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Nondestructive Raman Analysis of Polyacetylenes in Apiaceae Vegetables

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ABSTRACT: Food plants from the Apiaceae family have been found to demonstrate health-promoting properties. Polyacetylenes are bioactive compounds that are considered to contribute substantially to the beneficial properties of Apiaceae plants. This study applied a Raman mapping technique in the investigation of polyacetylene spatial distribution in fresh roots of some Apiaceae species. Fresh root sections were measured directly without any preliminary preparation. For three Apiaceae species, that is, parsnip (*Pastinaca sativa* L.), celeriac (*Apium graveolens* var. *rapaceum* L.), and parsley (*Petroselinum crispum*), the presence of polyacetylenes was confirmed due to the detection of strong and well-separated Raman signals of symmetric −C≡C—C≡C− stretching vibration in the range of 2200−2300 cm^{-1}. The spectra were used for generation of two-dimensional maps applying the integration and cluster analysis methods. The Raman maps visualized the distribution of total polyacetylenes as well as individual compounds. Heterogeneous and tissue-specific occurrence of polyacetylenes in roots is shown.

KEYWORDS: falcarinol, falcarindiol, Raman mapping, in situ, cluster analysis, spectroscopy

INTRODUCTION

A high consumption of vegetables has beneficial effects on human health by protecting against certain types of cancer and other serious diseases.[1] The health-promoting properties of vegetables were primarily attributed to plant components such as vitamins, minerals, fibers, and antioxidants, but recently polyacetylenes have also received attention as important health-promoting compounds, and in particular those of the falcarinol type present in vegetables of the Apiaceae family.[2,3] Polyacetylenes of the falcarinol type are secondary metabolites that have demonstrated various bioactive properties and are not desired in human diet in high concentrations due to their toxic effects, but they are beneficial in low concentrations.[4] This phenomenon is called hormesis or the biphasic effect.

Naturally occurring polyacetylenes are biosynthesized by plants as important protective compounds against pathogens and pests. They are formed from unsaturated fatty acids and are built up from acetate and malonate units.[3] Falcarinol-type polyacetylenes (Figure 1), for instatnce, are synthesized from oleic acid.

Aliphatic C_{17}-polyacetylenes of the falcarinol type are widely distributed in the Apiaceae and Araliaceae, although they have also been detected in the Solanaceae family such as tomatoes and aubergines, in which they appear to act as phytoalexins.[5] However, polyacetylenes are present in plants in low concentrations.

Falcarinol and falcarindiol exhibit antifungal activity in many Apiaceae plant species.[6] Falcarinol-type polyacetylenes are present permanently in plants, although their concentration can increase during infections. Additionally, falcarinol and falcarindiol have shown anti-inflammatory and antiplatelet-aggregatory properties[7] as well as antibacterial effects in nontoxic concentrations.[8] Polyacetylenes are also known to be inhibitors of some enzymes.[9] Also, investigations on herbal drugs indicated that some extracts obtained from Apiaceae and Araliaceae plants have been found to be highly cytotoxic against numerous cancer cell lines in vitro, for example, human gastric adenocarcinoma.[10] Additionally, falcarinol was demonstrated to have in vivo anticancer effect in rats.[11] Falcarindiol has also been found to have cytotoxic and antimutagenic activities, although this compound seems to be less bioactive compared to falcarinol.[12]

Identification and quantification of bioactive polyacetylenes in food plants require chromatographic methods.[2,3,13,14] However, chromatographic methods do not allow study of polyacetylenes nondestructively or determination of the compound distribution at cellular level. These limitations can be overcome by the use of Raman spectroscopy, which has already been successfully applied for the investigation of various secondary metabolites[15−17] including polyacetylenes in various plants.[18−22] It can provide valuable information on the cellular distribution of polyacetylenes for producing cultivars and to the agricultural industry with regard to taste- and health-promoting effects.

