Molecular Rotational Resonance Spectroscopy - Chiral Analysis without Chromatography

Brooks H. Pate[1, 2], Justin L. Neill[2], and Matt T. Muckle[2]

[1.] Department of Chemistry, University of Virginia, McCormick Rd., Charlottesville, VA 22904
[2.] BrightSpec, 770 Harris Street, 104B, Charlottesville, VA 22903

A new spectroscopy technique for determining the absolute configuration of molecules and quantitatively measuring the enantiomeric excess of a sample has been developed in the field of molecular rotational spectroscopy. The technique is fundamentally different than optical rotation methods, like vibrational circular dichroism, and produces signals that are on the same order as the regular 'achiral' measurement. Rotational spectroscopy offers extremely high spectral resolution and has a strong sensitivity to the mass distribution. These features offer the potential for high-speed chiral analysis that can be performed directly on complex sample mixtures without the need for chromatography.

A chiral molecule has a structure that cannot be superimposed upon its mirror image. Many of the physical properties of the mirror images, or enantiomers, are identical. However, the physical interactions between chiral molecules can be strongly influenced by the handedness of the molecules. These effects are of central importance when chemistry takes places in the homochiral environments of biological systems. In pharmaceutical applications this means that enantiomers of a drug can have different effects in the body. A significant fraction of small drug pharmaceuticals are chiral and there is a general goal of producing the final active pharmaceutical ingredient in an enantiopure form to improve drug potency, drug safety, or to extend intellectual property protection through the strategy of chiral switching.

Chiral analysis in pharmaceutical applications presents major challenges to analytical chemistry, especially in the case where the molecule has multiple asymmetric carbons. Each asymmetric carbon has a local geometry that can be either left or right handed, usually indicated using the R/S convention. Therefore, for a molecule with N chiral centres, there are 2^N possible stereoisomers. In the most general case, there will be 2^{N-1} distinct molecular geometries, known as diastereomers, and each of these diastereomers is chiral so that it has a pair of enantiomers. An example is shown in *Figure 1* for ohmefentanyl, an opioid analgesic, which has three chiral centres. This drug illustrates the challenges in pharmaceutical chemistry posed by chirality. The potency of the drug and its potential for addiction depend strongly on both the diastereomer geometry and enantiomeric form [1]. For example, the diastereomers labelled F9202 and F9204 are both highly potent, 1500 and 6400 times more potent than morphine, respectively. However, F9202 is found to be about 600 times less addictive in mice. The mirror images, or enantiomers, of these compounds are 2000 and 100 times less potent.

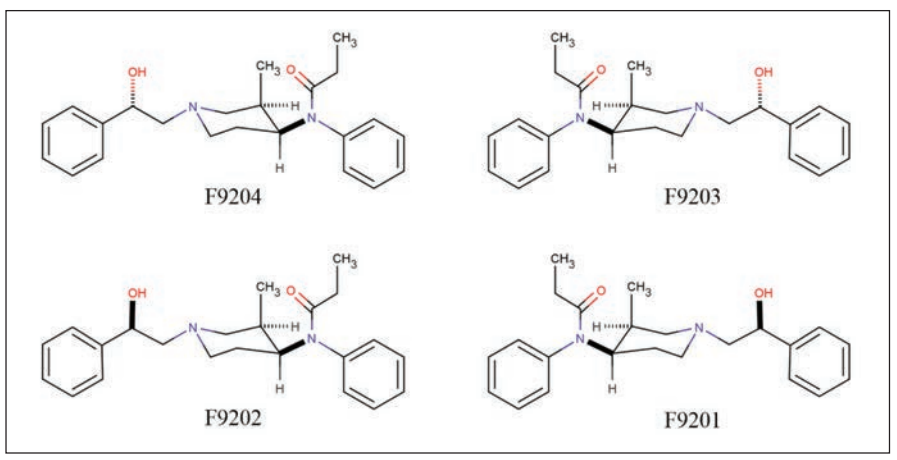

Figure 1. The molecular structures of four stereoisomers of ohmefentanyl are shown. The names used for the structures are consistent with the nomenclature of Ref. 1.

