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ORIGINAL ARTICLE

Raman spectroscopy enables highly accurate differentiation between young male and female hemp plants

Samantha Higgins[1] · Russell Jessup[2] · Dmitry Kurouski[1]

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Abstract

Main conclusion **Hand-held Raman spectroscopy can be used for highly accurate differentiation between young male and female hemp plants. This differentiation is based on significantly different concentration of lutein in these plants.**

Abstract Last year, a global market of only industrial hemp attained the value of USD 4.7 billion. It is by far the fastest growing market with projected growth of 22.5% between 2021 and 2026. Hemp (*Cannabis sativa* L.) is a dioecious species that has separate male and female plants. In hemp farming, female plants are strongly preferred because male plants do not produce sufficient amount of cannabinoids. Male plants are also eliminated to minimize a possibility of uncontrolled cross-fertilization of plants. Silver treatments can induce development of male flowers on genetically female plants in order to produce feminized seed. Resulting cannabinoid hemp production fields should contain 100% female plants. However, any unintended pollination from male plants can produce unwanted males in production fields. Therefore, there is a growing demand for a label-free, non-invasive, and confirmatory approach that can be used to differentiate between male and female plants before flowering. In this study, we examined the extent to which Raman spectroscopy, an emerging optical technique, can be used for the accurate differentiation between young male and female hemp plants. Our findings show that Raman spectroscopy enables differentiation between male and female plants with 90% and 94% accuracy on the level of young and mature plants, respectively. Such analysis is entirely non-invasive and non-destructive to plants and can be performed in seconds using a hand-held spectrometer. High-performance liquid chromatography (HPLC) analysis and collected Raman spectra demonstrate that this spectroscopic differentiation is based on significantly different concentrations of carotenoids in male vs female plants. These findings open up a new avenue for quality control of plants grown in both field and a greenhouse.

Keywords Hemp · Sex determination · Raman spectroscopy · Chemometrics · HPLC · Lutein

Introduction

Hemp (*Cannabis sativa* L.) is a broadly cultivated plant that is used to produce (i) fiber, (ii) psychologically active delta-9-tetrahydrocannabinol (Δ-9-THC) and most recently (iii) cannabidiol (CBD) and cannabigerol (CBG) (Hartsel et al. 2016; Appendino et al. 2008; Borrelli et al. 2013). In the first ideotype, plants contain very little if any cannabinoids. Hemp is considered to be marijuana in the US if it contains more than 0.3% Δ-9-THC (Sanchez et al. 2020a, d). THC-dominant Cannabis is broadly cultivated around the world. It is consumed by ~ 147 million people, which is around 2.5% of the world population (Havelka 2017). Over the past decade, substantial efforts have been made to develop hemp varieties with high concentration of CBD and CBG. These cannabinoids have distinct pharmacological activity. They are strong suppressors of bacterial activity, inflammation, and tumor cell growth. Further evidence indicates that CBD relieves chronic pain, lowers blood pressure, suppresses anxiety and treats depression (Mouslech and Valla 2009; Darkovska-Serafimovska et al. 2018). It can also mitigate side effects of cancer treatment, such as nausea and vomiting.

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✉ Dmitry Kurouski
dkurouski@tamu.edu

[1] Department of Biochemistry and Biophysics, Texas A&M University, College Station, TX 77843, USA

[2] Department of Soil and Crop Sciences, Texas A&M University, College Station, TX 77843, USA

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Hemp is a dioecious species that has separate male and female plants. If fiber is produced by both male and female plants, Δ-9-THC, CBD, CBG and other cannabinoids are produced in trichomes predominantly in only female plants (Sanchez et al. 2020a, d). Therefore, farmers that grow hemp for medical and pharmacological purposes, as well as cannabis growers, eliminate male plants on their farms. The use of male plants can be eliminated on the level of seeds if female plants are sprayed with colloidal silver in order to induce male flowers and pollen. Derived seeds from crossing only female plants are called "feminized" and genetically female plants. Only female plants are desired in production fields in order to maximize cannabinoid yields, and any nearby male hemp can cross-pollinate female plants.

Differentiation between male and female specimens can be achieved by subjective visual analysis of plants at the state of their flowering. This is a highly laborious process because each plant has to be inspected individually. Recent increase in the agricultural areas that are used for hemp production requires automated approaches that can be used for confirmatory differentiation between male and female plants prior to their flowering. PCR-based markers have been identified specific to male plants (Mandolino and Carboni 2004) and are routinely used by service laboratories; however, these tests are costly and time consuming.

