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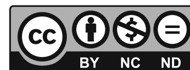
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APPLICATION OF VIBRATIONAL SPECTROSCOPY FOR PLANT TISSUE ANALYSIS – CASE STUDY

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Key words: FT-Raman spectroscopy; specific leaf weight; spectra decomposition; phenolic compounds, carotenoids.

Abstract

Raman spectroscopy is a particularly advantageous method in plant biology, allowing simultaneous examination of various compounds and evaluation of molecular changes in plant tissues subjected to different stress factors. The purpose of our research was to investigate to what extent the differences in the physical properties of leaves of *Alnus viridis*, *Hieracium bifidum* and *Platycerium bifurcatum* allow us to reliably determine qualitative and quantitative changes in their chemical composition. We proved that if we employed the FT-Raman spectroscopy method direct comparison of the obtained results might be difficult or even impossible. Normalization of the spectra in some situations may help in the results interpretation. However, to study the global impact of the stress factors on the tissue we suggest preparing a tablet obtained from lyophilized and powdered leaves, that avoids the inhomogeneity of the sample. Additionally, the decomposition procedure of the overlapped peaks is necessary to obtain reliable quantitative results.

Introduction

There are a great deal of scientific reports concerning the analytical determination of pigments and biologically active substances such as phenolic compounds (BABARINDE and ADEOLA 2023, BOUYAHIA et al. 2022). One of the very convenient method of their analysis is Raman spectroscopy which is a very widespread technique in plant biology. Research includes

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algae, cyanobacteria and higher plants (Gierlinger and Schwanninger 2007, Kula et. al.2016, Kula et. al. 2014, Prats-Mateu et. al. 2018, Stawoska et. al. 2021, ZEISE et al. 2018). In case of the latter ones, both seeds (Labanowska et. al. 2016, Skoczowski et al. 2011, Troc et al. 2009), leaves and fruits (Boyaci et al. 2015, Chylinska et. al. 2014, Stawoska et al. 2023) have been examined. This method has gained recognition, among others, due to the fact that it is non-invasive and does not require prior preparation of samples for the measurement. In the vast majority of cases, experiments are carried out directly on plant tissue. Raman spectroscopy has found a lot of applications – from simple measurements, allowing the determination of the occurrence of a particular type of compounds in a living organism (qualitative analysis), to a quantitative approach to the problem (Payne and Kurouski 2021, Schulz 2014). It has been proved that the discussed spectroscopic method allows the detection of substances responsible for the processes of photosynthesis (e.g. chlorophylls), photoprotection or mitigation of the effects of oxidative stress, especially carotenoids and phenolic compounds (Schulz and Baranska 2007). Raman spectroscopy is often used to look for and explain differences in the chemical composition of plant tissues, caused by various types of stresses (Payne and Kurouski 2021). Among others, the effects of abiotic stress, such as drought (Altangerel et al. 2017, Rys et al. 2015), herbicides (Saja et al. 2016) and wounding (Lukaszuk et al. 2017) are examined. Many experiments are also performed on the leaves of plants subjected to biotic stress (Rys et al. 2014). Furthermore, by performing the mathematical analysis (e.g. decomposition) of the spectra or their selected bands, it is possible to analyze e.g. the secondary structure of proteins in the tested samples (Stawoska et al.2020, Stawoska et al. 2009). Some scientists, however, consider the advantages of the technique overestimated and the results obtained in this way as not always reliable (Dong and Zhao 2017). There are also other non-invasive and quick methods of direct analyses of photosynthetic pigments (Cerovic et al. 2012). However, Raman spectroscopy is not suitable only for chlorophylls and flavonoids, but also for other biochemical components such as polysaccharides, fatty acids and proteins (Czamara et al. 2015, Farber et al. 2019, Saletnik et al. 2021, Stawoska et al. 2020, Talik et al. 2020).

On the other hand, as with other experimental methods, this one also has its limitations. The fundamental phenomenon seems to be fluorescence. Some samples excited by visible radiation can induce electronic transitions, which in turn cause autofluorescence. In this way, the so-called fluorescent background makes the identification of Raman bands difficult or even impossible. The laser as a light source, introduced in the 1960s,

definitely contributed to the increased popularity of the method. Lasers emitting UV, Vis or NIR (with wavelengths up to 830 nm) light are usually used in dispersive Raman spectrometers. When lasers with wavelengths of 532 nm and 785 nm are used, the fluorescence signal is so strong that it often obscures the actual Raman signals of the substance. Therefore, a special solution was developed – a spectrometer with a laser with a wavelength of 1064 nm. Reducing the energy of laser radiation allowed the fluorescence signal to be reduced to such an extent that it was possible to identify substances whose spectra previously obtained with lasers were completely unreadable.

Although using the NIR excitation wavelength in FT-Raman is more suited compared to lower wavelength excitation, one can be still aware that autofluorescence is not eliminated at all and still remains a nuisance.

The next problem concerning the application of the FT-Raman spectroscopy method on a larger scale in plant research, however, is often the type of tissue used to measure. As mentioned earlier, the preparation of the material for analysis is not demanding. Thus, a quick analysis of the chemical composition may be easily performed. However, the natural environment plant growth conditions (e.g. light intensity and its spectral composition, various temperature condition, wind) strongly influence, among others, the structure and the physical properties of tissues (e.g. colour, leaf area and thickness). This, in turn, impacts the quality of the spectra obtained, and ultimately also the limitations (like lack of homogeneity of the tissues, possible problems with repeatability or changes in the presence of other compounds that may disturb the Raman spectra), should be taken into account during their analysis (ALTANGEREL et al. 2017, BARANSKA et al. 2006, BAUER 2018, DONG and ZHAO 2017, KRIMMER et al. 2019). As we have observed, also the specific leaf weight (SLW) could be essential for the accuracy of the quantitative analysis. SLW is a physical parameter that affects the registered signal intensity. If plants of the same species grow in different thermal and light conditions in the field (under either shade or direct sunlight), they will have significantly different SLW. As a consequence, detected variations in spectral intensities are likely to be derived from different light absorption and scattering of tested leaves. It should be also pointed out that physical factors influencing the morphology of the leaves affect their chemical composition. Hence, the obtained results cannot be interpreted based on "raw data" and direct comparison of the obtained Raman spectra may lead to erroneous results. Solving this issue seems to be crucial also because scientists use the results obtained by Raman spectroscopy for chemometric analyses (cluster analysis) in which the intensity of the obtained signal is the most important (RYS et al. 2020).

