Evaluating naturals

Application of Vibrational Spectroscopy Methods in Essential Oil Analysis

A review of NIR, IR and Raman spectroscopic methods applied to the analysis of various essential oils

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ear-infrared (NIR) spectroscopy, which is related to the first experiments performed by Herschel in 1800, has been widely used in agricultural and food sectors. The first methods in this specialized field of application were developed by Norris et al. in 1951.¹⁻⁴ However, a real breakthrough of NIR spectroscopy as a routine technique was first obtained in the early 1980s, when efficient chemometric algorithms were successfully introduced.

Generally, the statistical support improved the interpretation of the NIR data dramatically and contributed strongly to this spectroscopy tool becoming one of today's fastest growing analytical technologies. While numerous NIR spectroscopy methods have been established in the field of agriculture for more than four decades, IR (infrared) spectroscopy has been primarily used as a structure-elucidation technique; only limited application can be found in literature for characterization of liquid samples, such as fruit juices or other plant extracts. Until recently, Raman spectroscopy was restricted mostly to academic research. However, in the last 10 years, the development of Fourier transform methods involving the replacement of the dispersive element with an interferometer gained broader industrial application. Most attempts to get specific Raman signals from plant tissues failed because fluorescence, with a quantum yield several orders of magnitude larger than that of the Raman effect, concealed the whole Raman spectrum. Using excitation by an Nd:YAG laser at 1064 nm disturbing fluorescence of the enzymes and coenzymes, resulting usually by application of all other Raman techniques with excitation wavelengths in the visible range and even at 785 as well as 830 nm, is avoided.

At a glance

A review of NIR, IR and Raman spectroscopic methods applied to the analysis of various essential oils is presented. Generally, the described measurements can be performed directly on plant material as well as on the volatile fraction isolated by hydro-distillation or solvent extraction. Whereas NIRS data can be interpreted only by application of chemometric algorithms, IR and Raman spectra obtained from the individual essential oils are mostly well-structured and present characteristic key bands. Based on such marker bands produced by individual volatile substances, spectroscopic analyses in principle allow discrimination of different species, and even chemotypes among the same species. Combination of vibrational spectroscopy and hierarchical cluster analysis provides a fast, easy and reliable method for chemotaxonomy characterization. In most cases, determination of main oil components applying the PLS algorithm results in comparatively high- R² and low- SECV values. The ability to rapidly monitor various essential oils makes it possible to efficiently select high-quality single plants from wild populations as well as progenies of crossing experiments. Furthermore, the vibrational spectroscopy methods described here can also be used in the flavor and fragrance industry in order to perform fast quality checks of incoming raw materials as well as continuous controlling of distillation processes.

Generally, IR, NIR and Raman spectra of essential oils can be observed as a consequence of molecular vibrations of the individual components (e.g. mono- and sesquiterpenes, alcohols, aldehydes, aromatic substances) present in the volatile fraction. Due to the different excitation conditions of the mentioned spectroscopic techniques, the relationship between absorption intensities and the addressed functionalities of the analytes vary significantly.

NIR spectroscopy measures a plant's absorption of electromagnetic energy over a wavelength range of 800-2,500 nm (12,500-4,000 cm⁻¹). The resulting spectra are dominated by broad, overlapping absorption bands that involve mainly overtones and combination vibrations of the C-H, O-H or N-H fundamental vibration modes.

Scanning IR and NIR spectrometers operate with a polychromatic light source from which the sample absorbs specific frequencies corresponding to the individual molecular vibrational transitions. Contrary to that, in Raman spectroscopy the sample is illuminated with monochromatic laser light (in the visible or NIR wavelength range); it has been observed that most of the scattered light is of the same frequency as the illuminating light (Raleigh or elastic scattering).⁵ Only a small segment of the excited molecules ($< 10^{-6}$) is scattered at different frequencies compared to the incident light (Raman scattering). The result is something similar to an IR spectrum with a shift scale from about 4,000 to 20 cm⁻¹. Contrary to IR spectroscopy, the selection rule for Raman signals is that, during vibration of a chemical bond, a change of polarizability must be observed.

NIR Applications: Measurements on Essential Oil Plants

Spice extracts and essential oils are obtained from various parts of plants including bulbs, rhizomes or roots, barks, leaves, flowers, fruits and seeds. Usually, routine analysis of essential oil constituents is performed by GC-FID methods and identification of unknown substances is achieved by applying hyphenated techniques such as GC/MS and GC-IR. In aromatic plants, essential oils can be present as isolated cells (organelles or idioblasts) or, closer to the surface of the plant material, as glandular hairs (as in *Labiatae* leaves) in cavities or special channels (as in umbelliferous fruits).

Caraway and fennel: The first attempts to perform non-destructive NIR reflectance measurements on Umbelliferae fruits were made by Toxopeus and Bouwmeester using an NIRSystems 6500 and InfraAlyser 500.⁶ The authors developed calibration models for the prediction of total essential oil and carvone contents in caraway fruits. Similar studies were done on caraway samples by another working group, resulting in a clearly higher prediction quality.^{7,8} Additionally, these scientists established NIR calibrations for fennel fruits using numerous single plants with a wide range of essential oil content and different essential oil substances such as carvone, fenchone and anethole.^{8,9} Approximately 2 g of the fruits were transferred into a standard reflectance cup without performing any clean-up procedures; the NIR spectra were collected with an InfraAlyzer 500 in the range of 1100-2500 nm. The described NIR method was found to be a suitable approach to select high-quality single plants during the breeding process.

