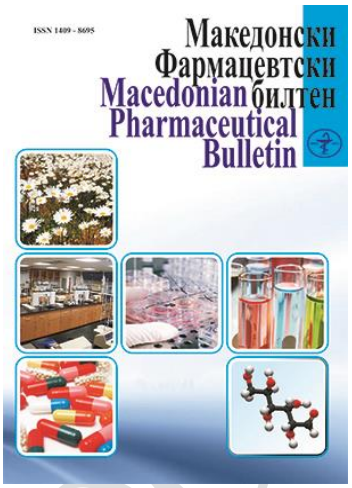
doi: *Original scientific paper*

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo, copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

UNEDITED PROOF

* Corresponding author e-mail: vsgjorgievska@ff.ukim.edu.mk

Accepted Manuscript



Title: Validation of an in-house method for quantification of cannabinoids in *Cannabis extractum spissum*: a comparative study with official methods

Authors: Veronika Stoilkovska Gjorgievska*, Ivana Cvetkovikj Karanfilova, Iskra Davkova, Ana Trajkovska Matevska, Gjoshe Stefkov, Marija Karapandzova

Ss. Cyril and Methodius University, Faculty of Pharmacy, Ss. Cyril and Methodius University, Mother Theresa 47, 1000 Skopje, Republic of North Macedonia

DOI:
Received date: February 2026
Accepted date: April 2026
UDC:
Type of paper: Original scientific paper
Mac. Pharm. Bull. 72 Vol. (2) 2026

Please cite this article as:

UNEDITED PROOF

* Corresponding author e-mail: vsgjorgievska@ff.ukim.edu.mk

doi: *Original scientific paper*

Validation of an in-house method for quantification of cannabinoids in *Cannabis extractum spissum*: a comparative study with official methods

Veronika Stoilkovska Gjorgievska*, Ivana Cvetkovikj Karanfilova, Iskra Davkova, Ana Trajkovska Matevska, Gjoshe Stefkov, Marija Karapandzova

Ss. Cyril and Methodius University, Faculty of Pharmacy, Ss. Cyril and Methodius University, Mother Theresa 47, 1000 Skopje, Republic of North Macedonia

Abstract

Cannabis is a chemically complex herbal matrix rich in phytocannabinoids demanding sensitive and robust analytical methods for reliable quality control of its' medicinal products. While the European Pharmacopoeia (Ph. Eur.) provides a harmonized HPLC procedure for cannabinoid determination in *Cannabis flos*, no official pharmacopoeial method is currently available for *Cannabis extractum spissum*.

In this study, effective and practical in-house HPLC-DAD method was developed and validated for cannabinoid potency determination in *Cannabis extractum spissum*, operating under identical chromatographic setup and conditions as the established pharmacopoeial method for *Cannabis flos*. Validation was performed in accordance with ICH Q2(R2) and ISO/IEC 17025 requirements and included evaluation of system suitability, specificity, linearity, accuracy, precision, and robustness. The method demonstrated excellent linearity for CBD, CBDA, CBN, Δ^9-THC, and Δ^9-THCA over the concentration range of 0.0001–0.2500 mg/mL ($R^2 \geq 0.9999$), together with low limits of detection (0.0001–0.0002 mg/mL) and quantification (0.0002–0.0005 mg/mL). Accuracy studies based on spiking experiments showed consistent recoveries with low associated variability, confirming trueness of measurement and efficient analyte recovery in the extract matrix.

Comparative assessment of several validated procedures, including UNODC-recommended and pharmacopoeial methods, demonstrated statistical comparability of analytical results for

* Corresponding author e-mail: vsgjorgievska@ff.ukim.edu.mk

Cannabis extractum spissum. Differences observed for *Cannabis flos* were attributed to variations in extraction design and capacity rather than chromatographic performance.

Keywords: *Cannabis sativa* L., hemp flowers, crude extract, HPLC method validation, cannabionoids potency.

Introduction

Cannabis (*Cannabis sativa* L.) has re-emerged as an important medicinal plant within the pharmaceutical sector, driven by expanding clinical interest in phytocannabinoids and the increasing regulatory demand for standardized, quality-controlled herbal preparations (Hossain and Chae, 2024). Over the last decade, the therapeutic potential of major cannabinoids, particularly Δ9-tetrahydrocannabinol (Δ9-THC) and cannabidiol (CBD) and their acidic forms, as well as minor cannabinoids such as cannabichromene (CBC), cannabigerol (CBG), cannabigerolic acid (CBGA), cannabinol (CBN) and cannabinolic acid (CBNA) has been demonstrated in various clinical and preclinical models, including chronic pain, epilepsy, neurodegenerative disorders (Huntington's and Parkinson's disease), seizure models, and anxiety-related conditions (Citti et al., 2018; Stone et al., 2020). As a consequence, the global market for medicinal cannabis products has expanded rapidly, necessitating strict regulatory control and robust analytical methodologies to ensure product quality, safety, and batch-to-batch consistency (Karanfilova et al., 2022; Stefkov et al., 2022).

To address these regulatory requirements, the European Pharmacopoeia (Ph. Eur.) has established harmonized quality standards for *Cannabis flos*, including a dedicated monograph (07/2024:3028) defining macroscopic and microscopic characteristics, purity tests, and validated analytical procedures for the quantification of cannabinoids (European Pharmacopoeia Commission, 2024). The official Ph. Eur. method enables the identification of nine cannabinoids and quantification of total CBD, total CBN and total Δ9-THC, by high-performance liquid chromatography (HPLC) (European Pharmacopoeia Commission, 2024). Although Pharmacopoeial methods are considered fully validated by the European Directorate for the Quality of Medicines & HealthCare (EDQM), individual quality control laboratories are required to perform method verification before routine implementation. Verification ensures that the the

UNEDITED PROOF

* Corresponding author e-mail: vsgjorgievska@ff.ukim.edu.mk

analytical procedure remains fit for its intended purpose when transferred to a new laboratory environment.

In contrast to *Cannabis flos* (CFl), *Cannabis extractum spissum* (CEx) is currently not covered by a specific Ph. Eur. monograph; however, it represents a widely used pharmaceutical dosage form due to its convenient administration, flexible dosing, and good patient compliance (Hazekamp, 2018). CEx is produced by extracting resin and cannabinoids directly from cannabis biomass using solvent-based or supercritical CO_2 extraction techniques, resulting in a concentrated lipophilic extract in which cannabinoid levels are significantly enriched compared to those in the raw plant material (Hazekamp, 2018; Suárez-Jacobo et al., 2023).

The absence of a dedicated pharmacopoeial procedure for CE*x* highlights the need for a validated analytical method capable of addressing the specific challenges of this matrix. This creates a clear rationale for the development and validation of a practical in-house method for reliable cannabinoid determination in CEx.

In this context, the aim of the present study was to develop and validate practical in-house method for the determination of cannabinoids in CEx, designed to operate under the same HPLC setup and chromatographic conditions as the pharmacopoeial method described for *Cannabis flos*. The validation was performed in accordance with regulatory requirements to confirm the suitability, reliability, and robustness of the method for routine quality control. In addition, comparative assessment to different well-known official analytical methods was undertaken to contextualize the performance of the validated in-house assay. Through this validation strategy, the study seeks to establish a harmonized and reliable method for quantitative determination of cannabinoids across solid and liquid cannabis matrices suitable for routine quality control.