The purpose of our work was to investigate to what extent the differences in the physical properties (namely SLW parameter) of leaves of three different plant species (*Alnus viridis*, *Hieracium bifidum*, *Platycerium bifurcatum*) allow to reliably determine not only qualitative but also quantitative changes in their chemical composition, using FT-Raman spectroscopy. In our research, we used leaves of plants belonging to different species, (a shrub, an herbaceous plant and a tropical fern) to propose an unambiguous and constant way of the preparation of the plant material, the spectra analysis and the interpretation of results.

Experimental

Materials and methods

Materials

The research was conducted for three plant species: *H. bifidum*, *A. viridis* and *P. bifurcatum*. To carry out the described experiments, leaves of the same plant species but with significantly different SLW parameter values were used. The differentiation of SLW values resulted from different plant growth conditions in the natural environment. Abiotic factors causing differences in the morphological structure of the leaves were strong sunlight, high temperature fluctuations of the day/night or differences in the intensity of light combined with the change of spectral composition. Naturally growing species, *H. bifidum* and *A. viridis*, were harvested from both shade or direct sunlight places. At least 10–20 leaves were collected from each growth place and 13 mm diameter roundels were cut out from them.

The nest leaves of *P. bifurcatum* (grew in a greenhouse of University of the National Education Commission, Kraków) were used as an experimental model possessing leaves with differential SLW parameter values (starting from the base to the apex of the leaf blade). The assay was performed for 2 morphologically different leaf parts. At least 4 leaves were collected from each growth places and again 13 mm diameter roundels were cut out from of them.

None of the tested plant specimens has been deposited in a publicly available herbarium. Detailed information is given below.

Alnus viridis. The plant specimens of *A. viridis* were obtained from a wild population located in the Bieszczady National Park. They were collected after obtaining permission from the park management (the topic of

the research no. 19/11, the license no. 48/12). Formal identification of *A. viridis* was done by Associate Professor Krzysztof Oklejewicz (Chair of the Botanic Department, University of Rzeszów). Green alder leaves – *A. viridis* came from positions located at various heights above sea level. The first harvest locality was at the top of Połonina Wetlińska (1215 m a.s.l.). The alder that existed here was a single shrub, growing in an open, well-lit area, on a ridge. The shrub was exposed to naturally changing weather conditions (strong sunlight and wind, large amplitude of daily temperatures) and was characterized by higher values of specific leaf weight (SLW – the method of determination is described below in the Methods section). The second locality was in post-agricultural areas, near the village Łobozew (568 m a.s.l.), in a location that was naturally a "protective umbrella" for the plant against strong sunlight, intense wind, or sudden rainfall. Shrubs in this locality were characterized by morphologically different leaves from those growing in Połonina Wetlińska and showed significantly lower values of SLW [42].

Hieracium bifidum. The leaves of *H. bifidum* were obtained from plants growing in their natural environment, in the Nature Reserve "Sokole Góry", in the Kraków – Częstochowa Upland. This is an area under partial protection, and the places where the research was carried out do not require any additional permission to conduct scientific investigations. Formal identification of *H. bifidum* was done by Professor Zbigniew Szeląg (ORCID ID: 0000-0002-7017-2823). Plants that grow in two different localizations were selected for experiments according to the conditions of full sunlight (plants from the southern wall of the rock, HL-plants, with higher value of SLW) and to the conditions of limited access of light (the shaded part, between the trees, LL-plants, with lower value of SLW).

Platycerium bifurcatum. The studies were performed using leaves of a six-year-old staghorn fern *(P. bifurcatum*). The plant specimens of *P. bifurcatum* came from the collection of the Institute of Biology and Earth Sciences, University of the National Education Commission, Kraków. They have been cultivated for years and are constantly available for research purposes. Formal identification of *P. bifurcatum* was done by Professor Andrzej Skoczowski (ORCID ID: 0000-0003-0334-9358). Plants were grown under a natural photoperiod in a greenhouse. Sporophyte has two types of leaves: the nest and the sporotrophophyll leaves. The nest leaves attach the plant to the trunk of the tree and collect minerals and water. Sporotrophophyll leaves are responsible for both assimilation and reproduction since they produce sporangia with spores. The Raman spectroscopic analyses were conducted using selected parts of blades of nest

leaves, namely the apex (with lower SLW values) and the base parts (with higher values of SLW).

Reagents and Solvents. Na_2CO_3 (p.p.a.), acetone (p.p.a.), methanol (p.p.a.), glacial acetic acid (p.p.a.) – Avantor Performance Materials Poland S.A., trans – ferulic acid, Folin and Ciocalteu's reagent – Sigma-Aldrich.

Methods. SLW Parameters Calculation

13 mm diameter roundels were cut out from the leaf blade of each of the tested plants (or its fragment – for *P. bifurcatum*), using a cork borer. Their area (a) was considered and then after the lyophilization, they were weighted, and their mass values (m) averaged. Based on the equation (1), the average density of the prepared roundels (Specific Leaf Weight) was calculated.

$$\mathrm{SLW} = \frac{m}{a} \quad [\mathrm{g\ dm^{-2}}] \tag{1}$$

where m means mass of the lyophilized roundels, a means area of the lyophilized roundels.

