Abstract: This paper demonstrates the special advantages of FT-Raman spectroscopy for in situ studies of several carotenoids that occur ubiquitously in the plant kingdom. Spectra obtained from various tissues of a range of plant species indicate that the wavenumber location of C=H stretching vibrations is mainly influenced both by the length as well as by the terminal substituents of the polyene chain of carotenoids and by their interaction with other plant constituents. The obtained results show also the usefulness of Raman spectroscopy in the investigation of cis–trans isomerization of carotenoids during processing. Additionally, 2-D Raman mappings present a unique possibility to evaluate the individual distribution of carotenoids in the intact plant tissue; in this context different 7-, 8-, and 9-double bond conjugated carotenoids can be analyzed independently in the same sample. Furthermore, the use of Raman spectroscopy for in situ detection of unstable substances such as epoxycarotenoids is discussed. © 2005 Wiley Periodicals, Inc. Biopolymers 77: 212–221, 2005

Keywords: carotenoids; xanthophylls; epoxycarotenoids; in situ; Raman mapping
INTRODUCTION

More than 400 carotenoids have been found in higher plants, algae, and bacteria of which α-, β-carotene, and lutein are uncounted most frequently. The carotenoid color from plants is a precursor for pigmentation in marine animals, egg yolks, and fat globules and serves as a source for vitamin A for mammals. Other biological functions attributed to carotenoids, such as prevention of cancer, cardiovascular disease, and macular degeneration, have been mainly attributed to their antioxidant property. The ability of quenching singlet oxygen is related to the conjugated double-bond system and it has been found that maximum protection is given by those having 9 or more double bonds. Thus, in recent years, studies related to human health have focused mainly on 11-conjugated lycopene whereas zeaxanthin and lutein have been investigated in the prevention of age-related macular degeneration.

Carotenoids occurring in plants are usually C₄₀ tetraterpenoids built from eight C₅ isoprenoid units and belong to the class of hydrocarbons (carotenes) or their oxygenated derivatives (xanthophylls). Their distinctive characteristic is a long central chain with a conjugated double-bond system, which is a light-absorbing chromophore responsible for yellow, orange, or red color of these compounds. Although these natural pigments occur in plants as minor components at the ppm level a very sensitive detection can be achieved by Resonance Raman in the visible region, when the wavenumber of the laser excitation coincides with an electronic transition of the individual carotenoid. NIR-FT-Raman spectroscopy also gives a strong enhancement of carotenoids due to the known pre-resonance effect; furthermore, the disturbing fluorescence effect of biological material usually observed when laser excitation is performed in the visible wavelength range, is avoided. Strong bands of carotenoids are observed in the Raman spectrum within the 1500–1550 and 1150–1170 cm⁻¹ ranges due to in-phase C=C (ν₁) and C–C stretching (ν₂) vibrations of the polyene chain. Additionally, in-plane rocking modes of CH₃ groups attached to the polyene chain and coupled with C=C bonds are seen as a peak of medium intensity in the 1000–1020 cm⁻¹ region. The wavenumber location of these bands, and in particular the ν₁ band, is strongly dependent on the length of the carotenoid chain, but the influence of substituents should also be considered.

Until now, spectrophotometric and liquid chromatographic methods have been extensively applied for plant pigment analyses. Today, high-performance liquid chromatography (HPLC) is mostly used in this context due to its high reproducibility and low detection limit. However, application of HPLC requires a long process of sample preparation, including solvent extraction of the pigments, which are usually strongly bonded to other plant constituents (e.g. proteins), and therefore the analysis results may not represent the real (authentic) carotenoid content. Moreover, solvents and also other factors such as high temperature or light may cause changes in the carotenoid conformation, leading to the formation of cis-isomers. In nature carotenoids occur primarily as more stable all-trans-isomers, thus the presence of cis-isomers that are described in the literature as natural products is often overestimated and can be considered to be artefacts. Only a few plant carotenoids are known to be present in the cis form, such as phytoene, phytofluene, or bixin. Therefore, the in situ analysis of carotenoids that would not affect their conformation is principally of great interest. On the other hand, the described conformational changes of the investigated molecules can be easily monitored by the use of Raman spectroscopy that has already been successfully demonstrated for example in a study of drug polymorphism.

In light of the foregoing consideration, NIR-FT-Raman spectroscopy, which is known as a fast and nondestructive method, can be a useful and powerful tool for in vivo plant analysis.

