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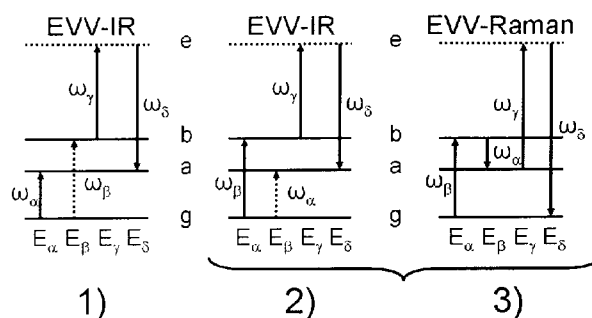
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Fig 1



(57) Abstract: A method is provided for detecting an interaction between chemical groups in a sample. The method comprises obtaining an electronic vibration vibration (EVV) 2-dimensional infrared (2DIR) spectrum of the sample, disregarding signals in said spectrum that are caused by chemical bonds present in the sample and identifying whether any additional signals are present in the spectrum, indicating the presence of such an interaction.

Method of Detection

This invention relates to a method of detection. It further relates to a method of structural analysis. In particular it relates to detection and determination of the geometry of complexes in which atoms and molecules interact without the formation of a chemical bond.

Background

There are several approaches for analysing the structure and composition of complex substances. One well known technique is Nuclear Magnetic Resonance (NMR). One of the advantages of NMR is its ability to measure the coupling strengths between nuclear spins and from this to deduce the distance between those spins. This capability stems from the relatively simple physics that determines spin-spin coupling, which enables distances to be inferred therefrom. In essence, spin-spin coupling follows a dipolar distance dependence. However 2D NMR is a relatively insensitive technique. In particular, a long throughput and large amount of material are required in order to detect interactions within a complex using 2D NMR. Furthermore, the spectra produced by NMR typically do not provide great detail about the interactions within a complex being studied.

The apparent similarities between 2DNMR methods and its optical analogues such as Coherent Two-Dimensional Infra-Red Spectroscopy (2DIR) have fuelled the hope that geometric information might be obtained from these techniques also. It is certainly true that analysis of known structures, rather than structural determination or analysis of unknown substances, can be greatly assisted by 2DIR methods, as the cross-peaks in 2DIR spectra can be very sensitive to molecular structure and vibrational cross-peaks can be accurately calculated from known structures described in reference (1) to (4) set out in the

reference list forming part of this description, all of which are incorporated by reference herein.

However a drawback with 2DIR techniques is that the coupling physics that connects molecular vibrations with each other is more complex than the Hamiltonians that, to a first approximation, are responsible for nuclear spin coupling. In essence there are two types of anharmonicity that result from vibration-vibration coupling: mechanical coupling and electrical coupling. Mechanical coupling, or mechanical anharmonicity, can generally be regarded as being the “mass on a spring” effect that arises when two atoms or molecules vibrate in the vicinity of one another. In contrast, electrical coupling or electrical/electronic anharmonicity, arises due to the change in electron cloud motion surrounding two atoms or molecules when they vibrate in the vicinity of one another. When chemical bonds are present in a substance it is usually, although not necessarily always the case, that mechanical anharmonicity dominates.

There is at present no known way to take the data from a 2DIR experiment and directly calculate or otherwise obtain structural information of a substance. As indicated above, one apparent factor in this problem is the complex relationship between cross-peaks in a 2D IR spectrum and mechanical anharmonicity due to the presence of chemical bonds. It has not been possible according to known techniques to identify a relationship or correspondence between measured parameters and molecular geometry in such substances. A further complexity arises in 2DIR spectra in that the spectrum is often heavily congested, so detection of particular peaks is difficult, let alone identification or determination of the associated structure. Two dimensional infrared spectroscopy techniques are thus not currently employed for detection or structural analysis of complex substances. Therefore alternative techniques

such as NMR, which itself has several disadvantages as detailed above, are relied upon.

The invention is set out in the claims.

Because EVV 2DIR is employed, a weak interaction between species that creates electrical anharmonicity, rather than mechanical anharmonicity, can be detected. Furthermore, those weak interactions can be easily identified from the output EVV 2DIR spectrum as the corresponding signals will not be present either in the EVV 2DIR spectra for the individual (i.e. monomer) species in the sample under study, or in other spectral outputs for the sample, for example Raman or FTIR spectra.

By employing a mathematical model, characteristics of the signals expected from the weak interactions in a sample can be predicted. This enables the user to focus on a particular portion of the EVV 2DIR spectrum when looking for signals corresponding to weak interactions, and/or to focus on signals of a particular strength.

By comparing the pulse-ordered spectra according to different pulse configurations, the method can confirm the absence, or negligible presence, of mechanical anharmonicity in a sample under study. This serves to validate the use of EVV 2DIR spectroscopy for such a sample, and any assumption made in the mathematical model.

Because a spectrum can be calculated for EVV 2DIR, rather than experimentally obtained, the presence of weak interactions in a sample can be confirmed without the need for experimental tests. Furthermore, the predicted spectrum can be used to focus experimental spectroscopy on the sample.

By comparing aspects of the signals present due to weak interactions in a sample to a reference signal, it has been shown that structural properties of the sample can be inferred. This has not been previously shown with any other 2DIR technique. Hence a newly sensitive and accurate structural analysis tool is provided. The structural analysis can again employ a mathematical model such as a multipole model to guide the determination of structural properties from the visible signals on EVV 2DIR spectra.

The methods according to the present invention are described below with respect to particular embodiments or sample classes, but it will be understood that the principles apply more globally to any class of substance in which electrical anharmonicity dominates over mechanical anharmonicity. This leads to the provision of a highly sensitive, accurate detection and analysis tool that has not been provided, nor even attempted, according to previously known spectroscopic techniques.

Description of Figures

Fig 1 depicts 3 wave mixing energy level diagrams depicting the four wave mixing EVV processes;

Fig 2 is an EVV 2DIR spectra of a short peptide (YLRRYSLG) measured for two pulse orderings, respectively E_α first (left side spectrum) and E_β first (right side spectrum). The spectra are measured with the same relative delays differences of 1 ps ($T_{12}=1$ ps, $T_{23}=1$ ps) and are represented with the same intensity scale;

Fig 3 depicts a scheme showing the molecular properties involved in the dipole-dipole model used in the text;

Fig 4 depicts the functional relationship between ϕ and signal ratio of two polarization setups, red curve for mode 1 as IR step, green for mode 2, drawn with $k = 2.65$;

Fig 5 shows the optimized geometry of Ph-CCH and Ph-CN complex at MP2/6-31+G(d,p) level;

Fig 6 shows the calculated EVV 2DIR spectra for Ph-CCH and Ph-CN complex;

Fig 7 shows the experimental EVV 2DIR spectra for pure benzonitrile, pure phenylacetylene, 50%-50% mixture of benzonitrile and phenylacetylene;

Fig 8 shows the experimental EVV 2DIR spectra for 50%-50% mixture of benzonitrile and phenylacetylene at PPP, reversed pulse ordering and PPS setups;

Fig 9 shows the FTIR spectra of the three samples in Figs 7 and 8; and

Fig 10 is a table showing the spectral parameters used in the $C_6H_5CCH-C_6H_5CN$ system

Overview

In overview, the present approach recognises that, despite the potential problems in using 2DIR as a generic tool for structural analysis and the technical prejudice to use alternative spectroscopic techniques, there is a class of structures for which 2DIR can be used as a highly sensitive tool for structural detection and analysis, even if low concentrations of sample are used. The class of substances to which the embodiments of the present invention apply are those in which electrical/electronic anharmonicity dominates. Domination of electrical anharmonicity over mechanical anharmonicity occurs, *inter alia*, in molecule-molecule interactions and molecular complex formation where no strong bond, i.e. no covalent bond, is formed. These types

of interactions are particularly prevalent in biological systems, for example intra protein, protein-protein and protein-ligand interactions.