The distribution of individual polyacetylenes investigated by Raman spectroscopy mapping has already been reported only in various cultivated and wild carrots.[18] In the presented work this approach has been applied to the study of other Apiaceae species containing polyacetylenes. The utilization of Raman spectroscopy provided additional information on the distribution of these compounds in intact root tissues as the measurements were nondestructive to plant material.[19,20]

MATERIALS AND METHODS

Plant Material. Three species of the Apiaceae family, parsnip (*Pastinaca sativa* L.), celeriac (*Apium graveolens* var. *rapaceum* L.), and

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parsley (*Petroselinum crispum* Mill. Nyman ex A.W. Hill), were used. The seeds were sown in soil, and the plants were grown in flat beds at the experimental station in Prusy near Krakow, Poland. The roots were harvested between 60 and 90 days of vegetation and gently washed to remove the soil directly before Raman measurements. To conform to the physical limitations of the spectrometer, only small roots were selected.

Raman Spectroscopy. Raman spectra from at least 10 roots of each species were recorded using a Bruker Multiram FT-Raman spectrometer (coupled with a Ramanscope III) equipped with a Nd:YAG laser, emitting at 1064 nm, and a germanium detector cooled with liquid nitrogen. The instrument was equipped with an *xy* stage, a mirror objective, and a prism slide for redirection of the laser beam. Root slices were mounted between two glass slides to avoid sample movement and deformation during the measurement.

Two-dimensional (2D) Raman maps were obtained point by point from at least five root slices by moving the *xy* stage; *x* and *y* directions of the accessory were automatically controlled by the spectrometer software. All parameters used for micro-Raman measurements, such as mapping area, step size (increment), laser power, and number of scans for each measured point, are given in the figure captions. All spectra were obtained with a resolution of 4 cm^{-1} in the wavenumber range from 100 to 4000 cm^{-1}. Mapping measurements were carried out with various mapping areas, 14800 × 12900, 5600 × 4900, 47000 × 41900, 20100 × 15900, 7000 × 14500, and 36400 × 7300 μm, and with different increments, 450, 300, 1230, 500, 350, and 450 μm, for a transversal section of parsnip root, quarter of parsnip root, celeriac root, parsley root, part of parsley root, and a longitudinal section of parsley root. The spectra collected from the mapped areas were smooth corrected and processed by the Bruker Opus/map software package v. 6.5 (Etlingen, Germany). The maps were obtained by the integration of a specific signal characteristic for the individual analyte and colored according to the Raman intensity or by using cluster analysis. Data preprocessing was performed with first derivative, using the standard method implemented in OPUS and Ward's algorithm.

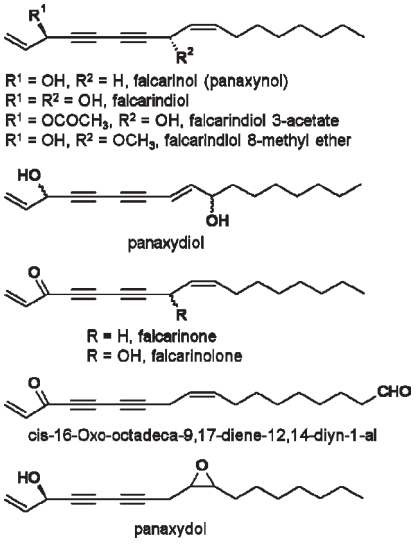

Figure 1. Structures of polyacetylenes identified in Apiaceae species.

RESULTS AND DISCUSSION

Identification and Distribution of Polyacetylenes in Parsnip Root. Parsnip is a food and feed plant with edible roots and leaves.[3] Five polyacetylene compounds have been identified in this plant, with falcarinol and falcarindiol occurring in the highest concentrations (1600 and 5770 mg/kg freeze-dried material, respectively).[23] Falcarinone and falcarinolone, the ketone derivatives of falcarinol and falcarindiol (Figure 1), have also been identified in parsnip; however, the concentration of each has yet to be determined.[3] Moreover, parsnip seeds contain polyacetylenic C_{18} ketoaldehyde[24] (Figure 1), which is considered to be a precursor of falcarinone.[25] It has been found that the IR spectrum of ketoaldehyde extracted from parsnip seeds shows characteristic bands at 2200 or 2230 cm^{-1} when measured in CS_2 or CCl_4, respectively.[24]