In this paper, NIR-FT-Raman spectroscopy is used to analyse carotenoids in situ in intact plant material in which the pigments are present as trace components. It is aimed to obtain spectra that provide reliable information with regard to the structure of the analyzed carotenoids. Moreover, the Raman mapping technique is applied to obtain deeper knowledge of the individual carotenoid distribution in various intact plant tissues.

MATERIALS AND METHODS

Sample Material

Naturally occurring carotenoids were analyzed in raw plant tissues of various species listed in Table I. Vegetables (orange carrot roots, red tomato fruits, green French bean pods, broccoli inflorescence, orange pumpkin, corn, and red pepper) as well as fruits (nectarine, apricot, and watermelon) were obtained from the local market. Yellow carrot and basil were cultivated in the experimental garden of the BAZ, whereas flowers of marigold and chamomile, leaves of ivory, begonia, and Euonymus fortunei Turcs. were collected from locally growing plants. Saffron stigmas were obtained from Worlee (Hamburg, Germany) and annatto
seeds were supplied by Martin Bauer & Co. KG GmbH (Vestenbergsgreuth, Germany). Additionally, canned 28% tomato puree (Russo, Italy) was bought in a supermarket. Pure carotenoid standards (α-carotene, β-carotene, and lutein) were purchased from Sigma–Aldrich (Taufkirchen, Germany).

Table I  Wavenumber Positions of $\nu_1$, $\nu_2$, and $\nu_3$ Modes of the Predominant Carotenoids Obtained from Measurement of Various Fresh Plant Tissues, Tomato Puree, and Pure Standards by FT- Raman Spectroscopy$^a$

