

Analysis of plant raw materials and extracts applying various vibrational spectroscopy techniques – possibilities and limitations

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Abstract New methods using MIR, NIR and Raman spectroscopy in combination with various chemometric algorithms are presented which allow monitoring numerous plant samples very efficiently within a short time. Today portable FT-IR spectrometers are available which only need sample amounts of a few microliters or milligrams. In most cases, the measurements can be performed directly and non-destructively on the individual plant tissues or plant extracts. Generally, with IR and Raman spectroscopic techniques spectra are obtained which present characteristic key bands of individual plant components. These bands provide important information about the chemical composition of the investigated samples. Based on such markers spectroscopic analyses in principle allow the discrimination of different species, and even to classify chemotypes among the same species. Combination of vibrational spectroscopy and hierarchical cluster analysis provides a fast, easy and reliable method for chemotaxonomic characterization. The ability to rapidly monitor various plant components makes it possible to efficiently select high-quality single plants from wild populations as well as progenies of crossing experiments. Furthermore, the vibrational spectroscopic methods can also be used by the processing industry in order to perform fast quality checks of incoming raw materials as well as continuous controlling of production processes.

Keywords: NIRS, Raman, ATR-IR, chemometrics, quality control, in-process control, plant, breeding, cultivation.

1 Introduction

Quality control of cultivated plant species as well as products derived from them usually comprises correct botanical identification of the plant material as well as quantification of the individual active principles. Furthermore, residues (e.g. organic solvents) and contaminants (e.g. pesticides and heavy metals) are determined applying various sophisticated analytical techniques. For this, testing of plant material such as phytopharmaceutical products is usually performed in accordance with validated standard methods described in the Food Chemical Codex, the European Pharmacopeia, the United States Pharmacopoeia and others. Contrary to this approach, there is some need to apply also various rapid high-throughput methods aiming to characterise simultaneously several quality parameters and to reduce efforts for sample preparation to a minimum. In this context new vibrational spectroscopy methods (MIR, NIR and Raman spectroscopy) in combination with various chemometric algorithms are presented which allow efficient monitoring of numerous plant samples within a short time. Especially Raman spectroscopy has been found to be a reliable and non-destructive method for rapid discrimination of different plant species or chemotypes if characteristic key bands can be observed in the spectrum. But also NIR and ATR-IR spectroscopy have made the handling of powdered as well as liquid samples very quick and simple. Today portable IR and Raman spectrometer systems are available which only need sample amounts of a few microliters or milligrams for analysis. In most cases, vibrational measurements can be performed directly on plant tissues as well as on fractions isolated from the plant material by hydro-distillation or solvent extraction. Based on individual marker bands, spectroscopic analyses in principle allow the discrimination of different species, and even to classify chemotypes among the same species. Combination of vibrational spectroscopy and hierarchical cluster analysis provides a fast, easy and reliable method for chemotaxonomic characterization. The ability to rapidly monitor various plant components provides the possibility to efficiently select high-quality

single plants from wild populations as well as progenies of crossing experiments. Today, vibrational spectroscopy is already introduced in industry in order to perform fast quality checks of incoming raw materials and continuous controlling of production processes.

This review presents an overview of some selected applications for NIR, ATR-IR and Raman spectroscopy useful to characterize plant raw materials and extracts produced therefrom.

2 Proteins and amino acids

Several Raman vibrational modes support the interpretation of various amino acids and proteins occurring in plant tissue. In this context, the following three signals are of main interest for the identification of different protein backbone conformations: amide I to be detected between $1680\text{--}1600\text{ cm}^{-1}$ (stretching vibration of C=O), amide II observed in the range between 1580 and 1480 cm^{-1} and amide III to be found between 1300 and 1230 cm^{-1} (both associated with coupled C-N stretching and N-H bending vibrations of the peptide group). Also IR spectroscopy was successfully applied to investigate amino acids and proteins in the plant material as for instance to analyze the distribution of lysine in barley [1].

Applying Raman microspectroscopy the individual content of protein in wheat kernels was measured with high spatial resolution [2]. Specific amino acid structure elements such as S-S and S-H groups of cystine and cysteine, aromatic rings of tryptophan, tyrosine and phenylalanine and the imidazole ring of histidine also provide helpful information for a reliable interpretation of the registered Raman and IR spectra. Identification of compounds containing disulfide bonds can be successfully obtained by using FT-Raman spectroscopy, because the S-S stretching band is polarized and very prominent in the Raman spectra while the IR intensity is usually weak due to its nonpolar nature [3]. Also sulfhydryl groups show comparatively strong S-H stretching modes in the Raman spectrum which occur in the region between 2550 and 2580 cm^{-1} .