Raman spectroscopy (RS), in contrast, is a label-free, non-invasive, non-destructive spectroscopic method that can be used to probe the structure and composition of analyzed samples (Cardona 1975). The Raman effect is based on inelastic scattering of photons. In this case, photons interact with molecules of the sample bringing them to higher vibrational or rotational states (Kurouski et al. 2015). This energy exchange can be used to determine chemical structure of molecules. Therefore, RS is broadly used in material science, food chemistry[11], electrochemistry, forensic analysis of gun-shot residues and body fluids (Almeida et al. 2010; Zeng et al. 2016; Virkler and Lednev 2009; López-López et al. 2013). Our own experimental results, as well as findings reported by other groups showed that RS could be used to diagnose biotic and abiotic stresses in plants (Farber and Kurouski 2018; Gupta et al. 2020; Mandrile et al. 2019; Payne and Kurouski 2021; Sanchez et al. 2020b, c, e). Furthermore, RS can be used to identify different plant species and their varieties (Farber et al. 2020a, b). Recently, our group demonstrated that RS can be used for confirmatory differentiation between hemp and cannabis (Sanchez et al. 2020a, d). This differentiation is based on quantitative assessment of psychologically active cannabinoids present in the plant material. We also showed that RS can be used to differentiate between cannabis, industrial hemp and CBD-rich hemp with high accuracy, as well as identify different varieties of hemp and cannabis (Sanchez et al. 2020a, d).

Raman-based analysis of plant material can be done using hand-held spectrometers that can be used directly in a greenhouse or a field (Sanchez et al. 2019a, 2019b, 2020a; Yeturu et al. 2016). Our findings, as well as technological development of RS sparked an interest of farmers, plant breeders and basic plant biologists in utilization of this technology for analysis of a cannabinoids profile of hemp. The question to ask is whether RS can be used to differentiate between male and female hemp plants. To answer this question, we collected Raman spectra from leaves of hemp plants 3weeks prior to flowering. After these plants produced flowers, we assigned spectra collected from male and female plants into two classes. Next, we used chemometrics to investigate differences between spectra from these two classes (male and female), as well as to determine the accuracy with which such spectral assignment can be made.

Materials and methods

Plants

Plants of a dioecious hemp population developed at Texas A&M University were grown in the greenhouse located in College Station, TX, USA. After 2 weeks of post-germination, plants were examined by Raman spectroscopy. At this stage ("young plants"), plants exhibited no flowers, which allows for visual differentiation between male and female hemp plants. In total, five spectra were collected from each plant. In parallel, 2–3 leaves of the plant were taken and placed in Ziploc bags for the subsequent HPLC analysis. These leaves were kept at − 20 °C. Next, the same plants were examined by Raman spectroscopy at the stage of flowering (4 weeks) to acquire spectra from "adult plants". The experiment was repeated twice; in every experiment, ~ 20 plants were used. All plants were kept at the same vegetation conditions (light intensity, irrigation, and temperature).

Raman spectroscopy

Raman spectra of hemp leaves were collected using a hand-held Resolve Agilent spectrometer equipped with an 831-nm laser. The following experimental parameters were used for all collected spectra: 1 s acquisition time, 495 mW power, and baseline spectral subtraction by device software. We collected 2–3 spectra from each leaf on its adaxial side. Next, 30–60 spectra collected form each class of plants (male and female) were averaged using Matlab and presented in the Fig. 1.