The prediction quality obtained with a dispersive and a Fourier transform spectrometer (NIRSystems 5000 and IFS 55 EQUINOX) showed only small differences with regard to the above mentioned secondary metabolites occurring in caraway and fennel fruits.¹⁰ Therefore, it is assumed that, more than the optical configuration itself, the sampling schemes seem to be of major concern for the reliability of the calibration results. In order to establish reliable NIR calibration statistics it is generally very important to use samples that show a very high variability with regard to the individual quality parameters.

In order to establish an NIR spectroscopic network, the accuracy and transferability of the obtained individual calibrations for estimating the content and composition of the volatile fraction in fennel fruits (as an example of an essential oil plant) was investigated.²⁴ In this context, 345 fennel samples were measured on a dispersive grating and scanning diode array system to gain insight into the influence of sample presentation and scanning techniques. For all instruments, calibrations with standard errors in the range of the reference GC method were achieved, and methods of overcoming different spectral responses were described.

Mentha: The potential of NIR spectroscopy was also investigated for the determination of secondary metabolites in leaves of different Mentha species.¹¹ The results prove that simultaneous prediction of the oil content and its main terpenoid components menthol and menthone can be reliably achieved in dried peppermint (Mentha x piperita L.) (T-1). For those components that usually occur in smaller amounts (for example menthofuran, isomenthone and pulegone), a semi-quantitative determination is possible, at the very least. The amounts of essential oil, menthol, menthone, isomenthone and 1,8-cineole can also be reliably predicted on fresh plant material. So, this NIR method can be applied as a useful tool to define the optimal harvest time with respect to essential oil and menthol content. The peppermint oil spectra are dominated by overtones or difRange, mean and NIRS correlation statistics for the essential oil content (mL/100 g) and composition (percent) in the dried leaves of *Mentha piperita* (n = 82); SECV = standard error of cross validation; R^2 = multivariate coefficient of determination

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Parameter	Range	Mean	SECV	R ²
essential oil content	0.63 - 3.63	2.13	0.19	0.93
limonene	0.00 - 6.47	1.25	0.62	0.82
1,8-cineole	1.15 - 6.04	3.59	0.47	0.80
menthofuran	0.00 - 4.20	1.01	0.64	0.62
menthone	10.10 - 56.90	30.91	4.00	0.88
isomenthone	2.02 - 8.44	4.16	0.98	0.53
menthol	15.90 - 58.60	38.13	3.93	0.86
menthyl acetate	0.77 - 7.89	3.78	1.11	0.57
pulegone	0.00 - 5.77	0.85	0.63	0.64
piperitone	0.41 - 2.38	1.25	0.34	0.60

ferent combinations of C-H-stretching and bending vibrations. The spectra recorded from dried leaves show additional strong absorption bands of water (maxima seen at 1450 and 1940 nm, respectively), in addition to other main matrix constituents such as carbohydrates (F-1).

Sage and marjoram: NIR measurements performed on sage leaves (*Salvia officinalis* L.) showed similar high prediction quality.¹² As presented in T-2, the individual essential oil and thujone contents can be analyzed, not only in the dried herb, but also in fresh leaves within a few seconds. Since these two quality parameters have been found to be genetically

Near-infrared spectra obtained from dried leaves (1) and isolated essential oil (2) of *Mentha piperita* (v = stretching vibration, δ = bending vibration); a: 3x v(CH); b: 2x v(CH) + δ (CH); c: 2x v(CH); d: v(CH) + δ (CH); e: 3x δ (CH); f: v(CH) + v(CC)



determined, a fast and reliable selection of appropriate sage genotypes within breeding experiments is principally possible. For several years, the composition of marjoram oil and its occurrence in various marjoram cultivars (*Origanum majorana* L.) have been extensively studied.¹³⁻¹⁵ During hydro-distillation, *cis*-sabinene hydrate and *cis*-sabinene hydrate acetate are transferred on a bigger scale to artifacts such as terpinene-4-ol, α -terpinene and γ -terpinene. In order to get a more authentic description of the phytochemical plant status, marjoram herb was carefully extracted with isooctane and subsequently analyzed by GC; based on the obtained reference data, an NIR calibration equation was successfully established.¹⁶

Rosemary: Major essential oil substances of rosemary leaves (*Rosmarinus officinalis* L.), such as 1,8-cineole, α -pinene and camphor, have also been quantified by NIRS measurements.^{17,18} Accordingly, different genotypes and plants cultivated in various Mediterranean countries such as France, Spain and Morocco could be successfully discriminated via their varying essential oil composition (for example, North African oils from Morocco are comparatively high in cineole, whereas oils originating from Spain present the highest camphor and α -pinene content).

The most important terpenoids, including the nonvolatile component carnosic acid, can be accurately predicted by a described NIR procedure, resulting in coefficients of determination mostly > 0.90 and standard errors of calibration similar to errors observed in the reference analysis methods (volumetric determination of the essential oil, GC and HPLC).^{17,18} Near infrared calibrations for the essential oil content and for the terpenoid composition were also carried out using a portable spectrometer, operated by a 12 V car battery. In this context it was observed that the prediction quality was generally lower in comparison to that received with a stationary NIR spectrometer system. These quality losses were mainly related to the limited wavelength range (950 to 1700 nm) and the lower spectral resolution (6 nm) of the used diode array spectrometer system. It should be noted that the

intensive bands caused by $\gamma(\rm CH)$ and $\delta(\rm CH)$ combination vibrations of the terpenoids in the range of 2200 to 2400 nm are not recorded by the portable NIR spectrometer. Practical use of the presented NIR method exists in the efficient selection of high-quality single plants from natural habitats and in the characterization of crossing progenies.