The present approach further recognises that not every 2DIR spectroscopy technique can be employed for detection and structural analysis for structures in which electrical anharmonicity dominates. Most 2DIR and other 2D spectroscopy techniques are difference spectroscopies, which rely on the energy differences (ΔE) between successive energy levels in a mechanical anharmonic oscillator system being different from one another. In contrast, the present invention relies on Electronic Vibration (EVV), which is not a difference spectroscopy and therefore is sensitive to pure electrical anharmonicity in substances, without relying on the presence of mechanical anharmonicity as well.

The methods described herein demonstrate that electrical anharmonicity alone can be responsible for the appearance of new cross-peaks in EVV 2DIR spectra when two molecular species are brought close together without the formation of a chemical bond. It demonstrates further that the dipolar approximation for the coupling between two interacting but non-covalently bonded molecular species is a good representation of the underlying physics. The dipolar approximation can be used to predict where new cross-peaks are likely to occur in an EVV 2DIR spectrum and further to predict the strength of those cross-peaks as formed by the dipolar couplings. In addition, the polarization dependence of the cross-peaks can be used to deduce the angle between the molecular species in question and to estimate the distances between the two interacting species via the intensities of the intermolecular cross-peaks.

The electrical anharmonicity interaction will typically be a weak interaction and will still be detectable, and will be in addition or secondary to the interaction that forms chemical bonds.

It will be noted that in some instances features created by chemical bonds are not disregarded, when they are non interaction affecting, for example where the chemical bonding causes no or little mechanical anharmonicity and the vibrational-vibrational coupling that results is purely electronic, as discussed in reference (3).

Detailed Description

Mechanical and Electrical Anharmonicity

For any molecular system there are two intrinsic types of anharmonicities, upon which every implementation of nonlinear spectroscopy depends in one way or the other for their signals. Mechanical anharmonicity is defined as the

expansion coefficients $\frac{\partial^3 V}{\partial Q_i \partial Q_j \partial Q_k} (V_{ijk}^{(3)})$ of the lowest-order anharmonic terms

in the expansion of molecular potential energy surface V :

$$V = V_0 + \frac{1}{2} \sum_i \frac{\partial^2 V}{\partial Q_i^2} Q_i^2 + \frac{1}{3!} \sum_{i,j,k} \frac{\partial^3 V}{\partial Q_i \partial Q_j \partial Q_k} Q_i Q_j Q_k + \frac{1}{4!} \sum_{i,j,k,l} \frac{\partial^4 V}{\partial Q_i \partial Q_j \partial Q_k \partial Q_l} Q_i Q_j Q_k Q_l + \dots$$

The Q_i s are a set of molecular normal mode coordinates. Electrical anharmonicity is defined as the lowest-order nonlinearity $\frac{\partial^2 \mu}{\partial Q_i \partial Q_j}$ in molecular electrical dipole moment as in the expansion:

$$\mu = \mu_0 + \sum_i \frac{\partial \mu}{\partial Q_i} Q_i + \frac{1}{2} \sum_{i,j} \frac{\partial^2 \mu}{\partial Q_i \partial Q_j} Q_i Q_j + \dots$$

Although mechanical anharmonicities are essential for many implementations of nonlinear spectroscopy, such as photon echo, a unique feature of EVV 2DIR spectroscopy is that electrical anharmonicity also plays an important, sometimes even dominating role in generating nonlinear spectral signals. It is therefore possible to use EVV 2DIR spectra for the determination of relative geometries of interacting chemical groups in situations where electrical anharmonicity dominates.

The molecular systems in which EVV 2DIR can be employed for structural detection and determination are those involving two or more chemical groups which, though not directly bonded to each other, lie close enough to interact. In this case, the vibrational modes of each functional group would be expected to be well localized around the group itself, and the through-space coupling between two vibrational modes localized separately on the two functional groups can only take place through electrostatic interaction. This coupling can in principle be anharmonic, thereby coupling these two modes and leading to EVV 2DIR cross-peaks. Numerous candidates of this kind of systems can be found in published X-ray crystal structures of proteins in the form of interacting side chains, such as the F19-Y63 pair in p53-mdm2 complex.

It is known that electrostatic interactions between two spatially interacting groups can also lead to mechanical anharmonicities. However this effect is expected to be much weaker, as demonstrated in the example below. As shown by Choreference (5), mechanical anharmonicities generated between two interacting dipoles *A* and *B* in this way can be expressed as:

$$V_{ij}^{(3)} \propto -\frac{1}{2} \left(\frac{\partial^2 \mu_A}{\partial Q_i^2} \right) \cdot \mathbf{T}_{AB} \cdot \left(\frac{\partial \mu_B}{\partial Q_j} \right)$$

$$V_{ij}^{(3)} \propto -\frac{1}{2} \left(\frac{\partial \mu_A}{\partial Q_i} \right) \cdot \mathbf{T}_{AB} \cdot \left(\frac{\partial^2 \mu_B}{\partial Q_j^2} \right)$$

Where $\mathbf{T}_{AB}$ is the interaction tensor between A and B defined as:

$$\mathbf{T}_{AB} = \frac{3\mathbf{R}\mathbf{R} - \mathbf{I}R^2}{R^5}, \text{ with } \mathbf{R} \text{ being the distance vector pointing from } A \text{ to } B. \text{ These}$$

expressions for induced mechanical anharmonicities involve, as a prerequisite, second order dipole moment derivatives of A and B , which are small by themselves. In contrast, and as detailed below, the expression for electrical anharmonicity from the same interaction (Equ.1) only involves first order derivatives of dipole moments and polarizability. Thus it is reasonable to assume that these induced mechanical anharmonicities are negligible and that EVV 2DIR signals originate entirely from the induced electrical anharmonicity.

With EVV 2DIR spectroscopy it is possible to verify the assumption that mechanical anharmonicity is negligible as set out above, by measuring the relative contributions of electrical and mechanical anharmonicity by changing pulse ordering using different pulse configurations during EVV 2DIR spectroscopy applied to a particular sample as described below.

EVV 2DIR spectroscopy experimental set up

The configuration of EVV 2DIR apparatus will be well known to the skilled reader such that detailed description is not required, and is further described in references (1), (2) and (6) which are incorporated herein by reference. At a general level, however, in EVV 2DIR, three independently tuneable and independently timed picosecond pulsed laser beams are brought together on a

sample in a phase matched configuration. Two of these beams are in the infrared and are used to excite molecular vibrations, while the third is a visible beam and is essentially used to resolve the polarisation created by the two IR beams such that the coupling strength of the two vibrations can be ‘read-out’ by the detection of visible photons. The signal is detected at the frequency $\omega_\delta = \omega_\gamma + \omega_\beta - \omega_\alpha$ with ω_α and ω_β the IR frequencies and ω_γ the visible beam frequency as shown in Fig. 1. Also in Fig. 1, E_α and E_β are the accordable infrared electric fields, E_γ the visible incident electric field and E_δ the detected four wave mixing EVV electric field. When E_α and E_β are resonant with coupled vibrational modes (levels a and b respectively) the FWM EVV signal is multiplicatively enhanced.

The final “read-out” step in EVV 2DIR is essentially half of a conventional Raman Scattering event, but coherent and phase matched. One of the consequences of this is that the electronic properties of the molecular system also affect the signal and that the experiment can be made triply resonant if the visible “probe” beam is tuned towards an electronic resonance.

The IR beams in an EVV 2DIR laser set up can be independently scanned in frequency and when they are in resonance with coupled vibrational modes the detected signal is also multiplicatively enhanced. The spectra output in EVV 2DIR represent the level of signal detected at ω_γ as a function of the IR frequencies showing cross-peaks at the specific IR frequencies corresponding to coupled vibrational modes in the sample under study. Two delay stages control the timing between the pulses: conventionally, T_{12} and T_{23} denote the delay of the IR pulse at ω_β relative to ω_α and of the visible pulse relative to the IR at ω_β , respectively.

One of the most unusual aspects of EVV 2DIR is that it not only involves the excitation of molecular vibrations with infrared laser pulses, but also the direct polarisation of electrons via a “half-Raman” scattering step. This makes EVV 2DIR effectively a hybrid Raman-IR method where the final signal strength depends on both IR and Raman processes. Another important property is that EVV 2DIR experiments can involve direct excitation of vibrational combination bands, usually in the near-infrared.

