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*Professional paper*

# LC-MS/MS method for detecting trace levels of aflatoxin B1 in cultivated cannabis from Republic of North Macedonia

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## Abstract

This study describes a sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for detection and quantification of aflatoxin B1 in dried cultivated cannabis flowers from R.N. Macedonia. Aflatoxin B1, a highly carcinogenic mycotoxin produced by *Aspergillus* species, poses significant health risks, particularly in agricultural products such as cannabis, where contamination levels must be carefully monitored. Traditional methods for detection have difficulties due to the complex matrix of cannabis, which is rich in phytocannabinoids and other bioactive compounds. Therefore, a matrix-matched calibration approach was used and high sensitivity and specificity of the applied LC-MS/MS method were achieved. Validation of the method demonstrated excellent linearity ($R^2$=0.999), precision (CV% < 20%), and recovery rates between 103-112%. When applied to twelve dried flower samples of cannabis cultivated in the Republic of North Macedonia, the method accurately quantified aflatoxin B1 within permitted level (< 2 µg/kg) set by the European Pharmacopoeia. This validated approach confirmed adherence of the investigated samples to the accepted safety standards respecting aflatoxin B1 and could be used as a reliable tool for aflatoxin B1 determination in cannabis production.

**Key words:** Aflatoxin B1, cannabis flos, QqQ-MS analysis

## Introduction

Aflatoxins are potent mycotoxins produced by certain species of *Aspergillus* fungi. Aflatoxins pose a significant health risk, particularly through contaminated agricultural products. Of all the mycotoxins that can impact cannabis, aflatoxins are particularly worrisome due to their high toxicity and extensive occurrence. These compounds are carcinogenic, genotoxic, and capable of suppressing immune function. Aflatoxin B1 (AFB1), in particular, is the most prevalent and dangerous among aflatoxins, with strong evidence linking it to liver cancer, inhibited growth, and immune system disruption (Al-Jaal et al., 2019; Di Nardo et al., 2020; Marchese et al., 2018).

While the presence of AFB1 in food and feed has been extensively studied, its occurrence in cannabis plants and derived products is gaining attention as the medicinal and recreational use of cannabis expands globally (Kumar et al., 2017; Turner et al., 2009). Commonly used techniques for aflatoxin detection include methods based on electrochemical and optical principles, such as thin layer

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chromatography (TLC), high-performance liquid chromatography (HPLC), and gas chromatography-mass spectrometry (GC-MS). Immunological methods, like radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), and immunochemical screening with immunoaffinity columns coupled to fluorometry, are also widely employed due to their sensitivity and specificity (Espinosa-Calderón et al., 2011; Miklós et al., 2020; Nie et al., 2019). The majority of control laboratories use these bioassays in routine practice, but there is an increasing need for sensitive and robust methods capable of accurately analyzing AFB1 in cannabis plant material to verify adherence to the European Pharmacopoeia's regulatory limits and guarantee consumer safety (Gámiz-Gracia et al., 2020). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has emerged as a powerful tool for detecting contaminants like mycotoxins and pesticide residues due to its high sensitivity, selectivity, and ability to analyze complex matrices (Zweigenbaum and Pierri, 2020). However, cannabis and particularly hybrid strains tend to produce high tetrahydrocannabinol (THC) levels, but exhibit diverse terpenoid composition, often containing terpenes such as myrcene, limonene and caryophyllene (Casano et al., 2011), making them challenging matrix regard analysis of contaminants. The most significant issue is sample preparation. Given the importance of AFB1 as a critical safety marker within the total aflatoxin group (B1, B2, G1, and G2), along with the pressing need for a highly sensitive and accurate analytical approach, this study aimed to validate LC-MS/MS method capable of quantifying trace levels of AFB1.

## Material and methods

*Materials*

This study utilized twelve dried flowers from cannabis cultivated in the southwestern region of the Republic of North Macedonia. The subject strain, Critical Orange Punch (Dutch Passion), is an Indica/Sativa hybrid with predominantly Indica genetics known for its high THC content (15-22%) and minimal cannabidiol (CBD) levels, but diverse terpenoid composition.