3 Lipids and fatty acids

Applying discriminant analysis (DA) and principal component analysis (PCA) it has been shown that commercial vegetable oils such as extra virgin olive oil, groundnut oil, corn oil, grape seed oil, olive oil, rape seed oil, sunflower oil, and walnut oil can be properly identified. Furthermore, it has been found that the frequency of concrete absorption bands in the fingerprint region ($700\text{--}1500\text{ cm}^{-1}$) provides direct information about the ratio between saturated and cis monounsaturated fatty acid acyl groups. Stretching vibrations of trans and cis olefinic double bonds have been observed around 3025 and 3006 cm^{-1} , respectively. Beside C-H stretching vibrations between 3000 and 2850 cm^{-1} , the C=O group of triglycerides shows strongest absorption bands at approx. 1746 cm^{-1} [4]. A partial least squares (PLS) calibration model for the prediction of the peroxide value was developed based on spectral information in the range between $3750\text{--}3150\text{ cm}^{-1}$ which exhibits the characteristic hydroperoxide absorption bands, centered at 3444 cm^{-1} [5]. The reproducibility of this FTIR method was found to be more reliable than the usually applied chemical titration method.

The intensities of Raman bands near 1660 cm^{-1} and 1670 cm^{-1} have been assigned to the individual cis and trans isomer contents, present in various edible oils [6]. It has been also reported that the ratio of scattering intensity arising from the C=C stretching vibration (1600 cm^{-1}) to that obtained from the CH₂ scissoring mode (1444 cm^{-1}) was used to reliably predict the iodine values of triglycerols and unconjugated vegetable oils. These Raman measurements are extremely useful for quality control purposes in the food industry, particularly the option to perform remote on-line control measurements through optical fibers to monitor simultaneously the degree of unsaturation and isomer formation during hydrogenation processes of edible oils.

Raman spectroscopy has been also applied to study the arrangement of the acylglycerol molecules, which leads to different melting behavior and other properties of oils. Especially the C-H stretching region (signals at 2850 cm^{-1} and 2885 cm^{-1}) in the Raman spectrum provides valuable information on the environment of hydrocarbon chains in lipids due to different liquid-crystalline lipid-water phases [7].

4 Carbohydrates

Most FT Raman spectra obtained from measurements of various mono-, di-, oligo- and polysaccharides show characteristic bands which can be used for discrimination purposes. The spectrum of sucrose presents the characteristic bands of α -glucose (847 cm^{-1}) and β -fructose (868 cm^{-1}); in the spectrum of maltose besides α -glucose signals the band due to β -glucose (898 cm^{-1}) can be seen, whereas cellobiose shows only a signal at 885 cm^{-1} representative for the β -anomer. Distinctive bands at 1462 cm^{-1} , 1126 cm^{-1} , 840 cm^{-1} , due to sugar vibrational modes, can be used to determine the distribution of these components in various plant tissues such as carrot roots [8]. ATR-IR spectroscopy combined with PLS algorithm allows the quantification of the three main monosaccharides (glucose, maltose and fructose) in glucose syrups [9]. Furthermore, infrared spectroscopy has been successfully applied for monitoring wine fermentation. A reliable PLS calibration model was developed and proved to be effective for analyzing cv. Cabernet Sauvignon for e.g. glucose and fructose content [10].

Generally, FT Raman spectroscopy was found to be a powerful tool for investigation of higher plant cell walls and their components providing complementary information to that obtained by FT-IR microspectrometry [11].

Amylose and amylopectin can be also successfully analyzed by application of Raman spectroscopy. It has been reported that the structural differences of both starch materials can be detected in the C-H stretching region between 2700 and 3100 cm^{-1} [12].

5 Phenolic substances

Most studied plant secondary metabolites are flavonoids, which show a wide distribution in each part of vascular plants. Here, individual flavonoid types can be modified by hydroxylation, methylation, acylation, and glycosylation.

