Identification of secondary metabolites in medicinal and spice plants by NIR-FT-Raman microspectroscopic mapping†

Malgorzata Baranska,a,b Hartwig Schulza,a Petra Rösch,a Marion A. Strehlec and Jürgen Poppb

a Federal Centre for Breeding Research on Cultivated Plants (BAZ), Institute for Plant Analysis, Neuer Weg 22-23, D-06484 Quedlinburg, Germany. E-mail: H.Schulz@bafz.de; Fax: 49 3946 47234; Tel: 49 3946 47231
b Faculty of Chemistry, Jagiellonian University, Ingarden 3, 30-060 Krakow, Poland. E-mail: M.Baranska@bafz.de
c Institut für Physikalische Chemie, Friedrich-Schiller-Universität Jena, Helmholtzweg 4, D-07743 Jena, Germany

Received 6th April 2004, Accepted 21st June 2004
First published as an Advance Article on the web 21st July 2004

This paper demonstrates the special potential of vibrational NIR FT Raman microspectroscopy for the study of fennel fruits, chamomile inflorescence and curcuma roots to obtain detailed information about their microstructure and chemical composition. Microscopic Raman maps of fennel fruits demonstrate that anethole, which is the main essential oil component, is present in the whole mericarp with highest concentration at the top of the fruit. In situ measurements obtained of the essential oil cells are dominated by two bands observed at 1657 cm⁻¹ and 1609 cm⁻¹ which are characteristic for anethole. Raman images of chamomile inflorescence show that spiroethers, identified by significant bands between 2150 and 2250 cm⁻¹, are accumulated in the middle part of the flower head. Due to the intense curcumin bands in the Raman spectrum of curcuma root, the distribution of this dyeing substance can be clearly determined; highest concentration of curcumin was observed on the core of the root.

1 Introduction

Usually the morphology and biochemical composition of plant single cells are characterized by (electron) microscopy, fluorescence spectroscopy and radiography. However, these techniques need extensive sample preparation and in most cases the analytical conditions do not allow in vivo measurements. Therefore some attempts have been made during the last decade to apply Raman microspectroscopy as an alternative technique in order to overcome these limitations. Due to the increasing detection sensitivity it is principally possible to obtain Raman spectra from single biological cells; Puppels et al. demonstrated for the first time that confocal Raman microspectroscopy with laser excitation at 514.5 nm can be applied to study intact cells and chromosomes. In this context some problems occurred relating to photodegradation or intrinsic fluorescence of the biological tissue. That is the reason why especially NIR-FT-Raman spectroscopy has been used for rapid and non-destructive plant analysis during the last years.5

The goal of our research programme is to get a deeper understanding of the Raman spectral features of different medicinal and spice plants on the cellular level. In this paper, we present some selected Raman microspectroscopic maps which allowing 2- and 3-dimensional images of the investigated plant samples to be obtained. The whole mapping area can be as big as 75 mm × 50 mm with increments of at least 5 μm. Considering the diameter of the laser beam (approx. 100 μm) and the size of the plant materials to be investigated, Raman maps presenting high resolution can be achieved within a reasonable measurement time with increments of 50 μm. The 2-dimensional maps can be directly compared to the corresponding visual images obtained from a microscope. However, the Raman mapping technique provides additional detailed information regarding the distribution of specific compounds, e.g. secondary metabolites, occurring in the surface layer of the plant tissue. Generally, it was aimed at interpreting the measurements collected from the plants in comparison with the spectra of the corresponding pure standard substances.

In this publication we report examples of some micro FT-Raman experiments for the characterization of natural compounds in fennel fruits (Foeniculum vulgare Mill.), chamomile inflorescence (Chamomilla recutita L.) and curcuma roots (Curcuma longa L.). Fennel fruits are predominantly used as a natural remedy, mainly in order to support the digestive organs. It is applied to the production of phytopharmaceuticals and food teas as well as for various spice mixtures. The essential oil isolated from two different fennel varieties (Foeniculum vulgare var. dulce and F. vulgare var. vulgare) is mainly produced in the Mediterranean countries. Major components of both oils are trans-anethole which occurs in sweet fennel oil (var. dulce) in amounts of >80% and fenchone which may be present especially in bitter fennel oil (var. vulgare) in comparatively high concentration of approx. 25%. Both fennel oils are used in food flavours and in small quantities also in perfumery. Chamomile is one of the most important medicinal plants worldwide. Dried flowers collected from chamomile plants are predominantly used for the production of herbal teas as well as aqueous–ethanolic extracts. Pharmacological tests have revealed relevant spasmylytic and antiphlogistic properties which are mainly related to several flavonoid components.6