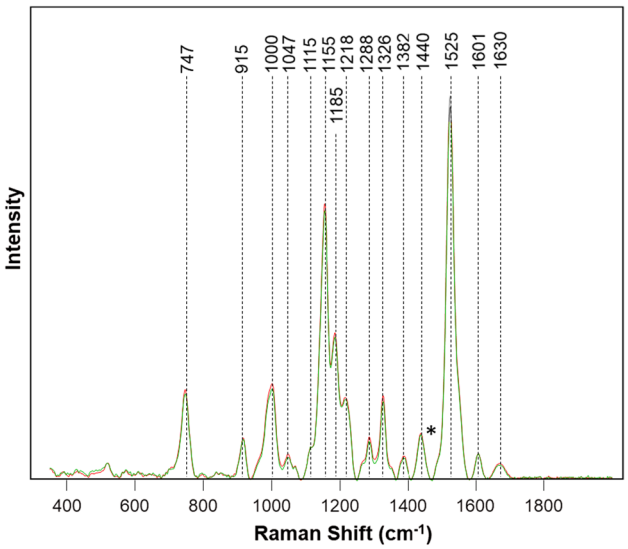

Fig. 1 Averaged Raman spectra collected from leaves of male (green) and female (red) young hemp plants. For each spectrum, 30–60 individual spectra collected from leaves of ~ 10 male and 10 female plants were averaged. Vibrational bands that correspond to certain chemicals present in the leaves are labeled and discussed in the Table 1

Carotenoid extraction

Collected leaves (~ 150 mg) were homogenized using pestle and mortar. After a sufficient homogenization was achieved, plant material was re-suspended into 1.5 mL of a mixture of chloroform and dichloromethane (2:1, v/v) and shaken at 500 rpm at 4 °C for 30 min. Next, 0.5 mL of 1 M sodium chloride solution was added to the plant extract, mixed by inversion, and centrifuged at 5000*g* for 10 min[27]. The aqueous and organic phases were separated. The aqueous phase was subjected to another round of separation by adding 0.75 mL of chloroform and dichloromethane (2:1, v/v), followed by centrifugation at 5000*g* for 10 min. The second organic phase was collected and pooled with the first batch and dried by the centrifugal evaporation method. Dried pellet was re-dissolved in 200 µL of methanol/tert-methyl butyl ether (MTBE) (60/40, v/v) prior to injection into HPLC. At least three independent extractions of the plant material were performed for both male and female plants. These triplicates were processed by HPLC to quantify the carotenoids content in the plant leaves.

HPLC analysis

Leaf extracts were analyzed by reversed phase HPLC using Waters 1525 pump equipped with 2707 auto sampler and 2489 photodiode array detector (PDA). Carotenoids were separated on a reverse-phase C30, 3 µm column (250 × 4.6 mm) (Thermofisher, part number 075723) using mobile phases consisting of (A) methanol/ water (95:5, v/v) and (B) tert-methyl butyl ether. The gradient elution used with this column was 97% A and 3% B at 0–6 min with a linear increase of B to 100% at 20 min and return to initial conditions at 23 min. The column temperature was maintained at 20 °C. The eluting peaks were monitored at 450 nm using PDA (Fig. 2). Determination of the peak area for each of the analyzed carotenoid in the corresponding HPLC profiles was performed using Breeze software. Results of three independent HPCL measurements were then used to determine standard deviations of the peak area, as reported by the error bars in the Fig. 3.

Multivariate statistical analysis

PLS_Toolbox software was used to perform partial least squares discriminant analysis (PLS-DA) for all collected spectra. Our previously reported experimental findings,

Table 1 Assignments of vibrational bands observed in the spectra collected from the leaves of hemp plants

Band	Vibrational mode	Assignment
747	γ(C–O–H) of COOH	Pectin (Synytsya et al. 2003)
915	ν(C–O–C) In plane, symmetric	Cellulose, lignin (Edwards et al. 1997)
1000	–C=C– (in plane)	Carotenoids (Schulz et al. 2005)
1047	ν(C–O) + ν(C–C) + δ(C–O–H)	Cellulose, lignin (Edwards et al. 1997)
1115	–C=C– (in plane)	Carotenoids (Edwards et al. 1997)
1155	–C=C– (in plane)	Carotenoids (Schulz et al. 2005)
1185	ν(C–O–H) Next to aromatic ring + σ(CH)	Carotenoids (Dou et al. 2021)
1218	δ(C–C–H)	Carotenoids (Dou et al. 2021)
1288	δ(C–C–H)	Aliphatics (Yu et al. 2007)
1326	δCH_2 bending	Aliphatics, cellulose, lignin (Edwards et al. 1997)
1382	δCH_2 bending	Aliphatics (Yu et al. 2007)
1440	$\delta(CH_2) + \delta(CH_3)$	Aliphatics (Yu et al. 2007)
1525	–C=C– (in plane)	Carotenoids (Devitt et al. 2018; Adar 2017)
1601–1630	ν(C–C) Aromatic ring + σ(CH)	Lignin (Kang et al. 2016; Agarwal 2006)