Materials and methods

Samples, standards, solvents and reagents

THC-rich and CBD-rich *Cannabis extracta spissa*, as well as dried *Cannabis flos* samples from a THC-dominant strain (Banjo) and a CBD-dominant strain (Charlotte's Angel), were kindly provided by a licensed local cannabis producer for the purposes of this study. Cannabidiol chemical reference substance (CRS) and *Cannabis flos* herbal reference standard (HRS) used for system suitability testing were obtained from the European Directorate for the Quality of Medicines & HealthCare (EDQM). Solutions for determination of detection and quantification

UNEDITED PROOF

* Corresponding author e-mail: vsgjorgievska@ff.ukim.edu.mk

limit were prepared by using the following certified reference materials (CRMs): cannabidiol solution (1 mg/mL in methanol) (purity 99.90%), cannabinol solution (1 mg/mL in methanol) (purity 99.60%), (-)-△9-tetrahydrocannabinol solution (1 mg/mL in methanol) (purity 99.00%), △9-tetrahydrocannabinolic acid A solution (1 mg/mL in acetonitrile) (purity 98.17%) and cannabidiolic acid solution (1 mg/mL in acetonitrile) (purity 97.50%) were purchased from CHIRON (USA). Linearity was evaluated using CRM standard mixture solution containing CBDA, CBD, CBN, Δ9-THC, and Δ9-THCA, obtained from Cayman Chemical (USA). Trifluoroacetic acid (≥99%), reagent grade, methanol HPLC grade and acetonitrile HPLC grade were purchased from ThermoFisher Scientific (UK). Ethanol 96%, Ph. Eur. grade was purchased from Alkaloid AD Skopje (North Macedonia).

Sample preparation for the in-house method for Cannabis extractum spissum

The stock test solution was prepared by accurately weighing 0.20 g of CE*x* and transferring it into a 50 mL wide-neck erlenmeyer flask fitted with a suitable glass stopper. Subsequently, 20 mL of ethanol (96%) R was added and the mixture was sonicated for 15 min. The stopper was rinsed with 20 mL of ethanol (96%) R, and the solution was quantitatively transferred into a 100.0 mL volumetric flask. The Erlenmeyer flask was further rinsed twice with 20 mL portions of ethanol (96%) R, and the washings were combined in the same volumetric flask. The solution was made up to volume with ethanol (96%) R, resulting in a final concentration of 2.0 mg/mL, and filtered through a 0.22 µm RC membrane filter.

Test solution (a) was prepared by diluting 5.0 mL of the stock test solution (2.0 mg/mL) to 10.0 mL with ethanol (96%) R in a volumetric flask, yielding a concentration of 1.0 mg/mL.

Test solution (b) was obtained by further diluting 1.0 mL of the stock test solution (2.0 mg/mL) to 10.0 mL with methanol R in a volumetric flask, resulting in a final concentration of 0.20 mg/mL.

System suitability solutions

System suitability was performed using CBD reference solutions at concentrations of 0.20 mg/mL (Reference solution (a)), 0.05 mg/mL (Reference solution (b)), and 0.08 mg/mL (Reference solution (c)), as well as CFl for system suitability testing HRS solutions corresponding

* Corresponding author e-mail: vsgjorgievska@ff.ukim.edu.mk

to 5 mg/mL (Reference solution (d)) and 0.5 mg/mL (Reference solution (e)) of plant material (European Pharmacopoeia Commission, 2024).

Analytical setup of the in-house method for Cannabis extractum spissum

Cannabinoid potency determination was performed using an in-house HPLC–DAD analytical setup based on an isocratic chromatographic system. Chromatographic analyses were carried out using an Agilent 1260 Infinity II HPLC system (Agilent Technologies, USA). The mobile phase consisted of 0.1% trifluoroacetic acid (TFA) in water and acetonitrile (ACN) in a 41:59 (*v/v*) ratio, delivered at a flow rate of 2.0 mL/min. The injection volume was 5 µL, and the column compartment temperature was maintained at 35°C throughout the analysis.

Separation was achieved using a CORTECS Shield RP18 chromatographic column (150 mm × 4.6 mm i.d., 2.7 µm; Waters, USA) equipped with a VanGuard™ pre-column cartridge (3.9 mm × 5 mm i.d., 2.7 µm; Waters, USA). Detection was performed using a diode array detector (DAD) at 228 nm.

Method validation of in-house method for determination of cannabinoids in Cannabis extractum spissum

The in-house method for the determination of cannabinoids (total CBN, total CBD and total Δ9-THC) in CEx was fully validated according ICH guideline Q2(R2) and the OMCL guideline for validation/verification of analytical procedures, PA/PH/OMCL (13) 82 R5 (ICH, 2005; OMCL Network/EDQM, 2020).

Specificity

Specificity was evaluated by injecting methanol and ethanol blanks at the beginning and end of each chromatographic sequence to confirm the absence of interfering peaks at the retention times of the target cannabinoids in both native, spiked samples and system suitability solutions.

System suitability

System suitability criteria were defined to ensure adequate chromatographic performance of the analytical system and were consistently applied within the in-house method. System suitability testing using reference solution (d) requires a minimum resolution of 2.0 between the peaks of cannabigerolic acid (CBGA) and Δ9-tetrahydrocannabinol (Δ9-THC), a peak-to-valley ratio of at least 1.5 between cannabigerol (CBG) and cannabidiolic acid (CBDA), and a peak-to-valley ratio

* Corresponding author e-mail: vsgjorgievska@ff.ukim.edu.mk

UNEDITED PROOF

of at least 5.0 between cannabinolic acid (CBNA) and cannabichromene (CBC). System suitability using reference solution (e) requires a minimum resolution of 2.0 between cannabidiol (CBD) and cannabidiolic acid (CBDA). Peak identification is performed using chromatograms of reference solution (b) for CBD and chromatograms obtained from the CFl system suitability HRS and reference solution (d) to identify Δ9-THC, Δ9-THCA, CBDA, CBN, CBNA, CBC, CBG, and CBGA. These requirements apply equally to both CEx and CFl, ensuring consistent chromatographic performance.

Range, linear response (linearity) and lower range limits (detection limit - DL and quantification limit - QL)

The working range of the method was established based on the validated concentration ranges for dominant cannabinoids defined in the European Pharmacopoeia for CFl (0.2–32% per cannabinoid), while extending applicability to higher cannabinoid levels typical for extract matrices, reaching up to approximately 80% per cannabinoid.

Linearity of the method was evaluated using a series of calibration standards. For CBD CRS, nine concentration levels in the range of 0.0001- 0.20 mg/mL were analyzed, while for the standard mixture containing CBD, CBDA, CBN, Δ9-THC and Δ9-THCA, ten concentration levels in the range of 0.0001-0.25 mg/mL were prepared and injected in triplicate. Calibration curves were constructed by plotting peak area versus concentration, and linear regression analysis was applied (International Conference on Harmonisation (ICH), 2024). The detection (DL) and quantification (QL) limit were determined experimentally using the signal-to-noise (S/N) approach. The DL was defined as the lowest concentration providing an S/N ratio of approximately 3:1, while the QL corresponded to the lowest concentration yielding an S/N ratio of approximately 10:1, as calculated by the chromatographic system software.