<table>
<thead>
<tr>
<th>Plant name</th>
<th>Sample</th>
<th>$\nu_1$ (cm$^{-1}$)</th>
<th>$\nu_2$ (cm$^{-1}$)</th>
<th>$\nu_3$ (cm$^{-1}$)</th>
<th>Predominant carotenoids</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saffron Crocus sativus L.</td>
<td>Dry spice-stigma</td>
<td>1536</td>
<td>1165</td>
<td>1020</td>
<td>Crocetin (7)</td>
<td>1,25,26</td>
</tr>
<tr>
<td>Marigold Calendula officinalis L.</td>
<td>Petal</td>
<td>1536</td>
<td>1157</td>
<td>1007</td>
<td>Auroxanthin (7)</td>
<td>45</td>
</tr>
<tr>
<td>Marigold Calendula officinalis L.</td>
<td>Petal/pollen</td>
<td>1531–1529</td>
<td>1157</td>
<td>1004</td>
<td>Flavoxanthin (8)</td>
<td>45</td>
</tr>
<tr>
<td>Marigold Calendula officinalis L.</td>
<td>Pollen</td>
<td>1529</td>
<td>1157</td>
<td>1006</td>
<td>Luteoxanthin (8)</td>
<td></td>
</tr>
<tr>
<td>Chamomille Chamomilla recutita L.</td>
<td>Pollen</td>
<td>1524</td>
<td>1157</td>
<td>1004</td>
<td>Lutein (9)</td>
<td>45</td>
</tr>
<tr>
<td>Nectarine Prunus perica L. var. nucipersica (Sucrow) C. Schneid</td>
<td>Fruit</td>
<td>1527</td>
<td>1157</td>
<td>1005</td>
<td>β-Cryptoxanthin (9)</td>
<td>1</td>
</tr>
<tr>
<td>Carrot Daucus carota L.</td>
<td>Yellow root</td>
<td>1527</td>
<td>1157</td>
<td>1006</td>
<td>Lutein (9)</td>
<td>35</td>
</tr>
<tr>
<td>Carrot Daucus carota L.</td>
<td>Leaf</td>
<td>1526</td>
<td>1157</td>
<td>1004</td>
<td>Lutein (9)</td>
<td>1</td>
</tr>
<tr>
<td>Ivy Hedera helix L.</td>
<td>Leaf</td>
<td>1526</td>
<td>1157</td>
<td>1004</td>
<td>Lutein (9)</td>
<td>1</td>
</tr>
<tr>
<td>Euonymus fortunei Turcs.</td>
<td>Leaf</td>
<td>1525</td>
<td>1156</td>
<td>1004</td>
<td>Lutein (9)</td>
<td>1</td>
</tr>
<tr>
<td>Basil Ocimum basilicum L.</td>
<td>Leaf</td>
<td>1525</td>
<td>1158</td>
<td>1005</td>
<td>Lutein (9)</td>
<td>1</td>
</tr>
<tr>
<td>Begonia Begonia x semperflorens-cultorum Hort.</td>
<td>Leaf</td>
<td>1525</td>
<td>1157</td>
<td>1005</td>
<td>Lutein (9)</td>
<td>1</td>
</tr>
<tr>
<td>Broccoli Brassica oleracea var. italica L.</td>
<td>Flower</td>
<td>1524</td>
<td>1157</td>
<td>1005</td>
<td>Lutein (9)</td>
<td>1,46</td>
</tr>
<tr>
<td>Corn Zea mays L.</td>
<td>Seed</td>
<td>1522</td>
<td>1157</td>
<td>1005</td>
<td>Lutein (9)</td>
<td>1,47</td>
</tr>
<tr>
<td>Pumpkin Cucurbita pepo L.</td>
<td>Fruit</td>
<td>1524</td>
<td>1157</td>
<td>1009</td>
<td>Zeaxanthin (9)</td>
<td>1</td>
</tr>
<tr>
<td>Apricot Prunus armeniaca L.</td>
<td>Fruit</td>
<td>1524</td>
<td>1156</td>
<td>1003</td>
<td>β-Carotene (9)</td>
<td>1</td>
</tr>
<tr>
<td>Carrot Daucus carota L.</td>
<td>Orange root</td>
<td>1520</td>
<td>1156</td>
<td>1007</td>
<td>β-Carotene (9)</td>
<td>1,28</td>
</tr>
<tr>
<td>Annatto Bixa orellana L.</td>
<td>Seed</td>
<td>1518</td>
<td>1154</td>
<td>1011</td>
<td>cis-Bixin (9)</td>
<td>1,19,20</td>
</tr>
<tr>
<td>Pepper Capsicum annuum L.</td>
<td>Seed in chloroform</td>
<td>1523</td>
<td>1155</td>
<td>1008</td>
<td>trans-Bixin (9)</td>
<td></td>
</tr>
<tr>
<td>Watermelon Citrullus lanatus Thumb.</td>
<td>Fruit</td>
<td>1510</td>
<td>1158</td>
<td>1008</td>
<td>Lycopene (11)</td>
<td>1</td>
</tr>
<tr>
<td>Tomato Lycopersicon esculentum Mill.</td>
<td>Fruit</td>
<td>1510</td>
<td>1156</td>
<td>1004</td>
<td>Lycopene (11)</td>
<td>1,30</td>
</tr>
<tr>
<td>Standard Powder</td>
<td>Pure</td>
<td>1510</td>
<td>1156</td>
<td>1006</td>
<td>Lycopene (11)</td>
<td></td>
</tr>
<tr>
<td>Standard Powder</td>
<td>Powder</td>
<td>1522</td>
<td>1157</td>
<td>1008</td>
<td>Lutein (9)</td>
<td></td>
</tr>
<tr>
<td>Standard Powder</td>
<td>Powder</td>
<td>1521</td>
<td>1157</td>
<td>1006</td>
<td>α-Carotene (9)</td>
<td>3</td>
</tr>
<tr>
<td>Standard Powder</td>
<td>Powder</td>
<td>1515</td>
<td>1156</td>
<td>1007</td>
<td>β-Carotene (9)</td>
<td>3</td>
</tr>
</tbody>
</table>

$^a$The number of double bonds in conjugated system is shown in parentheses.
Raman Measurements

Raman spectra were recorded using a Bruker NIR-FT-Raman spectrometer (model RFS 100) equipped with a Nd:YAG laser, emitting at 1,064 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. Compared with the standard vertical sampling arrangement, the samples were mounted horizontally.

Spectral measurements were taken from pure carotenoid standard and detached, intact plant organs or their sections. All spectra were measured with a spectral resolution of 4 cm\(^{-1}\) in the range from 100 to 4000 cm\(^{-1}\). Measurement of fruits and vegetables were performed with 512 scans and an unfocused laser beam of 300 mW, whereas 128 scans and a laser power of 100 mW were used for spectral analysis of leaves, saffron stigmas, and annatto seeds. Carotenoid standards were measured with 128 scans and an unfocused laser beam of 50 mW (for \(\alpha\)- and \(\beta\)-carotene) and of 6 mW (for lutein), respectively.