Hops: For cultivation of hop (*Humulus lupulus* L.), mainly used for production of beer, knowledge of quality parameters such as levels of α -/ β -acids, moisture, cohumulone and various essential oil components is very important. So-called "aroma" hop varieties are generally low in cohumulone content; furthermore, it is known that pleasant aroma is usually associated with a low proportion of myrcene in the essential oil fraction. In contrast, higher levels of humulene, caryophyllene and farnesene contribute to a desirable quality of good hop aroma.

First attempts to determine these parameters simultaneously by NIRS were made in 1981 and 1982.¹⁹⁻²¹ The milled and sieved hop samples were measured in reflectance, and based on GC reference analyses of the volatile hop aroma substances, reliable correlations between chromatographic data and NIR predictions could be achieved. As mentioned by the authors' attempts, NIR reflectance spectroscopy appeared to be very useful in determining the proportion of the most valuable quality parameters in hop varieties. Thus, the described method is of value to both the hop breeder and hop producer.

Thyme: Thyme (*Thymus vulgaris* L.) is mainly cultivated for essential oil production. It is also utilized in spice mixtures and as additive for pet food due to its antibacterial properties. Its spicy-phenolic odor is mainly related to the volatile components thymol and carvacrol. The oil content, as well as both main valuable phenolic substances, can be quantified in dried leaves applying NIRS measurements.²²

Basil: NIR spectra received from air-dried leaves of basil (*Ocimum basilicum* L.) predominantly show strong bands of water at 1450 and 1940 nm; also shown are spectral responses of other main plant constituents such as cellulose — signals of the volatile fraction can be observed between 2250 and 2350 nm. A comparatively high correlation has been found for the linalool content determined in cv. Genoveser (linalool type) between reference GC values and the predicted data received from the NIRS calibration set (N = 72, R² = 0.967, SECV = 0.008).²³

NIRS Measurements on Isolated Essential Oils

Today, most of commercially traded essential oils are specified by the International Organization for Standardization (ISO); furthermore, product quality of various essential oils is described in individual national Pharmacopoeia. Generally, the individual analytical methods described in these specifications (e.g. density, refractive index, optical rotation, titrimetric parameters such as acid number, ester number or ester number after saponification, gas chromatography) are Range, mean and NIR correlation statistics for the amounts of essential oil (mL/100 g in the plant material) and thujone (g/100 g in the essential oil) in different sage genotypes; all NIR predictions are based on GC analysis results received from dried leaves

		Essential oil	Thujone
Dried leaves	range	0.33 - 3.46	1.01 - 27.11
	mean	1.63	4.75
	SECV	0.17	1.27
	R ²	0.92	0.89
	SEV	0.18	1.21
	BIAS	0.02	0.25
Fresh leaves	SECV	0.21	2.01
	R ²	0.88	0.79
	SEV	0.24	1.79
	BIAS	0.03	2.21

time-consuming and cost a lot of money.

Due to the expense of quality checks, there is growing interest in reliable, rapid and simple methods. Furthermore, there is demand for rapid spectroscopic techniques, such as NIR or Raman, for on-line control of distillation processes. The first attempts to characterize and distinguish various essential oils were made by Schulz and Lösing.²⁵ A qualitative model was successfully used to present the well-separated clusters of the analyzed products (essential oils of rosemary, sage, basil, tarragon, origanum and clary sage) in a 2-dimensional factor plot. The authors found that those oils could be differentiated by PCA, which present a similar pattern of main constituents.

Mentha: Samples of the most important commercial Mentha oils isolated from Mentha x piperita L., Mentha spicata L., Mentha arvensis var. piperascens Holmes., Mentha citrata (Ehr.) and Mentha pulegium L. were measured in the transflection mode using a diffuse gold reflector (path length = 0.2 mm). Although peppermint and dementholized cornmint oil form a common factor space, a discrimination of these two very similar oil types is generally possible.^{11,26} Subsequent studies demonstrate that the most important essential oil components can be reliably predicted, if the NIR data are interpreted by suitable chemometric algorithms.

Mint oil spectra are dominated by overtones or different combinations of C-H stretching and deformation vibrations. Most significant and intensive absorptions occur 1-2

in the region of 2250 to 2500 cm^{-1} . The statistical results received by the described procedure show that in most cases the individual standard errors of cross validation (SECV) are not significantly higher than the standard errors of the more common GC method. The multivariate coefficients of determination (R^2) are always >0.94, representing a good correlation with the reference data. Only in pulegone and piperitone, which occur in the oil in amounts of 1-2 g/100 g, are the R² values below 0.90. Aside from application in quality control, this technique can be successfully used for on-line control of distillation processes of various mint oils. A sophisticated NIR method has already been introduced in the flavor industry to perform on-line control during the production of L-menthol.²⁷ Unwanted by-products, such as iso-menthol, neo-menthol and neo-iso-menthol, can be detected if these substances occur in amounts of > 50 mg/kg. The author of the study established a three-dimensional qualitative model that presented L-menthol and the three previously mentioned menthol isomers in separate clusters.