The processes that contribute to the total EVV signal are shown in Fig 1, wherein the pathway 1) is for E_α arriving first on the sample, and the pathways 2) and 3) are for E_β arriving first.

It can be seen that pathway 3 is essentially the same as Coherent Anti-stokes Raman Spectroscopy (CARS), but with the involvement of real excited states rather than the purely virtual states of a CARS experiment. Pathways 2 and 3 are only accessible if the near-infrared pulse which excites the combination band comes first. Thus it is possible to switch pathways on and off by manipulation of the pulse timings. Therefore EVV 2DIR offers spectral decongestion, as compared to Raman and FTIR spectroscopies. Experimental parameters such as delay between pulses and beam polarisation choices offer additional control of spectral congestion in EVV 2DIR.

As discussed above, EVV 2DIR is not a difference spectroscopy. As a consequence of this, cross-coupling can be observed even in the case where the mechanical anharmonicity is either too small to shift ground and excited vibrational states significantly or is entirely absent.

Domination of Electrical Anharmonicity

A good example of a case where electrical anharmonicity dominates is the methyl and methylene asymmetric stretch and scissor deformation modes. Calculations suggest that these modes have either very weak or zero mechanical anharmonic coupling but significant electrical anharmonicity. Both methyl and methylene features appear as strong and highly reproducible features in EVV 2DIR spectra of peptides and proteins.

In order to demonstrate that these features are indeed the result of pure electrical anharmonicity, the independent pulse timings available in EVV 2DIR can be used to extinguish the signal via interference of pathway 2) and pathway 3) (Fig 1). If the time ordering of the IR pulses is reversed (E_β first) from the timing usually used (E_α first), then pathway 1) no longer contributes and the signal is the sum of the contributions of pathways 2) and 3). Pathways 2) and 3) have opposite sign and therefore if the combination band is unshifted by the interaction of modes a and b, as is the case when there is zero mechanical anharmonicity, then the signals will totally cancel. The spectra shown in Fig 2 demonstrate this process: when only pathway 1) is opened (E_α first) CH_2 and CH_3 cross-peaks are present in the spectrum whereas when the IR pulses order is reversed (E_β first) these two cross-peaks disappear. If there is a level shift, then splitting of the cross-peaks is observed as pathways 2) and 3) are no longer degenerate.

The cancellation of pathways 2 and 3 in the case of pure electrical anharmonicity appears to be more or less total - with less than 3% of the signal remaining for the CH_2 cross-peak. Hence the assumption that mechanical anharmonicities are dominated in intermolecular non-covalent interactions appears valid for a large class of systems, in particular biological systems

including protein side chain interactions with other side chains or with small molecules.

Dipole Approximation

As discussed above, the present invention recognises that a dipole approximation of a multipole system can be used to model, detect and analyse electrical anharmonicity in a system. As a relatively simple theoretical model, one can consider two uncharged functional groups interacting with each other, each carrying one vibrational mode of interest, labelled as 1 and 2 respectively. The Hamiltonian of the interacting pair can be written as (referring to reference (7)):

$$H = H_0 + H'$$

with H_0 being the non-interacting Hamiltonian of the pair, and H' the interaction potential between them. Referring to reference (7), H' can be explicitly written for uncharged systems as:

$$H' = -\boldsymbol{\mu}_1 \mathbf{T}_{12}^{(2)} \boldsymbol{\mu}_2 - \frac{1}{3} \boldsymbol{\mu}_1 \mathbf{T}_{12}^{(3)} \boldsymbol{\Theta}_2 + \frac{1}{3} \boldsymbol{\mu}_2 \mathbf{T}_{12}^{(3)} \boldsymbol{\Theta}_1 + \frac{1}{9} \boldsymbol{\Theta}_1 \mathbf{T}_{12}^{(4)} \boldsymbol{\Theta}_2 + \dots$$

$\boldsymbol{\mu}_i$ and $\boldsymbol{\Theta}_i$ are the dipole and quadrupole moments of molecule i . $\mathbf{T}_{12}^{(j)}$ is the interaction tensor of order j . When $j = 2$, the expression for $\mathbf{T}_{12}^{(2)}$ is the same as given above for $\mathbf{T}_{AB}$. For uncharged molecules, dipole moments are their first non-vanishing multipole moments. So as an approximation, we take only the first term in the above equation as H' . By taking the derivatives of the total energy of the system with respect to external electric field and normal mode coordinates of 1 and 2 successively, an expression for the induced electrical anharmonicity between the two vibrational modes, induced by dipole-dipole interaction, can be obtained:

$$\left(\frac{\partial^2 \boldsymbol{\mu}}{\partial Q_1 \partial Q_2} \right)_{ind} = \frac{\partial \boldsymbol{\alpha}_1}{\partial Q_1} \cdot \mathbf{T}_{12}^{(2)} \cdot \frac{\partial \boldsymbol{\mu}_2}{\partial Q_2} + \frac{\partial \boldsymbol{\mu}_1}{\partial Q_1} \cdot \mathbf{T}_{12}^{(2)} \cdot \frac{\partial \boldsymbol{\alpha}_2}{\partial Q_2} \quad \text{Equ.(1)}$$

Where α_i is polarizability associated with the dipole moment. This is essentially the same as the expression given by Hahn et al in reference (8).

Equ. (1) above determines the size and direction of electrical anharmonicity, while all the geometrical information between the two molecules is incorporated in $\mathbf{T}$.

The complete procedure of calculating the third-order nonlinear susceptibility $\chi^{(3)}$ of EVV 2DIR signals in the presence of mechanical and electrical anharmonicities has already been given by Kwak et al in reference (3). However, when there is only electrical anharmonicity involved, $\chi^{(3)}$, after being orientationally averaged for an isotropic media, can be written in a much simpler form of vector/tensor products as:

$$\chi_{PPP}^{(3)} \propto \text{Tr} \left(\frac{\partial \alpha}{\partial Q_2} \right) \left(\frac{\partial^2 \mu}{\partial Q_1 \partial Q_2} \cdot \frac{\partial \mu}{\partial Q_1} \right) + 2 \left(\frac{\partial \alpha}{\partial Q_2} \cdot \frac{\partial^2 \mu}{\partial Q_1 \partial Q_2} \right) \cdot \frac{\partial \mu}{\partial Q_1} \quad \text{Equ. (2)}$$

apart from a line-shape function involving bandwidths and frequencies of the modes.

Equ. (2) was obtained for a polarization arrangement or pulse configuration with the three laser pulses for EVV 2DIR spectroscopy all parallel to each other (denoted PPP), and mode 1 was chosen as ω_α as discussed in reference (1). Another possible polarization arrangement or pulse configuration in EVV 2DIR spectroscopy is denoted PPS, wherein the polarization plane of the visible laser pulse is perpendicular to the other two. For this arrangement, $\chi^{(3)}$ is:

$$\chi_{PPS}^{(3)} \propto 2 \text{Tr} \left(\frac{\partial \alpha}{\partial Q_2} \right) \left(\frac{\partial^2 \mu}{\partial Q_1 \partial Q_2} \cdot \frac{\partial \mu}{\partial Q_1} \right) - \left(\frac{\partial \alpha}{\partial Q_2} \cdot \frac{\partial^2 \mu}{\partial Q_1 \partial Q_2} \right) \cdot \frac{\partial \mu}{\partial Q_1} \quad \text{Equ. (2')}$$

It has been recognised according to the present model that the dot-product formulations in Equ.s (2) and (2'), involving dipole moment μ_i and polarisability α_i , underlines the dependence of cross peak intensity in a 2DIR EVV spectrum on geometrical relationships among those two vectors and on the interaction tensor T. Together with Equ. (1), they provide a complete description of the dependence of EVV 2DIR signal to the relative geometry between the two interacting groups under study. No known spectroscopic technique has utilised the dot products of these vectors, or polarisation changes at all, to determine the structure of a sample.

Example

As an illustrative example, one can consider the special case of two interacting linear functional groups and the coupling between their stretching modes. We assume that: 1) each stretching vibrational transition dipole is aligned properly along its bond axis, as well as the largest component of its Raman transition polarizability tensor; 2) that the two smaller components of the same Raman transition polarizability negligible. The effect of the two approximations is discussed below.