The ideal analytical technique for chiral analysis would be able to perform the following three measurements: 1) quantitative measurement of the yields of diastereomers produced in the synthesis of a molecule with multiple chiral centres, 2) quantitative measurement of the enantiomeric excess in a sample, and 3) accurate determination of the absolute configuration (or handedness) of the enantiomer that is in excess. Furthermore, rapid measurement times and the ability to perform the measurement directly on a complex reaction mixture would be desired. Currently there is no single technique that can meet this set of challenges and physical separation of the diastereomers and enantiomers by chromatography is still a generally necessary step of the analysis process.

In a recent landmark paper, Patterson, Schnell, and Doyle introduced a new enantiomer-selective measurement technique that uses molecular rotational resonance spectroscopy [1]. Subsequent experiments by these researchers have shown that three wave mixing rotational spectroscopy experiments are a sensitive method for measuring enantiomeric excess [3-6]. The implementation of these techniques in a manner similar to NMR spectroscopy techniques has been described theoretically [7] and was recently implemented experimentally [8]. Molecular rotational resonance spectroscopy, which performs spectroscopy on the quantised energy levels that result from the kinetic energy of overall molecular rotation, has been recognised as a sensitive probe of the molecular three-dimensional geometry for many years [9,10]. The spectral patterns are governed by the principal moments-of-inertia (expressed as the rotational constants: A, B, C [11]) and are, therefore, sensitive to the mass distribution in the molecule. Recent instrument advances have made it possible to study the structure of larger molecules using broadband, chirped-pulse Fourier transform rotational spectroscopy of molecules cooled to temperatures of about 2 K in the adiabatic expansion of a pulsed jet source [12,13]. The spectra recorded in these instruments have exceptionally high spectral resolution so that complex mixtures can be directly analysed. The combination of high-sensitivity, enantiomer-specific measurement capabilities, excellent chemical selectivity, and high spectral resolution for direct mixture analysis offers new opportunities for chiral analysis in chemistry.

Experimental

The results from a recent experiment showing enantiomer-specific spectroscopic signatures in solketal using three wave mixing Fourier transform molecular rotational resonance (FT-MRR) spectroscopy are presented [8]. Solketal was purchased from Sigma-Aldrich in both enantiomerically pure forms and as a racemic mixture. The sample was introduced into the spectrometer vacuum chamber by heating it and entraining the vapour in a flow of neon carrier gas at 1 atm. pressure. A pulsed valve creates a free jet expansion that produces a 2K gas sample. The rotational spectrum of solketal was measured using a CP-FTMW spectrometer as shown in *Figure 2*. The signal-to-noise ratio in the measurement is higher than 1,000:1 so that it was possible to analyse the spectra of the singly substituted ^{13}C and ^{18}O isotopologues in natural abundance and this information was used to determine the experimental structure of solketal that is shown in *Figure 2*.

The spectroscopic information from this measurement was used to perform a three wave mixing FT-MRR spectroscopy measurement using the principles introduced by Patterson, Schnell, and Doyle [2-6] and further developed by Grabow [7]. The basic physical principle of this measurement was presented by Giordmaine who showed that three wave mixing (i.e. sum and difference frequency generation) is possible from a bulk sample as long as it has an enantiomeric excess [14]. For rotational spectroscopy, the important physical insight is that a somewhat subtle feature can distinguish enantiomers: the product of the signs of the components of the molecular dipole moment along the principal axis system of molecular rotation is enantiomer-specific. This idea is illustrated in *Figure 3*.

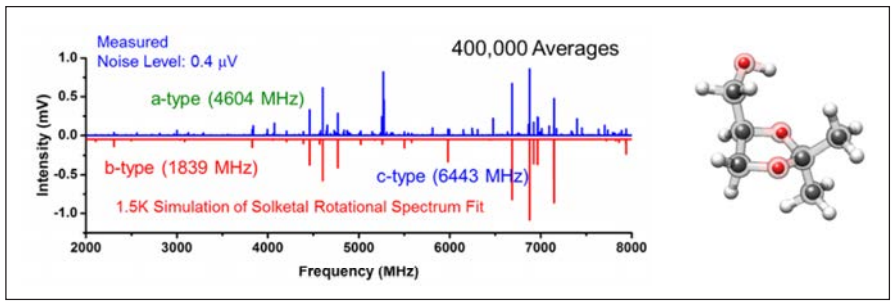

Figure 2. The molecular rotational resonance spectrum of solketal (2,2-Dimethyl-1,3-dioxolan-4-yl-methanol) in the 2-8 GHz frequency region is shown in blue. The red spectrum shows the simulation of the rotational spectrum calculated using the spectroscopic fit parameters and a sample temperature of 1.5 K. The spectra of the ^{13}C and ^{18}O single-substituted isotopologues was measured in natural abundance were also analysed and this information is used to determine atom positions of these nuclei. These positions, shown as the small, solid spheres, are compared to the theoretical structure calculated at the M06-2X/6-311++G(d,p) level of theory on the right side of the figure.