Citrus: Detailed interpretation of NIR data have also performed on various citrus oils.²⁸ The measured citrus oils obtained from grapefruit (Citrus x paradisi MacF.), orange (Citrus sinensis (L.) Osbeck), mandarin (Citrus deliciosa Ten.), lemon (Citrus limon (L.) Burm. f.), bitter orange (Citrus aurantium L.), and lime [Citrus aurantifolia (Christm. et Panz.) Swingle] were discriminated on the basis of their individual spectral data using principal component analysis by the first three PCA factors (98.3 percent of the variation explained). The highest influence was found for factor 1 in accordance to high (orange, grapefruit) or low (lime) limonene contents. In the case of lime oil, discrimination between cold-pressed and distilled products could be achieved. Since the optical rotation is strongly correlated with the limonene content, this physical parameter was also precisely predicted in all citrus oils. In addition to the individual terpenoid components (limonene, myrcene, α -pinene, β -pinene, sabinene, γ -terpinene and terpinolene), the total aldehyde content, ranging from 0.3 to 3.2 g/100 g, could be simultaneously determined. The lower NIR prediction quality of this parameter is related to the higher standard error of the reference titrimetric determination. A new NIRS method has been developed to find an efficient approach to determine

the 1,8-cineole content in eucalyptus oil.²⁹ The official assay described in the British Pharmacopoeia (BP) is a freezing-point method involving the addition of o-cresol to the eucalyptus oil, whereupon the phenolic substance and 1,8-cineole form a solid complex.³⁰ The mean accuracy for the NIRS method differed only by 1.01 percent or less; the mean BIAS was found to be +/- 0.33 percent or less. Therefore, the authors proposed to use NIR spectroscopy as an alternative method for the determination of 1,8-cineole in eucalyptus oils. The same research group analyzed several lemongrass and lemon oils for their citral content via NIRS, employing a reflection vessel for sample presentation.³¹ The obtained NIR data were correlated with the results of the official titration method described in the BP. According to the calculated calibration statistics, the new NIRS method may prove beneficial for the accurate determination of the citral content of lemongrass oils and *approximate* citral content of lemon oils.

Basil, *milfoil*: Feasibility studies were also performed to characterize the essential oils isolated from various basil (*Ocimum* sp.) and milfoil (*Achillea* sp.) gene bank accessions.²³ For NIR transflection measurements, sample amounts of approximately 600 μ L were found to be sufficient. The authors developed calibration sets for the most relevant essential oil components, and were able to demonstrate high correlations between NIR spectral data and GC reference results (T-3).

Pepper: Recently, a study demonstrated that most pepper oil substances in amounts of > 0.1 total GC peak area percent can be analyzed by NIRS. Furthermore, it was proven that NIRS measurements present sufficient reliability to predict the main essential oil substances directly in ground black and white pepper.³² According to the different amounts of main essential oil components such as caryophyllene, sabinene, δ -3-carene and limonene, it was possible to clearly discriminate three pepper types based on principal compound analysis of the spectral data.

Chamomile: Essential oils, hydro-distilled from numerous gene bank accessions of chamomile, were also used for NIR calibration development. The results showed that the main components of chamomile oil (β -farnesene, α -bisabolol, bisabololoxides A and B, and chamazulene) could be precisely predicted by NIR measurements.^{33,34} Based on these data, the main chamomile chemotypes could be easily discriminated.

Labiatae species: All relevant substances occurring in amounts of > 1 g/100 g in various *Origanum* oils could be reliably analyzed by NIR transflection spectroscopy.²² The authors proceeded from the assumption that NIR spectroscopy is also applicable for the rapid analysis of various other essential oils receiving a similar prediction quality. The authors hypothesized that in the near future several GC analyses performed in quality control and in-process control laboratories will be replaced by the cheaper and much faster NIR spectroscopy methods.

Thymol and carvacrol, which occur in numerous

species of the Labiatae family, present significant differences in the spectral range between 2300 and 2500 nm. These absorption bands are mainly related to combination bands of C-H-stretching and deformation vibrations. It is assumed that the good prediction quality obtained for thymol and carvacrol concentration in various thyme genotypes is predominantly influenced by these spectral features.²² According to the results described for marjoram and citrus oils, the NIR spectra of isolated basil oils are dominated by overtones and combinations of C-H-stretching and bending vibrations, which can be seen as comparatively sharp signals in the range between 1600 and 1800, and 2200 and 2500 nm. A study has demonstrated that the individual basil chemotypes (e.g. eugenol type, linalool type, methyleugenol type, estragole type, citral type, camphor type, caryophyllene type, elemicin type) present distinctive differences within these wavelength intervals.³⁴

IR and ATR-IR Applications: Measurement on Essential Oil Plants and Isolated Essential Oils

While mid-infrared spectroscopy was historically used as a qualitative technique for the identification and verification of pure essential oil substances, there are only a few applications in this area that use multivariate calibration techniques to extract the quantitative data from the information-rich and well-structured mid-IR signals. But in combination with the attenuated total reflectance (ATR) technology, the handling of liquid samples has become more convenient and quick; furthermore, portable FT-IR systems that require only sample amounts of several µL are now available.

Essential oil plants: Generally, isolation of the essential oil from various parts of plants is a time-consuming process that sometimes may lead to changes with regard to composition and quality. In order to reduce the efforts for quality analysis, the ATR-IR technique can be used in combination with efficient chemometric algorithms for rapid characterization of the volatile fraction in various ground pepper samples.³² Generally, good calibration results have been obtained for total essential oil content ($R^2 = 0.88$) and most terpenoids (R^2 :0.97 for β -pinene, 0.94 for myrcene, 0.93 for α -phellandrene and limonene, and 0.91 for α -pinene). Some volatiles show lower prediction quality, which is mainly related to the low concentration level of these substances in the peppercorn or to coincidences with the spectral contribution of other plant constituents.