As a summary, Fig. 3 shows schematically those molecular properties and structural parameters relevant to this model. If a coordinate system with the origin at the centre of molecule 1 is chosen, the intermolecular vector $\mathbf{R}$ defining the z axis, and $\mathbf{R}$ along with $\frac{\partial \mu_1}{\partial Q_1}$ defining the xz plane, then Equ. (1) can be reduced to obtain the three components of the induced electrical anharmonicity vector as [Equation (3)]:

$$\begin{aligned} \left(\frac{\partial^2 \mu_x}{\partial Q_1 \partial Q_2} \right)_{ind} &= -\frac{\cos \psi \sin \theta_1 \sin \theta_2 + 2 \cos \theta_1 \cos \theta_2}{R^3} \left(\frac{\partial \alpha_1}{\partial Q_1} \frac{\partial \mu_2}{\partial Q_2} \sin \theta_1 + \frac{\partial \alpha_2}{\partial Q_2} \frac{\partial \mu_1}{\partial Q_1} \cos \psi \sin \theta_2 \right) \\ \left(\frac{\partial^2 \mu_y}{\partial Q_1 \partial Q_2} \right)_{ind} &= -\frac{\cos \psi \sin \theta_1 \sin \theta_2 + 2 \cos \theta_1 \cos \theta_2}{R^3} \frac{\partial \alpha_2}{\partial Q_2} \frac{\partial \mu_1}{\partial Q_1} \sin \psi \sin \theta_2 \\ \left(\frac{\partial^2 \mu_z}{\partial Q_1 \partial Q_2} \right)_{ind} &= -\frac{\cos \psi \sin \theta_1 \sin \theta_2 + 2 \cos \theta_1 \cos \theta_2}{R^3} \left(\frac{\partial \alpha_1}{\partial Q_1} \frac{\partial \mu_2}{\partial Q_2} \cos \theta_1 - \frac{\partial \alpha_2}{\partial Q_2} \frac{\partial \mu_1}{\partial Q_1} \cos \theta_2 \right) \end{aligned}$$

Note here the α s and μ s are used as scalars. As shown in Fig. 3, three angles, θ_1 , θ_2 and ψ are needed to define the spatial relationship between the two molecules, with ψ being the dihedral angle around **R**. Alternatively, an angle ϕ defined as the angle between the two linear axes can also be used and is related to ψ as:

$$\cos \phi = \sin \theta_1 \sin \theta_2 \cos \psi - \cos \theta_1 \cos \theta_2$$

With ϕ , the magnitude of electrical anharmonicity can be written as:

$$\left| \frac{\partial^2 \mu}{\partial Q_1 \partial Q_2} \right|_{ind} = \frac{\cos \phi + 3 \cos \theta_1 \cos \theta_2}{R^3} (k^2 + 1 + 2k \cos \phi)^{1/2} \frac{\partial \alpha_1}{\partial Q_1} \frac{\partial \mu_2}{\partial Q_2} \quad \text{Equ. (4)}$$

As proposed and confirmed by Okumura et al. Cho and Hahn et al. in references (9), (5) and (8) respectively, electrical anharmonicity should be inversely proportional to R^3 . Equ.s (3) and (4) above are clearly consistent with this conclusion. k in Equ. (4) is a dimensionless constant defined as:

$$k = \left(\frac{\partial \alpha_2}{\partial Q_2} \frac{\partial \mu_1}{\partial Q_1} \right) / \left(\frac{\partial \alpha_1}{\partial Q_1} \frac{\partial \mu_2}{\partial Q_2} \right).$$

Combining Equ. (3) with Equ.s (2) and (2'), we can then obtain an expression directly connecting third order non-linear susceptibility $\chi^{(3)}$, and thus cross peak intensity, with the geometrical parameters defined in Fig.3. Hence measurement of absolute cross peak intensity on an EVV 2DIR spectrum can directly give information on the geometry of the interacting but non-covalently bonded species in a sample under study.

As an alternative, instead of directly measuring the absolute intensities of cross-peaks in an EVV 2DIR spectrum, the ratio of cross peak intensities measured from PPS and PPP polarization setups discussed above provides an even more convenient way to determine the intermolecular angle ϕ .

In the case of linear molecules, this ratio is expressed as:

$$\left| \frac{\chi_{\text{PPS}}^{(3)}}{\chi_{\text{PPP}}^{(3)}} \right|^2 = \left| \frac{2 + k \cos \phi - \cos^2 \phi}{1 + 3k \cos \phi + 2 \cos^2 \phi} \right|^2 \quad \text{Equ. (5)}$$

Fig. 4 shows the relationship between signal ratio $\left| \chi_{\text{PPS}}^{(3)} / \chi_{\text{PPP}}^{(3)} \right|^2$ and angle ϕ as expressed by Equ. (5). Therefore angle ϕ , which is the angle between the two linear axes of the two functional groups under study, can be experimentally determined by simply measuring the ratios of signal intensities from the two polarization schemes employed.

To then determine the distance R between the interacting pair, the absolute magnitude of $\chi^{(3)}$ has to be measured but, as will be appreciated by the skilled person, this is easily done experimentally if an intermolecular cross-peak of known intensity can be used as an internal standard. Such internal standards are used in several conventional spectroscopic techniques, although not previously for this particular purpose.

Experimental verification of Model

According to an embodiment, an EVV 2DIR laser system can initially be set up to PPP configuration as discussed in reference (10). Any suitable EVV 2DIR cross-peaks generated from weak interactions, i.e. electrical anharmonicity between the concerned molecular species, e.g. sidechains, should then be located. As described above, the dipolar model can be employed to predict those where those cross-peaks should occur in the EVV 2DIR spectrum and/or

how strong the signal at those peaks might be. The peaks can be located in any suitable manner as will be known to the skilled reader, for example scanning through a suitable frequency range in which the cross-peaks are or might be expected. Once the peaks are located, this confirms that at least one weak interaction between species in the complex has in fact been detected. The signal intensity of the crosspeaks can then be measured.

In order to determine the relative orientation between the interacting groups in the weakly bound complex, the next step in the method is to change the EVV 2DIR laser system configuration from PPP to PPS. As set out above, the ratio between the measured signal intensity for PPP and PPS respectively is directly related to the relative orientation between the interacting groups, as shown in Equ. (5) above. Furthermore, absolute intensity of the signal obtained, or relative intensity when using an internal reference, can then be used to obtain a direct measure of the distance (R) between the interacting species thus solving a complete structure analysis. It will be appreciated that if two cross-peaks are detected in the EVV 2DIR spectrum due to weak interactions between the concerned molecular species, it is possible to use the equations above to give the relative angles θ_1 and θ_2 . Alternatively, the presence of three cross-peaks enables the solution of the equations above to give θ_1 and θ_2 and ψ . Alternatively, it is possible to use two cross-peaks and use simple assumptions such as assuming a structure consistent with simple rules of physical chemical interaction based on chemical understandings about the nature of the weak interactions, which provide extra constraints on the possible values of the angles in order to obtain all three angles from the two cross-peaks. Further alternatively, as discussed in detail below, an angle ϕ to find the angle between the two linear axes of linear interacting groups can also be used.

As an example, EVV 2DIR spectra were calculated using Gaussian03, a well-known quantum chemistry software for a dimer formed by benzonitrile (BN) and phenylacetylene (PA) to illustrate the model described above. The vibrations of interest according to this illustrative example are the CC and CN stretching modes. The detailed procedure of calculating EVV 2DIR spectra will be familiar to the skilled person and is described elsewhere (see for example references (1) to (3)), so it will not be repeated here. Geometry optimization for the dimer led to a configuration as shown in Fig. 5 which is similar to the displaced parallel configuration obtained for benzene dimers. The distance between CC and CN bond centres is about 3.54 Å, and the angle between their bond axes is about 3°. First order derivatives of dipole moments and polarizability were calculated separately for the two monomers BN and PA from their optimized geometries.

