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Application of Raman spectroscopy in the analysis of plant tissues

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On February 28, 1928, at the Indian Association for the Cultivation of Sciences (IACS), Kolkata (India), C V Raman and his associates for the first time experimentally showed that when monochromatic light is scattered by transparent media, the scattered light contains not only the original colour, but also other colours. This effect was named as Raman effect [1] and it belongs to one of the four most important discoveries in physics with the highest impact to applications in modern life [2].

My first contact with vibrational spectroscopy, (which employs IR and Raman Spectroscopy), was during my Ph D thesis, where I used IR and Raman spectroscopy to identify newly synthesised geometric isomers of various complex substances of the type OsX_nY_{4-n} ox (X,Y= Cl, Br, J; n = 1, 2, 3). The symmetry-related differences of the individual isomers were mainly evident in the Os-halogen vibrational region. According to the selection rules, predominantly symmetrical vibrations of the molecules could be observed in the Raman spectrum and assigned to the individual vibrational modes.

Later, inspired by Prof Bernd Schrader (Germany), I had the opportunity to examine different plant species for their individual valuable substances on a larger scale for the first time. Until then, isolated plant extracts such as essential oils could be characterised very well with Raman spectroscopy, but it was not yet possible to measure plant tissue directly because the plant cells were destroyed and the Raman spectra were very often overlaid by strong fluorescence effects. Only by excitation with the radiation of a Nd:YAG laser emitting at 1064 nm could these undesirable effects be overcome and a global optimum for the non-destructive recording of Raman spectra of "living cells" be achieved.

Within the past 20 years Raman spectroscopy has become increasingly important in the field of plant analysis aiming to provide a fast and mostly non-destructive classification of plant tissues but also to get new insights into biophysical and biochemical processes and a more detailed knowledge concerning plant diseases at the molecular level. Today, even portable Raman spectrometers are available which only need sample amounts of a few microliters or milligrams. In most cases, measurements can be performed directly on plant tissues as well as on fractions isolated from the plant material by micro-hydro-distillation or solvent extraction [3]. 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, Raman spectroscopy 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 various production processes. Besides MS and NMR measurements, Raman spectroscopy today is a very important analytical tool in the field of plant metabolic fingerprinting. It offers an unbiased, global screening approach to classify samples that change in response to genetic background, various plant diseases or environmental influences (e.g. various stress factors). Unlike infrared spectroscopy, fresh plants can be analysed without any sample pre-treatments as the

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low polarisability index of water produces negligible Raman signal intensity. Using surface-enhanced Raman scattering (SERS) technique, the detection sensitivity can be enhanced up to 6-10 orders of magnitude over conventional Raman spectroscopy providing new challenges to detect individual plant components even when they occur in lower concentration. In this context generally, the presence of the gold colloids enabled strong SERS spectra, whereas the spectra of silver colloids were weaker and exhibited more noise than those registered for gold. In order to describe the individual gene functions, protein regulation and the production of small molecular weight metabolites, the variation within the biological systems has been studied at the genomic, proteomic and metabolomic levels [4].

At present, it is estimated that the number of metabolites detected in the plant kingdom may reach a number up to 200,000. For targeted and non-targeted analysis and quantification as well as metabolomic fingerprinting, techniques such as Raman and infrared spectroscopy are valuable analytical tools beside NMR and MS because they provide the opportunity for unbiased high-throughput measurements which in comparison to other techniques are comparatively inexpensive. In this context, a fast and reliable quantification method for the determination of indigo in various plants tissues was developed and the Raman spectrum of dihydro-indigo, an important intermediate product in the indigo dyeing process, was also discussed in detail for the first time, (Figs 1a & 1b) [5].

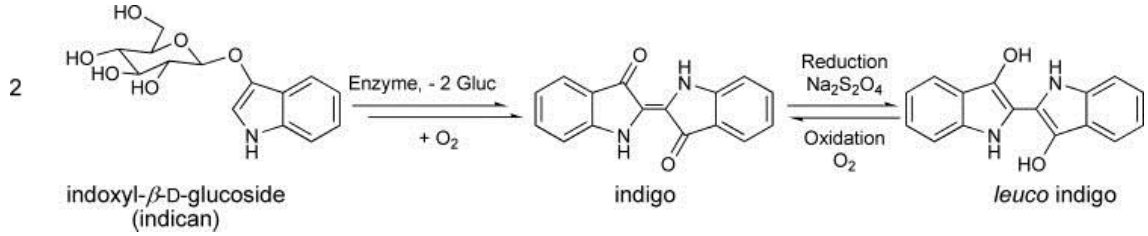

Fig 1(a). Formation of indigo from the naturally occurring glucoside indican and reduction to the soluble dihydro form, leuco indigo.