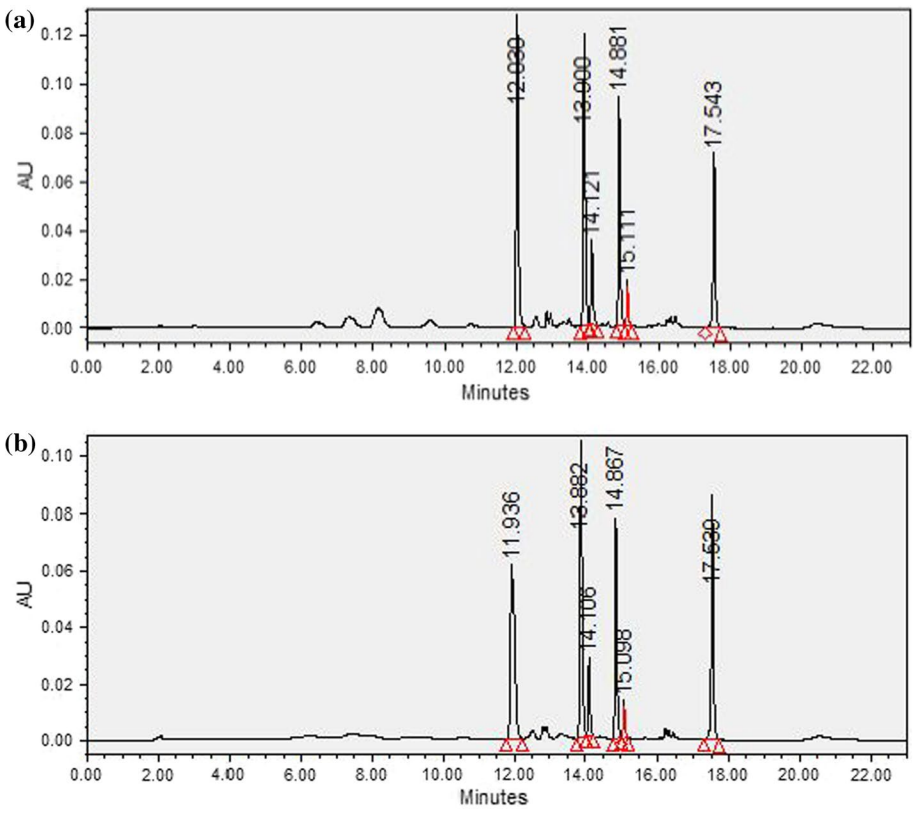

Fig. 2 HPLC profiles of leaves of mature female (**A**) and male (**B**) plants that show presence of LUT, CHR, LYC, BCR, ZEA and BCA in the extracted plant material. Retention times of these compounds are labeled

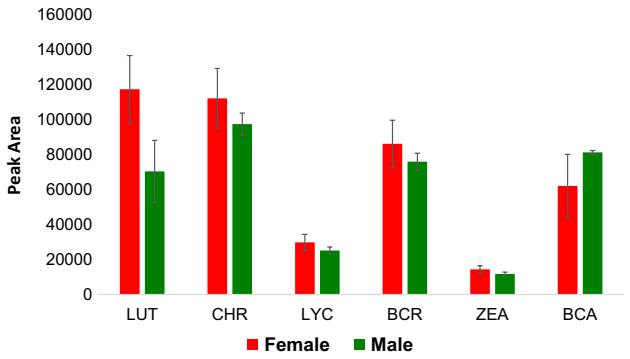

Fig. 3 Histogram of the peak areas of LUT, CHR, LYC, BCR, ZEA and BCA according to the HPLC profiles of mature male and female plants shown in the Fig. 2

as well as results reported by other groups, demonstrate that PLS-DA performs equally well or better than other chemometric methods, such as linear discriminant analysis (LDA) or soft independent modeling by class analogy (SIMCA) (Morey et al. 2020, 2021; Farber et al. 2021; Sanchez et al. 2020e; Mandrile et al. 2019). Consequently, we selected PLS-DA for statistical analysis of spectra collected in this work. The spectra were pre-processed by taking the second derivative of all intensity values (2nd polynomial order and a filter length of 15) and then centered on the mean and median. A true positive rate (TPR) was reported for each category based on the accuracy rate of the predictions.

