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Raman specroscopy as appropriate tool for chemical discrimination of wild hemp oil seed samples

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SUMMARY

Wild hemp (*Cannabis sativa* L. ssp. *sativa* var. *spontanea* Vavilov), as a whole plant, especially its seeds are highly valued, with significant place in the production of oil-based paints, in creams as a moisturizer, for cooking, animal and bird feed. This work represents an innovative report focused on the evaluation of the phytochemical composition of wild hemp seeds grown in two different locations in Serbia, using Raman microspectroscopy in combination with multivariate data analysis. Some significant differences were found between the analyzed oil samples in terms of their chemical profile. The Raman spectra of the wild hemp seed samples indicate the predominance of unsaturated (1657 and 1268 cm^{-1}), then saturated fatty acids (1443, 1306 and 1084 cm^{-1}) and fatty acid esters (1745 cm^{-1}) due to the higher intensity of the bands. The relative intensities of these bands and results of main principal components analysis (PCA) may indicate that the seed oil of wild hemp from two different locations differs in the content of unsaturated and saturated fatty acids, as shown by the differences in the relative intensities of 1657 and 1268 cm^{-1}.
Keywords: unsaturated (UFA), saturated fatty acids (SFA), vibration spectroscopy, PCA.

INTRODUCTION

Wild hemp (*Cannabis sativa* L. ssp. *sativa* var. *spontanea* Vavilov) is a variety of industrial hemp (*Cannabis sativa*, fam. *Cannabaceae*) which is believed to have been developed by acclimatization of industrial hemp to the conditions of ruderal habitats of Eastern Europe (Connell, 1981). This species is an annual herbaceous plant, which is widespread in Serbia as a weed on crop lands, but also in non-crop habitats, primarily in the area of Vojvodina and in the valleys of large rivers (Vrbničanin et al., 2015). Mature seeds are relatively small (<3.8 mm), easily separated from the pedicel, with irregularly pigmented areas with mottled or marbled appearance (Koren et al., 2020). Seed production is up to 2000 seeds per plant, and the seeds contain 30-35% oil (Vrbničanin et al., 2015). Despite the fact that the content and composition of fatty acids in industrial hemp oil has been thoroughly investigated (Alonso-Esteban et al., 2020; Zhao et al., 2022; Alonso-Esteban et al., 2023), there is little information on the composition of fatty acids in wild hemp seeds. Špirović Trifunović et al. (2021) analyzed the fatty acids in the seeds of two populations of wild hemp from Serbia using capillary gas chromatography and determined the presence of 15 different fatty acids. Their results indicate the dominant presence of unsaturated (88%) in relation to the presence of saturated (about 12%) fatty acids, which indicates the significant nutritional value of this seed.

Various analytical methods can be used for the analysis of fatty acids in oil samples, e.g. liquid chromatography, gas chromatography with flame ionization detector (GC-FID), near-infrared spectroscopy, etc. Compared to standard analytical methods, the various vibrational spectroscopic techniques are faster, simpler, non-destructive and time-saving. This technique has been used for certification, authentication, investigation of adulteration of products, fatty acid determination, etc. (Karacaglar et al., 2019; Zao et al., 2022). Depending on the number of C-H, C=C bonds and degree of unsaturation, fatty acids can be identified by Raman spectroscopy. Due to the complexity of oil samples, Raman spectroscopy can be used in combination with chemometrics for rapid and valuable evaluation of oils. The aim of this study was to evaluate Raman spectroscopy as a successful tool for chemical description and discrimination of wild hemp oil seed samples.

MATERIAL AND METHODS

In order to examine the possibility of using Raman spectroscopy for chemical discrimination of wild hemp seeds, two samples of seeds of this species were analyzed: CS1 - originating from the area of South Banat and CS2 - originating from the Braničevo district. The seeds were collected at maturity and stored at room temperature (20-25°C) until analysis. Homogenization was performed using a blender (Pbi International, Sumbeam), and oil extraction with hexane was performed according to the protocol described by Špirović Trifunović et al. (2021).

Raman microspectroscopy of extracted hemp seed oil samples was performed using a Horiba Jobin Yvon XploRA Raman spectrometer equipped with an Olympus BX 41 microscope. The experiment was based on a diode laser at 785 nm wavelength focused on the sample and an objective lens 50 LWD (Olympus, Tokyo, Japan). Raman scattering was performed with a 600 lines mm^{-1} grating, the spectra were recorded in the 700-1800 cm^{-1} spectral range, the same spectral range was selected for the chemometric investigations of the Raman spectra.