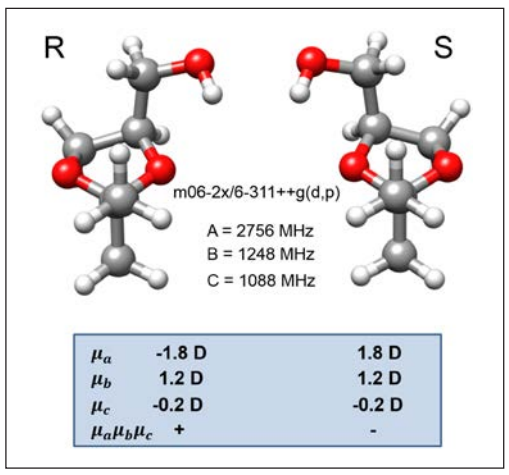

Figure 3. The mirror image enantiomer structures of solketal are shown. The theoretical estimates of the rotational constants used to predict the quantised rotational kinetic energy levels are shown. The shaded box gives the calculated electric dipole moment of solketal. The dipole moment is a vector quantity, and it can be specified by giving the components in the orthonormal coordinate system of the principal rotational axes of the molecule – a molecule fixed coordinate system. The sign of the product of these vector components is an intrinsic property of the enantiomer, and it can be used to distinguish the R and S forms.

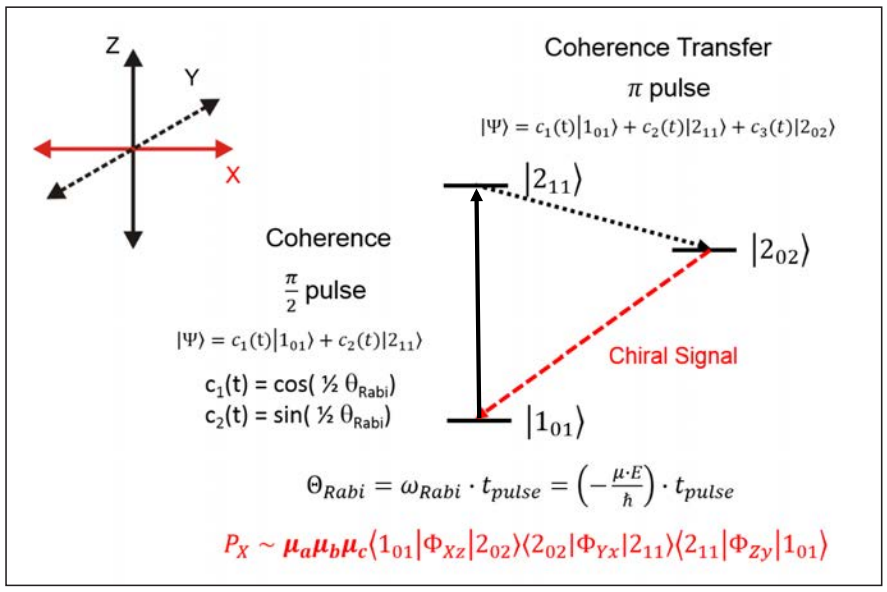

Figure 4. A schematic representation of the energy level diagram for the rotational kinetic energy levels of solketal is shown. The energy levels are labelled with the quantum numbers for rotational energy, JKaKc, and this example shown the 1_{01}, 2_{11}, and 2_{02} quantum levels.[11] Applying two excitation pulses, the first using the μc dipole component to drive the transition between 1_{01} and 2_{11} and the second using the μb dipole component to drive a transition between 2_{11} and 2_{02} levels, creates a time-dependent quantum state that will emit coherent light at the difference frequency of the transition between 2_{02} and 1_{01} (with an intensity governed by the μa dipole moment component). The electric field polarisation of these three light fields will be mutually orthogonal.[7]

Three wave mixing spectroscopy is used to create an emission signal that is proportional to this product so that the sign, or phase, of the emitted light identifies the enantiomer. This works because the molecular rotational spectrum is actually composed of three distinct spectra with intensities controlled by the three dipole moment vector components denoted m_a, m_b, and m_c. These are called the a-type, b-type, and c-type spectra [11]. In order to generate the chiral emission signal, the electric field vectors of the two excitation pulses need to by orthogonal and the electric field polarisation of the coherent emission signal will be mutually orthogonal to both excitation pulse polarisations.