Table 2 Confusion table for spectra collected from young male and female plants

	Number of spectra	TPR (%)	Predicted as male	Predicted as female
Male	33	90.6	31	2
Female	67	89.7	1	66

Results and discussion

Raman spectra collected from young male and female plants exhibit vibrational bands that can be assigned to carotenoids (1000, 1115–1218 and 1525 cm^{-1}), aromatic compounds (1601–1630 cm^{-1}), cellulose (747, 915 and 1047 cm^{-1}), as well as aliphatic vibrations that cannot be assigned to any specific classes of biological molecules (1288–1440 cm^{-1}) (Fig. 1 and Table 2). We found that intensities of carotenoid vibrations in the spectra collected from female plants were higher the in the spectra collected form male plants. We also found that intensities of vibrational bands that can be assigned to cellulose (747, 915 and 1047 cm^{-1}), as well as intensities of

aliphatic vibrations (1288–1440 cm^{-1}) are slightly higher in the spectra collected from female than male plants.

These findings suggest that concentrations of carotenoids in male and female hemp plants are different. To examine this difference, we extracted carotenoids using previously reported protocol and analyzed their concentrations by HPLC. Chromatographic analysis of hemp leaves revealed presence of lutein (LUT), chlorophyll (CHR), lycopene (LYC), β-cryptoxanthin (BCR), zeaxanthin (ZEA) and β-carotene (BCA) (Fig. 2).

We measured peak area of these six identified carotenoids in the acquired HPLC profiles (Fig. 3). Our results show that young male and female plants have very similar concentrations of LYC, BCR and ZEA. Differences between the concentrations of CHR and BCA were found to be insignificant. However, we found a significant difference between the concentration of lutein in young male and female plants (Table S1).

In our previous study, we found that CHR is highly fluorescent and, therefore, cannot contribute the Raman spectra of plants[27]. We have also found that vibrational frequency of 1525 cm^{-1} band in BCA is shifted to 1515 cm^{-1}. Therefore, BCA is unlikely to contribute to the observed changes in the intensity of carotenoid vibrations between male and female plants. However, the vibrational frequency of LUT was exactly at 1525 cm^{-1}.[27] Furthermore, changes in the concentration of lutein were associated with the spectroscopic fingerprint of citrus greening disease. Thus, we can conclude that different concentrations of lutein in young male and female plants is the underlying biochemical origin of spectroscopic differentiation between these plant species. This difference in the concentration of lutein in young plants may have far reaching implications in adult species. One may expect that higher concentration of lutein in female plants may be considered as a physiological preparation for the eventual greater levels of shading in female vs. male hemp plants.

Finally, we used chemometrics to investigate the accuracy of differentiation between young male and female plants. We build binary PLS-DA model that demonstrates 90.6% accurate identification of male and 89.7% accurate identification of female plants based on their Raman spectra (Table 2). We have also collected Raman spectra from the same groups of plants at the stage of flowering. Similar differences in the Raman spectra of male vs female plants were observed. Chemometric analysis of these spectra showed that sex of mature plants can be differentiated with ~ 94% accuracy (Table 3). These findings confirm that RS can be used to differentiate between male and female plants at different stages of their vegetation.

Table 3 Confusion table for spectra collected from mature male and female plants

	Number of spectra	TPR (%)	Predicted as male	Predicted as female
Male	37	94.3	33	4
Female	68	94.3	2	66

Conclusion

Our experimental results show that RS can be used for a label-free, non-invasive and non-destructive differentiation between male and female hemp plants prior to their flowering. This differentiation is based on differences in their biochemical profiles. Specifically, we found that female plants have significantly higher concentrations of lutein, a physiologically important carotenoid. If coupled to chemometrics, RS enables ~ 90% accurate differentiation between young and ~ 94% accurate differentiation between mature male and female hemp plants. A portable nature of this analytical approach, as well as intrinsic sensitivity of RS towards cannabinoid consent in hemp, opens up new avenues for the use of RS directly in farms to control and monitor hemp production. Validation across greater numbers of diverse genotypes is warranted in the future in order to assess potential breadth of the proposed RS application.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00425-022-03865-8.

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Data availability The data will be made available on reasonable request to the corresponding author.

Declarations

Conflict of interest All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

Author contribution statement S.H. collected plant material, performed Raman and HPLC analysis, performed chemometric analysis of data, wrote the manuscript; R.J. grown plants, performed visual analysis of plants, wrote the manuscript. D.K; supervised the work, wrote the manuscript.

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