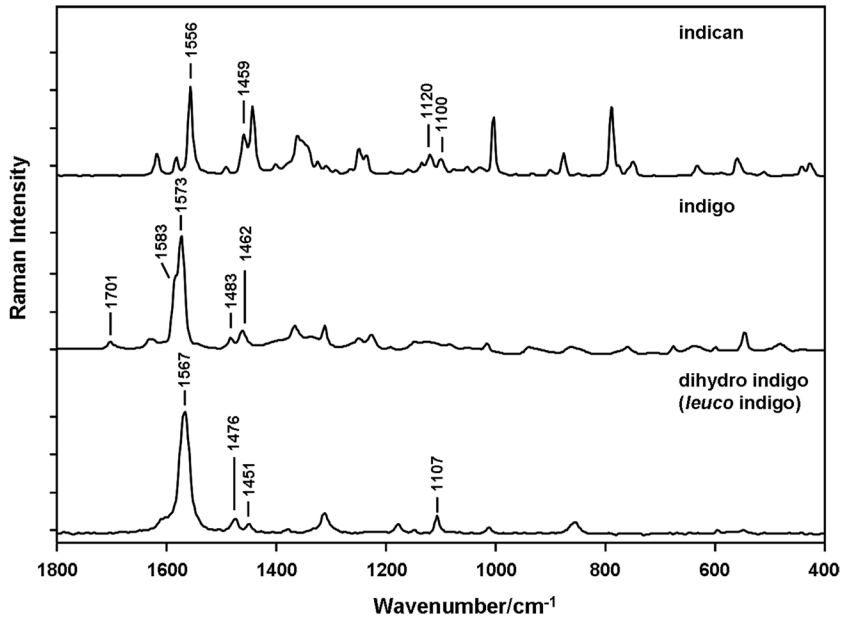

Fig 1(b). Comparison of the Raman spectra of indican (indoxyl-β-D-glucoside), indigo and *in situ* synthesized dihydro indigo.

Furthermore, numerous alkaloids, which have a wide range of different chemical structures and most of which have significant biological activity, were analysed using Raman spectroscopy. Especially caffeine, theophylline and theobromine show characteristic strong features between 1310 and 1340 cm^{-1} and between 1690 and 1710 cm^{-1}, assigned as C-N stretching and C=N stretching vibrations, have been detected in cacao seeds and their extracts applying FT-Raman spectroscopy. Raman microspectroscopy proved to be a very efficient tool to distinguish different structurally similar naphthylisoquinoline alkaloids in plants, showing characteristic signals of C=C stretching and C-H bending vibrations at 1356 and 1613 cm^{-1}, respectively [6].

Raman spectra of essential oils also show characteristic key bands that can be used as marker bands to distinguish between different plant species, cultivars and chemotypes. It could be shown that in some cases even a chemotaxonomic differentiation of plant species is possible by measuring the respective fresh plant tissues, Fig 2 [7].