Preparation of tablets. The lyophilized leaves (160 mg) were grinded in a mortar. Next, the tablet was made using the equipment ABL&E-JASCO Polska Sp. Z o.o. (the diameter 13 mm, the pressure 200 atm). The prepared tablets were stocked in an exsiccator till the time of measurement.

Fourier transform raman spectroscopy and curve fitting. FT-Raman measurements were performed on dry leaves (or tablets obtained from lyophilized and powdered leaves of the same investigated plants) of *A. viridis*, *H. bifurcatum* and *P. bifurcatum* using a Nicolet NXR 9650 FT-Raman spectrometer equipped with a $Nd{:}YAG^{3+}$ laser, emitting a beam at 1064 nm wavelength, and a germanium detector cooled by liquid nitrogen. The measurements were performed at room temperature, an aperture of 80 and a spectral resolution of 4 cm^{-1}. Spectra were recorded with a laser power of 0.4 W (it was monitored if it did not injure studied samples) and analyzed in the range 700–1800 cm^{-1}. Sixty-four scans were performed for each spectrum and each measurement was done in 4 to 8 repeats. The analysis of spectra was carried out using Omnic 8 and OriginPro 2017.

To determine the real contents of carotenoids and phenolic compounds, the band localized at 1480–1670 cm^{-1} was decomposed using PeakFit 4.12. The analysis started with a baseline correction that used a linear function.

In the next step, a second derivative of each measured spectrum was obtained, in order to find the number of components that built the band as well as their localization. Finally, a mathematical algorithm, employing Gaussian and Lorentzian functions was used, to iteratively estimate parameters using the method of least squares. In this method, the areas of selected peaks correspond to chemical vibrations that correlate with specified types of chemical moieties. For each obtained decomposition, the correlation coefficient was higher than 0.9986.

The biochemical assays of the total content of carotenoids, chlorophylls and phenolic compounds. The total content of carotenoids (and chlorophylls) in *P. bifurcatum* and *H. bifidum* was estimated according to the previously described (LICHTENTHALER 1987) and slightly modified procedure. In order to assess the quantity of these pigments, the extraction procedure was performed with the mixture of acetone: water (v/v: 98/2). The lyophilized plant sample (20 mg) was grinded in a mortar and the solution (1 mL) was added. The extraction was supported by ultrasonication for a higher yield (6 min, in an ice-water bath). In the next step the supernatant was collected, centrifuged (10 min, 3000 × g), and then decanted. The absorption spectra of the extract were recorded (3 repetitions) in the same solution, acetone: water (v/v: 98/2), in 1 cm path length quartz cuvette, in the spectral region 350–900 nm, with 0.2 nm precision, using spectrophotometer UV-VIS Analytic Jena, Germany. The absorption values were read at 470 nm, 644.8 nm and 661.6 nm and on the basis of their values, the chemical contents of chlorophyll a, C_a, chlorophyll b, C_b, and carotenoids, C_x, were estimated, according to the equations (2–4):

$$C_a = 11.24 \cdot A_{661.6} - 2.04 \cdot A_{644.8}, \quad (2)$$

$$C_b = 20.13 \cdot A_{644.8} - 4.19 \cdot A_{661.6}, \quad (3)$$

$$C_{x+c} = (1000 \cdot A_{470} - 1.90 \cdot C_a - 63.14 \cdot C_b)/214. \quad (4)$$

The chlorophylls contents, Cc, were estimated only in order to calculate the total carotenoids content according to the equations given above. Therefore, the changes in the chlorophylls contents were not discussed in this manuscript.

All the preparation and measurements steps were done under the dim light because of the extremely high light sensitivity of the pigments. The quantitative assay was carried out immediately after the preparation of the extract.

The total content of phenols was estimated via the spectrophotometric method as well, using the Folin – Ciocalteu reagent. In the first step, the liquid extraction supported with ultrasonication was conducted. The lyophilized material (20 mg) was grinded in a mortar and then treated with

1 mL of the solution glacial acetic acid: methanol (v/v: 1/99). The mixture was closed under the nitrogen and then it was ultrasonicated within 6 min in an ice-water bath. Then the supernatant was decanted and centrifuged (10 min, 3000 × g). Subsequently, the extract (20 µL) was taken and introduced to the test-tube and then water (1.58 mL) and Folin – Ciocalteu reagent were added (100 µL). After 5 min the saturated solution of Na_2CO_3 (300 µL) was added. The reaction mixture was incubated under the dim light for 30 min in 40°C. In final step, the absorbance measurements were carried out (3 repetitions) at 760 nm according to the blank probe using the spectrophotometer mentioned above. On the basis of the previously determined calibration curve for ferulic acid, the total content of phenols was estimated in the analyzed plant sample. During the extraction and other stages of the assay, the sample was protected against light and oxygen.

Results and Discussion

The FT-Raman spectroscopy measurements were carried out using the above-mentioned plant species. The variety of forms and species (shrub and herbaceous plants – both of them gathered in their natural habitat, epiphytic tropical fern) were selected so that within each of them it was possible to have plant material of different SLW. The measurements were performed on lyophilized intact leaf blades possessing different SLW values and on tablets obtained from lyophilized and powdered leaves in order to develop a uniform analytical procedure.

Additionally, in order to verify the accuracy of FT-Raman spectroscopic analyses, the biochemical determinations of the total contents of carotenoids and phenols were carried out using the leaves of two selected plant species: *H. bifidum*, representing a plant living in the natural environment, and *P. bifurcatum* grown in greenhouse conditions. For *A. viridis* it was not possible to perform biochemical analyses because of the further unavailability of the plant in its natural conditions, in places where field research was originally carried out.