Accuracy

Accuracy was evaluated using four complementary approaches. Standard addition (spiking) was performed by adding CBD reference standard solution to the sample matrix THC rich extract, at three concentration levels (80, 100 and 120%) relative to the nominal CBD content in the CBD rich strain, and recoveries together with an appropriate confidence interval (CI) (%) were calculated. Independent sample preparation with standard mix spiking was assessed by preparing three test solutions from the same sample and spiking each with a mixed cannabinoid standard solution (CBD, CBDA, CBN, Δ9-THC and Δ9-THCA) at three concentration levels (0.005, 0.010

* Corresponding author e-mail: vsgjorgievska@ff.ukim.edu.mk

and 0.050 mg/mL). Evaluation was performed using the method of standard additions to calculate the true concentrations of spiked solutions and recovery was calculated afterwards. Recovery experiments using CBD CRS were conducted to assess the efficiency of the extraction procedure and to confirm the trueness of the analytical method. Orthogonal procedure comparison was conducted by comparing the results obtained using the in-house method and the United Nations office of Drugs and Crime (UNODC) recommended method for the Identification and Analysis of Cannabis and Cannabis Products.

Precision

Method repeatability was assessed using six independently prepared test solutions (a) and test solutions (b), and expressed as RSD (%) of the quantified cannabinoids (CBDA, CBD, CBN, CBNA, Δ9-THC and Δ9-THCA).

Robustness

Robustness of the method was evaluated by introducing intentional small variations in analytical conditions, as described in the validation protocol. The flow rate was varied from the nominal value to 1.8 mL/min and 2.2 mL/min, the column temperature was adjusted to 33 and 37°C, and the mobile phase composition was modified to 40:60 and 42:58 (0.1% aqueous trifluoroacetic acid : acetonitrile, *v/v*).

Analytical performance assessment of the Europaean pharmacopoeial method for Cannabis flos

The analytical performance of the pharmacopoeial method for cannabinoid determination in CFl was assessed under local laboratory conditions in accordance with the principles of the International Council for Harmonisation Q2(R2) guideline and the OMCL Network/EDQM recommendations for validation and verification of analytical procedures. Method performance was confirmed through evaluation of system suitability criteria, specificity, precision, and accuracy. Accuracy was evaluated by recovery experiments using cannabidiol chemical reference substance (CBD CRS), whereby known amounts (10 mg) of CBD were added to the sample matrix prior to extraction and analysis under the established chromatographic conditions. Recovery was calculated by comparing the measured concentrations with the theoretical spiked amounts. In addition, a comparative assessment was performed using analytical data obtained from THC- and CBD-rich cannabis strains analyzed by the pharmacopoeial procedure and an orthogonal method

* Corresponding author e-mail: vsgjorgievska@ff.ukim.edu.mk

UNCORRECTED PROOF

based on the German Pharmacopoeia (DAB), enabling evaluation of relative analytical performance.

Statistical analysis

Linearity was evaluated by linear regression analysis and expressed as the coefficient of determination (R^2). Precision was assessed using relative standard deviation (RSD, %), while accuracy was evaluated by recovery (%) calculations. Detection limit (DL) and quantification limit (QL) were determined based on signal-to-noise (S/N) ratios obtained from the chromatographic system.

Comparison of two means per sample was performed using the Student's t-test to evaluate statistically significant differences between independently prepared test solutions, with a confidence level of 95% ($p < 0.05$).

Results and discussion

An in-house method was fully validated for cannabinoid determination (total CBN, total CBD and total Δ9-THC) in *Cannabis extractum spissum*. The method was designed to operate under an identical HPLC setup and chromatographic conditions to the Ph. Eur. procedure for *Cannabis flos*, thereby ensuring simplicity, practicality, and ease of implementation in routine laboratory workflows. Analytical validation was performed in accordance with ICH Q2(R2) requirements and included assessment of specificity, linearity, accuracy (evaluated by spiking studies and orthogonal method comparison), precision, robustness, detection and quantification limits. The validation results confirmed the suitability of the in-house assay for routine quality control of CEx and supported its comparative evaluation against established official methods.

Specificity/Selectivity

Specificity of the method was confirmed by analyzing blank solvents (methanol and ethanol), system suitability solutions, standard mixtures, and THC and CBD predominant sample solutions of *Cannabis extractum spissum*. No interfering peaks were observed at the retention times of the target cannabinoids, demonstrating adequate selectivity (Figs. 1 and 2).

UNEDITED PROOF

* Corresponding author e-mail: vsgjorgievska@ff.ukim.edu.mk

A 35-minute methanol blank run should be applied immediately after injections of CFl system suitability solutions Ref. sol. (d) and (e) to ensure complete elution of late-retained components and to prevent carry-over in subsequent analyses.

Fig. 1

Fig. 2

System suitability

System suitability testing confirms consistent chromatographic performance of the analytical system for both CEx under the applied conditions. System precision, evaluated through repeated injections of CBD CRS reference solutions, demonstrates stable retention times and peak areas with low variability, indicating reliable injection repeatability and detector response (Table 1). The obtained precision parameters confirm that the system operates within acceptable limits prior to sample analysis. Peak identification was verified by calculation of relative retention times (RRt) for individual cannabinoids using the system suitability herbal reference standard (d) and CBD reference solution (b). The experimentally obtained RRt values showed good agreement with literature values, confirming correct peak assignment for all monitored cannabinoids throughout the chromatographic run (Table 2). Chromatographic separation was further evaluated through resolution and peak-to-valley ratios for critical peak pairs. All assessed separation parameters met the predefined acceptance criteria, confirming adequate selectivity and baseline discrimination between closely eluting cannabinoids (Table 3).

Table 1

Table 2

Table 3

Linear response (linearity), detection limit (DL) and quantification limit (QL)

Strong linearity was obtained for CBD, CBDA, CBN, Δ9-THC, and Δ9-THCA over the investigated concentration range of 0.0001–0.2500 mg/mL, as shown in Table 5, with coefficients of determination $(R^2) \geq 0.9999$ for all analytes, indicating a highly proportional detector response within the working range. Sensitivity of the in-house method was experimentally evaluated at low

* Corresponding author e-mail: vsgjorgievska@ff.ukim.edu.mk

concentration levels (0.0001–0.0050 mg/mL) using signal-to-noise ratios derived directly from the chromatographic system (Table 4). Detection limits were achieved at concentrations of 0.0001–0.0002 mg/mL, corresponding to S/N ratios ≥ 3, while quantification limits ranged from 0.0002 to 0.0005 mg/mL with S/N ratios ≥ 10 (Table 4). The obtained DL and QL values confirm adequate method sensitivity for reliable detection and quantification of cannabinoids across the validated concentration range.

Table 4

Table 5

Accuracy

For *Cannabis extractum spissum*, recoveries of CBD, CBDA, CBN, Δ9-THC, and Δ9-THCA obtained at three concentration levels (0.005-0.05 mg/mL) were within acceptable limits (95.0-105.0%), with low RSD values, confirming adequate trueness and precision across the working range. Additional spiking at 80, 100, and 120% of the assay content using a CBD standard solution further confirmed consistency of recovery, while spiking with CBD CRS standard substance demonstrated satisfactory extraction efficiency of the applied sample preparation procedure. Accuracy data from spiking studies are summarized in Table 6 and orthogonal method comparison data are presented in Table 7.