Measurement on isolated essential oil: Whereas NIR spectroscopic data can be interpreted only by application of chemometric algorithms, IR spectra obtained from the isolated essential oils present characteristic key bands that can be used as marker bands to discriminate different plant species and chemotypes.³⁵ Usually, the analysis can be based on the ATR-IR spectra of the available pure terpenoid compounds as well as characteristic group frequencies published

NIR correlation statistics for most valuable substances occurring in the essential oil of milfoil (Achillea millefolium L.) (N=74)

Oil component	Range (g/100 g)	SECV (g/100 g)	R ²
sabinene	0.22-11.65	0.2	0.995
β-pinene	0.42-16.63	0.4	0.995
1,8-cineole	0.31-25.30	0.4	0.998
artemisia-ketone	0.21-30.47	0.3	0.999
camphor	0.32-28.54	0.7	0.995
borneol	0.56-13.36	0.5	0.974
anethole	0.25-69.61	0.4	0.999
chamazulene	0.26-18.69	0.6	0.993

in several spectroscopic books.³⁶⁻⁴⁰ In T-4, assignments of the most characteristic bands occurring in the IR spectra of some terpenoids are presented.

Significant differences are seen, for example, for isomeric compounds like thymol, carvacrol, α -terpinene and γ -terpinene. In the ATR-IR spectrum the most intense bands are seen at 804 cm⁻¹ for thymol and at 811 cm⁻¹ for carvacrol. These bands can be attributed to out-of-plane CH wagging vibrations, which are the most significant signals used in distinguishing different types of aromatic ring substitution.³⁹ These bands are usually weak in Raman, but very intense in the IR spectrum. Likewise, key bands are seen for the cyclohexadiene isomers: γ -terpinene can be identified by CH and CH₂ wagging vibrations at 781 cm⁻¹ and 947 cm⁻¹, whereas α -terpinene shows only one intensive signal at 823 cm⁻¹. The bicyclocamphane compound 1,8-cineole demonstrates three characteristic signals in the ATR-IR spectrum at 984 cm⁻¹, 1079 cm⁻¹ and 1214 cm⁻¹. Terpinen-4-ol, occurring as the main component in tea tree oil, can be identified by characteristic CH and CH₂ wagging vibrations at 887 cm⁻¹ and 924 cm⁻¹. For all compounds presented in T-4, it is possible to find marker bands in the ATR-IR spectrum, which can thus be used for identification purposes.

Marjoram and oregano: The spectra of the individual oils more or less show profiles similar to their main components; all their key bands are found to be characteristic for the individual species and chemotypes, as has been shown for oils isolated from various species of the genus *Origanum.*³⁶ If the main oil component is present in relatively high percentages (at least 50 percent), as can be seen for

Compounds	FT-Raman (cm ⁻¹)	Assignment	ATR-IR (cm ⁻¹)	Assignment
carvacrol	1623 760	ν (C=C) δ (ring)	811	ω (C-H)
1,8-cineole	652	δ (ring)	1374 1214 1079 984 843	$δ_{sym}$ (CH ₃ (CO)) v_{as} (C-O-C) v_{s} (C-O-C) ω (CH ₂)
citronellal	1725 1674 1382	ν (C=0) ν (C=C) δ _{svm} (CH ₃)	1725 1116	v (C=0)
citronellol	1674 1382	v (C=C) δ_{sym} (CH ₃)	1377	δ (C-O-H)
p-cymene	1613 1208 804	ν (ring) δ (ring) δ (ring)	1515 813	ω (C-H)
geranyl acetate	1679	v (C=C)	1738 1365 1227 1021	v (C=0) δ_{sym} (CH ₃ (C=0)) v_{as} (C-0-C) v_{s} (C-0-C)
limonene	1678 1645 760	ν (cyclohexene C=C) ν (ethylene C=C) δ (ring)	1644 886	ν (ethylene C=C) ω (C-H)
myrcene	1672 1634 1293	ν (C=C) ν (C=C) τ (CH ₂) ₂	1637 1595 989 890	ν (C=C) ω (CH ₂) ω (C-H)
phenylethyl phenylacetat	e 1605 1203 1003	ν (ring) δ (ring) δ (ring)	1736 1131 816 698	ν (C=O) ν (C-O-C) ω (C-H) δ (ring)
α -pinene	1659 666	ν (C=C) δ (ring)	1658 886 787	ν (C=C) ω (C-H)
β-pinene	1643 645	ν (C=C) δ (ring)	1640 873	v (C=C)
sabinene	1655 652	ν (C=C) δ (ring)	1653 861	v (C=C)
<i>cis</i> -sabinene hydrate	748	δ (ring)	926	ω (CH ₂)
trans-sabinene hydrate	755	δ (ring)	920	ω (CH ₂)
thymol	740	δ (ring)	804	ω (C-H)
terpinen-4-ol	1679 730	ν (cyclohexene C=C) δ (ring)	924 887	ω (CH ₂) ω (C-H)
α -terpinene	1611	v (conjugated C=C)	823	ω (C-H)
γ-terpinene	1701 756	ν (nonconjugated C=C) δ (ring)	947 781	ω (CH ₂) ω (C-H)

vibrations: <code>v-stretching</code>, $\delta\text{-deformation},$ $\omega\text{-wagging}$