As can be seen in Figure 2, the Raman spectra obtained from various points of the transversally cut parsnip root show distinct signals in the region of 2180−2270 cm^{-1}. The spectra

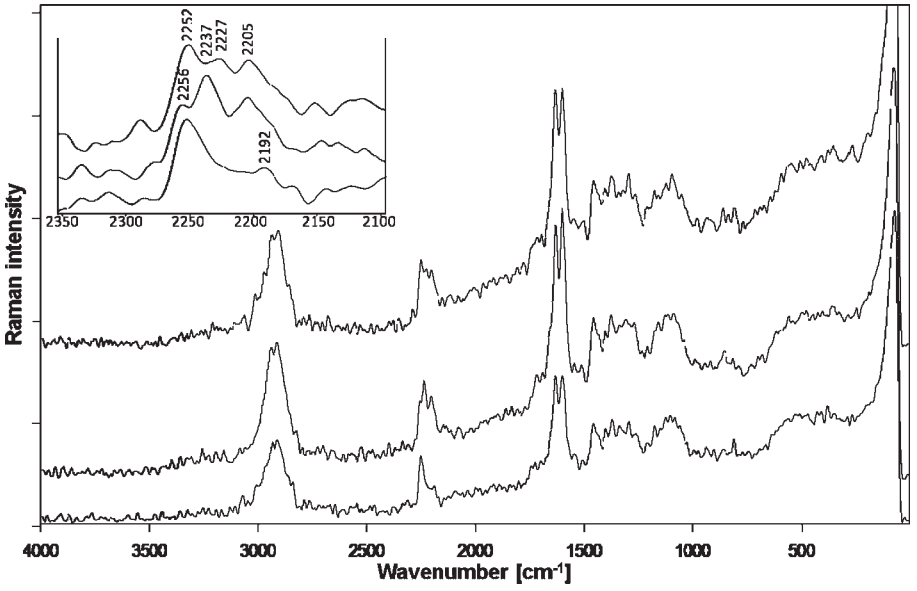

Figure 2. FT-Raman spectra of the parsnip root measured from three different spots in the range from 100 to 4000 cm^{-1}. (Inset) Same spectra in the region of 2100−2350 cm^{-1}.

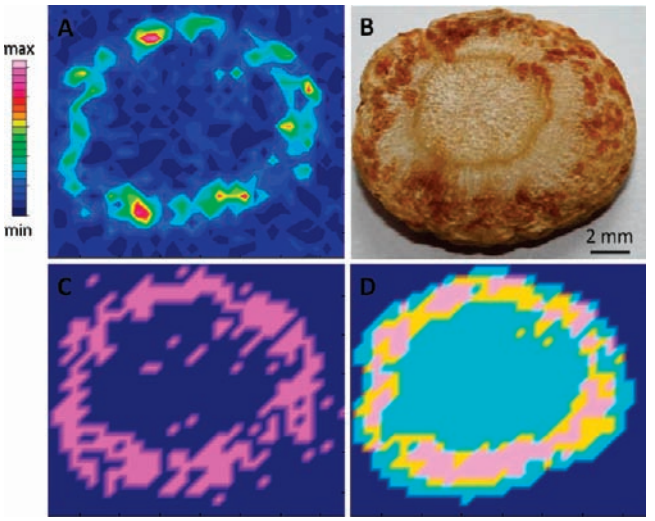

Figure 3. Transverse section of (B) parsnip root, Raman mapped using (A) integration method and (C, D) cluster analysis. Integration of the 2160−2280 cm^{-1} region produced the intensity scale represented by (A). Two clusters were assigned using (C) the 2220−2280 cm^{-1} region, and four clusters were assigned using (D) the 100−4000 cm^{-1} range.