2-D Raman maps of flat samples were obtained point by point moving the xy stage; \(x\) and \(y\) directions of the accessory were controlled by the spectrometer software. Traces or 2-D surface areas of the mapped samples were processed by the Bruker Opus/map software package. Raman mappings of a \(E.\) \textit{fortunei} leaf were performed at areas of 16 \(\times\) 12 mm and 5.5 \(\times\) 3.5 mm and marigold flower was mapped at an area of 26 \(\times\) 17 mm with spatial resolutions of 250, 100, and 250 \(\mu\)m, respectively. These samples were irradiated with a focused laser beam of 100 mW with a diameter of about 0.1 mm, with a spectral resolution of 4 cm\(^{-1}\); four scans were collected at each measured point.

RESULTS AND DISCUSSION

FT-Raman spectroscopy was used for in situ analysis of naturally occurring carotenoids. The chemical structure of predominant carotenoids present in the investigated samples are presented in Figure 1.

Effect of the Number of Conjugated Double Bonds on the Wavenumber Position of \(C\!=\!C\) Stretching Vibration

Three naturally occurring carotenoids, crocetin, \(\beta\)-carotene, and lycopene, were chosen as examples to investigate the relationship between \(v_1\) wavenumber and the number of conjugated carbon–carbon double bonds present in the polyene chain. For this purpose, FT-Raman measurements were taken from those fresh plant tissues, which are known to contain these pigments as main carotenoids.

Crocetin is a unique 7-conjugated \(C\!=\!C\) carotenoid substance occurring in stigmas of crocus (\textit{Crocus sativus} L.) flower, which are used as saffron spice. Its golden-yellow coloring matter has long been employed for a food flavoring, whereas, in the Middle Ages, saffron was employed as an artist’s colo-
As can be seen in Figure 2A, the $v_1$ band in the FT-Raman spectrum of saffron appears at 1536 cm$^{-1}$. This band of crocetin has been previously reported at 1537 cm$^{-1}$ in the Resonance Raman spectrum and at 1540 cm$^{-1}$ when excited in a near infrared region. The second intense band in the FT-Raman spectrum of saffron is seen in Figure 2A at 1165 cm$^{-1}$ and can be attributed to a C–C stretching vibration of the carotenoid skeleton. The in-plane rocking mode of CH$_3$ groups attached to the polyene chain is observed as a peak of medium intensity at 1020 cm$^{-1}$.

$\beta$-Carotene, a 9-conjugated C=C carotenoid compound, is the most widespread of all carotenoids in cultivated plants, usually accompanied by $\alpha$-carotene at a much lower concentration. Rich sources of $\beta$-carotene are orange carrot roots and pumpkin fruits. In Figure 2B the FT-Raman spectrum of orange carrot root presents strong bands at 1520, 1156, and 1007 cm$^{-1}$, which can be clearly assigned to $\nu$(C=C), $\nu$(C–C), and $\pi$(CH$_3$) of $\beta$-carotene, respectively.

The principal pigment of red tomato fruits is lycopene, an acyclic 11-conjugated carotene. Figure 2C presents the FT-Raman spectrum of tomato; the three most intense bands are seen at 1510 ($v_1$), 1156 ($v_2$), and 1004 cm$^{-1}$ ($v_3$). The signal at 1510 cm$^{-1}$ looks asymmetrical and it can be assumed that the shoulder to be seen at higher wavenumbers (about 1520 cm$^{-1}$) is due to $\beta$-carotene, which is also present in tomato but in lower amounts. The assignment of the band registered at 1510 cm$^{-1}$ is confirmed by Raman measurement of tomato puree rich in lycopene (see Table I). Furthermore, spectra measured in orange tomatoes have shown a higher intensity band near 1520 cm$^{-1}$ with a shoulder at 1510 cm$^{-1}$ that corresponds to higher amounts of $\beta$-carotene (1520 cm$^{-1}$) in comparison to lycopene (1510 cm$^{-1}$), which is reflected also in the color of this vegetable (spectra not presented).