Table 6

Table 7

Precision

Method precision (repeatability) was confirmed by the results for the RSD (≤10%) for CEx samples (Table 8).

Robustness

Robustness of the method was evaluated by deliberate variations of key chromatographic parameters, including flow rate (1.8 and 2.2 mL/min), column temperature (33 and 37°C), and mobile phase composition (40:60 and 42:58, *v/v*, 0.1% TFA:ACN), relative to nominal conditions.

UNEDITED PROOF

* Corresponding author e-mail: vsgjorgievska@ff.ukim.edu.mk

Under all tested conditions, system suitability criteria were consistently fulfilled, with resolution between CBGA and Δ9-THC remaining ≥ 2.0, peak-to-valley ratios for CBG/CBDA ≥ 1.5 and for CBNA/CBC ≥ 5.0 (ref. sol. (d), and resolution between CBD and CBDA ≥ 2.0 (ref. sol. (e). These results demonstrate that small, deliberate variations in chromatographic conditions do not significantly affect critical separation parameters, confirming the robustness of the method for routine application.

Analytical performance of Ph. Eur. Cannabis flos assay

The method for determination of total CBN and assay of cannabinoids in *Cannabis flos*, as described in the European Pharmacopoeia monograph 07/2024:3028, was verified by system suitability testing, which confirmed consistent chromatographic performance of the analytical system under the applied conditions and fulfillment of the predefined acceptance criteria. Method specificity was confirmed by the absence of interfering peaks at the retention times of the monitored cannabinoids, ensuring unambiguous peak identification in the CFl matrix. Method accuracy was evaluated by recovery experiments using cannabidiol chemical reference substance (CBD CRS). Known amounts (10 mg) of CBD were added to the sample matrix prior to extraction and analysis under the established chromatographic conditions. Recovery values obtained from the spiking experiments demonstrated consistent agreement between measured and theoretical concentrations, confirming the suitability of the method for quantitative determination of cannabinoids in CFl. Accuracy data are summarized in Table 6 and orthogonal method comparison is summarized in Table 7. Quantitative results for total cannabinoids (total CBN, total CBD, and total Δ9-THC) obtained for different cannabis chemotypes are presented in Table 8. Within the European Pharmacopoeia sample preparation procedure for CFl, variations of the extraction step using 15 min mechanical mixing and 15 min ultrasonic bath were evaluated and showed no statistically significant differences in quantified cannabinoid contents, with paired t-test values of $p = 0.42$ and $p = 0.68$ for CBD and $p = 0.51$ and $p = 0.84$ for Δ^9-THC in CBD- and THC-predominant strains, respectively (Table 8).

Table 8

Table 9

* Corresponding author e-mail: vsgjorgievska@ff.ukim.edu.mk

In the absence of a dedicated pharmacopoeial procedure for CEx, the present work addresses this analytical gap through the full validation of a practical in-house method designed for this matrix. The analytical performance of this approach was subsequently confirmed through comprehensive evaluation of key performance characteristics, supporting its suitability for routine quality control of CEx preparations and its relevance for regulatory and safety-related applications. The development of the sample preparation procedure demonstrates that variation in sample mass between 0.05 and 0.20 g does not significantly influence quantitative results for major cannabinoids in CEx. Selection of a higher sample mass combined with controlled dilution was therefore driven by practical considerations, including improved robustness and avoidance of carry-over effects. Exclusion of very low concentration test solutions was justified by insufficient detectability and unreliable quantification, confirming that optimized concentration levels are essential for accurate and reproducible cannabinoid analysis in highly concentrated extract matrices.

Data from the comparative assessment to different well-known official analytical methods was undertaken to contextualize the performance of the validated in-house assay., including the in-house method and UNODC method, are summarized in Table 7. Statistical evaluation using paired two-sample t-tests showed no significant differences between methods for CEx samples ($p > 0.05$), confirming agreement across analytical approaches (in-house and UNODC methods).

In contrast, statistically significant differences were observed for CFl samples ($p < 0.05$) analized with European pharmacopoeial method (Ph. Eur.) and German Pharmacopoeial (DAB) method, revealing higher values of cannabinoids' contents obtained using the European Pharmacopoeia method. These differences are attributable to variations in sample preparation and extraction capacity between the applied procedures. The Ph. Eur. method employs a higher effective solvent-to-matrix ratio, involving triple extraction of 500 mg plant material with a total volume of 90 mL of 96% ethanol (40 mL extraction followed by two consecutive extractions with 25 mL), and a final dilution to 100 mL. In comparison, the DAB method applies extraction of the same amount of plant material using a total of 45 mL of 96% ethanol (20 mL extraction followed by two consecutive extractions with 12.5 mL), with a final dilution to 50 mL. The higher extraction volume and increased extraction capacity of the Ph. Eur. procedure likely enable more exhaustive recovery of cannabinoids from the plant matrix.

* Corresponding author e-mail: vsgjorgievska@ff.ukim.edu.mk

The observed higher cannabinoid contents using the Ph. Eur. extraction protocol are consistent with literature reports demonstrating that increased sample-to-solvent ratios and greater solvent exposure enhance extraction efficiency, thereby improving recovery of cannabinoids from solid plant matrices (Addo et al., 2022; Tzimas et al., 2024). These findings highlight the critical influence of extraction conditions on quantitative cannabinoid determination and underscore the importance of standardized sample preparation procedures when comparing analytical results across different methods.

Conclusion

This study describes the development and validation of a practical in-house HPLC–DAD method for cannabinoid potency determination in *Cannabis extractum spissum*, designed to operate under identical chromatographic setup and conditions as established by Ph. Eur. method for total CBN and assay of cannabinoids in *Cannabis flos*. By retaining the same instrumental configuration, mobile phase composition, and chromatographic parameters, the proposed approach offers a simple and operationally efficient solution for routine quality control laboratories without the need for method-specific instrumental reconfiguration. The in-house assay complied with all validation requirements, exhibiting adequate linearity, sensitivity, accuracy, precision, and robustness, ensuring reliable chromatographic performance. Comparative assessment of analytical results for *Cannabis extractum spissum*, performed using the validated in-house method and an independent UNODC-recommended procedure, demonstrated statistical comparability ($p > 0.05$). In contrast, comparative evaluation of *Cannabis flos* samples using pharmacopoeial methods (Ph. Eur. and DAB) revealed statistically significant differences ($p < 0.05$), which were attributable to differences in extraction design and capacity rather than chromatographic or detection performance, underscoring the dominant influence of sample preparation on quantitative outcomes.

Overall, the presented results establish a sensitive, accurate, and robust analytical framework suitable for routine regulatory quality control of *Cannabis extractum spissum* and *Cannabis flos*, supporting harmonized cannabinoid quantification across pharmaceutical cannabis matrices and offering strong applicability for method development, validation, and comparative studies.

* Corresponding author e-mail: vsgjorgievska@ff.ukim.edu.mk

Acknowledgement

The authors sincerely acknowledge the Center for Natural Products, Faculty of Pharmacy, Ss. Cyril and Methodius University, Skopje, for allowing the publication of the obtained data. Additionally, we extend our gratitude to the cannabis companies for providing the samples used in this study.