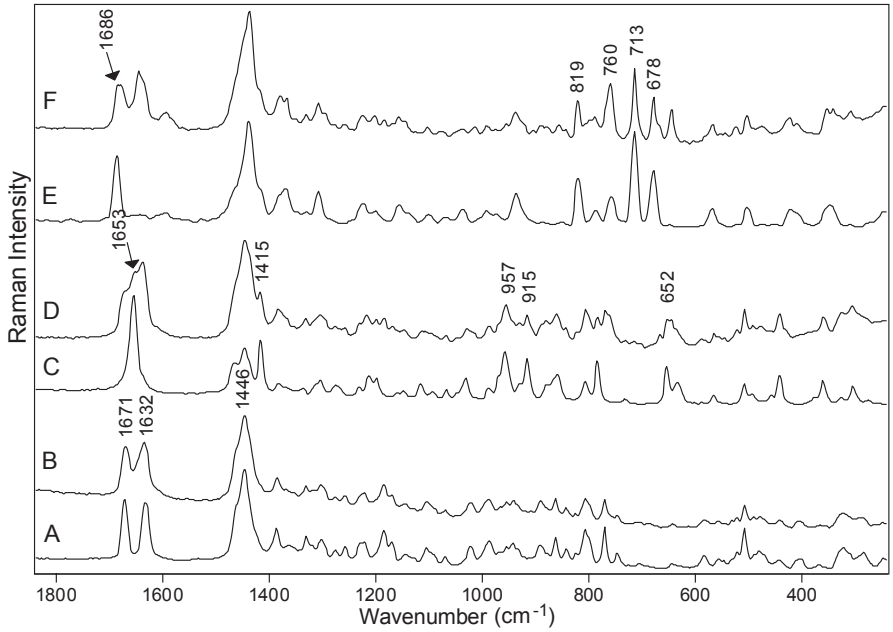

Fig 2. Raman spectra of different pepper oil types isolated by hydrodistillation from various commercial pepper samples: (A) pure caryophyllene standard; (B) pepper oil (caryophyllene type); (C) pure sabinene standard; (D) pepper oil (sabinene/caryophyllene type); (E) pure δ-3-carene standard; (F) pepper oil (δ-3-carene/caryophyllene/limonene type).

In addition, micro-Raman measurements carried out *in-situ* on the essential oil cells of various plant species, such as eucalyptus leaves, show the individual composition of the respective essential oil in the intact plant tissue, Fig 3.

Similarly, FT-Raman microspectroscopy of fennel fruits have been performed, revealing the distribution of anethole, the main component of fennel oil. *In-situ* studies of the fennel essential oil cells show two characteristic marker bands at 1657 and 1609 cm^{-1} (ring stretching modes of anethole) which were used for Raman mapping measurements [8]. Generally, plant pigments such as β-carotenoids show characteristic Raman bands assigned as double bond C=C stretching mode (1524 cm^{-1}), C-C in-plane single bond stretching mode (1157 cm^{-1}), and C-H bending mode (1008 cm^{-1}), respectively. In this context, it has been observed for the first time that the position of C=C stretching vibrations in the Raman spectrum

is mainly influenced both by the carotenoid polyene chain length and the structure of terminal substituents [10]. Based on this finding it is principally possible analysing simultaneously the distribution of different carotenoids in various plant tissues. Even unstable epoxycarotenoids such as auroxanthin and violaxanthin were detected with high sensivity and their distribution in plant tissue could be measured *in-situ* applying micro-Raman mapping. Furthermore, Raman imaging of carotenoid distribution was found to be a suitable approach showing possible changes in the plant metabolome induced by abiotic and biotic stresses as well as sunscald physiological disorder. Comprehensive Raman studies of various carrot cultivars present that quality parameters such as carotenoids, polyacetylenes, pectin and starch can be determined simultaneously in root samples [11]. Raman spectra of diacetylenes occurring in plants show a very strong and polarised band due to –C≡C–C≡C– symmetric stretching vibration in the region of 2200-2300 cm^{-1}. It has been observed that both, the number of conjugated -C≡C- bonds in polyacetylenes and substituents contribute to the number of Raman signals, their frequencies and intensities. Generally, polyacetylenes can be reliably quantified even if they occur in lower concentration in plant tissue. This observation is mainly due to the high scatter induced by -C≡C- bonds in a frequency region not interfering with other vibrational modes. Therefore, the distibution of polyacetylenes in plant tissues can be easily illustrated by *in-situ* Raman imaging. The special advantages of Raman mapping combined with cluster analysis have been successfully applied to discriminate flavonoids, anthocyanins, and carotenoids occurring in differently colored flower petals of various pansy cultivars [12].