The spectral resolution was ~3 cm^{-1} and the Si 521 cm^{-1} peak was used for calibration. The spectra were recorded with an exposure time of 10 s and scanning the sample 10 times. Data acquisition and instrument control were performed using LabSpec 6 software (Horiba Scientific, France). The assignment of the main bands was based on literature data.

The results of Raman spectroscopy were subjected to multivariate data analysis, PCA was performed on smoothed, baseline-corrected data normalized to the band with the highest intensity in the 200-1800 cm^{-1} range. The spectra were preprocessed using Spectragryph software (Menges, 2018). The spectra were base-corrected using Savitzky–Golay filters with 7 points and a second-order polynomial function was used for spectrum smoothing. PCA was performed using PAST software (Hammer et al., 2001). PC analysis was performed with ten spectra per cannabis oil sample, resulting in a total of 20 spectra.

RESULTS AND DISCUSSION

The lipid profile of hemp seed oil samples is characterized by the prevalence of polyunsaturated fatty acids (PUFA), followed by linoleic acid, α-linolenic acid , oleic acid, γ-linolenic acid, and palmitic acid (Abdollahi et al., 2020; Mandrioli et al., 2023). Similar to that, linoleic (45.3 and 47.5%) and α-linolenic acid (13.6 and 15.5%) (Špirović Trifunović et al., 2021) stand out as the most dominant fatty acids in wild hemp seeds from Serbia.

The Raman spectra of all recorded wild hemp oil samples in the 700-1800 cm^{-1} region are presented in figure 1, while characteristic bands and the related assignments are shown in table 1. The obtained spectra include bands that may be associated with the most important compounds found in seeds, such as fatty acids.

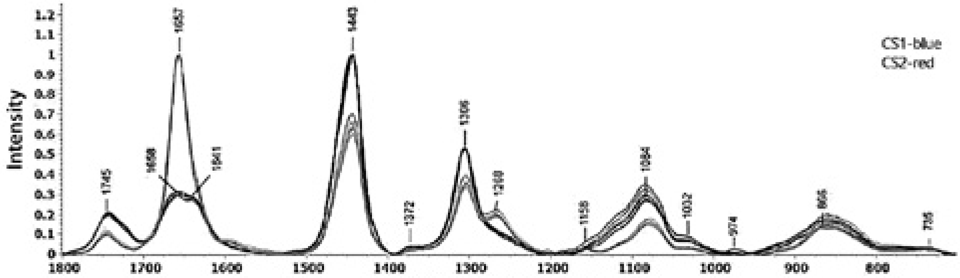

Figure 1. Normalized Raman spectra of two wild hemp seed oil samples (CS1, CS2), recorded in the fingerprint region (700 to 1800 cm^{-1}). All these samples show characteristic vibrational modes with higher intensity bands probably specific for unsaturated (1657 and 1268 cm^{-1}), saturated fatty acids (1443, 1306 and 1084 cm^{-1}) and fatty acids esters (1745 cm^{-1})

Slika 1. Normalizovani Ramanovi spektri dva uzorka ulja semena divlje konoplje (CS1, CS2), snimljena u oblasti "otiska prsta" (700 do 1800 cm^{-1}). Svi uzorci pokazuju karakteristične vibracione modove većeg intenziteta koji su verovatno specifični za nezasićene (1657 i 1268 cm^{-1}), zasićene masne kiseline (1443, 1306 i 1084 cm^{-1}) i estre masnih kiselina (1745 cm^{-1})

The highest intensity bands positioned at 1657 and 1443 cm^{-1} can be assigned to (C=C) of cis (RHC=CHR) represent unsaturated (Qiu et al., 2019; Pang et al., 2022) are due to the twisting and scissoring C–H vibrations of methylene (–CH$_2$) from saturated fats. Medium intensity bands at 1306 and 1084 cm^{-1} can be assigned to C–H bending twist and (C–C) stretching of the (CH$_2$)n group from UFA. The mode at 1268 cm^{-1} can be assigned to (=C–H) deformation of cis (R–HC=CH–R) (Pang et al., 2022) and represent shoulder of band positioned at 1306 cm^{-1}. This band is present only in CS2 and indicated on the difference between CS2 and CS1 in unsaturated fatty acids content (UFA). The band at 1747-1739 cm^{-1} was assigned to the stretching of C=O from triacylglycerol structure that was present in all Raman spectra of seed oil samples. The band is related to the presence of fatty acid esters (da Silva et al., 2008; Farber et al., 2020).