Anthocyanins belong to the most important group of plant pigments that are visible to human eyes. In general, their concentration in most fruits and vegetables varies between 0.1 and $1\text{ g}/100\text{g}$ dry matter. Resonance Raman (RR) spectroscopy has been applied to determine the

influence of glycosylation on the benzopyrylium part of the flavonoid molecule, and provided some characteristic spectral features of these phenomena [13,14]. In this context, characteristic spectral features were observed in the spectral range between 500 and 900 cm^{-1} . When the C(5) position of the anthocyanin molecule is glycosylated, significant perturbations of spectral features between 640 and 750 cm^{-1} are visible, but they depend also on the nature of the individual sugar. Resonance Raman spectra obtained directly from vacuoles of the skin of cv. Pinot noir wine berries (*Vitis vinifera*) showed that its main pigment is malvidin 3-glucoside, which occurs in the quinonoidal base form inside the skin whereas in the outer face of the skin it is mainly present in the flavylum form [15]. In the epidermis of petals of the common mallow (*Malva sylvestris*), only malvidin 3,5-diglucoside has been detected, entirely in the cationic flavylum form [15].

Several phenolic compounds biosynthesized in the phenylpropane pathway such as anethole, eugenol, carvacrol, and thymol, have been identified as major essential oil components. They are widely used in the production of perfumes, flavorings and phytopharmaceuticals or as additives for pet food relating to their antibiotic properties.

Both IR and Raman spectroscopies allow identifying these substances. They demonstrate strong IR bands due to C-H wagging vibration between 800 and 920 cm^{-1} , whereas in the FT-Raman spectrum ring deformation vibration is observed between 740 and 760 cm^{-1} [16–18]. Significant differences are seen for isomeric compounds like thymol /carvacrol in FT-Raman as well as in ATR-IR spectra. In FT-Raman spectra ring vibration of thymol is seen at 740 cm^{-1} , while for carvacrol this signal is shifted to 760 cm^{-1} . In the ATR-IR spectrum the most intense bands are seen at 804 cm^{-1} (thymol) and 811 cm^{-1} (carvacrol) [16,17].

FT-Raman spectroscopy was also used for investigation of curcumin, which is a valuable dyeing component of curcuma root (*Curcuma sativa*). The most intense bands appearing at 1630 and 1601 cm^{-1} can be assigned to the benzene ring, whereas bands at 1185 and 965 cm^{-1} are due to COC and COH vibrations [19]. 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 [20].

6 Terpenoids

Terpenes occurring in flowers, stems, leaves, and roots of numerous plant species are frequently used in perfume compositions as well as in flavours of food-stuffs or mouth care products. For numerous essential oil plants such as basil (*Ocimum basilicum*) [21], fennel (*Foeniculum vulgare*) [20, 22], oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*) [16–18, 23–25], marjoram (*Origanum majorana*) [16, 17], pepper (*Piper nigrum*) [26], chamomile (*Chamomilla recutita*) [20, 23], eucalyptus species [27] and various citrus fruits [28] calibration models have been developed which allow to quantify valuable components and to discriminate different chemotypes.

Acyclic monoterpenes show the most intense bands due to stretching vibrations of C=C bonds at about 1670 cm^{-1} in the Raman spectrum, whereas IR spectra are more miscellaneous. Both, monocyclic and bicyclic terpenes, demonstrate strong IR bands due to C-H wagging vibration between 800 and 920 cm^{-1} , however by using Raman spectroscopy differentiation between these groups is more clear. Ring deformation vibration observed in the FT-Raman spectrum of monocycles between 740 and 760 cm^{-1} , in the case of bicycles is shifted about hundred to lower wavenumbers and can be therefore recognized in the range between 645 and 666 cm^{-1} [16–18]. Significant differences are seen for structural isomers like α -terpinene/ γ -terpinene in FT-Raman as well as in ATR-IR spectra. In the FT-Raman spectrum characteristic C=C stretching vibrations appear at 1611 cm^{-1} for α -terpinene and at 1701 cm^{-1} for γ -terpinene reflecting the difference between a conjugated and a nonconjugated system, respectively.

More than 600 carotenoids have been found in plants, but only α , β and a few other carotenes (not lycopene and lutein) can be converted into vitamin A by human beings. 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 [29, 30]. FT-Raman spectroscopy also gives a strong enhancement of carotenoids due to the known pre-resonance effect; furthermore disturbing fluorescence effect of biological material usually observed when laser excitation is performed in the visible 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 (ν_1) and C-C stretching (ν_2) vibrations of the polyene chain. Additionally, in-plane rocking mode 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. It has been shown that the wavenumber location of these bands is strongly dependent on the length of the carotenoid chain, and generally, carotenoids with 11, 9, 8, 7 conjugated C=C bonds have their characteristic bands at about 1510, 1524, 1530, 1536 cm^{-1} [31,32].