*Chemicals*

The following chemicals were used: acetonitrile, CHROMASOLV™ ≥ 99.9%, for LC-MS (Honeywell Riedel-de Haën™, Seelze, Germany), 2-propanol, ROTISOLV® ≥ 99.95 %, Ultra LC-MS (Roth, Karlsruhe, Germany), formic acid 99%, RS For LC/MS (Carlo Erba, Val-de-Reuil, France), ammonium formate, HiPerSolv CHROMANORM® ≥ 99%, for LC/MS (VWR, Magna Park, United Kingdom), and standard of aflatoxin B1 in acetonitrile (Trilogy®, Washington, MO USA). Tubes for solid phase extraction were supplied from Supelco, Bellefonte, PA USA (SPE Tubes, Discovery® DSC-18, 500 mg, volume 6 mL). Ultra-pure water obtained from Millipore Direct-Q® 5 UV system (Tokyo, Japan) was used for preparation of all solutions. Recovery studies were performed using blank spiked cannabis samples.

*Apparatus*

The LC/MS/MS system utilized in this study included an Agilent 1200 Series vacuum degasser, a binary pump, a well-plate autosampler, thermostatted column compartment, and an Agilent 70 LC/TQ equipped with Jet Stream Technology (Agilent, Santa Clara, California).

*Chromatographic conditions*

The analysis was performed using a gradient method (Chen and Cappozzo, 2011) with modifications. The aqueous mobile phase consisted of 5 mM ammonium formate (0.03% *w/v*) and 0.1% formic acid (1:100 *v/v*) and organic phase was pure acetonitrile, with flow rate of 0.4 mL/min. The injection volume of the samples was 5 µL. Chromatographic conditions are summarized in Table 1.

Table 1. Chromatographic conditions for analysis of aflatoxin B1

| | | |
|---|---|---|
| Column: | Zorbax RRHD Eclipse Plus C18 2.1x100 mm, 1.8 µm p/n: 959758-902 | |
| Column temperature: | 40 °C | |
| Injection Volume: | 5 µL | |
| Mobile phase: | A) 5 mM ammonium formate & 0.1% formic acid<br>B) 100% acetonitrile | |
| Gradient: | Time (min) | %B |
| | 0 | 5 |
| | 5 | 100 |
| | 6 | 100 |
| MS/MS | Agilent 6470 LC/TQ | |
| Ion Mode: | ESI/Agilent Jet Stream, Positive ionization | |

Pure acetonitrile (100%) was employed for preparation of standard solutions and for extraction of AFB1 from cannabis flowers. Chromatographic data were processed using Agilent's MassHunter software (Agilent, USA), adhering to chromatographic analysis standards. The analyte transitions with precursor, product ion and corresponding collision energies are given in Table 2.

Table 2. Aflatoxin B1 transitions and collision energies

| **Aflatoxin B1** | |
|---|---|
| Precursor ion (m/z) | 313.1 |
| **Transitions/collision energies** | |
| Product ion (m/z) | 241,1; 285.1; 269,1 |
| Collision energy (eV) | 35; 20; 25 |