With these geometrical parameters and using the spectral properties listed in Table 1, which can be calculated in any appropriate manner for example using the Quantum Mechanical Calculation procedures described in reference (1). Equ. (1) was used to calculate the induced electrical anharmonicity between the two modes, also given in Table 1. Note that by using Equ.(1) but not Equ.(3), the complete transition polarizabilities as given in Table 1 were used, thus the only approximation involved is the proper alignments of the transition dipoles and polarizabilities. From the resultant electrical anharmonicity, an EVV 2DIR spectrum was calculated and combined with the calculated monomeric 2DIR spectra of BN and PA to obtain the EVV 2DIR spectrum for the BN-PA dimer as shown in Fig.5.

In Fig. 6 the cross peak at 2113/4226 cm⁻¹ (CC-CC cross peak) is the cross peak between CC stretching fundamental and first overtone, and similarly the one at 2239/4478 cm⁻¹ (CN-CN cross peak) is between CN stretching

fundamental and first overtone. The calculations are in accordance with the model detailed above, in that they predict there are two new cross-peaks at around 2113/4352 (CC-CN cross peak) and 2239/4352 cm^{-1} (CN-CC cross peak) which arise entirely from the extra electrical anharmonicity induced by the interaction between the CC and CN groups, with the CN-CC cross peak being 7.2 times stronger than the other one.

To further illustrate the methods described herein, EVV 2DIR spectra of pure BN, pure PA and a 50%-50% mixture of them were measured and shown in Fig. 7. Description of a suitable EVV apparatus and other experimental details can again be found elsewhere for example in reference (1), (2), (6) and (10). Compared with the spectra of pure liquids, two new cross-peaks did appear at 2225/4335 and 2110/4335 cm^{-1} in the spectrum of the mixture, as predicted by our calculations. As will be appreciated by the skilled reader the fact that the pair of cross-peaks do not exist in either of the two pure substances makes it unambiguously clear that they must come from the interaction between PA and BN. On the other hand, FTIR spectrum of the BN-PA mixture as shown in Fig. 9, is almost exactly the sum of the two pure substances. There is no any indication in the FTIR spectrum of the interaction as detected clearly by EVV 2DIR spectroscopy. This is again in accordance with the principles described above, since FTIR is a difference spectroscopy and thus not sensitive to electrical anharmonicities.

For all the three 2DIR spectra in Fig. 7, the pulse sequence used is that with pulse ω_α leading the other two pulses, ω_β and ω_γ . As discussed above, pulse sequences in EVV 2DIR spectroscopy can be changed to select three different nonlinear optical coherence pathways. With ω_α well ahead of the other two pulses, only one pathway will be included in the signal. On the contrary, if ω_β is well ahead of ω_α and ω_γ , the signal will come from the other two pathways.

However, unless there is a non-zero anharmonic shifting for the upper combination band, $\chi^{(3)}$ from these two pathways will always cancel out, leading to the disappearance of signals. If mechanical anharmonicities between CC and CN stretching modes are indeed negligible in the BN-PA mixture as assumed, the anharmonic shifting of CC+CN combination band will be very small too, thus the observed CC-CN and CN-CC cross-peaks should disappear upon pulse ordering changes. This was confirmed by the spectra shown in Fig. 8, suggesting that the assumption of negligible mechanical anharmonicity in this class of structures is valid.

Changing experimental polarization arrangement from PPP to PPS, we measured the PPS/PPP signal ratios as 0.417 ± 0.037 for the CC-CN cross peak and 0.368 ± 0.017 for the CN-CC cross peak. If Equ. (5) is directly applied to these ratios along with the value of k given in Table I, the angle between the CC and CN bond axes emerges as $46 \pm 2^\circ$ and $64 \pm 1^\circ$ respectively. However in obtaining Equ. (5) smaller components of the two transition polarizabilities were omitted. This approximation is easily accounted for. From Table I, theoretical PPS/PPP signal ratios for CC-CN and CN-CC cross-peaks can be calculated as 0.14 and 0.16 respectively, which lead to angles of 18° and 36° using Equ. (5). The deviation of these theoretical values from the geometrical one (3°) can be accounted for by the inclusion of smaller components of transition polarizabilities in calculating electrical anharmonicity, which also led to a bifurcation of the calculated angular values -- an error of 15° for CC-CN cross peak but 33° for CN-CC cross peak. The similar bifurcation of the theoretical values ($18 \sim 36^\circ$) and the uncorrected experimental values ($46 \sim 64^\circ$) indicates that they come from the same source of error. Thus if these theoretical errors were used as estimates of the errors in experimental values too, then the angle obtained experimentally can be corrected to 31° .

Apart from usual experimental errors, another potential source of “error” in the model described above is the approximation used in obtaining Equ. (5) that transition dipole moments and polarizabilities align properly with their bond axes. Since the two groups are interacting with each other, some deviations of these spectral properties from geometrical bond directions are actually well expected. In fact when this does happen, the concept of “geometrical” angles become ill-defined for optical spectroscopists since the only observables for them are these spectral parameters anyway. Instead, in this case ϕ in Equ. (5) should be interpreted as the angle between the transition properties of the interacting groups and it could be different from the visible “geometrical” value. Thus the experimentally-measured and theoretically-corrected value (31°) is considered to be a satisfactory measurement of the structure of the BN-PA mixture.

Given a known angle of interaction, determination of the distance R between the interacting CC and CN groups is straightforward. Since $\chi^{(3)}$ of the CN-CC and CC-CN cross-peaks should be inversely proportional to R^3 while the monomeric cross-peaks (CN-CN and CC-CC cross-peaks) should not, the intensity ratios between them should already carry information about R . This is fulfilled by the theoretically optimized distance (3.54 Å). The theoretical intensity ratio between CN-CC and CN-CN cross-peaks is calculated to be about 0.215. Then with an experimental value of the same ratio at about 0.732, a simple calculation gave a distance between the CC and CN groups at about 2.90 Å. Although ~ 0.6 Å shorter than the reference value, this is a good result, and illustrates well the usefulness of the model described above in detecting and determining the geometry of complex structures.

Advantages and Applications

The successful detection of new interaction-induced cross-peaks and subsequent determination of relative geometry of the interacting pair in the BN-PA mixture illustrates the validity of the theoretical model applied herein and its underlying assumptions. This dipole-dipole coupling model provides a clear physical picture about the origin of coupling between two functional groups. Although explicit expressions are provided herein connecting EVV 2DIR signal intensity with geometrical parameters for the special case of two linear molecules or functional groups, it will be appreciated that the same principles can be applied in order to obtain similar expressions for more complicated cases. . For example, for other chemical groups with different shapes, one might find other sets of geometrical parameters more appropriate to define their interaction geometry and explicit expressions can be deduced for them from the general expression of Equation .1

Like other 2DIR methods, EVV 2DIR spectroscopy depends on the coupling between different vibrational modes for its signals, but unlike other non-linear spectroscopic techniques, it is also very sensitive to electrical anharmonicity, which could arise from through-space electrostatic interaction. Electrical coupling between molecular groups or species is often not accompanied by any visible changes in IR or Raman vibrational spectroscopy, meaning that they are unable to detect such groups or species. As shown above, due to the unique feature of being sensitive to electrical anharmonicity, EVV 2DIR spectroscopy can be used as a method for the detection of weakly interacting complexes and also for their structural determination. The example given above is just a simple two-component liquid mixture, yet the interaction between the functional groups shows up clearly and distinctively as cross-peaks in the 2DIR spectra. The geometrical parameters determined subsequently from these cross-peaks, although crude at first sight, are correctable as detailed above by making

adjustments for approximations made in the model and can provide at least a semi-quantitative picture of the configuration of the complex.

Because EVV is a two dimensional spectroscopic technique, the spectrum produced is inherently less densely populated as compared to one dimensional spectroscopy techniques. Furthermore EVV 2DIR is particularly useful because the spectrum produced is more sparse than other 2DIR techniques, since EVV 2DIR can employ time delay of approximately 1 ps so as to only study long-lived states and, furthermore, it must satisfy both Raman and IR selection rules. Furthermore, EVV 2DIR can select to turn pathways on and off to decongest the spectrum further as discussed above.