A. viridis leaves deriving from the locality at an altitude of 568 m a.s.l. (post-agricultural areas, near the village Łobozew – a shadow locality sheltered from the wind) were thin and fragile with SLW = 0.57 g dm^{-2}, Table 1. Oppositely, leaves collected at an altitude of 1215 m a.s.l. (grassland Wetlina, a locality exposed to low temperature, strong wind and intensive sunlight) were smaller and their SLW was significantly higher and amounted to 1.43 g dm^{-2}. In turn, in the case of *H. bifidum* light intensity and spec-

tral composition strongly influenced leaf morphology. The plants growing in high light (not sheltered rock, HL-plants) developed leaves with SLW = 0.88 g dm^{-2}. At the same time, leaves from plants collected from shady localities (localities in the forest, LL-plants) were characterized by significantly smaller SLW equaled 0.40 g dm^{-2}, Table 1.

The nest leaves of *P. bifurcatum* were another tested object. Plants grew under constant light and temperature conditions, therefore the living circumstances were standardized for each specimen. The fact of different SLW values of the base and apex of the green nest leaf is in this case a consequence of the typical differentiation of morphological structure of the leaf blade. SLW for base and apex of the leaf equal 0.59 and 0.20 g dm^{-2}, respectively, Table 1.

Table 1

Specific leaf weight (SLW) parameters (±SD) of *Alnus viridis*, *Hieracium bifidum* and *Platycerium bifurcatum*

Locality/specimen	Specific Leaf Weight (SLW) [g·dm^{-2}]
Alnus viridis	
568 m a.s.l (Łobozew village – LL)	0.57 ±0.11
1215 m a.s.l (grassland Wetlina – HL)	1.43 ±0.13
Hieracium bifidum	
LL-plants	0.29 ±0.04
HL-plants	0.40 ±0.08
Platycerium bifurcatum	
Base of the leaf	0.59 ±0.16
Apex of the leaf	0.20 ±0.04

Explanations: LL-plants – plants with limited access of light; HL-plants – plants that grow in full sunlight

Measurements carried out using FT-Raman spectroscopy on the leaves of three plant species mentioned above allowed the vibration characteristics and distinction of the most important groups of chemical compounds present in all tested leaves, Figure 1 and Table 2. The spectra showed intensive bands typical for carotenoids, observed at frequencies 1005, 1158 and 1525 cm^{-1}, the so-called carotenoid triplet (SCHULZ 2014, VITEK et al. 2017). Each time, the band with a maximum at 1525 cm^{-1} was the most intensive. This band derives from C = C stretching of polyene chain, what also indicates the number of conjugated double bonds in these structures. The obtained values suggest that the main carotenoids present in the tested specimens were lutein, zeaxanthin and β-carotene, all having 9 conjugated double bonds in the molecules (BARANSKI et al. 2005, SCHULZ et al. 2005).

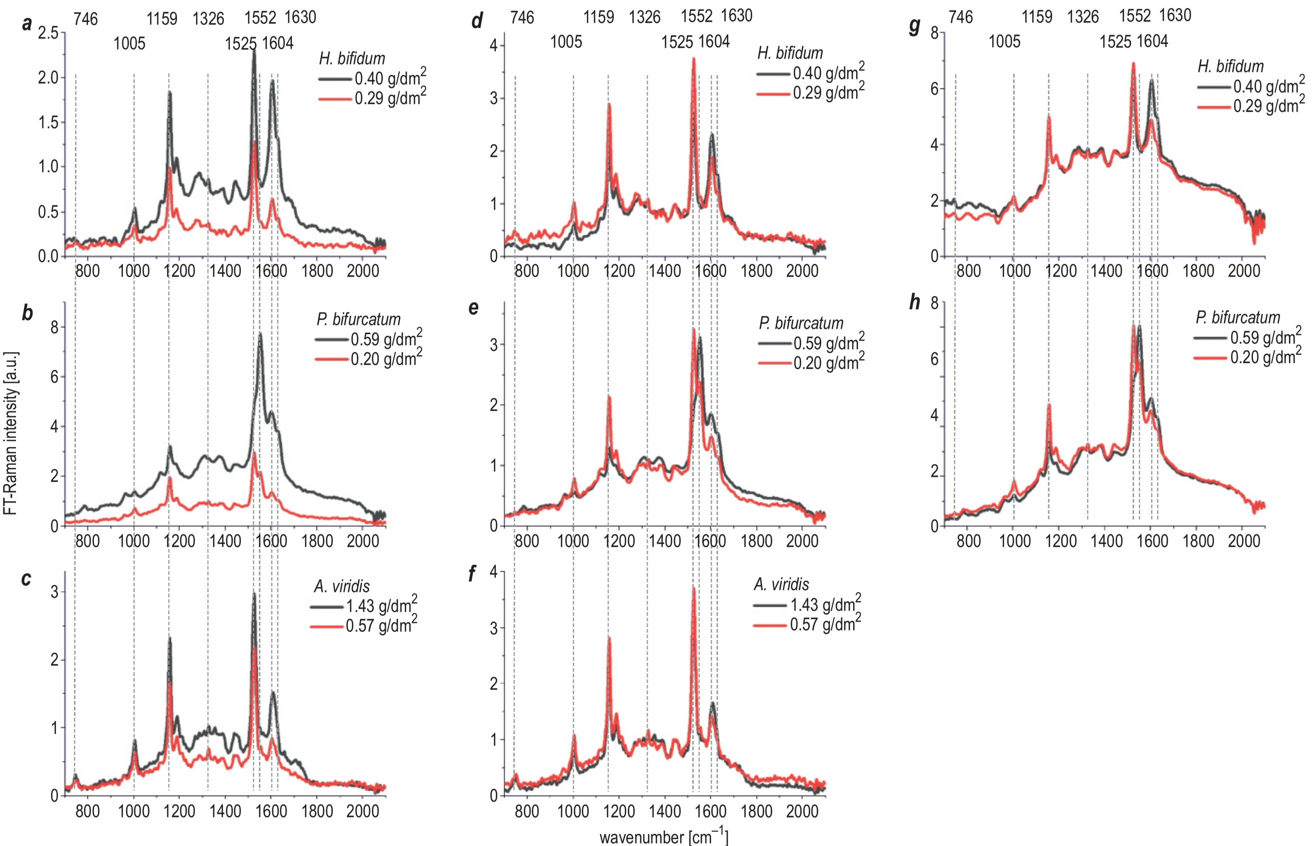

Fig. 1. Spectra of leaves and spectra of tablets: *a*, *b*, *c* – raw FT-Raman spectra obtained for lyophilized leaves of *Hieracium bifidum*, *Platycerium bifurcatum* and *Alnus viridis*, respectively; *d*, *e*, *f* – spectra after normalization according to 1444 cm^{-1}; *g*, *h* – spectra recorded using tablets prepared from lyophilized and powdered leaves of *Hieracium bifidum* and *Platycerium bifurcatum*, respectively. Presented results are the mean values from 4–8 replications