*Sample preparation*

AFB1 extractions from the examined dried cannabis flowers were conducted according to the procedure published by Stone et al. (2019). Briefly, one gram of chopped sample of cannabis flower was transferred to a 50 mL polypropylene centrifuge tube with two ceramic homogenizers. The tube was mechanically shaken for 5 minutes at high speed (2500 rpm on Multi Tube vortex, Harlow, United Kindom) and then 15 mL acetonitrile was added to the tube. Afterwards, the content of the tube was shaken again for additional 5 minutes at high speed for the extraction and then centrifuged (Rotofix 32 A, Tuttlingen, Germany) at 5000 rpm for 5 minutes. While centrifuging, SPE cartridges were placed on a manifold with a collection tube of 25 mL. The obtained supernatant was decanted into the SPE cartridge, and the solvent was passed through by gravity. The remaining plant material and pellets in the tube were mixed with 5 mL of acetonitrile, agitated to suspension, and shaken for 3 minutes. The contents were then poured into the same SPE cartridge, and the cleaned eluent was collected. Each time 5 mL of acetonitrile was added to ensure that the SPE cartridge surface was dry. An additional 5 mL of acetonitrile was added to the empty tube, vortexed for 30 seconds, and transferred to the SPE cartridge. Volume of the yielded extract was adjusted to exactly 25 mL by adding acetonitrile directly to the collection tube. A portion of 5-10 mL was filtered through Millex PTFE 0.22 µm syringe filter (Sigma-Aldrich, Tullagreen, Ireland). Then, 1 mL of the filtered eluent was diluted with 3 mL methanol/water (25:75% *v*/*v*) in a 7 mL glass eprouvette. The diluted content was vortexed for 20 seconds and prepared for LC/MS injection. The sample preparation procedure resulted in a 1/100 total dilution.

*Standard solutions and matrix matched solutions*

Stock solution containing 10 µg/L of AFB1 was prepared in pure acetonitrile. Due to the complexity of the matrix, a matrix matched standard calibration model was used. Hence, blank samples from dried cannabis flowers were extracted and used to dilute the stock solution of AFB1 standard. Concentration range of 0.01-1 µg/L (1-100 µg/L considering the dilution of 1:100 during sample preparation) was selected according to the newest Ph. Eur. on cannabis flower.

*Method validation*

The validation procedure involved whether this method comply with the acceptable criteria for following parameters: recovery, precision upon quantification, accuracy and optimal limits of detection and quantification according to International Conference of Harmonization for validation of analytical methods and the Directive 96/23/EC considering the performance of analytical methods and Committee for Medicinal Products for Human Use ICH Q2(R2) Guideline on validation of analytical procedures (Directive 96/23/EC, 2002; EMA/CHMP/ICH/82072/2006, 2023).

*Recovery studies*

Recovery studies were performed to evaluate the accuracy of the method used in this study. The results indicate how effectively the applied method extracted and quantified AFB1 from cannabis samples. For this aim, AFB1 standard solution was added to the blank extract of cannabis plant material to obtain concentrations of 0.01 µg/L, 0.02 µg/L, and 0.1 µg/L.

## Results and discussion

Legalized cultivation of medical cannabis in 2016 in Republic of North Macedonia prompted rapid industry's growth. By March 2024 nearly 70 privately owned companies in the country were registered for cultivating medical cannabis and producing cannabis buds and oil (Risk Bulletins - The Global Initiative Against Transnational Organized Crime, 2024). In view of the growing interest in cannabis and increased worldwide regulation, it is critical for cannabis products to be safe. Therefore, LC-MS/MS method for determination of aflatoxins in food matrices reported by Chen and Cappozzo (2011) was appropriately modified to detect and quantify AFB1 trace levels in dried cannabis flowers. Published studies have identified significant challenges in using LC-MS/MS for detecting AFB1 in complex matrices like cannabis. Matrix effects, such as ion suppression or enhancement, can disrupt ionization consistency, leading to signal intensity fluctuations that undermine the accuracy

and precision of quantitative analysis. Additionally, the chemical complexity of cannabis, demands meticulous sample preparation and chromatographic separation to minimize interference. Retention time variability caused by co-eluting matrix components further complicates reproducibility and accuracy. While advanced methods like matrix-matched calibration can reduce these issues, they require extensive validation to ensure consistent performance (Matuszewski et al., 2003; Serafimovska et al., 2021). These challenges were successfully addressed in this study, optimizing separation and detection conditions for AFB1 determination. Clean-up techniques like solid-phase extraction (SPE) combined with matrix-matched calibration mitigated interference from the complex cannabis matrix, ensuring high accuracy and reproducibility. By leveraging Multiple Reaction Monitoring (MRM), the applied method ensured both selectivity and specificity, being particularly helpful for AFB1 determination in hybrid cannabis strains with diverse chemical composition, including cannabinoids and terpenes (Casano et al., 2011; Zandkarimi et al., 2023; Zweigenbaum and Pierri, 2020). Furthermore, detection and quantification of AFB1 was performed in a single run, significantly enhancing the efficiency of quality control processes.