Based on Resonance Raman spectra of retinal, crocetin, $\beta$-carotene, lycopene, decapreno-$\beta$-carotene, and dodecapreno-$\beta$-carotene it has already been shown that the wavenumber of the $v_1$ band decreases with the extent of the conjugation length of the central polyene chain due to an electron–phonon coupling. Our experiment revealed that this relationship also occurs for carotenoids (i.e., crocetin, $\beta$-carotene, and lycopene) measured in situ by FT-Raman spectroscopy. The observed $v_1$ shift toward red is correlated with an increasing number of conjugated double bonds in the carotenoid chain, i.e., 1536 cm$^{-1}$ ($v_1$) → 1524 cm$^{-1}$ ($v_2$) → 1510 cm$^{-1}$ ($v_3$). Contrary to that, no correlation is observed for $v_2$ and $v_3$ modes (see Table I). A similar relationship is observed for other carotenoids as shown in Table I.

**Other Agents Influencing the Wavenumber Location of C=C Stretching Vibration**

Cyclization and other modifications during the biosynthetic pathway (hydrogenation, dehydrogenation, isomerization, introduction of hydroxyl groups, rearrangements, etc.) result in a variety of carotenoid structures. Most of them have 9 conjugated double bonds in the central chain. In this study we analyzed three examples of such carotenoids: $\beta$-carotene, $\alpha$-carotene, and lutein with different side groups. Bicyclic $\beta$- and $\alpha$-carotenes are formed in two separate branches of the biosynthetic pathway and differ only in the position of the double bond in one $\beta$-ionone ring whereas dihydroxylation of the latter results in lutein formation (see Figure 1).

In Figure 3 the FT-Raman spectra of the pure standards (C=B-carotene, A, $\alpha$-carotene, B, and lutein, C) are presented. The wavenumber positions of C=C stretching vibrations are different for the above-mentioned compounds, the lowest is seen for $\beta$-carotene at 1515 cm$^{-1}$, for $\alpha$-carotene at 1521 cm$^{-1}$, and at 1522 cm$^{-1}$ for lutein. All these carotenoids contain the same number of conjugated double bonds, so obviously the regarded wavenumber shift is not correlated with the length of the polyene chain. It can be therefore concluded that the side groups also influence the wavenumber location of $v_1$.

It was observed that the wavenumber position of the C=C stretching of $\beta$-carotene in a pure standard (1515 cm$^{-1}$) is moved in comparison to that obtained in situ measurement of carrot roots (1520 cm$^{-1}$). This phenomenon can be explained by the fact that...
Carotenoids in carrot roots are bonded to proteins, but the presence of other carotenoids such as β-carotene or lutein may also affect the band shift toward higher wavenumbers and therefore cannot be neglected in this context. Generally, chromatographic analyses of carotenoids (TLC, HPLC) in plant tissues include solvent extraction of the pigment. However, this procedure causes the destruction of the natural carotene–protein complexes. Contrary to that, FT-Raman spectroscopy allows the investigation of the carotenoids nondestructively in their natural environment.

In the next step, five carotenoids characterized by 9 conjugated double bonds in the main chain were measured in situ. Figure 4 shows the FT-Raman spectra of red pepper fruit (main carotenoid: capsanthin) (Figure 4A), nectarine fruit (main carotenoid: β-cryptoxanthin) (Figure 4B), yellow carrot root (main carotenoid: lutein) (Figure 4C), pumpkin fruit (main carotenoid: β-carotene) (Figure 4D), and corn (main carotenoid: zeaxanthin) (Figure 4E). For all of these pigments, the characteristic C=H stretching vibration can be found in the wavenumber range between 1517 cm⁻¹ (capsanthin) and 1527 cm⁻¹ (lutein and β-cryptoxanthin). The dispersion of the ν₁ wavenumbers of 9-conjugated systems is significant, however this wide range is still distinct and is located below 1536 cm⁻¹ (characteristic of the 7-conjugated system of crocetin) and above 1510 cm⁻¹ (characteristic of the 11-conjugated lycopene). These in situ measurements of carotenoids confirm the above-mentioned observation that the side groups of the central polyene chain also influence the position of the ν₁ wavenumber. The ν₁ shift can also be attributed to the fact that carotenoids usually bond to other compounds in plants. It is known that green leaves and vegetables contain unesterified hydroxy carotenoids, mainly lutein, whereas carotenols in fruit are esterified with fatty acids. In our results lutein can be seen at 1527 cm⁻¹ in yellow carrot root, in green leaves at 1525–1526 cm⁻¹, and in green vegetables at 1524 cm⁻¹ (see Table I). A similar situation is observed for β-carotene in orange carrot root (1520 cm⁻¹) and pumpkin or apricot (1524 cm⁻¹).