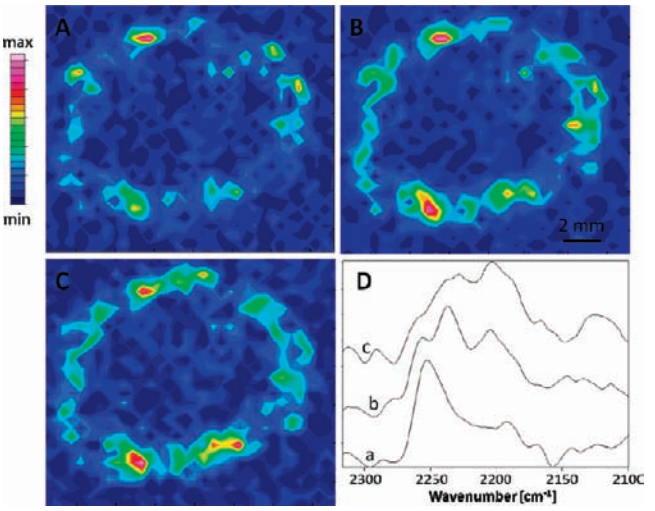

Figure 4. Transverse section of parsnip root, Raman mapped using integration methods. The regions of (A) 2245−2275 cm^{-1}, (B) 2215−2245 cm^{-1}, and (C) 2165−2215 cm^{-1} as well as Raman spectra (D) in the range of 2100−2320 cm^{-1} were obtained from the spots with high polyacetylene concentration in map A (a), B (b), and C (c).

demonstrate a specific pattern depending on the localization within the root. All polyacetylenes found in parsnip contain two conjugated triple bonds (Figure 1). The symmetric stretching of −C≡C—C≡C− is influenced only slightly by the substitution of this group. It is known that isolated forms of falcarindiol and falcarinol show Raman maxima at about 2252 and 2258 cm^{-1}, respectively.[12] Raman spectra obtained from the parsnip root in this work (Figure 2) had distinctive bands at about 2252 and 2256 cm^{-1}, so the former band can be assigned to falcarindiol, which occurs in parsnip in the highest concentration among all polyacetylenes,[23] and the latter band to falcarinol. Additional carbon triple-bond stretching signals were observed at about 2237, 2227, 2205, and 2192 cm^{-1}. Because Raman spectra of the other parsnip polyacetylenes have not been reported yet, the listed bands cannot be unequivocally assigned to the individual compounds. We can speculate that the band at about 2237 cm^{-1} can be related with the shift of the falcarinol signal observed already for in situ measurement in ginseng fresh roots in comparison to root extract that was correlated with the molecular modification of falcarinol.[26] A similar assumption respecting the origin of 2237 cm^{-1} band could be raised here; hence, it can be assigned to falcarinol upon some molecular change due to specific interaction with plant matrix.

According to Jones et al.[24] ketoaldehyde occurring in parsnip seed absorbs in the region of 2200 and 2230 cm^{-1}. Thus, the other signals observed in the spectrum of parsnip root below 2230 cm^{-1} can be associated with ketoaldehyde derivatives if they occur in this part of the plant. These signals were not assigned but are likely due to other polyacetylenes or polyacetylene−plant matrix interaction.

Distribution of polyacetylenes in parsnip root can be easily followed by means of Raman mapping due to the proportional relationship between Raman intensity and the concentration of the analyte.[22] Thus, the integration of Raman bands was applied to demonstrate the quantitative distribution of these compounds in the section of parsnip root. It can be seen in Figure 3A that the distribution of polyacetylenes is not uniform over a root section and that the compounds are accumulated in the phloem tissue. Accordingly, the inner part (the xylem tissue) contains only small amounts of polyacetylenes. Additionally, the accumulation of polyacetylenes is not uniform even within given tissue. Comparison of panels A and B of Figure 3 shows the correlation between the occurrence of polyacetylenes and dark colored spots seen in the root section. It seems that polyacetylenes may be responsible for that or they coexist with other compounds responsible for the darkening effect during exposure to light and oxygen.