Table 2
Functional groups and vibrational modes obtained for the FT-Raman spectra of tested leaves

Peak number	Wavenumber [cm^{-1}]	Components	References
1	746	chlorophyll	(SCHRADER et al. 1999, VITEK et al., 2017)
2	1005	carotenoid (tetraterpenes), δ($C\text{-}CH_3$)	(BARANSKA et al. 2005, SCHULZ 2014, VITEK et al. 2017)
3	1159	carotenoids (tetraterpenes) ν(C-C)	(SCHRADER et al. 1999, SCHULZ 2014, VITEK et al. 2017)
4	1189	chlorophyll ν(CC), γ(CH), polyphenols δ(CH)	(ANDREEV et al. 2001, VITEK et al. 2017)
5	1284	poliphenols δ(OH), chlorophyll – δ(CH), ν(CN)	(ERAVUCHIRA et al. 2012, VITEK et al. 2017)
6	1326	chlorophyll – pyrrole ring vibrations, δ(CH), ν(CN)	(BARANSKI et al. 2005, SCHRADER et al. 1998, 1999, VITEK et al. 2017)
7	1525	carotenoids (tetraterpenes) ν(C=C)	(BARANSKI et al. 2005, SCHULZ 2014, SCHULZ and BARANSKA 2007, SCHULZ et al. 2005, VITEK et al. 2017)
8	1552	chlorophyll – central 16-membered ring vibrations, pyrrole ring vibrations, phenolic compounds	(HEREDIA-GUERRERO et al. 2014, PASCAL et al. 2000, SATO et al. 1995, VITEK et al. 2017, ZENG et al., 2021)
9,10	1602, 1630	polyphenols (ν(C-C), ν(C=C) stretching ring)	(HEREDIA-GUERRERO et al. 2014, SCHRADER et al. 1999, SCHULZ and BARANSKA 2007, VITEK et al. 2017)

It should be noted that the band at 1159 cm^{-1}, associated with strong stretching C-C vibrations of the polyene chain arises from the imposition with another, weaker band resulting from deformation vibrations δ (CH) in phenyl rings (from the phenolic compounds) (VITEK et al. 2017). Additionally, at frequencies 746, 1284, 1326 and 1552 cm^{-1} vibrations characteristic of chlorophyll molecules are identified. Furthermore, perhaps the band at 1552 cm^{-1} origins from the superposition of not only chlorophylls but also phenolic compounds (HEREDIA-GUERRERO et al. 2014). In turn, peaks recorded in the range 1250–1400 cm^{-1} indicate the presence of stretching and deformation vibrations specific to the $-CH$, $-CH_2$ and $-CH_3$

groups building chains of fatty compounds (Muik et al. 2005, Schulz and Baranska 2007, Thygesen 2003). In all the spectra, the bands at 1602 (phenyl-ring stretching mode) and 1630 cm^{-1} (C=C stretching vibration) are also clearly marked, what again confirms the presence of phenolic compounds in the examined plant tissues (Vitek et al. 2017).

Characteristic bands derived from carotenoids, chlorophylls and phenolic compounds (as well as from lipids and fatty acids – not discussed in this work) are visible in Raman spectra recorded directly on the leaves of various plant species, regardless of SLW values. However, SLW values determine Raman signal strength. The greater SLW of the leaf is, the more intensive signal is recorded, Figures 1*a*, *b* and *c*. Therefore, the quantitative comparison of the raw results may not be possible.

Striving to elaborate a reliable procedure for quantitative analysis of the obtained results, the spectra were normalized to the most typical band present in each one at 1444 cm^{-1} (related to C-H vibrations originating most likely from the CH_3, CH_2, and CH functional groups in lipids, amino acid side chains of the proteins, and carbohydrates) (Farber et al. 2001), Figures 1*d*, *e* and *f*. Following this mathematical operation, spectra of each specimen with different SLW values were found to be largely identical in terms of frequencies and intensities of selected vibrational bands. However, large differences one can find for the bands assigned to carotenoids (1005, 1159, 1525 cm^{-1}) and phenolic compounds (1602, 1630 cm^{-1}). Alternative solution to the problem regarding samples with different SLW was also proposed, namely spectra registered on the previously prepared tablets, Figures 1*g* and *h*. This strategy may also be applied if it is not possible to directly register the spectrum on the plant tissue originating e.g. from shredded herbarium materials. Such an approach can also be an additional method allowing verification of the obtained results. However, one should keep in mind that this requires a time-consuming preparation procedure and leads to destruction of the plant tissue.

Considering the fact that, as it has already been indicated, some bands are the sum of vibrations coming from different components, we decided to decompose the band (registered both for lyophilized leaves – and tablets obtained from lyophilized and powdered leaves of the same plants) in the selected range, precisely 1480–1670 cm^{-1}, Figures 2–4. Peaks coming from C=C (1525 cm^{-1}) vibrations of carotenoid chains and those characteristic of the structure of chlorophylls or phenolic compounds are observed herein. Characteristic bands were selected and the change in peak intensity was analyzed to confirm possible differences in plant tissues (e.g. resulting from different environmental growth conditions). The intensity ratios of selected bands at 1602 to 1525 cm^{-1} (I_{phen}/I_{car}) were compared, Table 3.