References

Addo, P.W., Sagili, S.U.K.R., Bilodeau, S.E., Gladu-Gallant, F.-A., MacKenzie, D.A., Bates, J., McRae, G., MacPherson, S., Paris, M., Raghavan, V., Orsat, V., Lefsrud, M., 2022. Cold Ethanol Extraction of Cannabinoids and Terpenes from Cannabis Using Response Surface Methodology: Optimization and Comparative Study. Molecules 27. https://doi.org/10.3390/molecules27248780

Citti, C., Braghiroli, D., Vandelli, M.A., Cannazza, G., 2018. Pharmaceutical and biomedical analysis of cannabinoids: A critical review. J. Pharm. Biomed. Anal. 147, 565–579. https://doi.org/10.1016/j.jpba.2017.06.003

European Pharmacopoeia Commission, 2024. Cannabis flos (Monograph 07/2024:3028). In: European Pharmacopoeia, 11.5 ed. Strasbourg: EDQM, Council of Europe.

Hazekamp, A., 2018. The Trouble with CBD Oil. Med. Cannabis Cannabinoids 1, 65–72. https://doi.org/10.1159/000489287

Hossain, M.K., Chae, H.J., 2024. Medical cannabis: From research breakthroughs to shifting public perceptions and ensuring safe use. Integrative Medicine Research 13, 101094. https://doi.org/10.1016/j.imr.2024.101094

International Conference on Harmonisation (ICH), 2024. Validation of Analytical Procedures: Text and Methodology (ICH Q2(R2)). Geneva, Switzerland.

International Organization for Standardization, 2018. EN ISO / IEC 17025:2018 General requirements for the competence of testing and calibration laboratories. (order number 3681. from 03.07.2019).

Karanfilova, I.C., Gjorgievska, V.S., Stefkov, G., Karapandzova, M., Trajkovska, A., Davkova, I., Acevska, J., Kulevanova, S., Brezovska, K., 2022. DAB method transfer for potency of cannabinoids in dry cannabis flower. Maced. Pharm. Bull. 68(2). https://doi.org/10.33320/maced.pharm.bull.2022.68.02.003

UNEDITED PROOF

* Corresponding author e-mail: vsgjorgievska@ff.ukim.edu.mk

Stefkov, G., Cvetkovikj Karanfilova, I., Stoilkovska Gjorgievska, V., Trajkovska, A., Geskovski, N., Karapandzova, M., Kulevanova, S., 2022. Analytical Techniques for Phytocannabinoid Profiling of Cannabis and Cannabis-Based Products-A Comprehensive Review. Molecules 27, 975. https://doi.org/10.3390/molecules27030975

Stone, N.L., Murphy, A.J., England, T.J., O'Sullivan, S.E., 2020. A systematic review of minor phytocannabinoids with promising neuroprotective potential. Br. J. Pharmacol. 177, 4330–4352. https://doi.org/10.1111/bph.15185

Suárez-Jacobo, Á., Díaz Pacheco, A., Bonales-Alatorre, E., Castillo-Herrera, G.A., García-Fajardo, J.A., 2023. Cannabis Extraction Technologies: Impact of Research and Value Addition in Latin America. Molecules 28, 2895. https://doi.org/10.3390/molecules28072895

Tzimas, P.S., Petrakis, E.A., Halabalaki, M., Skaltsounis, L.A., 2024. Extraction solvent selection for Cannabis sativa L. by efficient exploration of cannabinoid selectivity and phytochemical diversity. Phytochem. Anal. 35, 163–183. https://doi.org/10.1002/pca.3282

UNEDITED PROOF

* Corresponding author e-mail: vsgjorgievska@ff.ukim.edu.mk

doi: *Original scientific paper*

Резиме

Валидација на интерен метод за квантификација на канабиноиди во *Cannabis extractum spissum:* компаративна студија со официјални методи

Вероника Стоилковска Ѓоргиевска*, Ивана Цветковиќ Каранфилова, Искра Давкова, Ана Трајковска Матевска, Ѓоше Стефков, Марија Карапанџова

„Универзитет „Св. Кирил и Методиј“, Фармацевтски факултет, Мајка Тереза 47, 1000 Скопје, Република Северна Македонија

Клучни зборови: *Cannabis sativa* L., цветови од коноп, суров екстракт, валидација на HPLC метод, потенција на канабиноиди.

Канабисот претставува хемиски комплексен растителен матрикс богат со фитоканабиноиди, што наметнува потреба од робусни, чувствителни и практично применливи аналитички методи за сигурна контрола на квалитетот на медицинските производи. Иако Европската фармакопеја (Ph. Eur.) обезбедува хармонизирана HPLC метода за определување на канабиноиди во *Cannabis flos*, во моментов не постои официјална фармакопејска метода за *Cannabis extractum spissum*.

Во оваа студија беше развиена и валидирана нов, ефикасен и практичен интерен HPLC–DAD метод за определување на потенцијата на канабиноиди во *Cannabis extractum spissum*, кој функционира со идентнична хроматографска поставеност и услови како и воспоставената фармакопејска метода за *Cannabis flo*s. Аналитичката валидација беше спроведена во согласност со барањата на ICH Q2(R2) и ISO/IEC 17025 и опфати евалуација на соодветност на системот, специфичност, линеарност, точност, прецизност и робусност. Методата покажа одлична линеарност за CBD, CBDA, CBN, Δ⁹-THC и Δ⁹-THCA во концентрациониот опсег од 0.0001–0.250 mg/mL ($R^2 \geq 0.9999$), заедно со ниски граници на детекција (0.0001–0.0002 mg/mL) и квантификација (0.0002–0.0005 mg/mL). Испитувањата на точноста базирани на спајкување покажаа конзистентни проценти на аналитички принос

UNEDITED PROOF

* Corresponding author e-mail: vsgjorgievska@ff.ukim.edu.mk

со ниска придружна варијабилност, што ја потврдува вистинитоста на мерењето и ефикасното извлекување на аналитите од испитуваниот матрикс.

Компаративната проценка спроведена со користење на независни валидирани процедури, вклучувајќи UNODC-препорачани и фармакопејски методи, покажа статистичка споредливост на аналитичките резултати за *Cannabis extractum spissum*. Разликите забележани кај *Cannabis flos* беа припишани на варијации во дизајнот и капацитетот на екстракцијата, а не на хроматографските перформанси.