Since EVV 2DIR spectroscopy has already been shown to be able to greatly reduce spectral congestion of complicated biological samples described in reference (2), (6) and (10), this additional ability to detect weak interactions between organic functional groups strongly enhances the usefulness of 2DIR spectroscopy EVV techniques for *inter alia*, protein and biomolecular research. In particular it can be employed both in the detection of structure activity relationship in intraprotein, protein-protein, protein-small molecule and protein-ligand interactions and in the determination of the geometry of these interactions.

As well as recognising the usefulness of EVV 2DIR spectroscopy for detection and structural analysis of substances in which electrical anharmonicity dominates over mechanical anharmonicity, it has been recognised that a particular application of this method is in protein side chain interactions. Furthermore a model has been devised to illustrate the physics between electrical coupling and relative geometry of the coupled groups, on the basis of which structural analysis can be carried out. In practice, the EVV 2DIR spectra

output for the PPP and PPS laser configuration respectively can be compared against one another to determine the relative angle between weakly interacting groups that are responsible for new cross-peaks on the EVV 2DIR spectra. Furthermore, the relative intensity of those cross-peaks can be used to obtain a measure of the distance between the two interacting species. The method as described herein can be used to predict the location and intensity of cross peak in an EVV 2DIR spectrum arising from interactions between non covalently bonded molecular species, in order to guide the user in searching for and detecting those cross-peaks. Alternatively, the user can scan through a range of EVV 2DIR spectra for a substance to look for such additional cross-peaks, which would not occur in conventional 2DIR spectra as discussed above. In either case, the equations set out above can be employed with respect to the detected crosspeaks to determine at least an approximation of the distance and relative angular position of the molecular species present.

According to the methods described herein, structural analysis and complex detection of biomolecules and other structures having weakly interacting but noncovalently bonded atomic/molecular species therein can be carried out. Such structures may include condensed phase materials, proteins, liquids and solids.

The methods described herein may be employed in a variety of applications including, *inter alia*, drug binding, drug discovery, drug design and toxicology screening. Furthermore, a compact laser system dedicated to EVV 2DIR may be implemented in an automated instrument, such that a device for carrying out the methods described herein could be employed as a black box by first line chemists for their research. Due to the high sensitivity of EVV 2DIR, in particular its usefulness with low concentration of sample and a short

throughput of material, a cost effective and accurate method for detecting weak interactions in a substance is provided.

In practice, the described methods can be run and controlled using any suitable hardware or software means. Instructions for carrying out the described methods may be recorded in a digital or analogue record carrier or computer readable medium. The record carrier may comprise optical storage means such as a readable disk or may be in the form of a signal such as a focussed laser beam. A magnetic record carrier such as a computer hard drive may also be used for storage of instructions for carrying out described methods. Alternatively, solid state storage or any suitable signal recording may be employed.

A computer or other suitable processing means may be programmed to execute instructions for carrying out the described methods. Such processing means may be used for display, manipulation, analysis, storage and/or transmission of the spectra and associated analysis and/or prediction results. Furthermore, a computer program may be provided for use in such a processing means in order to implement the described methods. Such computer implementation of the described methods may be used to provide automated detection, analysis and prediction. However, the described methods may be carried out using any suitable combination of computer and user implemented steps.

Although references are given above for one suitable EVV laser configuration, it will be appreciated that any suitable configuration for carrying out EVV 2DIR can be employed. Furthermore, although the methods described herein has been illustrated for liquid-liquid interaction, the principles of detection and analysis do not rely on any particular form of structure, but on the domination of electrical anharmonicity over mechanical anharmonicity for application of EVV 2DIR spectroscopy. Therefore the same principles apply for other phases of material.

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Claims

1. A method of detecting an interaction between chemical groups in a sample, the method comprising obtaining an electronic vibration vibration (EVV) two dimensional infra red (2DIR) spectrum of the sample, disregarding signals in said spectrum caused by chemical bonds present in the sample and identifying whether any additional signals are present in the spectrum, indicating the presence of said interaction.
2. A method as claimed in claim 1 wherein the step of disregarding the signals caused by chemical bonds present in the sample comprises comparing the sample EVV 2DIR spectrum to respective EVV 2DIR spectra of two or more individual species present in the sample.
3. A method as claimed in claim 1 wherein the step of disregarding the signals caused by chemical bonds present in the sample comprises comparing the sample EVV 2DIR spectrum to at least one of a Raman spectrum and a Fourier Transform Infra Red (FTIR) spectrum of the sample.
4. A method as claimed in any of claims 1 to 3 further comprising the step of employing a mathematical model to predict the presence of said interaction in the sample, wherein said prediction step includes predicting at least one of the location of a signal within the spectrum and the absolute or relative intensity of a signal within the spectrum.
5. A method as claimed in any of claims 1 to 4 comprising the step of obtaining a first EVV 2DIR spectrum of the sample using a first (PPP) pulse configuration and obtaining a second EVV 2DIR spectrum of the sample using

a second, different (PPP) pulse configuration and comparing the output spectra to detect mechanical coupling in the sample.

6. A method according to any preceding claim wherein the sample is one of a protein, a liquid, a solid and a condensed phase material.

7. A method according to any preceding claim wherein the interaction to be detected is one of: a protein-protein interaction, an intraprotein reaction, a sidechain interaction, a protein-small molecule interaction and a protein-ligand interaction.

8. A method of analysing the structure of a sample comprising obtaining a first electronic vibration vibration EVV two dimensional infra red 2DIR spectrum of the sample using a first (PPP) pulse configuration and obtaining a second EVV 2DIR spectrum of the sample using a second, different (PPP) pulse configuration, identifying a first signal present in the first spectrum due to the presence of an interaction in the sample and comparing the intensity of said first signal to a reference signal present in the first or second spectrum, to obtain structural information therefrom.

9. A method as claimed in claim 8 wherein said reference signal is one of: a signal in the second spectrum present due to the same interaction that causes said first signal in said first spectrum; and an internal standard signal in the first spectrum.

10. A method according to claim 8 or claim 9 further comprising modelling said interaction using the assumption that the interaction is electronic and a multipolar system.

11. A method according to any of claims 8 to 10 wherein analysing the structure of the sample comprises at least one of: determining a distance between two interacting species in the sample and determining an angular relationship between two species in the sample.

12. A method of predicting a characteristic of employing an interaction between species in a sample, comprising calculating an electronic vibration vibration (EVV) two dimensional infra red (2DIR) spectrum of the sample, disregarding signals in said calculated spectrum caused by chemical bonds present in the sample and identifying whether any additional signals are present in the calculated spectrum, predicting the presence of said interaction

13. A method according to claim 12 wherein said characteristic is at least one of the location of a signal within the spectrum and the absolute or relative intensity of a signal within the spectrum.

14. A method of detecting an interaction between chemical groups in a sample, the method comprising obtaining an electronic vibration vibration (EVV) two dimensional infra red (2DIR) spectrum of the sample, disregarding interaction-affecting signals in said spectrum caused by chemical bonds present in the sample and identifying whether any additional signals are present in the spectrum, indicating the presence of said interaction.

15. A method as claimed in claim 14 in which non-interaction-affecting signals include signals where the chemical bonding causes only non-interaction

affecting mechanical anharmonicity and results in substantially purely electronic vibration vibration coupling.

16. An EVV 2DIR apparatus, configured to carry out the method according to any of claims 1 to 15.

17. A processing means programmed and operable to execute instructions for carrying out the method according to any of claims 1 to 15.

18. A record carrier having instructions stored thereon for execution by a processing means to carry out the method according to any of claims 1 to 15.

19. The record carrier of claim 18 wherein said record carrier includes an optical, magnetic or solid state storage means or a readable signal.

20. A computer program including instructions executable by a processing means for carrying out the method according to any of claims 1 to 15.