The cluster analysis (CA) method using the spectroscopic range of polyacetylenes was applied to confirm the results obtained from the integration method, presented in Figure 3A. Two clusters were applied to show whether root parts where polyacetylenes are accumulated can be distinguished from other tissues and plant background. As can be seen in Figure 3C the distribution of polyacetylenes is almost the same as that presented in Figure 3A. However, CA gives no information about the concentration of the investigated compound. The application of the CA method to the whole range of spectra wavenumbers and the extension of the outcome number of clusters to four resulted in the map demonstrating the region where polyacetylenes should also occur (in purple in Figure 3D). The navy blue cluster is related to the background outside the root, whereas the light blue area shows the inner part of the root and the peel. The yellow color shows the area rich in lignin (characteristic Raman doublet with maxima at about 1600 and 1630 cm^{-1}). The map of polyacetylene spatial distribution obtained using the integration method is more accurate than that using CA, but both methods provide highly congruent results. The CA method seems to be more advantageous to distinguish regions differing in the content of those constituents for which information is poor, that is, neither the number of components nor their spectroscopic data are known.

Because several polyacetylenes occur in the parsnip root, it is possible to follow their individual distribution in the root separately. Panels A, B, and C of Figure 4 indicate the distribution of polyacetylenes associated with Raman signals in the regions of 2245−2275, 2215−2245, and 2165−2215 cm^{-1}, respectively.

As can be seen, all polyacetylenes are present in the phloem tissue, and their distributions are similar. However, they occur at various concentrations and ratios depending on the localization. This observation is confirmed by variation in spectra extracted from the presented maps in spots of high concentration of individual polyacetylenes (Figure 4D).

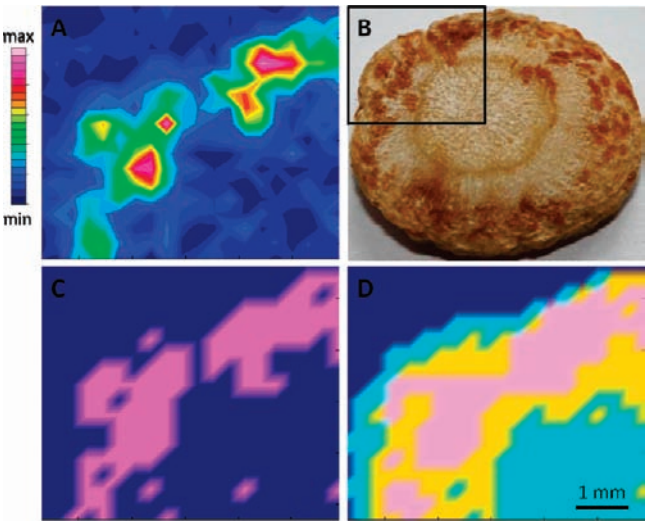

Figure 5. Transverse section of (B) parsnip root Raman mapped using (A) integration method and (C, D) cluster analysis. Integration of the 2160−2280 cm^{-1} region produced the intensity scale represented by (A). Two clusters were assigned using (C) the same spectral region, and four clusters were assigned using (D) the 100−4000 cm^{-1} range.

Figure 5 presents more detailed information about the distribution of polyacetylenes in a fourth of the transversally cut parsnip root section obtained with the spatial resolution of Raman mapping of 300 μm (which means that every 300 μm a whole spectrum was registered). As can be seen in Figure 5A, polyacetylenes are distributed only in the phloem tissue, and their highest amounts are restricted to small areas visible as bright spots. Comparison between the integration (Figure 5A) and CA (Figure 5B,C) methods clearly indicates that the latter reproduces the polyacetylene distribution in a satisfactory way.

Identification and Distribution of Polyacetylenes in Celeriac Root. Celeriac (*A. graveolens* var. *rapaceum*) is a root vegetable plant of the Apiaceae family that is widely cultivated and consumed and is closely related to another celery variety, *A. graveolens* var. *dulce*, of which the petioles (stalks) are used as condiments.[3] Several polyacetylenes were discovered in celeriac root using HPLC methods. Falcarindiol and falcarinol occurring at the highest concentration are accompanied by 8-*O*-methylfalcarindiol and panaxydiol (Figure 1) (2070−4580, 230−1620, 40−170, and 20−60 mg/kg freeze-dried material, respectively).[23] Two additional polyacetylenes known from the study on parsnip, that is, falcarinone and falcarinolone, were also