carvacrol, 1,8-cineole, thymol, γ -terpinene and *cis*-sabinene hydrate types, the corresponding oil spectrum is dominated by its characteristic key bands (see F-2). Those compounds, which occur in essential oils only in low percentages, do not significantly influence the ATR-IR spectrum. However, in the case of the terpinen-4-ol chemotype, the corresponding vibrational spectrum reflects the complex composition of this essential oil (F-2F), with the intensive bands attributed to: terpinen-4-ol (887 cm⁻¹), *cis*-sabinene hydrate (926 cm⁻¹), γ - terpinene (781 cm⁻¹) and α -terpinene (823 cm^{-1}) , which are present in the oil in amounts of approximately 25 percent, 14 percent, 14 percent and 8 percent, respectively. This example demonstrates that vibrational spectroscopy can be used not only for chemotype classification of marjoram and oregano essential oils, but also for identification of their major oil constituents.

Thyme, chamomile: Chemotaxonomic analysis based on IR spectra of the isolated essential oils has also been performed for thyme (*Thymus vulgaris* L.) and chamomile (*Chamomilla recutita* L.).^{41,42} The ATR-IR spectra obtained from the essential oil of two different chemotypes of thyme (*Thymus vulgaris* L.) showed characteristic differences that are mainly related to different amounts of the isomeric phenolic components thymol and carvacrol. Characteristic key bands of carvacrol occured at 995, 116 and 1176 cm⁻¹, whereas absorptions due to thymol vibrations were found at 944, 1090 and 1286 cm⁻¹. Therefore, qualitative discrimination of both chemotypes was possible

even by visual judgement without applying any chemometric algorithm. Calibration statistics established for a sample set of various thyme oils showed high precision quality for both phenolic isomers as well as several other monoterpenoids such as p-cymene and γ -terpinene.

Reliable discrimination of chamomile essential oils can also be performed on the basis of specific key bands. The spectrum of the bisabolol type is mainly characterised by vibrations relating to bisabolol and β -farnesol ($v_{C=C}$ at 1630 cm⁻¹, δ_{CH2} at 1375 and 1438 cm⁻¹, δ_{C-H} at 916 cm⁻¹). Contrary to that, the spectral profiles of the bisabolol oxide A and bisabolol oxide B type contain additional absorption bands at 977 and 891 cm⁻¹, respectively. Based on the spectral data sets, cluster analysis can be also established presenting the specific differences of the individual chemotypes.⁴¹ As demonstrated in F-3, cluster analysis assigned on the ATR-IR data basis of various chamomile oils shows a remarkable variation relating to the different genetic background of the plants (chemotypes) as well as individual manufacturing processes. Furthermore, it has been found that the GC composition of the investigated oils correlates very well with the statistic results (Ward's algorithm) calculated from

ATR-IR spectra obtained from marjoram and oregano essential oils of different chemotypes; A: carvacrol type, B: 1,8-cineole type, C: thymol type, D: γ-terpinene type, E: *cis*-sabinene hydrate type, F: terpinen-4-ol type



Cluster analysis (Ward's algorithm) based on the ATR-IR spectra (measurement range: 650-4000 cm-1) of different chamomile chemotypes and manufacturing processes



the spectral data.

Basil: The ATR-IR method has also been successfully applied for rapid and reliable discrimination of 11 basil (*Ocimum* sp.) chemotypes.²³ All spectra obtained from the essential oils of different basil plants (including the existing chemotypes) show individual fingerprints. Application of PLS algorithm has resulted in very good calibration results ($R^2 \ge 0.99$), with the individual SECV values in the same order of magnitude as the standard error of the applied GC reference method.

Mentha: Samples of the essential oil isolated from *Mentha* x *piperita* L., *Mentha arvensis* and *Mentha spicata* L. also demonstrated IR spectra with characteristic fingerprints.⁴³ The authors proved that quantitative predictions of the main essential oil substances were possible, receiving coefficients of determination above 0.92.

Pepper: IR analyses of pepper oils isolated by hydro-distillation from white and black peppercorns allow for the discrimination of three oil types due to varying main components: "caryophyllene," "sabinene/caryophyllene" and " δ -3-carene/caryophyllene/limonene."³² Generally it has been found that the correlation statistics obtained for the isolated oils (R² for most terpenoids above 0.97) show higher reliability than measurements performed directly at the dried pepper fruits (*vidé supra*).

Citrus: Recently Schulz et al. reported the application of ATR/IR spectroscopy for the classification and quantitative analysis of various citrus oils.^{38,43} Because limonene is a major component in grapefruit, orange, bitter orange (all approximately > 95 percent), and other citrus oils (amounts of 50-78 percent), it is not surprising that the IR spectra of these oils are dominated by the vibrational modes of this monoterpene substance, which are registered at 886 cm⁻¹ (out-of-plane bending vibration of the terminal methylene group), 1436/1453 cm⁻¹ (δ_{CH2}) and 1644 cm⁻¹ ($v_{C=C}$). The authors concluded that ATR/IR spectroscopy



F-4



achieved a better prediction quality than NIRS in terms of higher R² values for some parameters (e.g. myrcene and aldehyde content). Additionally, the SECV values, calculated from the PLS calibrations of the IR validation sets, are in most cases lower in comparison to the related NIR chemometrical results. This is mainly due to the fact that the IR spectra generally have larger signal intensities and higher resolution of the characteristic (fundamental) vibrations than NIR transflection spectroscopy. Finally, it should be noted that IR spectroscopy can be used for investigation of the aging of citrus oils by following the formation of p-cymene.⁴⁴