Table 3

The Raman intensity ratio (I_{phen}/I_{car}) of peaks belonging to phenols (1602 cm^{-1}) and to carotenoids (1525 cm^{-1}), respectively, obtained after decomposition of FT-Raman bands recorded for *Platycerium bifurcatum*, *Hieracium bifidum* and *Alnus viridis*

	Platycerium bifurcatum			
	SLW = 0.59 [g dm^{-2}]		SLW = 0.20 [g dm^{-2}]	
	leaf	tablet	leaf	tablet
I_{phen}/I_{car}	1.19 ±0.10	0.94 ±0.08	0.30 ±0.03	0.36 ±0.10
	Hieracium bifidum			
	SLW = 0.40 [g dm^{-2}]		SLW = 0.29 [g dm^{-2}]	
	leaf	tablet	leaf	tablet
I_{phen}/I_{car}	0.77 ±0.15	1.01 ±0.03	0.39 ±0.05	0.43 ±0.01
	Alnus viridis			
	SLW = 1.43 [g dm^{-2}]		SLW = 0.57 [g dm^{-2}]	
	leaf		leaf	
I_{phen}/I_{car}	0.36±0.04		0.20±0.04	

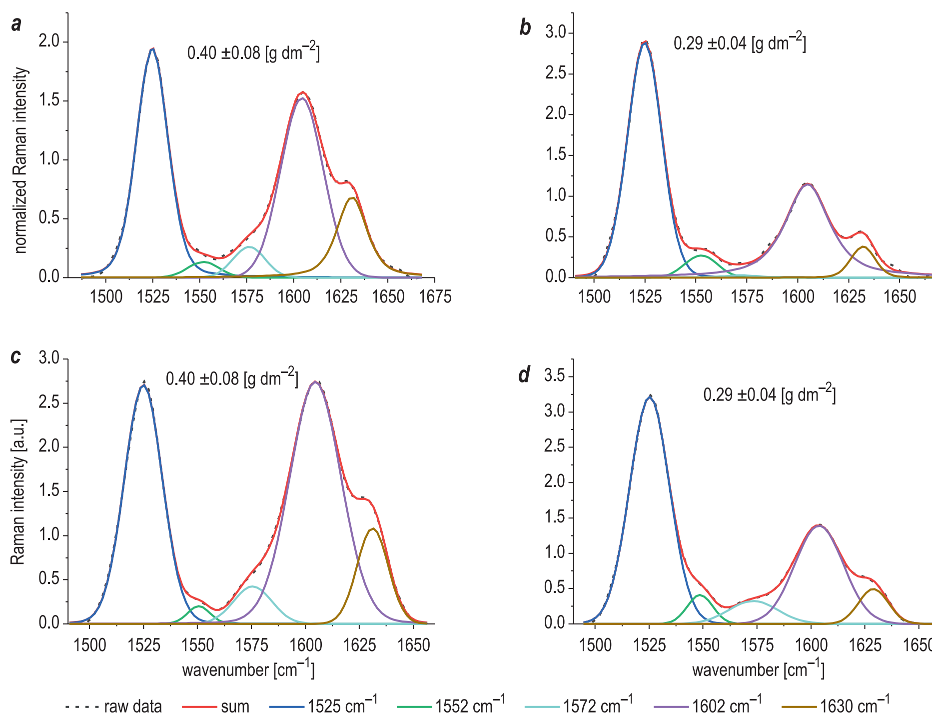

Fig. 2. Decomposition of the recorded FT-Raman spectra of *Hieracium bifidum* performed in the region 1480–1670 cm^{-1}, revealing the quantitative relationships of the phenolic compounds and carotenoids depending on the SLW parameters values: *a*, *b* – decomposition of spectra recorded using leaves with different SLW (values pointed on the pictures); *c*, *d* – decomposition of spectra recorded using tablets prepared from leaves with different SLW (values pointed on the pictures)

