Macedonian pharmaceutical bulletin, 68 (Suppl 2) 133 - 134 (2022)
ISSN: 1857 - 8969
DOI: 10.33320/maced.pharm.bull.2022.68.04.060
Short communication

Comprehensive methodology for characterization of *Cannabis sativa* L. (hemp) inflorescences through combined NMR and LC-based metabolite profiling

Petros S. Tzimas[1], Eleftherios A. Petrakis[1], Stavros Beteinakis[1], Panayiota Papastylianou[2], Ioanna Kakabouki[2], Daphne Chandrinou[3], Andrea L. Small-Howard[4], Dimitrios Bilalis[2], Maria Halabalaki[1], Leandros A. Skaltsounis*[1]

[1]*Division of Pharmacognosy and Natural Products Chemistry, Department of Pharmacy, National and Kapodistrian University of Athens, Panepistimioupoli Zografou, 15771 Athens, Greece*
[2]*Laboratory of Agronomy, Department of Crop Science, Agricultural University of Athens, 75 Iera Odos Str., 11855 Athens, Greece*
[3]*PharmaGnose S.A, Athens, Greece*
[4]*GbS Global Biopharma, Ottawa, Canada*

Introduction

Cannabis sativa L. is a plant widely known for its bioactive constituents, and especially for cannabinoids. Cannabinoids are accumulated in the inflorescences, which is considered the most valuable plant part (Hanuš et al., 2016). Fiber-type *C. sativa* (hemp) predominantly produces cannabidiol (CBD) and cannabidiolic acid (CBDA), both of which exhibit numerous pharmacological activities. Consequently, reliable analytical results about major cannabinoids would be important for the quality control of plant material. The contribution of minor cannabinoids in the pharmacological properties of cannabis has recently attracted significant interest (Cerrato et al., 2021). Other compound classes also exist in the plant, adding up to its chemical complexity. Still, the phytochemical composition may be affected by factors such as variety, harvesting year, geographic origin, and cultivation environment. This variability is inevitably translated into different pharmacological effects (Andre et al., 2016). In this regard, characterization and classification of hemp samples based on their metabolite profiles is crucial for standardization of products and consistency in therapeutic outcomes. Aiming for a comprehensive investigation and comparison of *C. sativa* phytochemical profiles, inflorescences belonging to different varieties were collected during consecutive harvesting years from various regions of Greece. Following an optimized sample preparation protocol, the ethanol extracts of the samples were analyzed by NMR and LC-based techniques aided by chemometrics. For a quick overview of metabolite classes, ^{1}H-NMR was employed. In parallel, UPLC-PDA and LC-HRMS were applied with focus on cannabinoids.

Materials and methods

Extraction and sample preparation - An optimized ultrasound-assisted extraction (UAE) method was implemented (Tzimas et al., 2021). Briefly, 204.0 mg of dried inflorescences were weighed and 20.0 mL of ethanol were added. The mixture was vortexed, sonicated (15 min, 40 °C) and, finally, centrifuged (5 min, 1930 × g). Aliquots of the supernatants were diluted with acetonitrile (ACN) for LC-PDA. For the remaining analyses, aliquots of 1.3 mL were withdrawn and dried; Methanol-d_4 was added to re-dissolve the aliquots before NMR analysis, while LC-MS grade methanol was used as diluent before LC-MS analysis.

^{1}H-NMR analysis - NMR spectra were acquired on a Bruker Avance III instrument equipped with an inverse detection probe with a z-gradient. The spectra were

*skaltsounis@pharm.uoa.gr

obtained by Fourier transformation (FT) of the free induction decay (FID). Data acquisition and processing was performed using TopSpin (version 4.0.6) software. Hexamethyldisiloxane (HMDSO) was added to methanol-d_4 as internal standard.

Liquid chromatographic analysis (LC-PDA & LC-HRMS) - Quantitative determination of target cannabinoids was carried out by UPLC-PDA using an Acquity UPLC system, under the control of Empower 3 software. A C18-PFP column was employed, while the mobile phase consisted of a gradient of ACN/water both containing 0.1% formic acid (FA). Selective chromatograms were obtained at 210 and 225 nm, acoording to the λmax of analytes.