The way that these measurement concepts are implemented is shown in *Figures 4 and 5*. First, the experiment requires a transition 'cycle' between three of the quantised rotational kinetic energy levels. The intensity of the transitions between each pair of energy levels, *Figure 4*, is governed by a different dipole moment component. The transition frequencies fall in the microwave region of the electromagnetic spectrum, and polarised excitation pulses can be created using horn antennae as shown in *Figure 5*.

The electric field polarisations of the two excitation pulses are orthogonal and the coherent signal generated through the three wave mixing process has an electric field that is orthogonal to both excitation pulses. The frequency of the emitted wave is the difference of the excitation frequencies in the level diagram of *Figure 4*. The coherent emission signal for the two different enantiopure samples of solketal are shown in *Figure 6* where it is seen that they have opposite sign, or phase, as expected from the sign of the product of the dipole moment components. The Fourier transform of the coherent signals is also shown in *Figure 6*. This figure also shows the measurement using the racemic sample where no signal is generated.

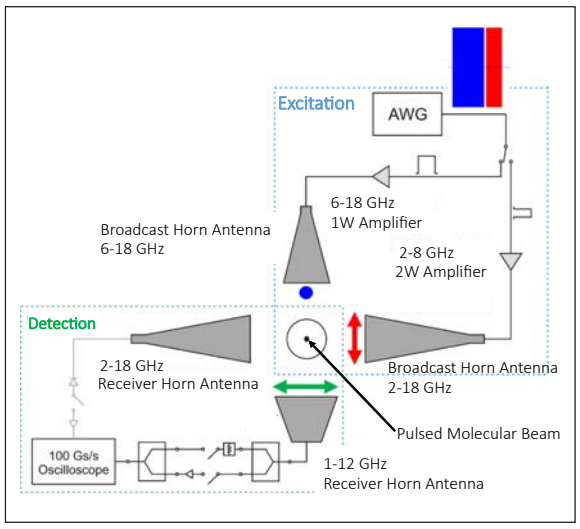

Figure 5. The experimental design to implement three wave mixing FT-MRR spectroscopy is shown. Microwave horn antennae are used to generate linearly polarised excitation pulses at the resonance frequencies of the energy level diagram in Figure 4. The electric field vectors of the blue and red excitation pulses are orthogonal. A coherent emission at the difference frequency will be generated if the sample has an enantiomeric excess and this signal is detected using a third horn antenna. The electric field polarisation of the signal wave (green) is mutually orthogonal to the excitation pulse polarisations.[8]