21. A method, apparatus or system as described herein or substantially as shown in the appended figures.

Fig 1

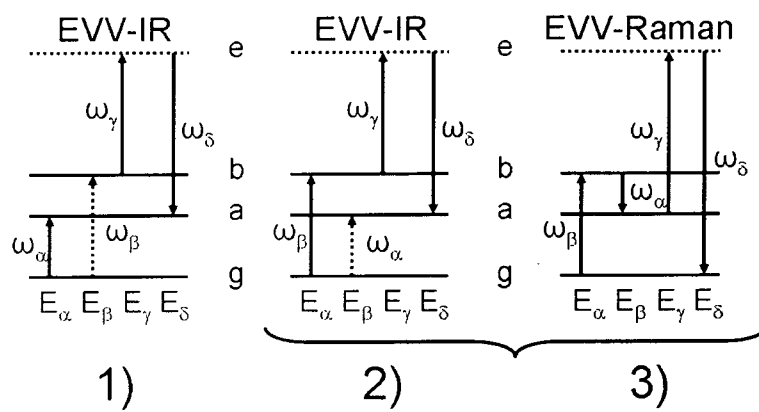


Fig 2

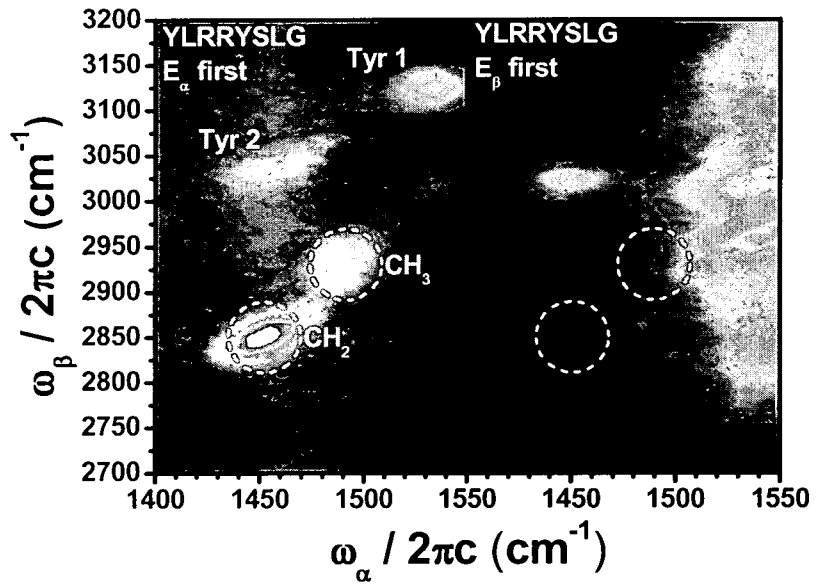


Fig 3

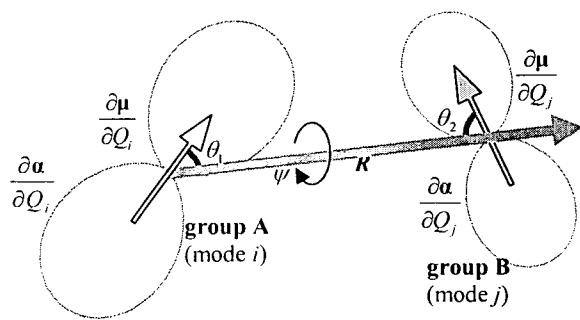


Fig 4

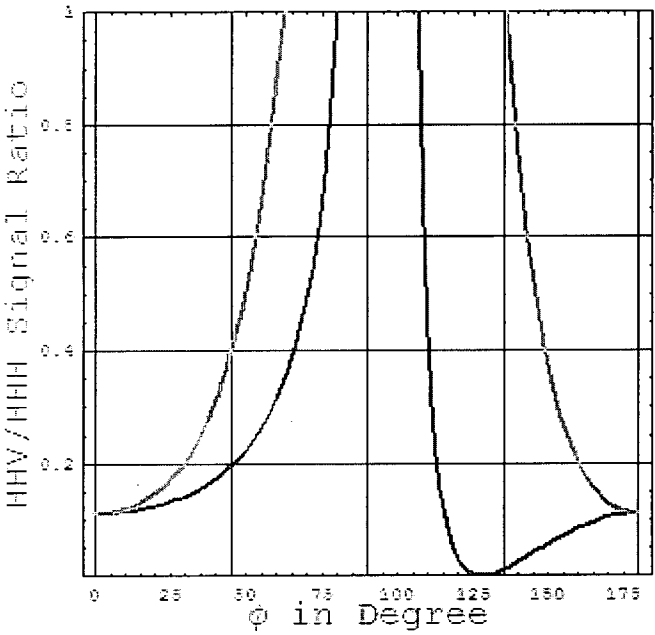


Fig 5

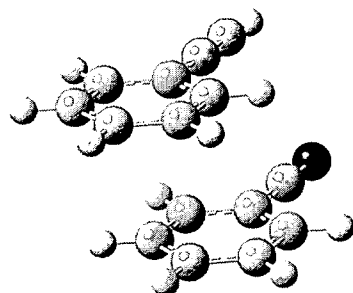


Fig 6

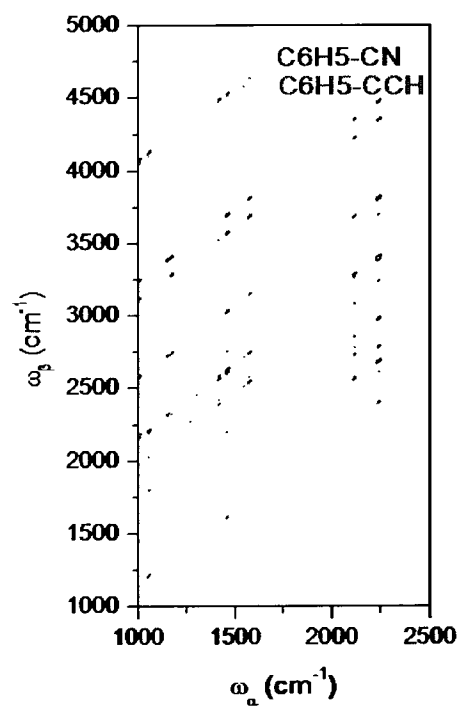


Fig 7

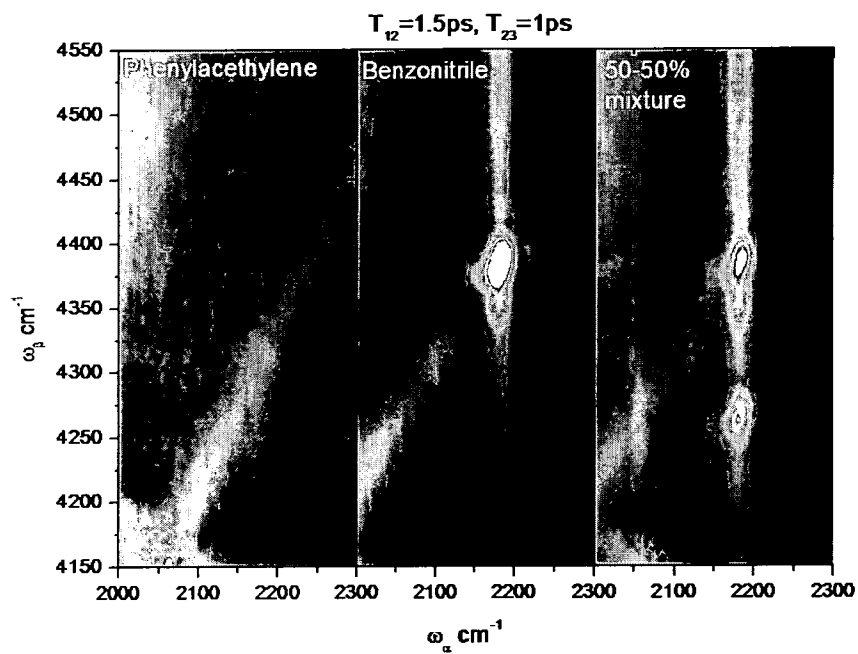


Fig 8

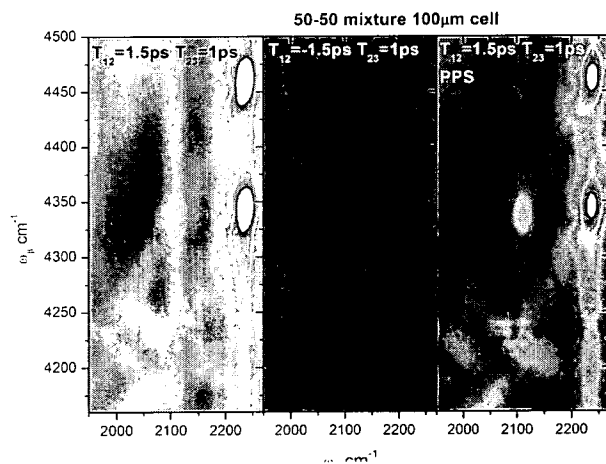


Fig 9

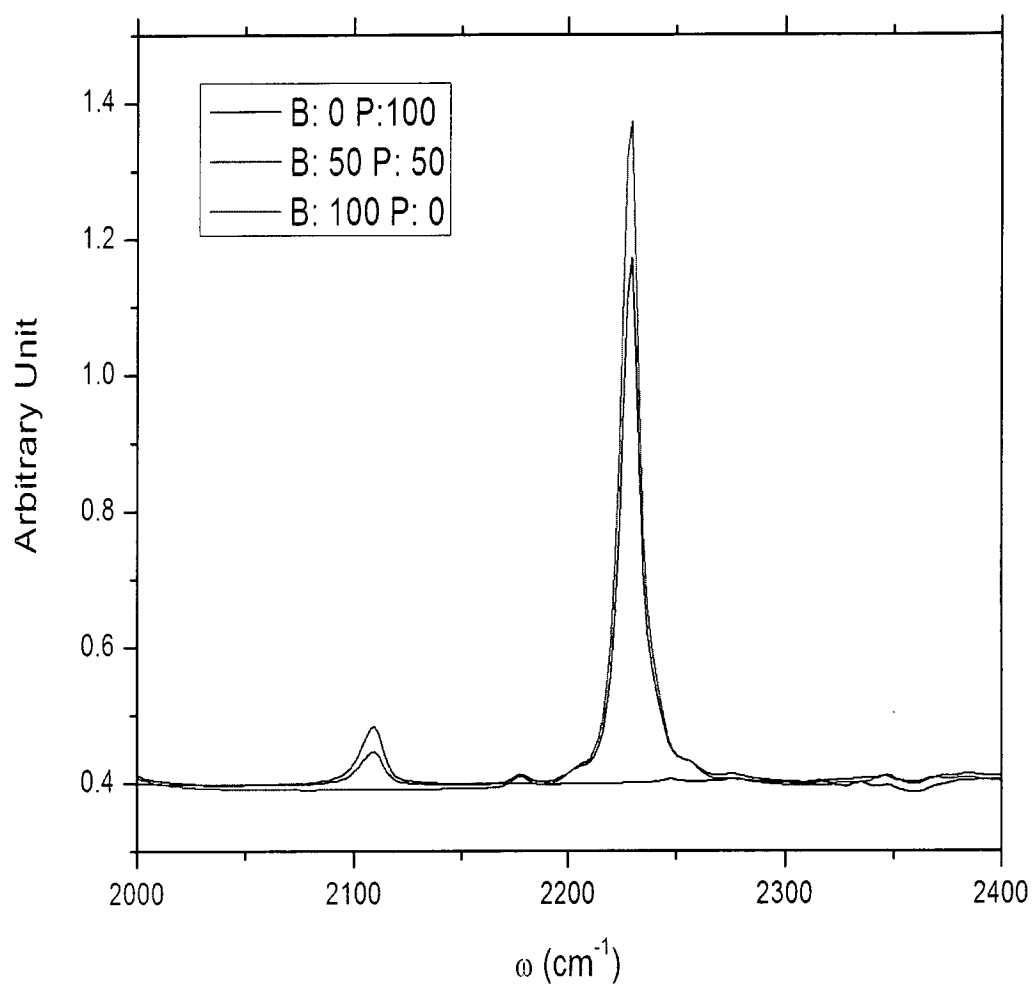


Fig 10

TABLE I Spectral parameters used in the C₆H₅CCH-C₆H₅CN system

	Ph-C≡N	Ph-C≡CH
frequency (cm ⁻¹)	2239	2113
reduced mass (amu)	12.6796	6.1124
$\frac{\partial \mu}{\partial Q}$	0.698	0.256
$\frac{\partial \alpha}{\partial Q}$	xx: 3.007 yy: 3.612 zz: 75.773	xx: 4.524 yy: 4.548 zz: 73.755
k	2.65	
$\frac{\partial^2 \mu}{\partial Q_i \partial Q_j}$	x: 0.0054 y: -0.0085 z: 0.1659	
$ \chi_{PPP}^{(3)} ^2$	7.78×10^{-31}	1.08×10^{-31}
$ \chi_{PPS}^{(3)} ^2$	1.25×10^{-31}	1.56×10^{-32}

^a calculated at B3LYP/6-311++G(d,p) level.^b in molecular-fixed coordinate systems, only non-zero components given.^c components given as in the coordinate system as defined in the text.^d with the CC and CN stretching modes as IR step respectively.^e in a.u. unless stated otherwise.

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2010/000350

A. CLASSIFICATION OF SUBJECT MATTER

INV. G01J3/433 G01N21/35 G01N21/65 G01N33/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

G01J G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DONALDSON P M ET AL: "Decongestion of methylene spectra in biological and non-biological systems using picosecond 2DIR spectroscopy measuring electron-vibration-vibration coupling" CHEMICAL PHYSICS, NORTH-HOLLAND LNKD-DOI:10.1016/J.CHEMPHYS.2008.02.050, vol. 350, no. 1-3, 23 June 2008 (2008-06-23), pages 201-211, XP022942206 ISSN: 0301-0104 [retrieved on 2008-03-04]	1-7, 12-21
Y	cited in the application the whole document ----- -/--	10, 11

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

9 August 2010

Date of mailing of the international search report

16/08/2010

Name and mailing address of the ISA/
