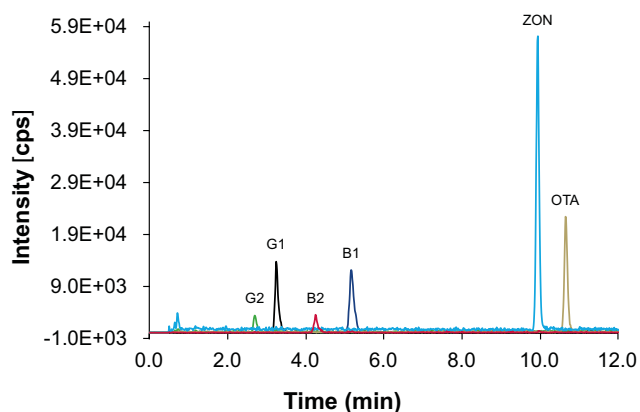


Fig.2 Overlay of XIC from quantifier transitions, mycotoxin mix standard at L2

Tab.2 Comparison of LOD/LOQ without matrix and limit values in ppb, *valid for animal feeds/foodstuff

Peak	LOD (S/N=3)	LOQ (S/N=10)	FDA*	AOAC	(EC) No 2023/915*
G2	0.160	0.540	20 (sum of G2/G1/B2/B1)	20 (sum of G2/G1/B2/B1)	4-15 (sum of G2/G1/B2/B1)
G1	0.024	0.080			
B2	0.024	0.080		5 (B1)	0.1-12 (B1)
B1	0.018	0.060			
ZON	0.210	0.690	-	-	20 - 400
OTA	0.027	0.090	-	20	0.5 - 80

For the determination of recovery rates for each sample and sample preparation procedure, the samples were spiked with mycotoxins using the concentration of L5 of the calibration solution (see Tab.1).

P1 - Solid liquid extraction (SLE)/ liquid-liquid extraction (LLE)

The first applied extraction procedure is the simplest and fastest one. The detailed preparation is described in the sample preparation section. Using this preparation method, the matrix removal was insufficient. Not all analytes could be recovered in the hemp pellets and seeds samples (Fig.3).

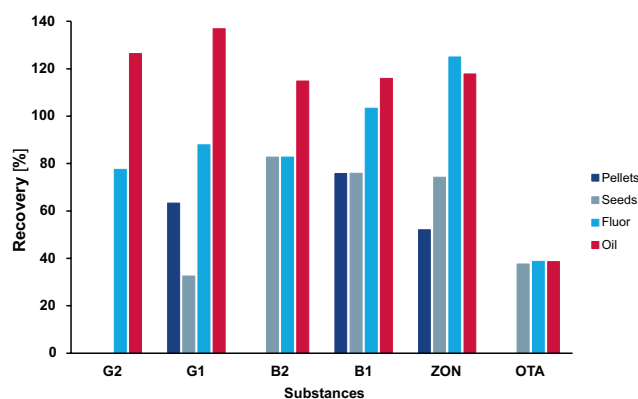


Fig.3 Recovery [%] of mycotoxins for all samples with sample preparation P1

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P2 - QuEChERS

Like the first sample preparation P1, with the standard QuEChERS approach not all analytes could be recovered in the hemp pellets and seeds samples (Fig.4).

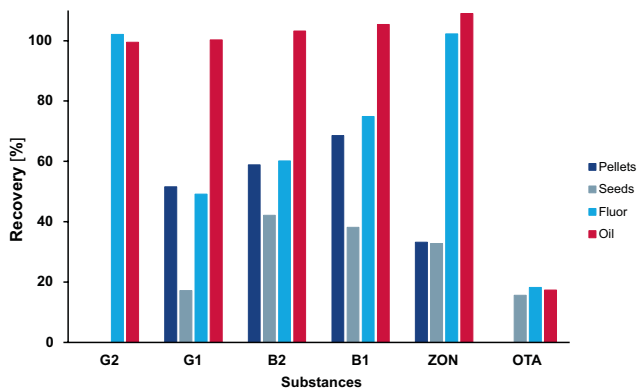


Fig.4 Recovery [%] of mycotoxins for all samples with sample preparation P2

P3 - CrossTOX

This preparation procedure is very similar to P1, but instead of the 0.2 µm nylon filter, CrossTOX filter columns were used (Fig.5). Using this preparation method, the matrix removal was insufficient and not all analytes could be recovered in the hemp pellets and seeds samples.