UNEDITED PROOF

* Corresponding author e-mail: vsgjorgievska@ff.ukim.edu.mk

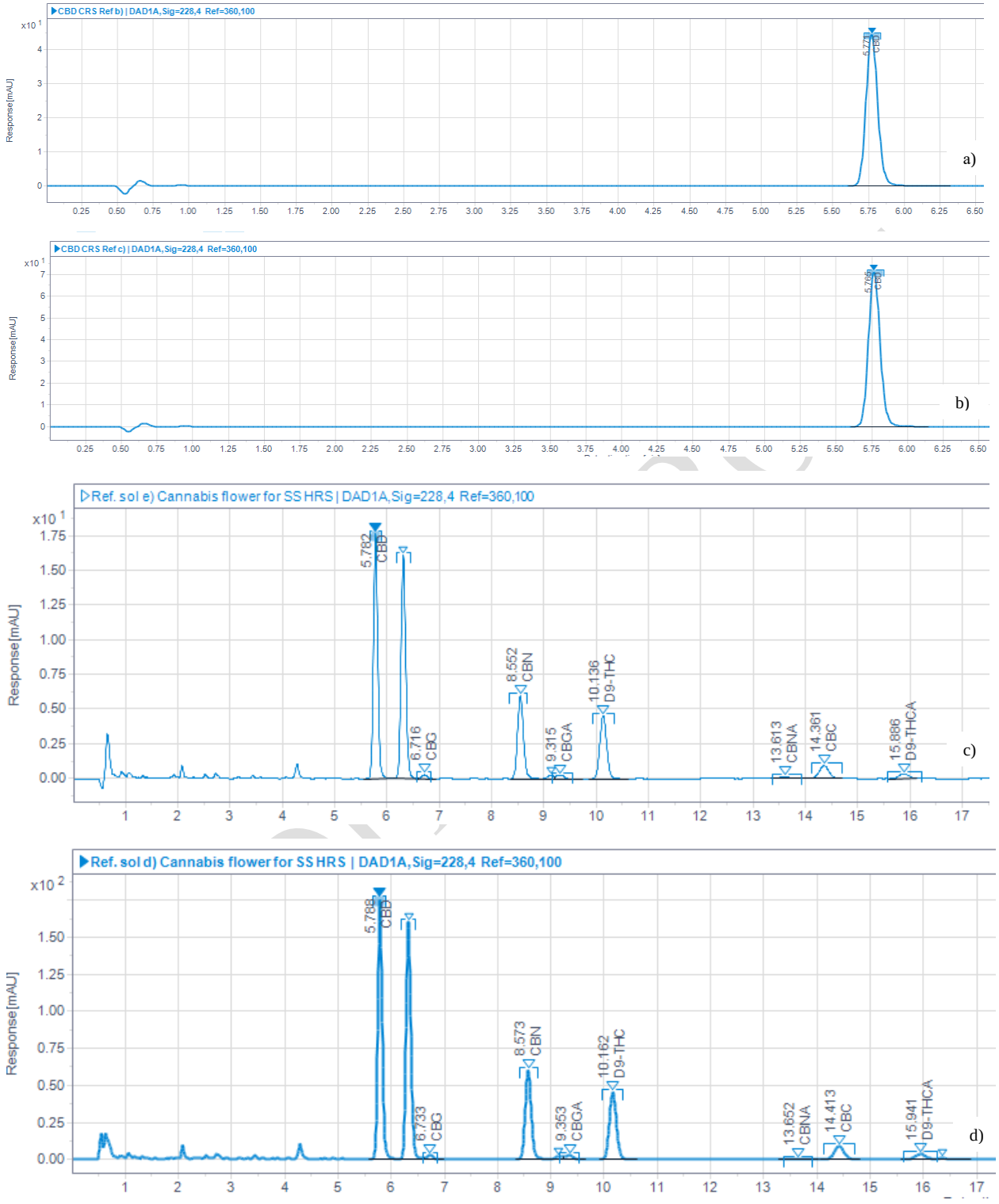
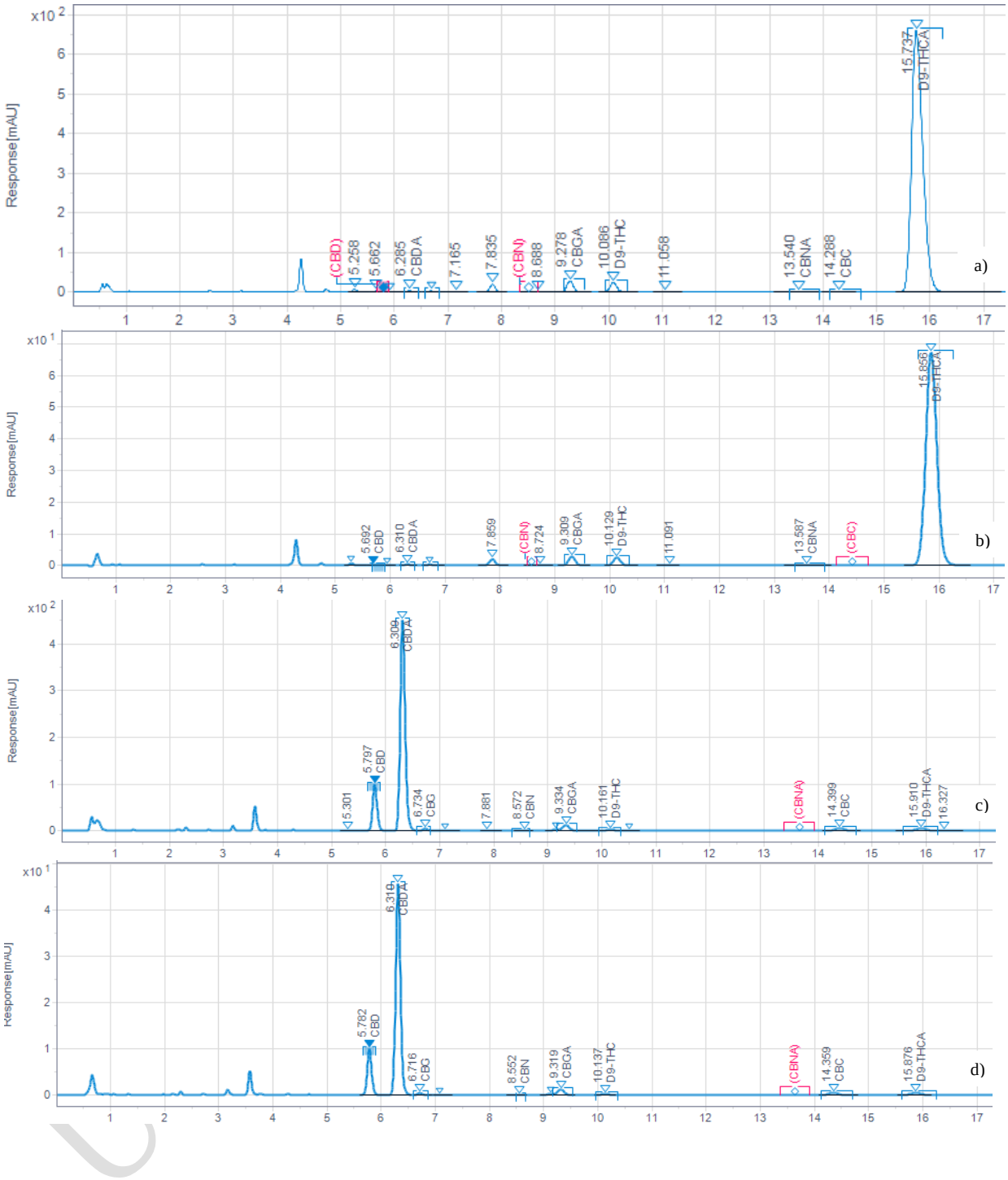


Fig. 1. Chromatograms from system suitability solutions: a) and b) CBD CRS Ref. sol. (b) and (c), respectively; c) and d) *Cannabis flos* for system suitability HRS Ref. sol (d) and (e), respectively.