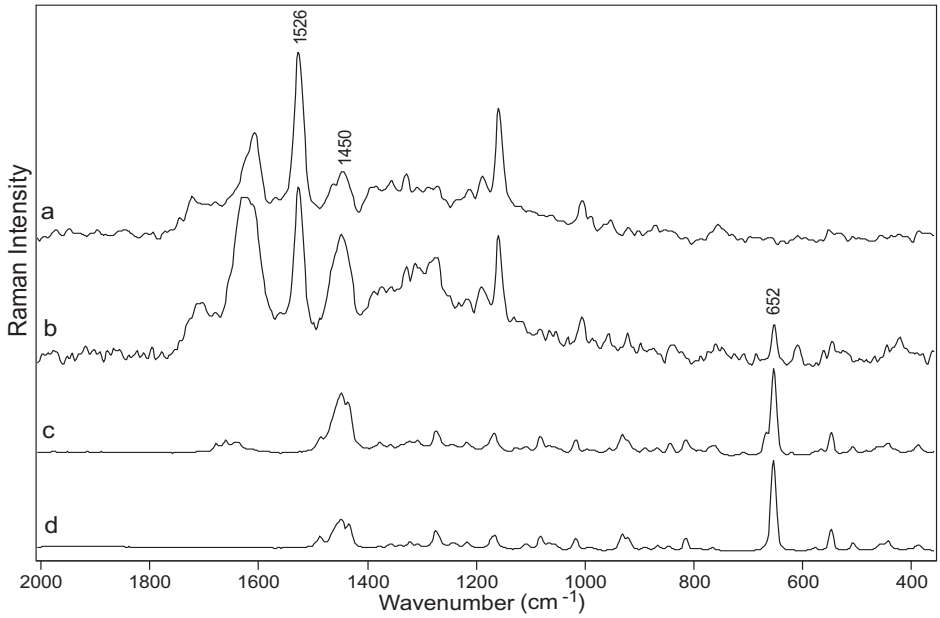

Fig 3. FT-Raman spectra taken from Eucalyptus globulus leaf (green area outside the oil droplet (a), oil droplet (b), essential oil isolated by hydro-distillation (c) and 1,8-cineole standard (d) [9].

Raman spectroscopy has been also applied to discriminate pollen grains of different tree species. In this context, single pollen Raman spectra are presented showing the individual key bands of carotenoids, proteins, nucleic acids, carbohydrates, and lipids. The classification of the different pollen species was based on Principal Component Analysis as well as Hierarchical Cluster Analysis and allowed to analyse various chemical classes of molecules simultaneously. Without any further sample pretreatment, carotenoids occurring in single pollen grains were examined achieving both, high detection sensitivity and structural selectivity.

Photo 1. Prof Vinod Rastogi (3rd from left) lighting the lamp at International Conference on Materials Science & Advanced Biomolecules, IMSAB-2012, Bishop Moore College, Mavelikara, Kerala.

Photo 2. Prof Vinod Rastogi presenting a memento to Prof Hartwig Schulz during 4th ICOPVS2013, at Bishop Moore College, Mavelikara, Kerala, India.

Photo 3. (from left) V V Tuchin, Vinod Rastogi, Hartwig Schulz and T Yamamoto during ICOPVS2020 at JNCAR, Bangalore, India.

Finally, I would like to express my sincere gratitude to Prof Vinod Rastogi (founder of series of ICOPVS conferences) for inviting me to several ICOPVS meetings at different places in India to present my latest research results on vibrational spectroscopic characterisation of different plant species. In this context, I hope to have made a small contribution to motivate especially younger scientists in India to work in this exciting field of research and to apply this fascinating spectroscopic technique in the analysis of plant tissues in Prof Raman's home country.

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Selected book chapters related to the topic:

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2. Schulz, H, Krähmer A, Naumann A, Gudi G, Infrared and Raman Spectroscopic Mapping and Imaging of Plant Materials, in: Infrared and Raman Spectroscopic Imaging, editors: Reiner Salzer & Heinz Siesler, 2nd edn, (Wiley-VCH, Weinheim), 2014, pp 227–293.
3. Schulz H, Qualitative and quantitative FT-Raman analysis of plants, in: Optical Spectroscopy and Computational Methods in Biology and Medicine, editor: Malgorzata Baranska, (Springer, Dordrecht), 2014, pp 253–278.

Hartwig Schulz studied chemistry in Bonn and Kiel (Germany). After completing his studies, he did research for several years in the field of human nutrition (vitamin E, phytosterols, plant lipids) at the University of Kiel, Germany. He then worked for 10 years as head of an analytical department at one of the world's largest perfume and aroma manufacturers (Symrise). From 1996 to 2019, he was director and professor of a research institute on behalf of the German Federal Ministry of Agriculture. Hartwig Schulz is author of numerous scientific articles and book chapters and, as President of the "German Society for Quality Research (DGQ)", was active in food research at national and international level for eight years. Since his retirement, he has been working as a consultant mainly in the field of "medicinal and aromatic plants" and supports various development aid projects on a voluntary basis.