These examples show that the wavenumber location of C=C stretching is influenced not only by the length of the polyene chain and the molecular structure of the terminal groups of carotenoids but also significantly by their interaction with other plant constituents (proteins, fatty acids, etc.). In this context FT-Raman spectroscopy is a potential tool to perform more detailed investigations of these molecular interactions in situ.

Cis–Trans Isomerization

Bixin (see Figure 1) is a unique carotenoid compound present in annatto seeds (Bixa orellana L.) and it is used in food industry as a natural colorant. Unlike most of the other carotenoids, bixin occurs in nature in the cis form, but after extraction in organic solvent it converts to the more stable trans form. This experimental observation has already been confirmed by theoretical calculation. The spectra of bixin obtained directly from seeds as well as related chloroform extracts are presented in Figure 5A and 5B, respectively. As can be seen, the ν₁ position of bixin occurring in the natural environment is found at 1518 cm⁻¹ whereas in organic solution a band at a higher (1523 cm⁻¹) wavenumber is detected that can be assigned to its two conformational forms, cis and trans, respec-

![Figure 4](https://via.placeholder.com/150)

**Figure 4** FT-Raman spectra of red pepper fruit (A), nectarine fruit (B), yellow carrot root (C), pumpkin fruit (D), and corn seed (E).

![Figure 5](https://via.placeholder.com/150)

**Figure 5** FT-Raman spectra of cis-bixin measured directly from annatto seeds (A) and trans-bixin in chloroform extract (B).
tively. However, some influence of plant constituents bonding to bixin (cis isomer) and the chloroform solvent (trans isomer) on the $v_1$ position should also be taken into consideration.

FT-Raman spectra of cis- and trans-crocetin, extracted from Crocus sativus L. stigmas and separated by an HPLC method have been previously reported. Stretching vibrations of C=H bonds were observed at 1535 and 1547 cm$^{-1}$ for trans and cis isomers, respectively. The assignment of these bands is opposite of that discussed above for bixin. Thus, the lower wavenumber of conjugated C=H stretching vibrations is observed for naturally occurring cis-bixin in annatto and trans-crocetin in crocus.

The difference in the $v_1$ position between cis and trans isomers in both cases is significant and can be easily detected using FT-Raman spectroscopy. It has already been reported that carotenoids in fruit and vegetables undergo isomerization during processing and/or storage and, as a consequence, a decrease in their color intensity and a reduction of their bioactivity occurs. Heat, light, acids, and adsorption on the metal surfaces promote trans–cis isomerization, e.g., an increase of cis-$\beta$-carotene can be observed in cooked carrot. Therefore, FT-Raman spectroscopy can be efficiently applied for the investigation of conformational changes of carotenoids, e.g., in the field of quality control.

Raman 2-D Maps of Carotenoid Distribution in Plants

FT-Raman spectroscopy can also be successfully applied to characterize the carotenoid distribution in the plant tissue at cellular level. The use of a horizontal stage with automatically controlled motion provides the opportunity to perform measurements from a specified area of living tissue. As a consequence, a detailed distribution of individual analytes within the measured region in a 2-D map can be visualized.

Euonymus fortunei Turcs. ‘Canadale Gold’ is a chlorophyll mutant with light green/yellow edge on leaf blades. The presence of yellow carotenoids, mainly lutein, and $\beta$-carotene is masked by green chlorophyll, therefore it is not possible to evaluate the carotenoid distribution without analysis. This is why we used Raman mapping to determine the carotenoid distribution in a leaf of Euonymus; the relative concentration of carotenoids was determined according to the intensity of the band at 1525 cm$^{-1}$ (Figure 6). As can be seen, the higher level of carotenoids is observed in dark green leaf regions, which corresponds to a higher amount of chlorophyll in that tissue. A more detailed view was obtained from a second Raman mapping performed over a smaller area of the same leaf but with a higher resolution (Figure 6D). Comparing this map with the related microscopic
image supplies a rapid overview of the individual carotenoid level corresponding to the anatomical structure of the leaf. This example confirms also the nondestructive feature of NIR-FT-Raman spectroscopy: repeated measurements can be taken from the same sample area several times without perceptible changes with regard to quality or reliability.