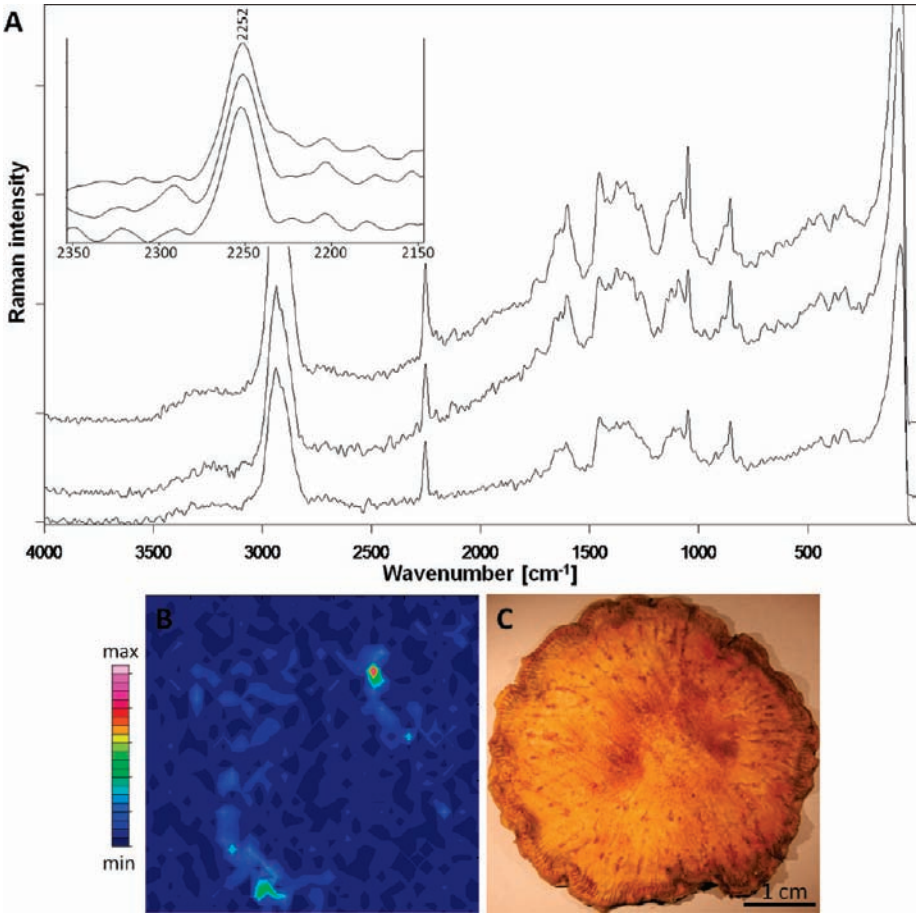

Figure 6. FT-Raman spectra of the celeriac root (A) measured from three different spots in the range from 100 to 4000 cm^{-1}. (Inset) Same spectra in the region of 2100−2350 cm^{-1}). (C) Transverse section of celeriac root that was Raman mapped using (B) integration method in the 2220−2280 cm^{-1} region.

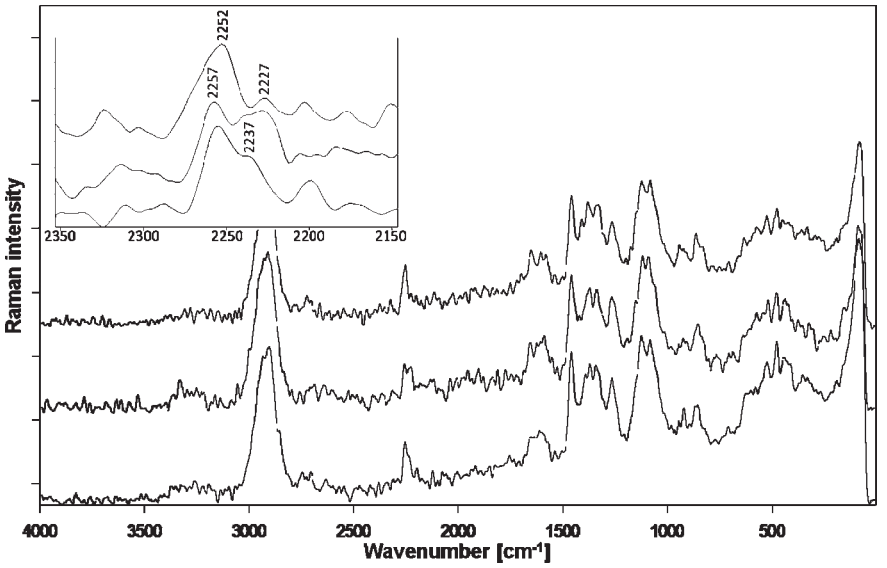

Figure 7. FT-Raman spectra of the parsley root measured from three different spots in the range from 100 to 4000 cm^{-1}. (Inset) Same spectra in the region of 2100−2350 cm^{-1}.