The blue-coloured essential oil obtained by steam distillation of the flowers and stalks finds extensive use in cosmetics and foodstuffs. Curcuma, also known as turmeric, belongs to the
Zingiberaceae family and is cultivated in tropical countries. In the fresh state, the root has an aromatic and spicy fragrance which is closely related to the presence of various sesquiterpenes such as turmerone and zingiberene. Curcuma is a very important spice especially in Asia and is used in all curry powders. The root contains several compounds with anti-inflammatory and antioxidative properties of which curcumin is the most prominent one. It was reported that curcumin can be used in antiviral therapy as well as skin protector against radiation and in treatment for cancer. Extracts of curcuma roots have been also used for yellow dyeing of textiles.

2 Experimental

2.1 Sample material and reference analysis

Chamomile and fennel plants were cultivated in the experimental garden of the Federal Centre for Breeding Research on Cultivated Plants (BAZ) in Quedlinburg, Germany. Curcuma roots were bought at a local market in Chiang Mai, Thailand. The isolated essential fennel oils were received from Paul Kaders GmbH in Hamburg (Germany). All reference analyses were performed by GC/FID using a Hewlett-Packard chromatograph 6890 series, fitted with a HP-5, 50 m $\times$ 0.32 mm fused silica column (film thickness: 0.52 μm). The percentage composition was computed from the GC peak areas without applying any correction factors.

Pure standard substances of anethole, fenchone and curcumin were purchased from Roth (Karlsruhe, Germany).

2.2 Raman measurements

Raman spectra were recorded using a NIR-FT-Raman Spectrometer of Bruker (Model RFS 100) equipped with a diode-pumped Nd:YAG laser, emitting at 1064 nm, and a germanium detector cooled with liquid nitrogen. Spectral measurements were performed on fresh plant material with a spectral resolution of 4 cm$^{-1}$ in the range of 200–3700 cm$^{-1}$ with a laser power of 10–50 mW. 2-D maps were achieved using a spectral resolution of 4 cm$^{-1}$ and 3D data processing. Compared to the standard vertical sampling arrangement, the sample is mounted in a horizontal manner when using the mapping stage. Therefore, the accessory requires the mirror objective to be exchanged for the standard collimating lens of the sample compartment. The mapping stage allows to perform surface mappings of TLC (Thin Layer Chromatography) plates or other flat samples. The x and y direction of the accessory are software-controlled, the z-direction (height) is adjusted manually. Traces or 2-dimensional surface areas of thin samples can be investigated by the Opusmap software package from Bruker, Germany.

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 listed in Table 1.

<table>
<thead>
<tr>
<th>Fig. no.</th>
<th>Mapping area (μm)</th>
<th>Increment/μm</th>
<th>Laser power/mW</th>
<th>No. scans per point</th>
</tr>
</thead>
<tbody>
<tr>
<td>1B</td>
<td>3700 $\times$ 3000</td>
<td>100</td>
<td>50</td>
<td>8</td>
</tr>
<tr>
<td>1C</td>
<td>1300 $\times$ 1900</td>
<td>50</td>
<td>50</td>
<td>8</td>
</tr>
<tr>
<td>1E</td>
<td>3500 $\times$ 6500</td>
<td>150</td>
<td>30</td>
<td>4</td>
</tr>
<tr>
<td>1F</td>
<td>1200 $\times$ 4750</td>
<td>50</td>
<td>30</td>
<td>4</td>
</tr>
<tr>
<td>3B,C</td>
<td>9000 $\times$ 9000</td>
<td>250</td>
<td>50</td>
<td>8</td>
</tr>
<tr>
<td>5B</td>
<td>9600 $\times$ 12600</td>
<td>300</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>5C</td>
<td>4400 $\times$ 4400</td>
<td>100</td>
<td>10</td>
<td>4</td>
</tr>
</tbody>
</table>