*Method validation*

As shown in Figure 1, the linearity of response was determined by calculating the ratio of AFB1 peak areas to its concentration in spiked samples, across a concentration range of 0.01-1 µg/L in acetonitrile. The calibration curve demonstrated good linearity with $R^2$ values of 0.999.

The detection and quantification limits were calculated using following equations: LOD = 3,3 x σ / S and LOQ = 10 x σ / S, where σ is the standard deviation of the response and S is the slope of the calibrating curve (EMA/CHMP/ICH/82072/2006, 2023; Wenzl et al., 2016). Experimental values of 0.00366 and 0.01222 µg/L were obtained for LOD and LOQ, respectively. Since AFB1 in cannabis flower is limited to maximum level of 2 µg/kg (Ph. Eur.) and the samples in this study were diluted 100 times, correlating level of AFB1 would be 0.02 µg/kg. Therefore, the theoretical LOQ level would be 0.01 µg/kg. This was confirmed through estimation of LOD via calibration approach, using 5 calibration levels including zero, and their responses obtained from MassHunter software.

The accuracy of the method was evaluated through recoveries of known amounts of the analyte added to a blank matrix. Blank matrix material of dried cannabis flowers was spiked with AFB1 at 3 different concentration levels of 0.01, 0.02, and 0.1 µg/L and measurements of AFB1 concentration were repeated at least 6 times. During the same day, recovery results were ranging between 103-112% and within three different days, 89-96%. Recovery rates ensured the method provides reliable results by confirming that the analyte can be accurately measured. The obtained results showing adequate precision, accuracy, and reproducibility of the described method are summarized in Table 3.

*LC-MS/MS analysis of aflatoxin B1 in real samples*

The new monograph Cannabis flower (3028) was published in Ph. Eur. Supplement 11.5 (European Pharmacopoeia, 2023), recently. However, the monograph for cannabis flower does not include specific parameters, such as mycotoxin contamination, for quality testing. Instead, general monographs for herbal drugs, extracts, and preparations provide guidelines for quality parameters not covered by individual monographs, requiring their combined application for compliance.

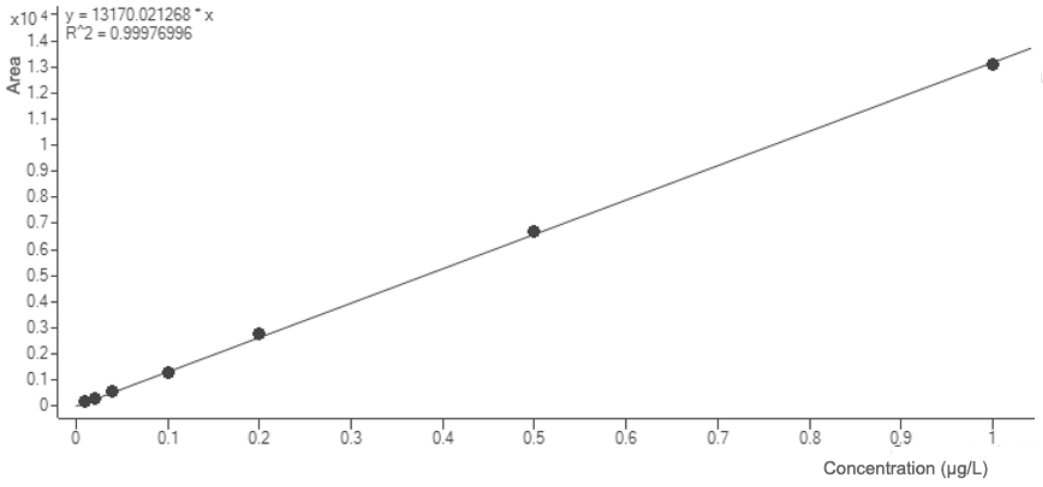


Fig. 1. Calibration curve of aflatoxin B1.