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Authorized officer

Spott, Thorsten

INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2010/000350

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2007/138267 A1 (IMP INNOVATIONS LTD [GB]; KLUG DAVID ROBERT [GB]; DONALDSON PAUL MURRA) 6 December 2007 (2007-12-06) page 4, line 12 - page 5, line 13; claims 1-3,5; figure 1	16-21
X	DONALDSON PAUL M ET AL: "Direct identification and decongestion of Fermi resonances by control of pulse time ordering in two-dimensional IR spectroscopy" JOURNAL OF CHEMICAL PHYSICS, AMERICAN INSTITUTE OF PHYSICS, NEW YORK, NY, US, vol. 127, no. 11, 21 September 2007 (2007-09-21), pages 114513-1, XP009133814 ISSN: 0021-9606 cited in the application	8,9
Y	the whole document	10,11
A		1,12,14
A	DOERR A: "Optical proteomics" NATURE METHODS, NATURE PUBLISHING GROUP, GB, vol. 5, no. 12, 1 December 2008 (2008-12-01), page 993, XP007913156 ISSN: 1548-7091 the whole document	1,8,12,14
X,P	GUO RUI ET AL: "Detection of complex formation and determination of intermolecular geometry through electrical anharmonic coupling of molecular vibrations using electron-vibration-vibration two-dimensional infrared spectroscopy" 20091014 LNKD- DOI:10.1039/B910804A, vol. 11, no. 38, 14 October 2009 (2009-10-14), pages 8417-8421, XP007913158 [retrieved on 2009-07-22] the whole document	1-7,12-21
A	FOURNIER ET AL: "Optical fingerprinting of peptides using two-dimensional infrared spectroscopy: Proof of principle" ANALYTICAL BIOCHEMISTRY, ACADEMIC PRESS INC, NEW YORK LNKD- DOI:10.1016/J.AB.2007.11.009, vol. 374, no. 2, 13 November 2007 (2007-11-13), pages 358-365, XP022477602 ISSN: 0003-2697 section "Discussion"	8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB2010/000350

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-7, 12-21

Disregarding chemical bond signals in an electronic vibration vibration (EVV) two dimensional infra red (2DIR) spectrum of a sample.

2. claims: 8-11

Obtaining two EVV-2DIR spectra of a sample, using two different pulse configurations.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/GB2010/000350

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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