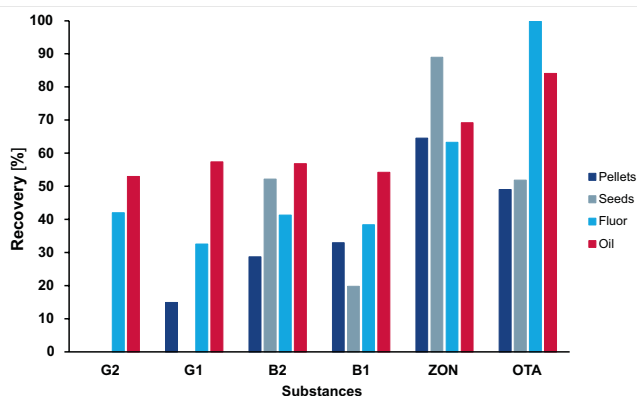


Fig.5 Recovery [%] of mycotoxins for all samples with sample preparation P3

P4 - Immunoaffinity chromatography solid phase extraction (IAC SPE)

This preparation procedure is the most time-consuming but also was the most effective. Using the IAC SPE, all mycotoxins could be detected in all spiked samples (Fig.6).

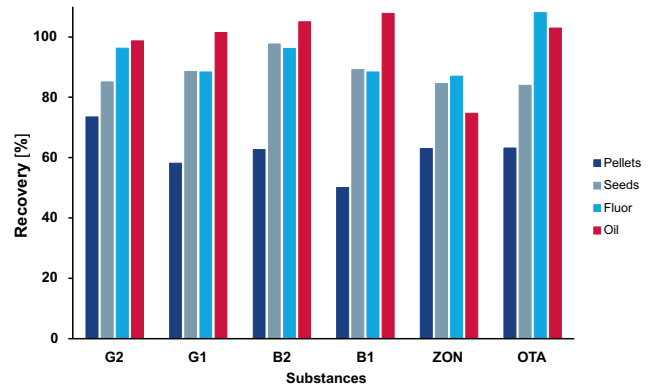


Fig.6 Recovery [%] of mycotoxins for all samples with sample preparation P4

Generally, the limit values were met for all sample preparation procedures. The determination of recovery rates was dependent on the complexity of matrix and on the complexity of sample preparation. Fig.7 displays the comparison of easy and complex sample preparation for the used hemp seed sample.

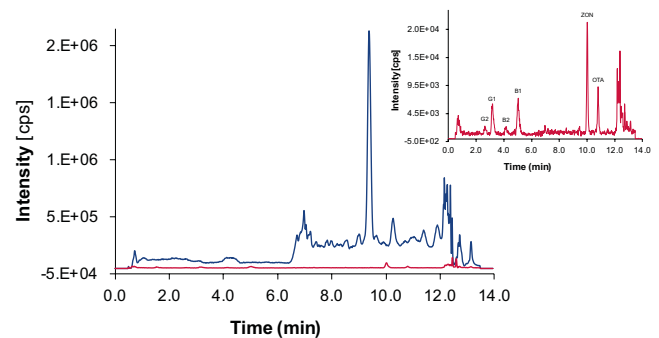


Fig.7 TIC of spiked hemp seed sample with different sample preparations; P1 - blue, P4 - red; right corner: XIC of P4 for hemp seeds

Tab. 3 summarizes the time and costs for the different procedures per sample. The solvent consumption during analysis was the same for all samples and is therefore not considered in the table below.

Tab. 3 Comparison of sample preparation parameters, * costs refer to used solvents and additional consumables (costs for working hours need to be considered individually)

Sample prep	Time [min]	Costs* [€]	Solvent consumption [ml] (preparation only)
P1 - SLE/LLE	30	6.80	5.5
P2 - QuEChERS	30 - 45	10.20	20.0
P3 - CrossTOX	30	7.60	10.0
P4 - IAC SPE	30 - 45	39.50	43.0

More steps in sample preparation required more additional consumables, like for QuEChERS or IAC SPE. Therefore, these types of preparations were more time consuming and expensive per sample. However, when it comes to reaching the LODs or LOQs, the more complex procedures were more effective. Simple sample preparation methods, such as SSL/LLE, may result in more matrix suppression and thus in lower recovery rates.

CONCLUSION

Generally, the limit values were met for all sample preparation procedures. The determination of recovery rates was dependent on the complexity of matrix and complexity of sample preparation. Challenging matrices like hemp and hemp products should be treated with more complex sample preparation procedures. The best results for these challenging matrices (pellets, seeds, flour) were obtained using the IAC SPE procedure. Nevertheless, the standard QuEChERS extraction showed a good cleanup of the samples, but recovery rates need to be optimized. A modification of the standard QuEChERS approach, for example a combination of QuEChERS and CrossTOX, could be meaningful.