Raman Applications: Essential Oil Plants

An approach similar to that presented for IR analysis of essential oils can be applied to Raman measurements. The Raman spectra obtained from essential oils demonstrate characteristic key bands that can be used as marker bands to discriminate different plant chemotypes. Interpretation is based on the spectra of pure substances (T-4) and characteristic group frequencies.^{36,37,39,40,45}

Measurement on essential oil plants, eucalyptus: Chemotaxonomic identification via Raman spectroscopy can be tentatively obtained directly from the living plant.^{46,47} As presented in F-4, a micro-Raman measurement performed in situ on the essential oil cells of Eucalyptus globulus leaf netted a general impression of the individual essential oil distribution.⁴⁸ Measurements were performed in the oil droplet (b) and in the outside green area of the leaf (a). Furthermore, the spectrum of the essential oil isolated by hydro-distillation was presented (c), in addition to the spectrum of 1,8-cineole, its main component (d). This bicyclocamphane compound demonstrated a strong ring vibration at 652 cm⁻¹ in the Raman spectrum, which could be used for its identification. This marker band was clearly seen in the spectrum taken from the oil droplet (b), proving that the *in situ* measurement was really performed in the essential oil cell. Both spectra taken from the leaf showed the presence of the plant matrix,; the significant bands were at 1525 and 1157 cm⁻¹, which were due to in-phase C=C (v_1) and C-C stretching (v_2) vibrations of the polyene chain of carotenoid vibration modes.⁴⁹ In comparison to that, a spectrum taken in the oil droplet clearly showed lower intensity of the carotenoid signals.

The presence of characteristic 1,8-cineole bands in the spectrum of eucalyptus oil droplets provided very good pre-conditions to apply Raman mapping for *in situ* investigation of a part of the eucalyptus leaf containing several oil cavities. This technique netted detailed information regarding the distribution of specific compounds, e.g. secondary metabolites, occurring in the surface layer of the plant tissue.⁴⁸⁻⁵² The Raman spectra were taken point by point from the defined area and integrated in the wavenumber range characteristic for the investigated component. F-5A and B demonstrate the oil distribution in an area of 1200 μ m x 1300 μ m of the *Eucalyptus* globulus leaf, colored according to the intensity of the two most intensive bands of 1,8-cineole at 652 cm⁻¹ (A) and 1450 cm⁻¹ (B).⁴⁸ Complementary to that, the Raman image obtained from the integration of the signal at 1525 cm⁻¹ (F-5C) showed the distribution of carotenoids in the measured area. Maximum amounts of carotenoids were seen outside the oil cavities, and very low amounts were found in the range of the essential oil cells.

Fennel: The special advantage of NIR-FT-Raman microspectroscopy has also been demonstrated for measurements of fennel fruits. The obtained Raman maps revealed the distribution of anethole, the main essential oil component, which is present in the whole mericarp with the highest concentration at the top of the fruit.⁵⁰ In situ measurement performed in the essential oil cells was dominated by two bands observed at 1657 and 1609 cm⁻¹, due to the ring stretching modes of anethole.

Raman spectra measured directly in the plant cell via excitation of the laser in the visible range were often masked by strong fluorescence. However, the use of a micro-Raman system in combination with Ag colloid enabled investigation of the essential oil in the glandular trichomes as well.^{46,47} This technique is called SERS (surface-enhanced Raman scattering), and allows enhancement of Raman signals from molecules absorbed on the metal surface to six orders of magnitude or more due to electromagnetic and chemical factors.⁵³ Additionally, fluorescence effects caused by impurities or plant enzymes are quenched. The SERS method has been applied for the study of various molecules of biological importance, including in situ investigation of the essential oils of Mentha piperita and Thymus vulgaris.^{46,47} The SERS spectrum of T. vulgaris showed an intensive band at 738 cm⁻¹, attributed to ring stretching of thymol, but the signals of carvacrol and p-cymene were not seen, probably due to their small concentration or low affinity towards the silver hydrosol.

One disadvantage of this method is that the high amounts of heavy metals required for SERS technique often lead to destruction of the sensitive biological samples. One approach to overcome this problem is the application of silver-coated glass fibre tips with a diameter of the sub- μ m size range as the SERS substrate.⁵⁴ Due to the small dimension of the fibre and the fact that the main light intensity emanates from

the fibre tip, the spatial resolution of this method is very high (200-500 nm); therefore, the laser power can be significantly reduced.⁵⁵ This method has been used for the investigation of the essential oil of Mentha piperita L. nm. citrata directly in the glandular trichomes on the plants. The Raman spectra obtained by using the "fibre tip method" showed better quality than the corresponding SERS spectra, and revealed some additional information. By using fibre tips it was possible to reduce the amount of heavy metals contaminating the sample; thus, the microalgae present on the plant surface were not destroyed and showed two characteristic β -carotene signals.⁵⁴

Isolated essential oils: In addition to IR and NIR, Raman spectroscopy can be successfully applied for analysis of isolated essential oils. However, up to now only a few reports can be found in the literature dealing with this interesting topic. Generally, the Raman technique can be applied complementary to the IR method, providing additional verification or confirmation of the obtained results. Most of the results presented here correlate very well with those previously discussed in the section devoted to IR applications.