Table 3. Precision and accuracy of the method

| Concentration measurements of AFB1 within same day | | |
|---|---|---|
| Concentration added (µg/L) | Measured concentration (µg/L) | |
| | Mean (µg/L) ± RSD (%) | Recovery (%) |
| 0.01 | 0.01083 + 0.0012 | 103.24 |
| 0.02 | 0.0229 + 0.0014 | 108.30 |
| 0.10 | 0.1141 + 0.0020 | 112.10 |
| Concentration measurements of AFB1 within 3 different days | | |
| Concentration added (µg/L) | Mean (µg/L) ± RSD (%) | Recovery (%) |
| 0.01 | 0.01040 + 0.0013 | 96.15 |
| 0.02 | 0.0221 + 0.0011 | 90.49 |
| 0.10 | 0.1117 + 0.0056 | 89.52 |

Therefore, regarding contaminants (like, for example, mycotoxins, pesticides, and microbiological quality), it is still required to refer to the general Ph. Eur. monograph Herbal Drugs. Aflatoxins, particularly aflatoxin B1, are recognized in the "Guideline on Specifications: Test Procedures and Acceptance Criteria for Herbal Substances" as potential contaminants in herbal drugs that may persist in final products. To address this, Ph. Eur. establishes maximum allowable limits for aflatoxins, ensuring adherence to stringent quality and safety standards. Analysis of all twelve dried cannabis flower samples using LC-MS/MS revealed AFB1 levels below the limit of quantification (LOQ) and well within the specified threshold of 2 µg/kg (Ph. Eur.), demonstrating full compliance with established quality and safety standards (EMA/HMPC/CHMP/CVMP/162241/20051, 2022; European Pharmacopoeia, 2023).

To further validate the findings, four randomly chosen samples from the investigated batch were spiked with AFB1 standard at a concentration of 0.1 µg/L. The absence of the AFB1 was confirmed by qualitative analysis of the non-spiked samples, which revealed no peaks at the retention time corresponding to AFB1. In the spiked samples, AFB1 was unambiguously identified, demonstrating the method's accuracy and sensitivity for its detection under these conditions. Representative chromatograms from the analysis of non-spiked sample and spiked one are shown in Figure 2.