3 Results and discussion

3.1 Occurrence and spatial distribution of terpenoids in fennel fruits

FT-Raman microspectroscopy was used for investigation of terpenoids occurring in fennel essential oil in single fruits. According to the GC results the essential oil of the sweet fennel type contains approx. 85% anethole and 3% fenchone, whereas the oil of the bitter type presents a significant lower anethole content (approx. 32%) but higher amounts of fenchone (approx. 9%). In Fig. 1 the Raman spectra of these two fennel oil types (C; D) as well as the spectra of pure anethole (A) and fenchone (B) standard substances are presented. The spectrum of anethole shows two intense bands at 1657 and 1609 cm$^{-1}$ due to ring stretching vibrational modes, whereas fenchone can be recognized by a characteristic ketone C=O stretching vibration to be observed at 1741 cm$^{-1}$. The spectrum of sweet fennel oil (Fig. 1C) is dominated by signals of anethole, which is the most abundant component in this product. Relating to Raman measurements of pure fenchone it can be assumed that this monoterpenoid component contributes to the Raman spectrum of bitter fennel oil (Fig. 1D), but the Raman intensity of this key band is comparatively low.

Fig. 1E and F present results of micro-Raman studies performed at a single fennel fruit as to be seen in Fig. 2. The chemical composition of the individual essential oil cells were investigated in situ by Raman spectroscopy and compared with the spectra of the relating pure standards and fennel oils isolated by hydro-distillation. The Raman spectrum measured in situ in the essential oil cell (Fig. 1E) is very similar to those obtained from sweet fennel oils (Fig. 1C). It can be clearly seen that the spectrum is dominated by the two bands at 1657 and 1609 cm$^{-1}$, which are characteristic for anethole; obviously there are no disturbing spectral influences of the plant matrix within this wavenumber range. This confirms that in situ measurements allows the localization of the essential oil. As can be seen in Fig. 1F, it is also possible to find spots with additional components present next to anethole. These plant substances can be tentatively characterized by two Raman bands to be observed at about 1740 and 1450 cm$^{-1}$, which may be attributed to fatty acids and/or fatty acid esters. Pure fenchone shows also signals in the similar range, but relating to the two intense bands between 600 and 700 cm$^{-1}$ (Fig. 1B) the spectral contribution of this compound seems to be comparatively low.

A similar inhomogeneity of mono- and sesquiterpene composition of individual peltate trichomes as well as changes of the essential oil composition during ontogenesis (= the process
of an individual organism growing organically) has been already reported for *Mentha × piperita*. Here a striking variation in monoterpene composition was observed by special GC measurements among individual trichomes and between different parts of the leaf. It is also known that the age of the glands strongly influence the composition of the oil that they contain; this effect can be clearly observed by comparison of older and younger plant material. However, difference in composition of fennel oil measured directly in the essential oil cells of a single fruit has not been reported.

Additionally, Raman mappings were performed on transverse and longitudinal sections of some selected fennel fruits, for which microscopic images are presented in Fig. 2A and D, respectively. In Fig. 2B and E are demonstrated Raman maps coloured according to the intensity of C–H stretching vibrations in the wavenumber range of 2800–3000 cm$^{-1}$, showing the distribution of essential oil as well as fatty acids (B and E, respectively) and Raman maps coloured according to the intensity of the Raman signal at 1609 cm$^{-1}$ related to anethole (C and F, respectively).

According to the literature, in fennel fruit the essential oil is stored in special oil ducts (so-called “vittae”) which are located in the outer parts of the mericarp. Based on Raman mappings, obtained for transverse and longitudinal sections of fennel fruits, the distribution of anethole, which is the main component in the essential oil of the analysed fennel chemotype, was determined. As can be seen in Fig. 2C, anethole is present in most essential oil cells in the whole mericarp, also in the endosperm, and the highest concentration of this compound can be found at the top of the fruit (Fig. 2F). To our best knowledge, the chemical composition of fennel oil measured directly in the single fruit has not yet been reported.

Results presented in this paper show the high potential of Raman microspectroscopy for *in situ* investigations of...
individual essential oil glands; furthermore this fast and sophisticated method can be used in addition to time-consuming analysis methods such as gas chromatography in order to get more detailed information concerning the spatial distribution of plant metabolites.