* Corresponding author e-mail: vsgjorgievska@ff.ukim.edu.mk



* Corresponding author e-mail: vsgjorgievska@ff.ukim.edu.mk

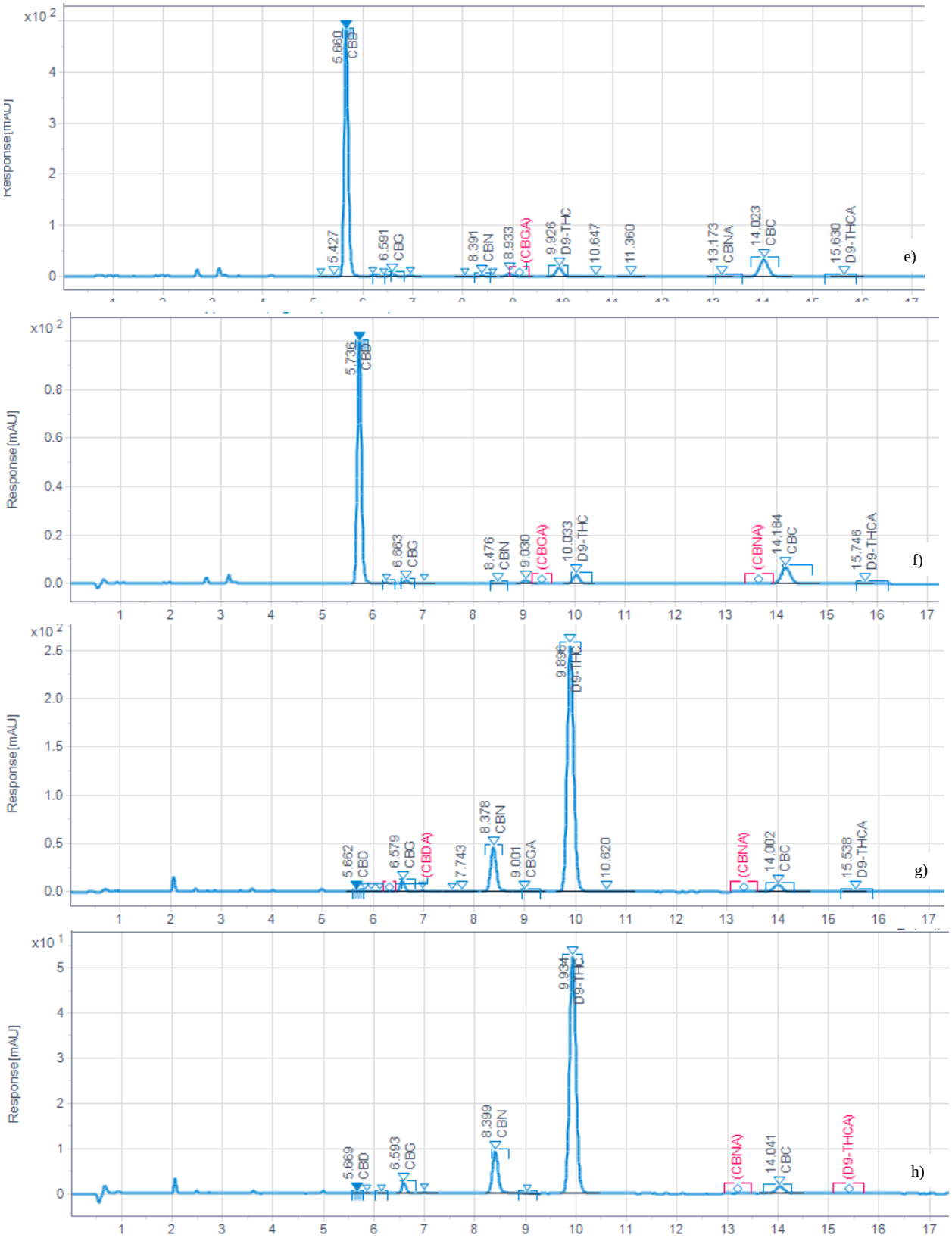


Fig. 2. Chromatograms from Test solution (a) and Test solution (b) from *Cannabis flos* and *Cannabis extractum spissum*, respectively. a) and b) Banjo strain Test sol. (a) and (b), respectively;

* Corresponding author e-mail: vsgjorgievska@ff.ukim.edu.mk

c) and d) Charlotte's Angels strain Test sol. (a) and (b), respectively; e) and f) CDDDA 22 CBD *Cannabis extractum spissum* Test sol. (a) and (b), respectively; g) and h) THC Buba Kush *Cannabis extractum spissum* Test sol. (a) and (b), respectively.

UNEDITED PROOF

* Corresponding author e-mail: vsgjorgievska@ff.ukim.edu.mk

Table 1. System precision

Reference solution	Analyte	Concentration (mg/mL)	Mean Rt* (min)	Mean peak area (mAu)	SD	RSD (%)
CBD CRS Ref. sol. b) (n=6)	CBD	0.05	5.77	254.03	0.49	0.19
CBD CRS Ref. sol. c) (n=6)	CBD	0.08	5.76	404.61	0.47	0.12

*Rt = retention time (min), n=6 – number of injections per solution.

UNEDITED PROOF

* Corresponding author e-mail: vsgjorgievska@ff.ukim.edu.mk

Table 2. Relative retention times of nine cannabinoids in *Cannabis flos* for system suitability HRS reference solution (d)

Reference solution	Compound	Rt (min)	RRt (observed)	RRt (literature)
Cannabis flos for SS HRS - Ref. sol. (d)	CBD	5.788	1.00	1.00
	CBDA	6.322	1.10	~1.10
	CBG	6.73	1.17	~1.17
	CBN	8.573	1.49	~1.48
	CBGA	9.353	1.62	~1.63
	Δ9-THC	10.162	1.76	~1.76
	CBNA	13.652	2.37	~2.38
	CBC	14.413	2.50	~2.48
	Δ9-THCA	15.941	2.76	~2.78

UNEDITED PROOF

* Corresponding author e-mail: vsgjorgievska@ff.ukim.edu.mk

Table 3. System suitability - resolution and peak-to-valley ratios

Reference solution	Criterion	Peak pair	Obtained value	Acceptance criterion
Ref. sol. (d)	Resolution	CBGA / Δ9-THC	3.0	≥ 2.0
	Peak to valley ratio	CBG / CBDA	3.1	≥ 1.5
	Peak to valley ratio	CBNA / CBC	17.2	≥ 5.0
Ref. sol. (e)	Resolution	CBD / CBDA	3.4	≥ 2.0

UNEDITED PROOF

* Corresponding author e-mail: vsgjorgievska@ff.ukim.edu.mk

Table 4. Results for detection limit (DL), quantification limit (QL) and signal to noise (S/N) ratio for in-house method for cannabinoid quantification with linear equations and R^2 value from single cannabinoids injected in lower range concentrations (0.0001 – 0.005 mg/mL range)

Component	Linear equation	R^2 *value*	LD conc. (mg/mL)	LD (mAu)	S/N ≥3	LQ conc. (mg/mL)	LQ (mAu)	S/N ≥10
CBD CRS	y = 5074.6x + 0.237	1.0000	0.001	0.62	3.5	0.0002	2.02	12.6
CBDA	y = 8949.6x + 0.0375	1.0000	0.0001	0.76	4.7	0.0002	1.97	11.8
CBN	y = 13422x - 0.3741	0.9998	0.0001	1.53	9.8	0.0002	2.49	14.6
Δ9-THC	y = 4872.3x + 0.0276	1.0000	0.0001	0.55	3.4	0.0005	2.51	14.6
Δ9-THCA	y = 8908.8x + 0.2682	0.9999	0.0002	1.85	5.8	0.0005	4.87	11.8