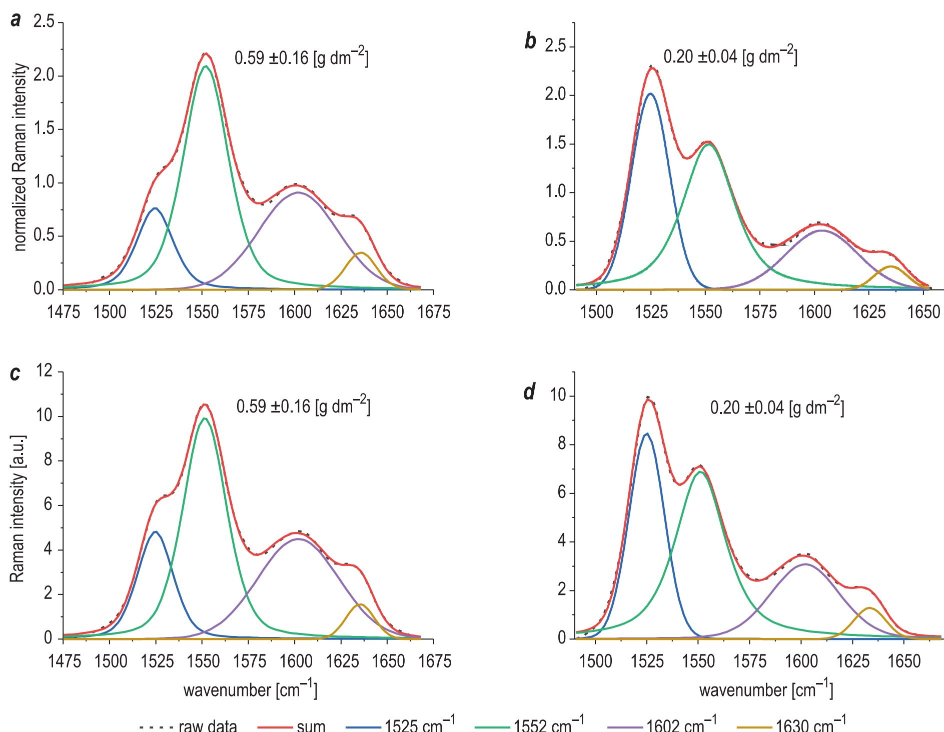

Fig. 3. Decomposition of the recorded FT-Raman spectra of *Platycerium bifurcatum* performed in the region 1480–1670 cm^{-1}, revealing the quantitative relationships of the phenolic compounds and carotenoids depending on the SLW parameters values: *a*, *b* – decomposition of spectra recorded using leaves with different SLW (values pointed on the pictures); *c*, *d* – decomposition of spectra recorded using tablets prepared from leaves with different SLW (values pointed on the pictures)

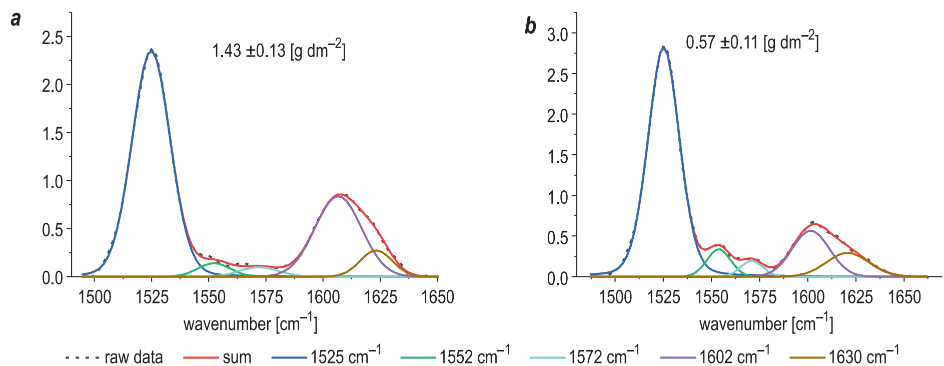

Fig. 4. Decomposition of FT-Raman spectra recorded for leaves of *Alnus viridis* performed in the region 1480–1670 cm^{-1}, revealing the quantitative relationships of the phenolic compounds and carotenoids depending on the SLW parameters values; *a*, *b* – decomposition of spectra recorded using leaves with different SLW (values pointed on the pictures)

This relationship between phenols and carotenoids was analyzed to illustrate quantitative changes that appear under stress factors (light, temperature, wind) affecting the plants. Both carotenoids and phenolic compounds are chemical substances possessing protective activity against stress factors and their contents in plant tissue is strictly connected with external features (QUIDEAU et al. 2011, STRZALKA et al. 2003, TANASE et al. 2019, WEBER et al. 2019).

The obtained results show that the ratios of the intensities of bands at 1602 to 1525 cm^{-1} (I_{phen} /I_{car}) in the spectra of leaves and in the spectra recorded for tablets obtained from powdered leaves of the same investigated plants are consistent what confirms the correctness of the methodology (not only normalization but also the decomposition procedure in order to select the proper bands). Additionally, to verify the results obtained in the spectroscopic investigations, the biochemical analyses of the total phenols and carotenoids were carried out in the case of two tested species: *P. bifurcatum* and *H. bifidum*. The obtained results are set in Table 4. In the case of *P. bifurcatum* the ratios C_{phen}/C_{car} equal 94.85 and 268.18 for leaves characterized with SLW of 0.20 g dm^{-2} and 0.59 g dm^{-2}, respectively. The leaves possessing SLW of 0.20 g dm^{-2} contain more carotenoids and less phenolic compounds. In the case of *H. bifidum* the ratios C_{phen}/C_{car} equal 1184.55 and 3267.49 for the plants growing in low light and high light, respectively (SLW = 0.29 g dm^{-2} and SLW = 0.40 g dm^{-2}).

Table 4

Mean values (±SD) of the contents of phenols and carotenoids assayed for leaves

A	SLW = 0.40 [g dm^{-2}]		SLW = 0.29 [g dm^{-2}]	
	phenols	carotenoids	phenols	carotenoids
Mean values of content [mg/g d.w.]	70.66 ±0.52	0.02 ±0.00	61.95 ±9.01	0.05 ±0.01
C_{phen}/C_{car}	3267.86 ±266.27		1184.55 ±342.27	
B	SLW = 0.59 [g dm^{-2}]		SLW = 0.20 [g dm^{-2}]	
	phenols	carotenoids	phenols	carotenoids
Mean values of content [mg/g d.w.]	134.12 ±38.09	0.52 ±0.20	74.76 ±10.86	0.83 ±0.06
C_{phen}/C_{car}	268.18 ±15.66		94.85 ±15.64	

Explanations: the presented values were obtained for lyophilized leaves of *A* – *Hieracium bifidum* (SLW = 0.40 [g dm^{-2}] and SLW = 0.29 [g dm^{-2}]) and *B* – *Platycerium bifurcatum* (SLW = 0.59 [g dm^{-2}] and SLW = 0.20 [g dm^{-2}]), [mg/g d.w.] – mg of compound on 1 gram of dry weight of leaves; C_{phen}/C_{car} – the ratio of phenols to carotenoids contents. The standard deviations were calculated on the basis of data obtained in 6 series of assays for *B* and 3–4 series of assays for *A*