detected, but there is no information on their concentration in celeriac.[3]

Figure 6A presents Raman spectra obtained from measurements of celeriac root. The bands obtained from different points of the root show the same pattern at about 2252 cm^{-1}. Thus, it seems that falcarindiol dominates in celeriac root. The earlier reported polyacetylenes were not detected in the material measured. The distribution of polyacetylenes in the celeriac root section was investigated by means of Raman mapping. As can be seen in Figure 6B, polyacetylenes are accumulated only in a few small areas occasionally localized near the peel in the root section.

Identification and Distribution of Polyacetylenes in Parsley Root. Parsley from the Apiaceae family is widely cultivated as a vegetable and condiment plant. The most desired parts of parsley are roots and leaves.[3] It has been reported that parsley roots contain four polyacetylenes.[3,23] Falcarindiol, widely occurring in the majority of Apiaceae species, is present in parsley root at the highest concentration (up to 2320 mg/kg freeze-dried material). Apart from falcarindiol and falcarinol, two other polyacetylenes were identified: 8-*O*-methylfalcarindiol and panaxydiol in amounts of 350 and 120 mg/kg freeze-dried material, respectively.[23]

Figure 7 presents the Raman spectra obtained from parsley root section. As can be seen, bands due to symmetric −C≡C—C≡C− stretching are present in the region of 2200 and 2300 cm^{-1}. Spectra extracted from various root points indicate the diversity of patterns and positions of the maxima. It seems that three main bands can be identified with maxima at about 2257, 2252, and 2227 cm^{-1}. Additionally, some shoulder bands can be observed near 2237 cm^{-1}. Signals at about 2257 and 2252 cm^{-1} can be assigned to falcarinol and falcarindiol, respectively. The remaining bands can be considered as signals related to other polyacetylenes that are present in the parsley root or to the effects discussed above for parsnip.

Polyacetylenes are accumulated rather uniformly in the phloem tissue near the secondary cambium (Figure 8A,B). However, spots with higher concentration of polyacetylenes are observed occasionally, particularly when mapping is performed

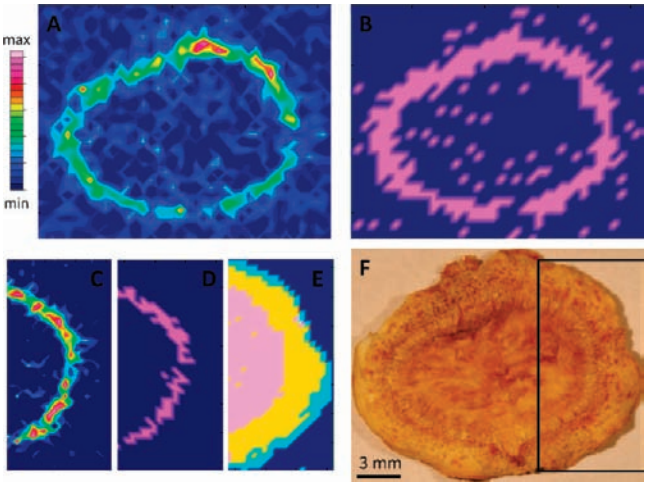

Figure 8. Transverse section of (F) parsley root was Raman mapped using (A, C) integration methods and (B, D, E) cluster analysis. Integration of the 2220−2280 cm^{-1} region produced the intensity scale represented by (A) and (C). Two clusters (B, D) were assigned using the same spectral region, and four clusters were assigned using (E) the 100−4000 cm^{-1} region.

with higher resolution (Figure 8C−E). The accordance between maps obtained using the integration (Figure 8A) and CA (Figure 8B) methods is satisfactory, although the latter shows more artifacts within the root boundary as well as outside of the root. This discrepancy is reduced in maps with higher resolution (Figure 8C,D). However, when CA was applied in the whole wavenumber range with four clusters (Figure 8E), the map showed a much broader area that comprised polyacetylenes and other plant constituents. Thus, the obtained polyacetylene distribution might overlap with the distribution of other components.