UNEDITED PROOF

* Corresponding author e-mail: vsgjorgievska@ff.ukim.edu.mk

Table 5. Linearity results obtained from the standard mixture containing CBD, CBDA, CBN, Δ9-THC and Δ9-THCA at ten concentration levels in the range of 0.0001 - 0.250 mg/mL, including regression coefficients and R^2 values for the in-house method

Component	Concentration range (mg/mL)	Linear equation	*R^2 value*
CBD	0.0001 – 0.250	y = 5087.6x + 1.5825	0.9999
CBDA	0.0001 – 0.250	y = 9259.7x + 2.4284	0.9999
CBN	0.0001 – 0.250	y = 11950x + 3.7919	0.9999
Δ9-THC	0.0001 – 0.250	y = 4727x + 1.64	0.9999
Δ9-THCA	0.0001 – 0.250	y = 8435.9x + 2.2058	0.9999

UNEDITED PROOF

* Corresponding author e-mail: vsgjorgievska@ff.ukim.edu.mk

Table 6. Results for accuracy with spiking studies (*Cannabis extractum spissum* and *Cannabis flos*, respectively)

Cannabis extractum spissum THC BUBA KUSH spike studies (for method validation)											
Concentration of spiked standard mix solution with Test solution (b)		Components									
		CBD		CBDA		CBN		Δ9-THC		Δ9-THCA	
		Recovery ± CI (%) (n = 3)	RSD (%) (n = 3)	Recovery ± CI (%) (n = 3)	RSD (%) (n = 3)	Recovery ± CI (%) (n = 3)	RSD (%) (n = 3)	Recovery ± CI (%) (n = 3)	RSD (%) (n = 3)	Recovery ± CI (%) (n = 3)	RSD (%) (n = 3)
0.005 mg/mL		100.41±1.80	0.72	100.06±0.61	0.25	99.72±1.36	0.55	100.64±1.87	0.75	99.40±0.97	0.39
0.01 mg/mL		98.84±0.77	0.31	100.11±2.79	1.12	100.41±3.44	1.38	102.77±1.57	0.61	99.62±1.40	0.57
0.05 mg/mL		100.08±1.06	0.43	100.03±0.82	0.33	100.00±1.11	0.45	100.30±2.84	1.14	99.99±0.53	0.21
Spiked with CBD standard solution (% of assay content of *Cannabis extractum spissum* CDDA22)	80%	100.39±4.36	1.75	/		/		/		/	
	100%	100.86±3.92	1.57	/		/		/		/	
	120%	99.40±3.40	1.39	/		/		/		/	
Spiked with 10 mg CBD CRS standard substance	0.01 mg/mL	103.59±0.33	0.13	/		/		/		/	
Cannabis flos Banjo spike studies (for method verification)											
Spiked with 10 mg CBD CRS standard substance	0.01 mg/mL	101.43±0.70	0.27	/		/		/		/	

UNEDITED PROOF

* Corresponding author e-mail: vsgjorgievska@ff.ukim.edu.mk

Table 7. Results for orthogonal method comparison (*Cannabis extractum spissum* and *Cannabis flos*, respectively)

	Cannabis extractun spissum (%w/w)				*Cannabis flos* (%w/w. expressed on dry drug)			
	CDDA 22 CBD In-house method	CDDA 22 CBD UNODC method	THC Buba Kush Δ9-THC In-house method	THC Buba Kush Δ9-THC UNODC Method	Charlotte's angel CBD Ph.Eur. method	Charlotte's angel CBD DAB method	Banjo Δ9-THC Ph.Eur. method	Banjo Δ9-THC DAB method
1	55.79	57.47	51.57	50.78	8.67	7.97	25.62	23.39
2	56.19	57.17	52.10	52.18	8.86	8.23	26.32	23.46
3	56.60	55.92	51.85	53.17	8.89	8.32	26.23	23.77
t-test: paired two sample for means	0.5206	significant difference if ($p < 0.05$)	0.7717	significant difference if ($p < 0.05$)	0.005	significant difference if ($p < 0.05$)	0.005	significant difference if ($p < 0.05$)

UNEDITED PROOF

* Corresponding author e-mail: vsgjorgievska@ff.ukim.edu.mk

Table 8. Cannabinoid content of CBD- and Δ9-THC-predominant *Cannabis flos* and CBD- and Δ9-THC-predominant *Cannabis extractum spissum*, respectively

Parameter	Referent values for Cannabis flower (*%w/w*) according to PhEur monograph	Cannabis flower Result (*%w/w*, n = 3)				Cannabis extract Result (*%w/w*, n = 6)			
		CBD dominant strain		THC dominant strain		CBD dominant extract		THC dominant extract	
		CBD Charlotte's Angel		Banjo					
		Sample preparation comparative study				CDDA22		THC Buba Kush	
		15 min. mixing	15 min. US bath	15 min. mixing	15 min. US bath				
Loss on drying	max. 12.0%	5.03	5.03	6.07	6.07	(%)	RSD	(%)	RSD
Total CBN	max 1.0	0.01	0.01	0.13	0.13	0.16	2.02	2.91	0.65
Total CBD	min 5.0 (CBD strain) max 1.0 (THC strain)	8.81	8.77	0.13	0.13	56.08	0.90	BLQ	/
Total Δ9-THC	min 5.0 (THC strain) max 1.0 (CBD strain)	0.37	0.38	26.06	26.32	3.48	1.44	51.80	0.43
t-test (significant difference if p < 0,05)		CBD *p = 0.42* Δ9-THC *p = 0.51*		CBD *p = 0.68* Δ9-THC *p = 0.84*		/		/	

BLQ - below limit of quantification; n – number of individual samples prepared.

UNEDITED PROOF

* Corresponding author e-mail: vsgjorgievska@ff.ukim.edu.mk

Table 9. Results of robustness testing under deliberately varied chromatographic conditions of the in-house method for determination of cannabinoids in *Cannabis extractum spissum*.

Parameter	Nominal value	Deliberate variation	Resolution CBGA / Δ9-THC (≥ 2.0, in ref. sol. (d)	Peak-to-valley ratio CBG / CBDA (≥ 1.5, in ref. sol. (d)	Peak-to-valley ratio CBNA / CBC (≥ 5.0, in ref. sol. (d)	Resolution CBD / CBDA (≥ 2.0, in ref. sol. (e)
Flow rate	2.0 mL/min	1.8 mL/min	2.9	2.7	11.6	3.6
		2.2 mL/min	3.0	3.4	6.8	3.3
Temperature	35°C	33°C	3.3	2.7	6.4	3.3
		37°C	3.9	4.1	5.5	3.0
Mobile phase	41:59 (0.1% TFA : ACN)	40:60 (0.1% TFA : ACN)	3.8	3.1	5.4	3.0
		42:58 (0.1% TFA : ACN)	3.3	3.8	6.5	3.3

UNEDITED PROOF

* Corresponding author e-mail: vsgjorgievska@ff.ukim.edu.mk