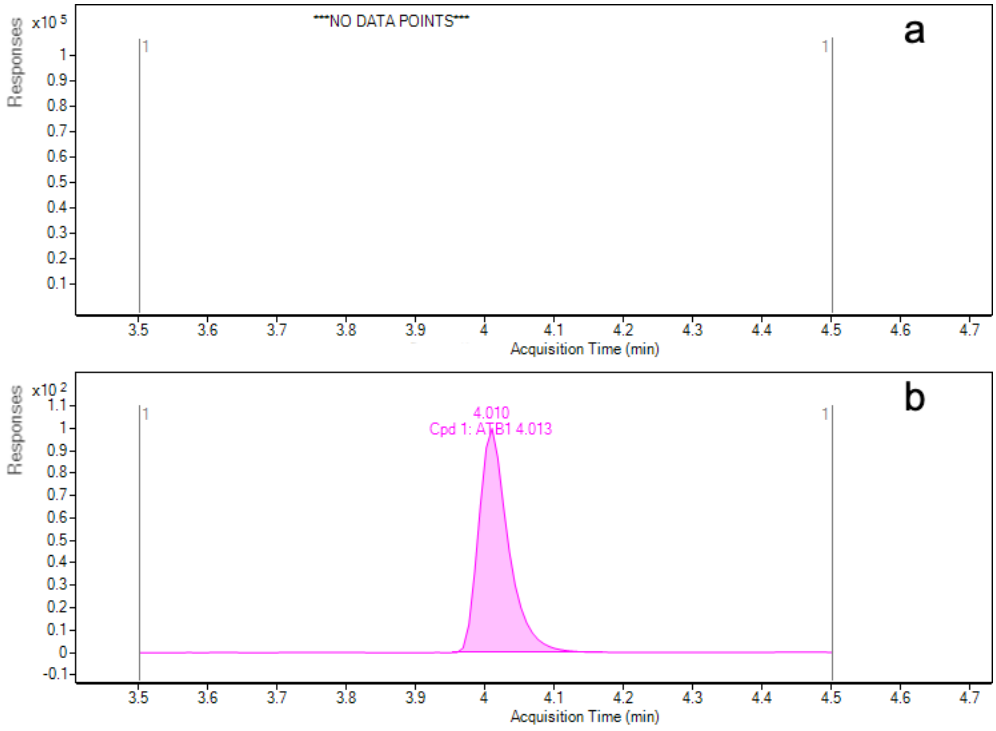


Fig. 2. Chromatograms obtained from (a) real sample and (b) spiked sample with AFB1.

Having reliable analytical tools for determining AFB1, a mutagenic and carcinogenic agent (Pitt et al., 2012), is critical for maintaining regulatory compliance and protecting consumer health because the possibility of contamination of cannabis material increases with industry growth. The suggested LC-MS/MS approach, which can evaluate trace levels of AFB1, may have a major impact on the safety of cannabis products because of the importance of AFB1 as a key indicator within the entire aflatoxin group (B1, B2, G1, and G2). This is not only relevant in the context of cannabis regulation to meet stringent legal standards, but it is crucial to mitigate serious health consequences and to maintain public trust, supporting a safer, and more responsible cannabis industry.

## Conclusion

In cannabis industry marked by rapid growth and regulatory evolution, the use of LC-MS/MS aligns with the need for rigorous analytical methodologies that prioritize consumer safety. A sensitive, specific, accurate and precise LC-MS/MS method for aflatoxin B1 determination in dried cannabis flowers was successfully validated. When it was applied to analyze twelve samples of dried cannabis flowers, it revealed that neither of the samples contained AFB1 above the maximum allowed levels. This method provides a benefit of using low amounts of solvents and has the advantage of practical applicability in laboratories engaged in cannabis testing and regulatory compliance. In addition to the analytical convenience, it is a cost-effective approach that may be optimal for regular cannabis safety monitoring, particularly in resource-limited settings. As a result, this research may significantly contribute to cannabis product safety.

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**Резиме**

# LC-MS/MS метод за определување на афлатоксин B1 во канабис одгледуван во Република Северна Македонија

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**Клучни зборови:** Афлатоксин B1, сув цвет од канабис, LC-MS/MS

Овој труд опишува осетлив метод на течна хроматографија спрегната со масена спектрометрија (LC-MS/MS) за откривање и квантитативно определување на афлатоксин B1 во суви цветови од канабис одгледуван во Република Северна Македонија. Афлатоксинот B1 е високо канцероген микотоксин произведен од видовите *Aspergillus* и претставува значителен здравствен ризик, особено во растителни производи како што е канабисот. Поради оваа причина, внимателно треба да се следи нивото на контаминација на канабисот со афлатоксин B1. Со оглед на тоа што канабисот е богат со канабиноиди и други биоактивни соединенија претставува комплексен примерок и воедно аналитички предизвик. Во овој труд е направено оптимизирање на условите за раздвојување и детекција на афлатоксин B1 користејќи модел на матрикс-усогласена калибрација. Оптимизацијата обезбеди висока сензитивност и специфичност на применетиот метод. Валидацијата на методот покажа одлична линеарност ($R^2$=0,999), прецизност (CV% < 20%) и принос во опсег 103-112%. Оптимизираниот LC-MS/MS метод беше применет на примероци од суви цветови на канабис одгледувани во Република Северна Македонија. Во испитуваните примероци афлатоксин B1 беше определен во концентрации пониски од максималното дозволено ниво (< 2 μg/kg) согласно Европската фармакопеја и затоа овој метод претставува сигурна алатка за контрола на неговото присуство како важен безбедносен критериум во производството на канабис.