According to intensities of all mentioned bands observed in fingerprint region indicated by the predominance of UFA and SFA in both hemp oil samples, as well as in differences in their content. These findings are consistent with results of fatty acid analyses in wild hemp seeds by capillary gas chromatography (Špirović Trifunović et al., 2021). Intensities of bands positioned at 1657 and 1268 cm^{-1} in CS2 are higher than in CS1. Meanwhile, the intensities of Raman bands at 1443, 1306, 1084 and 1745 cm^{-1} are higher for CS1. These results could indicate that oil from hemp seeds in CS2 has a higher content of UFA, while CS1 has a higher content of SFA. It is well known that the Raman spectra of linolenic, linoleic and oleic acids exhibit two specific bands at 1657 and 1268 cm^{-1} related to the presence of *cis* stretching vibration of C=C in the propanoic chain and the bending of C–H from unsaturated fats, respectively (da Silva et al., 2008; Schultz and Baranska, 2007; Jamieson et al., 2018) and differences in intensities of these bands indubitably distinguish UFA from SFA (Martini et al., 2018).

Table 1. Vibrational bands and their assignments observed from the wild hemp seed oil samples.
Tabela 1. Vibracione trake i njihovo objašnjenje uočene u uzorcima ulja semena divlje konoplje.

	Literature data/ Literaturni podaci	Vibrational mode Vibracioni režim	Chemical moiety Hemijski sastav	Reference Reference
1745	1730–1757	CH$_2$, C=O	fatty acid ester, lipids	Schulz and Baranska, 2007; Martini et al., 2018; Farber et al., 2020, Pang et al., 2022; Popović-Đorđević et al., 2022
1657-1641	1650-1661	C=C	cis-unsaturated fatty acid, lipids	De Gelder et al., 2007; Schulz and Baranska, 2007; daSilva et al., 2008; Agarwal et al., 2011; Wiercigroch et al., 2017; Martini et al., 2018; Farber et al., 2020; Pang et al., 2022; Popović-Đorđević et al., 2022
1443	1440	CH$_2$/CH$_3$ def. of aliphatic carbon chains	aliphatic chains of fatty acids, SFA	Martini et al., 2018; Pang et al., 2022; Popović-Đorđević et al., 2022;
1372	1387	CH$_2$	aliphatic chain	Farber et al., 2020
1306	1303, 1305	CH$_2$	lipids	Martini et al., 2018

1268	1262-1273	C-H	esterified unsaturated fatty acids, lipids	Da Silva et al., 2008; Sharma et al., 2015; Wiercigroch et al., 2017; Martini et al., 2018; Zeise et al., 2018; Farber et al., 2020; Pang et al., 2022; Popović-Đorđević et al., 2022
1158	1171	CH_2 rotation		Potcoava et al., 2022
1084	1074-1079	C–C	aliphatic stretches all trans, lipids	Martini et al., 2018
1032	/	/	/	/
974	969, 972	=C-H, C=C	Bending out-of-plane, UFA	Farber et al., 2020; Pang et al., 2022
866	800-920	C-C, CH_3, C=O	stretches and rocks at acyl and methyl terminals	Potcoava et al., 2022
734	727	=C-H	bending in-plane	Potcoava et al., 2022

*/-non identify

PCA WITH THE AIM OF ENABLING DIFFERENTIATION BETWEEN HEMP SEED OIL SAMPLES

Multivariate analysis based on PCA was performed of the Raman spectra of the wild hemp seed oil samples from different locations. Figure 2. shows the results and loadings of PCA1 versus PCA2. Figure 2a shows the clear separation into different object groups, with the first and second principal components describing 98.42% of the data variance. Loadings for each PC (Figure 2b) are analyzed to determine which bands had the greatest influence so that it can be determined which main chemical constituent influenced it the most. The score plot of PC1 versus PC2 (Figure 2a) shows a separation between the CS1 and CS2.

PC1 typically explains most of the variability, therefore the bands in the spectral range of 1000-1800 cm^{-1} are mainly assigned to UFA and SFA. The PC1 loading plots show high factor loadings associated with the UFA (negative loadings) at 1657 cm^{-1}, 1444, 1308 and 1085 cm^{-1} (positive loadings) probably associated to SFA (Martini et al., 2018; Farber et al., 2020; Pang et al., 2022; Popović-Đorđević et al., 2022). According to the analyzed loadings of PC1, CS1 might have higher content in SFA than CS2- and they are richer in UFA than CS1.

The loading plot of PC2 (Figure 2b) shows the bands responsible for similarity between CS1 and CS2, corresponding to the positive loadings originating predominantly from streaching vibration of C=C, C=O, and bending vibration of C-C-H, CH_2 and CH_3 of UFA and aliphatics (e.g. 1429, 1451, 1649, 1668, 1315, 1293 cm^{-1}) (Farber et al., 2020).