The LC-HRMS analysis was conducted on an Acquity UPLC system coupled to Velos Pro-Orbitrap Elite hybrid mass spectrometer, using a heated electrospray ionization source (HESI). The samples were analyzed under both negative and positive ESI modes. The chromatographic separation was also performed on a C18 column using a gradient of ACN/acidified (0.1% FA) water. Data acquisition and analysis were performed using Thermo Xcalibur software, Version 2.2.

Multivariate data analysis (MVDA) - Proton NMR data were binned, aligned and IS-normalized before import to SIMCA software (version 14.1) for multivariate analysis. LC-HRMS raw data were firstly processed using MZmine software (version 2.53), including steps for deconvolution, alignment and grouping of isotope patterns. The final feature list was imported to SIMCA. Principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA) and orthogonal projection to latent structures-discriminant analysis (OPLS-DA) were mainly used for model generation.

Results and discussion

Exploration of the NMR data highlighted the different compounds classes present in the samples. It was evident that the samples from the two harvesting years (2019 and 2020) showed different chemical profiles. Resonance signals for fatty acids mainly linoleic and linolenic were observed in 2019 while the concentration of minor phenolics was higher in 2020 samples. CBD and CBDA were the cannabinoids most easily detected, with CBD content being higher in 2019. So, the above could be tentative biomarkers for the harvesting year.

UPLC-PDA analysis enabled the accurate and high-throughput determination of cannabinoid contents and exploration of some basic patterns in the samples. The LC-HRMS based dereplication allowed the identification of many minor cannabinoids, belonging to various subclasses, in addition to other metabolites. These subclasses include CBE, CBC, CBG, THC, and CBN-types, among others. The multivariate analysis on the LC-MS datasets also led to the classification of hemp inflorescences according to important parameters and the identification of possible biomarkers.

Conclusion

^{1}H-NMR proved to be a quick and informative tool, providing a holistic view of metabolite classes present in hemp inflorescences, including lipids and minor phenolics. Analysis of target cannabinoids by UPLC-PDA provided useful quantitative results, which was expanded by an LC-HRMS based untargeted workflow with focus on minor cannabinoids. Multivariate techniques, such as PCA, PLS-DA and OPLS-DA revealed underlying patterns and class structure within the samples. The class-determining metabolites as pointed out by each platform were explored in search of efficient biomarker identification. Overall, the proposed strategy offered comprehensive characterization of *C. sativa* inflorescences and classification according to important parameters, a first of its kind for Greek samples.

Acknowledgements

The present work was co-funded by the European Regional Development Fund (ERDF) and Greek national funds through the Operational Program "Competitiveness, Entrepreneurship and Innovation", under the call “RESEARCH – CREATE - INNOVATE” (CannabisMED, project code: T1EDK-04301).

References

Andre, C. M., Hausman, J.-F., & Guerriero, G., 2016. Cannabis sativa: The Plant of the Thousand and One Molecules.Front. Plant Sci., *7*, 1-17.

Cerrato, A., Citti, C., Cannazza, G., Capriotti, A. L., Cavaliere, C., Grassi, G., Marini, F., Montone, C. M., Paris, R., Piovesana, S., & Laganà, A., 2021. Phytocannabinomics: Untargeted metabolomics as a tool for cannabis chemovar differentiation. Talanta, 230, 1–9.

Hanuš, L. O., Meyer, S. M., Muñoz, E., Taglialatela-Scafati, O., & Appendino, G., 2016. Phytocannabinoids: A unified critical inventory. Nat. Prod. Rep. 33, 1357–1392.

Tzimas, P. S., Petrakis, E. A., Halabalaki, M., & Skaltsounis, L. A., 2021. Effective determination of the principal non-psychoactive cannabinoids in fiber-type Cannabis sativa L. by UPLC-PDA following a comprehensive design and optimization of extraction methodology. Anal. Chim. Acta, 1150, 338200.