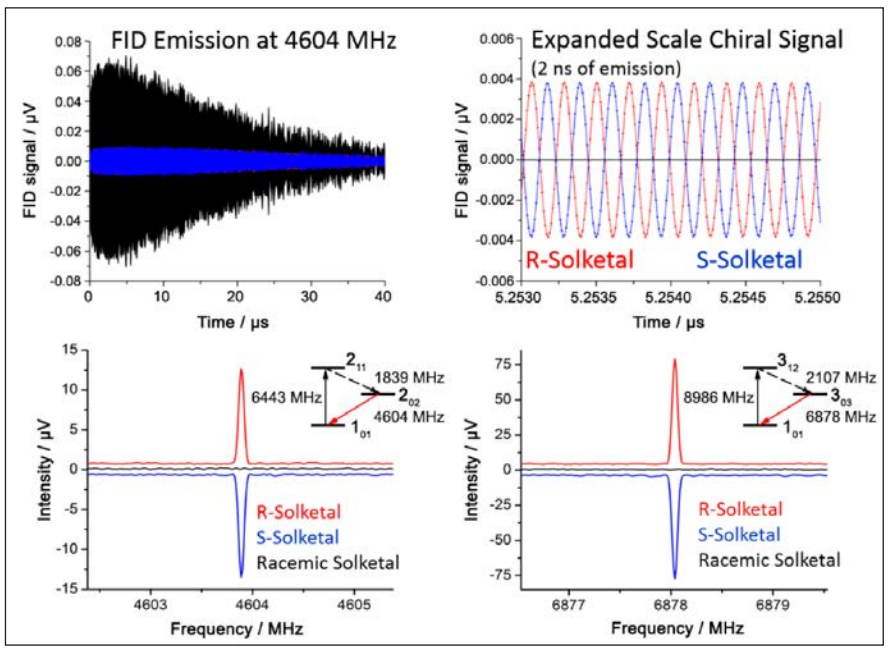

Figure 6. A summary of the chiral spectroscopy using the three wave mixing FT-MRR technique is shown. The upper left panel shows the time domain recording of the coherent difference frequency emission at 4604 MHz in blue ((S)-solketal) and red ((R)-solketal). These signals are so similar in amplitude that it is hard to discern the red data trace. The signal in black is the direct measurement of the transition using the normal, 'achiral' rotational spectroscopy. The important point is that the chiral signals are on the same scale as the achiral signals. The top right shows a very small time segment of the coherent emission signals when enantiopure samples of S-solketal (blue) and R-solketal (red) are measured. These signals are 180° out of phase, as expected from the difference in the product of the dipole moment components. A Fourier transform analysis of the time-domain signals is shown in the bottom two panels for two different chiral measurement cycles. The amplitudes of the enantiopure signals are the same. The black trace shows the results for a measurement using a racemic solketal mixture and this does not generate a chiral signal.

Results and Discussion

The spectroscopic results demonstrate that three wave mixing rotational spectroscopy produces an enantiomer-specific signature through the phase of coherent emission signal. This signal is produced at the sum or difference frequency of the two, resonant microwave pulses used to excite the sample and has a similar magnitude to the normal, 'achiral' signal.

This result is an important difference between three wave mixing FT-MRR spectroscopy and better known techniques like vibrational circular dichroism (VCD) [15]. These optical rotation methods rely on the interference of electrical and magnetic dipole moment contributions to the spectral intensity and these signals are generally 10^{-4} of the intensity of the 'achiral' vibrational spectrum due to the small size of the magnetic dipole moment. In the FT-MRR measurement, detection at the sum or difference frequency occurs against zero background giving high sensitivity even for small enantiomeric excesses.

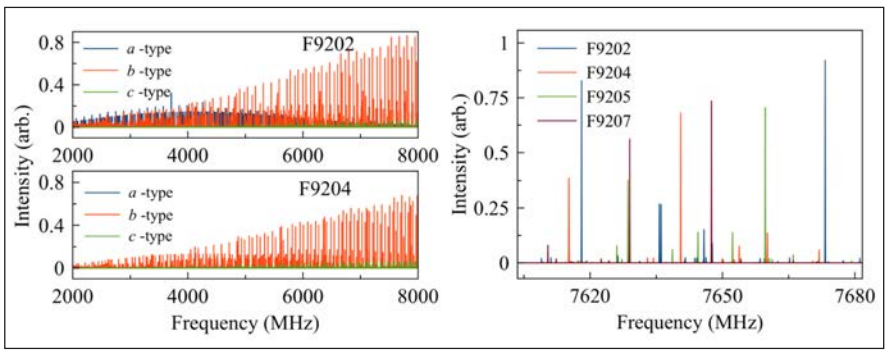

Figure 7. This figure shows simulations of the molecular rotational resonance spectra of the diastereomers of ohmefentanyl. The left panel shows simulations of the F9202 and F9204 stereoisomers (see Figure 1). These spectra can be easily distinguished by the different intensities of the separate a-, b-, and c-type rotational spectra [11]. In particular, F9204 has a very weak a-type rotational spectrum. The right panel shows a small frequency range of the rotational spectrum where all four distinct diastereomers rotational spectra are simulated using the experimental line widths. Molecular rotational resonance spectroscopy is capable of fully resolving these spectra. These simulated spectra have an intensity that is about half as strong as solketal in the 2-8 GHz frequency range.

There is an important limitation in the current measurement: it has not yet been possible to achieve absolute phase calibration of the signal so that the absolute configuration, or handedness, of the enantiomer in excess cannot be directly determined. However, the current instrument does give a measurement phase that is reproducible day-to-day and this indicates that it will be possible to achieve absolute phase calibration in future instrument designs. There may also be possibilities to determine the absolute configuration using internal molecular standards [5].