Pepper: As can be seen in F-6, main components of pepper oil can be clearly identified based on the fingerprint region of the individual Raman spectra.³² The Raman spectrum of the so-called "caryophyllene type" (F-6B) showed comparatively strong stretching vibration modes at 1632 and 1671 cm⁻¹ and a broad band at 1446 cm⁻¹, which is assigned to a CH₂-deformation vibration. The Raman spectrum of the "sabinene/caryophyllene type" (F-6D) presents the key bands of caryophyllene and additionally characteristic signals of sabinene ($v_{C=C}$ at 1653 cm⁻¹ and δ_{CH2} at 1415 cm⁻¹) are also observed here. Furthermore, several sabinene bands of minor intensity occurred in the range between 600 and 1000 cm⁻¹ (δ_{C-C} at 950 cm⁻¹ and ring deformation vibration at 652 cm⁻¹). The Raman spectrum of the " δ -3-carene/caryophyllene/limonen type" showed signals of all three main components — δ -3-carene (key bands at 1686, 819, 713 and 678 cm⁻¹), caryophyllene (key band at 1632 and 1671 cm⁻¹) and limonene (key band at 760 cm⁻¹) (5F). Cluster analysis applied to Raman data sets of pepper oil demonstrated a clear discrimination of the discussed sub-groups (F-7).

Raman spectroscopy has also been successfully used for chemotype classification of marjoram and oregano essential oils, as well as for identification of their major oil constituents.³⁶ The characteristic bands of the main terpenoids present in these oils are listed in T-4.

Basil: Raman spectra recorded from essential oils that were isolated from different basil plants allow for discriminating among 11 chemotypes.²³

Contrary to the above, Raman spectra obtained from various citrus oils were found to be more or less similar.³⁸ With the exception of two lime oil products (distilled and cold-pressed oils), all spectra differed only in the intensity of some minor bands. The dominating bands were due to limonene vibrations and were recorded at 760, 1435 and 1644/1678 cm⁻¹. The different citrus types and qualities could be discriminated very well applying principal component analysis (PCA). Using the first three PCA factors, 86 percent of the total variation could be explained. All relevant oil components, as well as sum parameters (total aldehyde content, optical rotation) of the various citrus oils, could be analyzed very precisely using the PLS algorithm. The coefficient of determination in most cases was larger than 0.98, and the related RMSECV

F-5

NIR-FT-Raman maps obtained from a Eucalyptus globulus leaf colored according to the intensity of the band at 652 cm-1 (A), 1450 cm-1(B) and 1525 cm-1 (C) showing the distribution of essential oil (A and B) and carotenes (C)



values corresponded more or less to the error of the individual reference method.

Generally, determination of main oil components using FT-Raman spectroscopy can be based on the intensity of the individual key band. Calibration curves can be built using reference data from GC-FID or GC/MS measurements, as has been shown for the quantitative analyses of α -pinene and β -myrcene in mastic gum oil.⁵⁶ The correlation coefficients were calculated to be 0.992 and 0.997, respectively. Nevertheless, it has to be stressed here, that this method was only successfully applicable if the analyte molecule was present in the oil in sufficient amounts and the selected key band did not coincide with vibration modes of other essential oil constituents.

Conclusion

This paper summarizes the results of recently published studies describing new applications of various spectroscopic methods for an efficient and non-destructive measurement of essential oil plants and isolated oils. Whereas NIR spectroscopy is increasingly applied in the flavor and fragrance industry for raw material quality control, IR and Raman spectroscopy are still primarily restricted to academic research. However, each method has the potential to replace standard procedures for commercial essential oil analysis. This is especially true for isolated essential oils, for which the correlation statistics present very high values for coefficients of determination.

All vibrational spectroscopy methods discussed here show several advantages over chromatographic characterization of essential oils in terms of efficiency. This is especially so for Raman spectroscopy, which was found to be an extremely useful technique for non-destructive analysis of essential oil cells in intact plant tissue, eschewing the need for sample clean-up steps. Raman spectroscopy combined with microequipment can provide detailed molecular information with high spatial resolution at the cellular level. Raman spectra can be obtained directly from the essential oil cell or by mapping of a specified plant area. Usually, measurements performed on the plant are interpreted in comparison with the spectra of corresponding pure standards. However, it should be pointed out that contrary to single spectrum measurements, Raman mapping needs an analysis time of several hours, depending on the selected sample area and step size.

Usually, the sensitivity of the discussed vibrational methods is lower in comparison to other analytic techniques and depends on the chemical structure of the analyzed plant components. In some cases, components occurring in low concentration can be analyzed (e.g. carotenoids at the mg/kg level), but generally the detection limit is in the mg/g range. However, increase in sensitivity can be provided by some special Raman techniques, like the SERS, or "tips," method, which

F-6

NIR-FT-Raman spectra of different pepper oil types isolated by hydro-destillation from various commercial pepper samples; A: pure caryophyllene standard, B: pepper oil (caryophyllene type), C: pure sabinene standard, D: pepper oil (sabinene/caryophyllene type), E: pure δ -3-carene standard, F: pepper oil (δ -3-carene/caryophyllene/limonene type)



produces a signal enhancement of a six-plus magnitude.

All these aspects should be taken into account when choosing a suitable method for essential oil analysis. Unfortunately, the price of some Raman spectrometers is still comparatively high, which presently limits access to this highly advanced technique. However, there exist several advantages over common GC methods:

- the ability to perform non-destructive measurements on living plant material
- the option to apply NIR- and Raman spectroscopy for continuous controlling of distillation processes
- the possibility to use the described highthroughput methods for quality control purposes in the flavor and fragrance industry

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