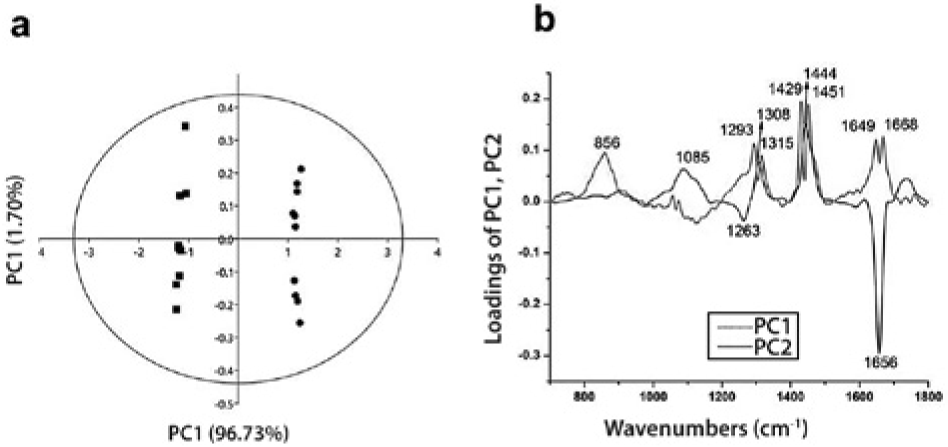

Figure 2. PCA applied to the data obtained from Raman spectra of wild hemp seed oil samples: (a) score plot, (b) loading plots. closed cycle-CS1, closed squares-CS2
Slika 2. PCA za podatke dobijene iz Ramanovih spektra uzoraka ulja semena divlje konoplje: (a) grafikon rezultata, (b) projekcije varijabli. zatvoreni krugovi-CS1, zatvoreni kvadrati-CS2

CONCLUSION

Raman spectroscopy is a suitable technique for the analysis of seed oils. It was shown that the examined oil content is a good source of unsaturated and saturated fatty acids, with the region below 1800 cm^{-1} in the Raman spectra being the most important for their characterization. The Raman spectra and additional chemometric analysis of the wild seed oil samples indicated the differences in the chemical profile of samples collected from two different locations in Serbia.

The most striking spectral features in CS1 for unsaturated fatty acids (UFA) are associated with the addition of C=C double bonds. A strong band at 1655 cm^{-1} (C=C stretching), which increased in sample CS2, indicates the increasing degree of unsaturation and probably a higher content of UFA. In addition, a similar trend was observed in sample CS2, where the relative intensity of the band at 1268 cm^{-1} (=C-H deformation) increased with increasing degree of unsaturation. On the other hand, the increasing relative intensity of the bands positioned at 1443, 1306 and 1084 cm^{-1} indicates the predominance of saturated fatty acids (SFA) in sample CS1.

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Primena Ramanove mikrospekroskopije za utvrđivanje razlika u sadržaja ulja u semenu divlje konoplje

REZIME

Divlja konoplja (*Cannabis sativa* L. ssp. *sativa* var. *spontanea* Vavilov) je cenjena biljna vrsta, koristi se u proizvodnji boja na bazi ulja, kao hidratantno sredstvo u kremama, za kuvanje, ishranu životinja i ptica. Cilj ovog rada je bio procena fitohemijskog sastava divlje konoplje uzgajane na dve različite lokacije u Srbiji, primenom Ramanove mikrospektroskopije u kombinaciji sa multivarijantnom analizom podataka. Između analiziranih uzoraka ustanovljena je značajna razlika u pogledu hemijskog profila. Pikovi visokog intenziteta u Ramanovim spektrima uzoraka semena divlje konoplje ukazuju na dominantnost nezasićenih (pikovi na pozicijama 1657 i 1268 cm^{-1}), zatim zasićenih masnih kiselina (1443, 1306 i 1084 cm^{-1}) i estara masnih kiselina (1745 cm^{-1}). Relativni intenziteti ovih traka i rezultati analize glavnih komponenata (PCA) ukazuju na to da se ulje iz semena divlje konoplje prikupljeno sa različitih lokaliteta razlikuje u sadržaju nezasićenih i zasićenih masnih kiselina što se uočava na osnovu razlika u relativnim intenzitetima traka na pozicijama 1657 i 1268 cm^{-1}.

Ključne reči: nezasićene, zasićene masne kiseline, vibraciona spektroskopija, PCA.