The leaves of HL-plants possess the lower content of carotenoids and the higher content of phenols in comparison to LL-plants. To conclude, in the case of HL-leaves (HL stress), the total content of phenols is higher whereas the total content of carotenoids is lower in comparison to LL-leaves (shadow conditions). This observation is in line with the results discussed in literature (ALTANGEREL et al. 2017). Shade grown leaves may possess more chlorophylls to increase light capture and less carotenoids and phenolic compounds because of the fact that photoprotection and antioxidant protection are not so demanding (ALTANGEREL et al. 2017, DEMMIGADAMS and ADAMS 1992, TUNGMUNNITHUM et al. 2018). ALTANGEREL et al. (2017) reports a crucial and relevant application of Raman spectroscopy in simultaneous and *in vivo* detection of polyphenols, and carotenoids being reactive oxygen–scavenging pigments. They confirmed the upregulation of phenolic compounds and degradation of carotenoids under abiotic stress (light, drought, salt, cold) occurrence. Additionally, we compared the ratio C_{phen}/C_{car} with the ratio I_{phen}/I_{car} estimated on the basis of the decomposition of FT-Raman spectra recorded directly on leaves and on tablets. Assuming 100% for the leaves with higher SLW parameters, the presented values are a percentage of the same values calculated for leaves of the same species but with lower SLW, Table 5.

We calibrated the FT-Raman method by quantifying carotenoids and phenolic compounds using classical biochemical methods. Therefore, we treat biochemical analyses as an indicator of the actual contents of phenolic and carotenoid compounds and their ratio showing the occurrence in the tissue. For both *H. bifidum* and *P. bifurcatum* the greater similarity of FT-Raman and biochemical results was observed considering the intensity of the peaks obtained by the decomposition of the complex bands registered for tablets (homogenous samples for which we do not observe the effect of SLW on the intensity of the measures Raman signal) instead of leaves, Table 5. This fact confirms the above statement that quantitative interpretation of the results is strongly influenced by tissue morphology. In the case of homogenous material (tablets), the intensities of Raman signals correlate well qualitatively and quantitatively with the ratio of phenols to carotenoids (C_{phen}/C_{car}) for the analyzed leaves, estimated on the basis of the biochemical assays. One should remember that for both tablets (used for FT-Raman measurements) and leaves (used for biochemical analyses) a large amount of plant tissues was employed. If, on the other hand, we make FT-Raman spectra on intact leaf blades, we detect the signal only from its small area. There is one more crucial aspect to be considered regarding the anatomy of the leaf blade. In various leaf blade layers, there is a differing concentration of particular biologically active

compounds (e.i. phenolic compounds are mainly present on the top cell layers of the epidermis whereas chlorophylls and carotenoids are very abundant in chlorenchyma – a deeper layer). As a consequence, we observe a better correlation of the biochemical results with those measured using a homogenous sample (tablet) since powdering the material abolishes these in-depth variations in composition. Therefore, it is the reason for the divergent results obtained between the discussed numerical values for leaves and biochemical determinations, Table 5. This discussed aspect is another limitation that should be considered.

Table 5

Ratios (±SD) of phenols to carotenoids contents estimated on the basis of FT-Raman spectra (leaves and tablets) decomposition (I_{phen}/I_{car}) and biochemical estimations (C_{phen}/C_{car})

Specification	Ratios (±SD) of phenols to carotenoids in [%]	
	Platycerium bifurcatum	*Hieracium bifidum*
I_{phen}/I_{car} (leaves)	25 ±5	51 ±16
I_{phen}/I_{car} (tablets)	38 ±14	42 ±2
C_{phen}/C_{car} (biochem.)	35 ±8	36 ±13

Explanations: the presented values were calculated for leaves with a lower SLW and are a percentage of the same values obtained for leaves with a higher SLW (100%)

In order to discuss the Raman spectra, many researchers use chemometric methods (e.g. cluster analysis), the purpose of which is to find significant and systematic differences in the recorded spectra (RYS et al., 2020). However, taking into account the fact of different SLW of leaves of the same species, cluster analysis should not be performed directly on the raw data obtained from FT-Raman measurements. First, the spectra should be averaged, normalized, and finally they might be further transformed (cluster analysis) (PAYNE and KUROUSKI 2021). Otherwise, we may run the risk of receiving erroneous results of the analyses performed.

In conclusion, it should be emphasized that the quantitative comparison of the results obtained for morphologically different leaves, with significantly different SLW values, is not possible.

Conclusions

The goal of our research was to investigate to what extent the differences in SLW of the leaves of the same species allow us to reliably determine qualitative and quantitative changes in their chemical composition, using FT-Raman spectroscopy. For the experiments, we used lyophilized leaves of three plant species (*A. viridis*, *H. bifidum*, *P. bifurcatum*).

Based on the obtained results, we proved that if we employed the FT-Raman spectroscopy method to study changes in the chemical composition of plant tissues subjected to stress factors, influencing the morphology of leaves, namely their SLW, direct comparison of the obtained results might be difficult or even impossible.

In such cases, it is suggested to normalize the spectra (which have already been presented and reported many times). It is necessary to remember that this mathematical calculation should be performed considering the one particular band that originates from vibrations, typical for all tested samples (e.g. aliphatic). The normalization procedure is necessary for chemometric analysis (cluster analysis), otherwise, the obtained results will not be reliable. Raman spectra performed on the leaf blade allow for the detection of the signal only from the small, specified area. Thus, if we want to study the global impact of stress on the tissue, the normalization procedure is not enough. The solution to this problem is preparing a homogeneous sample, e.g. in the form of a tablet obtained from lyophilized and powdered leaves. This procedure allows for the unification and standardization of samples and avoids the influence of SLW (in practice the thickness of the leaves) on the results of quantitative analysis. One should also take into account the overlap of peaks coming from vibrations characteristic for the analyzed chemical groups. In such a situation, normalization is not enough to obtain quantitative results. It is necessary to apply additional mathematical operation – decomposition, which allows to isolate the components of a given band responsible for the analyzed vibrations.

Summarizing, the application of FT-Raman spectroscopy in research into plants is indisputably essential and beneficial, however in case of inhomogeneous tissues particular limitations of this method should be considered and a special procedure of analyzing the spectra should be taken.

Conflict of interests. The authors declare no competing interests.

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