On the basis of HPLC investigations it is known that petals and pollen of marigold (*Calendula officinalis* L.) contain mostly flavoxanthin and luteoxanthin (see Figure 1). Luteoxanthin is formed from violaxanthin in the process of epoxidefuran oxide rearrangement, resulting in shortening of the chain to 8 conjugated double bonds. Further epoxidation results in the formation of auroxanthin with a 7-conjugated chain. Auroxanthin is present in marigold petals in high amounts whereas, in the pollen, lutein and antheraxanthin (both pigments are 9-conjugated carotenoids) are additionally detected. The spectra presented in Figure 7A are taken from marigold flower; they show the different location of the carotenoid $\nu_1$ bonds. The stretching vibration of C=C bonds of the diepoxy-carotenoid auroxanthin is observed at 1536 cm$^{-1}$, exactly at the same wavenumber as for crocetin, the other 7-conjugated system (Figure 7A, upper spectrum). Lutein and anthraxanthin signals are seen at about 1524 cm$^{-1}$, which is the characteristic range for 9-conjugated chains (Figure 7A, bottom spectrum). However, the most interesting carotenoids are flavoxanthin and luteoxanthin, as they possess 8 double bonds in the central chain, and, until now, Raman spectra of such systems were not reported. As expected, these carotenoids give strong Raman signals in the range between 1529 and 1531 cm$^{-1}$ (Figure 7A, middle spectrum). In Figure 7 additionally a photo of marigold flower (Figure 7B) and three Raman maps (Figures 7C, 7D, and 7E) showing the distribution of different carotenoids are presented. The maps were obtained according to the intensities of the characteristic C=C stretching vibrations for 7-, 8-, and 9-conjugated carotenoids, respectively.
8-, and 9-conjugated systems, respectively. The presented Raman maps reveal much higher accumulation of auroxanthin in the outer petals and its lack in the inner part of the flower, which is composed mainly of stigmas filled with pollen (Figure 7C). The distribution of lutein and antheraxanthin is opposite, which is in agreement with the performed HPLC measurements (Figure 7E). The derived luteoxanthin with 8-conjugated bonds can be allocated to all flower parts (Figure 7D). However, it is important to stress that the Raman mapping results show the concentration of carotenoids both at the surface and in the surface layer of the flower. The application of NIR laser excitation and lack of confocal arrangement implicate that Raman measurements of the sample may be recorded by penetrating a relatively thick outer layer. This is the reason why, in some parts of Calendula flower, where several petals are lying one on the other, a high concentration of carotenoids can be seen (see red spots in Figures 7C and 7D).

The spectrum of Calendula pollen with a strong band at about 1530 cm$^{-1}$ is similar to that measured before in chamomile pollen where the $v_1$ mode was observed at 1529 cm$^{-1}$ (see Table I). Therefore it is most likely that both flowers contain 8-conjugated epoxycarotenoids in their pollen. In conclusion, the presented Raman maps performed in situ allow the precise localization of various carotenoids in different parts of the plant tissue and principally a semiquantitative characterization of these pigments can be achieved. Raman spectroscopy also confirms the presence of 8- and 7-conjugated carotenoids in the Calendula flower, most likely flavoxanthin, luteoxanthin, and auroxanthin. These data also show the possibility that FT-Raman spectroscopy can be applied for the measurement of epoxycarotenoids in situ, which can be very helpful as these compounds have the tendency to easily undergo degradation and therefore are often underestimated in conventional food or plant analysis. Their occurrence in nature is also often questioned as some of them can be formed as artefacts during sample extraction or analysis. 

CONCLUSION

It has been found that FT-Raman spectroscopy can be successfully applied for the identification of carotenoids directly in the plant tissue without any preliminary sample preparation. Compared with the very intense carotenoid signals the spectral impact of the surrounding biological matrix is weak and therefore does not contribute significantly to the obtained results. The analysis of natural products (e.g., fruits, vegetables, flowers) can be performed fast, reliably, and nondestructively. The wavenumber locations of different carotenoid C=C stretching vibration are strongly correlated with the individual number of conjugated double bonds but are also influenced by the terminal groups of the polyene chain as well as their interaction with other plant constituents. Additionally, Raman spectroscopy provides structural information about conformational changes of cis–trans isomers that may occur during sample preparation or food processing. Furthermore, it is possible to measure unstable epoxycarotenoids in situ which may easily undergo degradation processes and are therefore underestimated when conventional analytical methods such as HPLC are applied. FT-Raman mapping allows the localization of carotenoids throughout the surface layer of the plant tissue as well as the performance of semiquantitative measurements. Generally, FT-Raman spectroscopy is a powerful and supplementary tool that, in addition to other analytical techniques, can provide very informative data of carotenoids in plant material as well as in related food products.

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