The distribution of polyacetylenes was also investigated within a longitudinal section of parsley root. As can be seen in Figure 9A, C, polyacetylenes are accumulated in the phloem tissue and

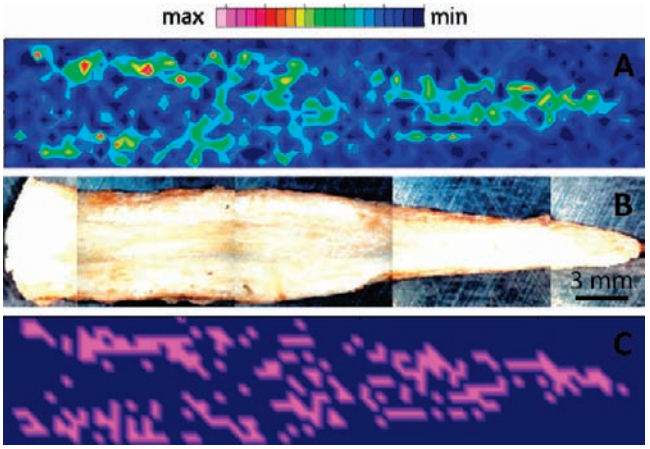

Figure 9. Longitudinal section of (B) parsley root was Raman mapped using (A) integration and (C) cluster analysis. Integration of the 2220−2280 cm^{-1} region produced the intensity scale represented by (A). Two clusters were assigned using (C) the same spectral region.

distributed throughout the whole length of the root. It can be seen that at the top of the parsley root (left side in the figures) polyacetylenes are accumulated near the peel due to more extended xylem tissue, whereas at the bottom of the root (right side) they are distributed through the whole root due to very thin xylem tissue. The accordance between maps obtained using the integration (Figure 9A) and CA (Figure 9C) methods is satisfactory.

The presented results show the usefulness of the Raman mapping technique for the in situ investigation of polyacetylenes in raw plant materials. The method is a powerful tool that can confirm the presence of a polyacetylene and provide detailed information about its distribution in the investigated plant tissue. In contrast to other analytical methods, which require polyacetylene extraction and thus sample destruction, Raman spectroscopy enables identification of these compounds directly in plant tissue. The results obtained provide information on the real occurrence and distribution of polyacetylenes in the individual tissues and are not biased by the presence of compounds that might occur as a result of structural changes or reactions during extraction. The results obtained for three Apiaceae species, that is, parsnip, celeriac, and parsley, indicate that each species may contain various polyacetylenes, differ in their spatial distribution, and have various ratios of individual compound contents depending on the root area being measured (with additional data for carrot). Besides the most abundant polyacetylenes, falcarinol and falcarindiol, some other polyacetylenes were also detected using Raman spectroscopy. However, due to the fact that the characteristic Raman signals from triple-bond stretching from only falcarinol and falcarindiol are known and not those for the other polyacetylenes present in the investigated vegetables, we were not able to confirm the presence of several polyacetylenes identified in parsley or parsnip roots by other authors using chromatographic methods. This discrepancy might be due to various compositions of materials used. Biological samples may differ considerably due to genetic background as well as environmental factors affecting plant metabolism during growth and after harvest. It is also possible that those polyacetylenes identified by other authors occurred at very low concentration in our materials, below the detection limit of the instrument used. The occurrence of polyacetylenes mainly in the outer part of the phloem tissue near the peel in the investigated root vegetables may support the statement of significance of these compounds toxic to microorganisms in the plant response reaction to biotic stress. The knowledge of the heterogeneous distribution of polyacetylenes in roots may also be important for industrial processing aiming to debitter agricultural products or extract these bioactive compounds.

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