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MATERIAL AND METHODS

Tab. 4 System configuration

Instrument	Description	Article No.
Pump	AZURA P 6.1L HPG, 5 ml, 1000 bar	APH35GA
Autosampler	AZURA AS 6.1L, cool/heat, 1240 bar	AAA11AA
Thermostat	AZURA CT 2.1	ATC00
LC Software	ClarityChrom 9.0.0 - Workstation, autosampler control included	A1670
MS	Sciex Triple Quad™ 5500+ System - QTRAP® Ready	-
MS Software	SCIEX OS 3.1	-
Column	Eurospher II 100-2 C18, 150 x 2 mm ID	15BE181E2F

Tab.5 LC method parameters

Parameter	Description																											
Eluent A	Water + 0.1% formic acid																											
Eluent B	Methanol + 0.1% formic acid																											
Flow rate	0.5 ml/min																											
Temperature	60 °C																											
Gradient	<table border="1"> <thead> <tr> <th>Time [min]</th> <th>% A</th> <th>% B</th> </tr> </thead> <tbody> <tr> <td>0.00</td> <td>60</td> <td>40</td> </tr> <tr> <td>5.60</td> <td>60</td> <td>40</td> </tr> <tr> <td>5.62</td> <td>40</td> <td>60</td> </tr> <tr> <td>11.20</td> <td>40</td> <td>60</td> </tr> <tr> <td>11.22</td> <td>0</td> <td>100</td> </tr> <tr> <td>12.20</td> <td>0</td> <td>100</td> </tr> <tr> <td>12.22</td> <td>60</td> <td>40</td> </tr> <tr> <td>15.00</td> <td>60</td> <td>40</td> </tr> </tbody> </table>	Time [min]	% A	% B	0.00	60	40	5.60	60	40	5.62	40	60	11.20	40	60	11.22	0	100	12.20	0	100	12.22	60	40	15.00	60	40
	Time [min]	% A	% B																									
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	12.20	0	100																									
	12.22	60	40																									
15.00	60	40																										
Injection volume	10 µl																											

Tab.6 MS source parameters

Source parameters			
CAD gas	10	Ion source gas 1	50 psi
Curtain gas	40 psi	Ion source gas 1	60 psi
Temperature	600 °C	Ion spray voltage	3500 V

Tab.7 MRM table

Group ID	Compound ID	Q1 mass [Da]	Q3 mass [Da]	Dwell time [ms]	DP [V]	EP [V]	CE [V]
Aflatoxin B1	Aflatoxin B1_1	313.061	285.056	75	110	10	35
Aflatoxin B1	Aflatoxin B1_2	313.061	241.045	75	110	10	53
Aflatoxin B2	Aflatoxin B2_1	315.11	287.238	75	135	10	39
Aflatoxin B2	Aflatoxin B2_2	315.11	259.09	75	135	10	42
Aflatoxin G1	Aflatoxin G1_1	329.056	243.071	75	110	10	39
Aflatoxin G1	Aflatoxin G1_2	329.056	199.049	75	110	10	57
Aflatoxin G2	Aflatoxin G2_1	331.087	313.152	75	80	10	36
Aflatoxin G2	Aflatoxin G2_2	331.087	245.14	75	80	10	44
OTA	Ochratoxin A_1	404.223	239.128	75	20	10	31
OTA	Ochratoxin A_2	404.223	358.139	75	20	10	21
ZON+	ZON_1+	319.148	283.147	75	65	10	20
ZON+	ZON_2+	319.148	301.103	75	65	10	17

REFERENCES

[1] Guidance for Industry: Action Levels for Poisonous or Deleterious Substances in Human Food and Animal Feed; <https://www.fda.gov/regulatory-information/search-fda-guidance-documents/guidance-industry-action-levels-poisonous-or-deleterious-substances-human-food-and-animal-feed#afla>, 15/04/2024

[2] COMMISSION REGULATION (EU) 2023/915 of 25 April 2023 on maximum levels for certain contaminants in food and repealing Regulation (EC) No 1881/2006.

[3] AOAC SMPR® 2021.010, Standard Method Performance Requirements (SMPRs®) for Quantitative Analysis of Mycotoxins in Cannabis Biomass and Cannabis-Derived Products