This field of spectroscopy is in its infancy and the early experiments have selected molecular targets that do not push the limits of Fourier transform molecular rotational resonance spectroscopy. For the technique to have an impact in applications related to small drug discovery it will be necessary to perform chiral analysis on larger molecules. The general limitations on molecules for successful orally-delivered drugs summarised by the well-known Rule of Five of Lipinski [16] and augmented to consider molecular flexibility by Veber [17] are favourable to the technique. In general, small molecule drugs have molecular masses less than 500 Da. They are fairly polar, as conveyed through the general result that the octanol-water partition coefficient is not larger than 5. There is a general preference for molecules with limited numbers of flexible bonds thereby limiting the conformational complexity of the molecule.

As an illustration of what might be possible as this technique develops, we consider simulations of the molecular rotational resonance spectra of ohmefentanyl *(Figure 1)*. A summary of the spectroscopic properties of the 8 stereoisomers that are obtained from quantum chemistry calculations [18] is given in *Table 1*. The rotational constants, related to the moments-of-inertia for the rotational motion, are identical for enantiomers (giving the same energy level structure) but differ for the diastereomers that have distinct geometries. The electric dipole moment varies for the diastereomers, especially in the vector components in the principal axis system of the molecular rotation. These results further reinforce the key physical insight that the sign of the product of the dipole moment components uniquely defines the enantiomer. Simulations of the spectra of the four diastereomers show that they would be easily identified in a measurement with a key feature being the change in the relative intensities of the three different a-, b-, and c-type spectra as shown in *Figure 7*. Furthermore, FT-MRR spectroscopy can fully resolve the spectra of all four diastereomers. In principle, quantitative diastereomer yields and enantiomeric excess measurements could be performed without the need for chromatography to separate the 8 stereoisomers.

Table 1. Stereoisomers of ohmefentanyl.

Isomer	Absolute Configuration1	A (MHz)	B (MHz)	C (MHz)	μa (D)	μb (D)	μc (D)	|μtot| (D)
F9201	(+)-cis-(3S,4R,2′S)	439.3	71.4	65.9	3.8	4.5	-1.2	6.0
F9202	(-)-cis-(3R,4S,2′R)	439.3	71.4	65.9	3.8	4.5	1.2	6.0
F9203	(-)-cis-(3S,4R,2′R)	442.2	68.9	65.5	0.6	-4.0	-1.3	4.3
F9204	(+)-cis-(3R,4S,2′S)	442.2	68.9	65.5	0.6	-4.0	1.3	4.3
F9205	(+)-trans-(3S,4S,2′S)	446.7	68.0	64.6	-1.1	-4.1	0.6	4.3
F9206	(-)-trans-(3R,4R,2′R)	446.7	68.0	64.6	1.1	-4.1	0.6	4.3
F9207	(+)-trans-(3S,4S,2′R)	445.9	69.8	66.0	4.3	-4.4	0.08	6.1
F9208	(-)-trans-(3R,4R,2′S)	445.9	69.8	66.0	4.3	-4.4	-0.08	6.1

Conclusions

A fundamental advance in physical chemistry has produced a new spectroscopy technique that can perform quantitative chiral analysis directly on complex chemical mixtures with the potential to make the measurements on time scales of minutes or less. As pointed out by Nafie in a companion article to the original discovery [19], this new field faces several challenges to create a useful tool for analytical chemistry but there is reason for optimism. Techniques will be required to volatilise large molecules, while retaining the ability to cool them through adiabatic expansion, so that they can be measured in the gas phase [20, 21]. When faced with a similar challenge the field of mass spectrometry produced volatilisation methods that gave incredible leaps in the power of the technique [22,23]. The rotational spectroscopy community needs to validate that quantum chemistry can accurately calculate the molecular parameters required in the analysis (the rotational constants obtained from the geometry and the electric dipole moment). In this case, however, the computational requirements are vastly lower than for the interpretation of VCD spectra [24]. Finally, like other methods including VCD [15,24] and NMR spectroscopy [25], the measurements and analysis will need to deal with conformational flexibility – an issue omitted from the ohmefentanyl discussion above. Conformational flexibility causes fewer problems in rotational spectroscopy because there is usually some cooling of the conformational energy in the pulsed jet expansion leading to spectral simplification [26] and the conformations are static on the time scale of the measurement so dynamic exchange effects are not present. As the rotational spectroscopy community works to develop this new technique, it welcomes input from the broader analytical chemistry community to help define the problems where the unique features of three wave mixing FT-MRR spectroscopy can solve problems that are unaddressed by current analytical chemistry techniques.

Acknowledgements

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