3.2 Spatial distribution of polyacetylenes and carotenoids in chamomile inflorescence

The essential oil of chamomile, depending on the individual chemotype, contains mainly chamazulene, α-bisabolol, bisabololoxides A and B as well as cis and trans-spiroethers. It is well known that spiropoethers occur in chamomile extracts in relatively high amounts. These substances, belonging to the chemical group of polyacetylenes, possess two \(-\text{C} = \text{C}\)- triple bonds in the molecule which cause strong Raman signals in the range between 2150 and 2250 \(\text{cm}^{-1}\). The Raman spectrum measured directly in the chamomile inflorescence in the marked point (Fig. 3A and B) shows clearly the presence of polyacetylenes in the investigated plant. The detection of polyacetylenes by Raman spectroscopy has been already reported before\(^1\) but up to now the exact distribution of these components was not known in detail.

In Fig. 3C and D Raman images of chamomile inflorescence are presented. Integration in the range of the triple bond stretching mode demonstrates that spiropoethers are accumulated in the middle part of the flower head (Fig. 3C). They can also be found in disk florets, receptacle and stem whereas ray florets are free of them. Antifungal, antifungal and antimicrobial activities of polyacetylenes were reported for many plants\(^17\)\(^-\)\(^20\) so the knowledge about their localization and concentration seems to be very important. In this context the Raman technique described in this paper can generally supply detailed information about the polyacetylene distribution in living plant material of various species.

Contrary to that, carotenoids which are characterised by typical bands occurring at 1525, 1156 and 1004 \(\text{cm}^{-1}\) (see Fig. 3B), are located at the top of the flower heads (Fig. 3D). Detailed investigation of this region under the microscope shows anthers full of pollen, whereas anthers, which are empty, do not produce carotenoid signals. Therefore, it can be supposed that in accordance with the results of earlier studies the yellow colour of chamomile disk florets is mainly related to the occurrence of flavonoids (e.g. apigenin-7-glucoside and luteolin-7-glucoside).\(^21\)\(^,\)\(^22\)

3.3 Mapping of curcuma roots

FT-Raman spectroscopy was also used for investigation of curcumin, which is a valuable dyeing component of curcuma root.

In Fig. 4 Raman spectra of a curcumin standard and a fresh curcuma root are presented. In spite of the fact that curcumin is present in the root in amounts of only 3–5%, it dominates in the Raman spectrum of the curcuma root and the individual key signals occur almost at the same positions as for the pure standard. The most intense bands appearing at 1630 and 1601 \(\text{cm}^{-1}\) can be assigned to the benzene ring, whereas bands at 1185 and 965 \(\text{cm}^{-1}\) are due to COC and COH vibrations.\(^23\)

The presence of characteristic curcumin bands in the spectrum of curcuma roots provide very good pre-conditions to apply selective Raman maps in order to determine the curcumin distribution in a sprouting curcuma root. Whereas the root demonstrates clearly the presence of curcumin, this substance is not detected in the sprout (Fig. 5). The highest concentration of the dyeing substance was observed in the core of the curcuma root; the outer layer of the root contains only slight amounts of curcumin.

4 Conclusions

This paper demonstrates that NIR-FT-Raman spectroscopy is a valuable tool for investigation of several secondary plant metabolites. It has been proven to be a non-destructive, fast and sensitive method requiring only minimal sample preparation. Single Raman spectrum can be obtained in seconds and as well the detection of the sample main components. In particular, microspectroscopy has been shown to provide special advantages in qualitative and semi-quantitative analysis of natural plant substances, even when occurring in low concentrations, as shown for polyacetylenes or carotenoids present in chamomile inflorescence. In such cases the minimum detectable quantity is at the ppm level. Contrary to single Raman spectrum Raman mappings are generally long measurements running on the sample area and step size and go on for hours. Based on the individual maps of the displayed area, the obtained Raman information allows to localize and identify numerous plant components relating to their different medicinal and spice properties. It was also shown that principally the distribution and chemical composition of secondary plant metabolites can be determined \textit{in situ}.

Acknowledgements

The financial support of the “Deutsche Forschungsgemeinschaft (DFG)” in Bonn, Germany (Grant No.: Schu 577/7-1 as well as Po 5634-1) is gratefully acknowledged.

References