7 Alkaloids

In spite of the fact that alkaloids show a broad range of different chemical structures only a few vibrational measurements of these plants substances have been published. FT Raman spectra obtained from green berries of pepper (*Piper nigrum*), ground black pepper and black pepper oleoresin predominantly show significant key signals of piperine [26]. Apart from the intense –C-H stretching vibrations between 2800 and 3100 cm^{-1} , the main Raman signals occur in the fingerprint range between 1100 and 1630 cm^{-1} . On the basis of ATR-IR and Raman measurements chemometric equations have been developed for calibration of piperine content in pepper samples, showing a comparatively high prediction quality [26].

Both, ATR-IR technique and FT Raman spectroscopy have been demonstrated to be very promising tools for fast and reliable determination of the main alkaloids (morphine, codeine, papaverine, thebaine and noscapine) occurring in poppy plant material and related pharmaceutical products [32]. Raman spectra in the fingerprint range between 700 and 1500 cm^{-1} show numerous sharp bands which are mainly assigned to deformation and stretching vibrations of the alkaloid ring system. Raman spectra obtained from poppy milk presents most relevant morphine bands (e.g. peaks at 631, 1620, 1642, 3044 and 3073 cm^{-1}) and aqueous-ethanolic extracts prepared from unripe poppy capsules show several specific peaks which can be assigned to vibrational modes of the mentioned alkaloid substances.

Guarana seeds, which represent an important product of the Amazonian rain forest, were also successfully analyzed by FT Raman spec-

troscopy aiming to determine the individual content of the main alkaloids (caffeine, theophylline and theobromine) [33]. The discrimination between anhydrous caffeine and its monohydrate form was presented as a key band at 1656 cm^{-1} and its relative intensity compared to the 1698 cm^{-1} signal, both of which are CO stretching modes. The occurrence of these bands in the Raman spectrum of guarana methanolic extracts confirms that this product contains anhydrous caffeine. Theobromine was distinguished from caffeine and theophylline by the presence of a band at 620 cm^{-1} , whereas the other two alkaloids have a strong feature at 556 cm^{-1} and a medium doublet for caffeine to be seen at 643 and 741 cm^{-1} [33].

8 Polyacetylenes

Generally, polyacetylene spectra show strong and polarized bands, due to the triple bonds in the molecule, in the region around 2200 cm^{-1} . As has been discussed earlier [34], the number of triple bonds as well as substituents influence the frequency of the polyacetylene $\text{-C}\equiv\text{C-}$ stretching modes. Thus, the spectral position of $\text{-C}\equiv\text{C-}$ vibrations and pattern of Raman bands usually provide enough information to recognize the type of substitution and to support the identification of polyacetylenes [3]. Generally, for compounds containing a $\text{-C}\equiv\text{C-C}\equiv\text{C-}$ grouping, the vibrational modes are described as asymmetric and symmetric $\text{-C}\equiv\text{C-C}\equiv\text{C-}$ stretching, and accordingly they are IR and Raman active, respectively. The characteristic, strong and polarized, symmetric stretch of the $\text{R-C}\equiv\text{C-C}\equiv\text{C-R'}$ structure should be seen in the interval of $2257\text{-}2251\text{ cm}^{-1}$ in the Raman spectrum [10].

It has been shown that by using Raman spectroscopy it is possible to distinguish the main polyacetylenes occurring in carrot as well as in ginseng roots. Carrot polyacetylenes possess a similar molecular structure with two adjacent triple bonds substituted with one -OH group (falcarinol) and two -OH groups (falcarindiol) and show their characteristic $\text{-C}\equiv\text{C-}$ mode in the Raman spectrum at 2258 and 2252 cm^{-1} , respectively [35,36]. In ginseng roots, beside falcarinol, its epoxy derivative (panaxydol) occurs in higher amounts, and can be characterized by the strong Raman signal at 2260 cm^{-1} [37]. Falcarinol and panaxydol are among the most bioactive polyacetylenes isolated from ginseng

and hence they are very important in relation to their anti-cancer effect and other pharmacological properties of ginseng roots. Furthermore, faltarinol and faltarindiol contribute strongly to the bitter taste of carrot [38,39], but may also have some negative effects when administered in high doses [40].

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