WHITE PAPER

IDENTIFICATION AND QUANTITATION OF HARD-TO-RESOLVE INDIVIDUAL COMPONENTS IN MIXTURES WITHOUT CHROMATOGRAPHY, CHEMOMETRICS, AND REFERENCE STANDARDS

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ABSTRACT

Accurate identification and quantitation of individual components in chemical mixtures is required to effectively control industrial chemical reactions. In complex mixtures, including those requiring stereochemical and/or chiral characterization, multiple analytical techniques are typically required to accomplish this task. In this white paper, we demonstrate that an emerging MRR technology enables unambiguous ID, stereochemical characterization, and quantitation of hard to detect and hard to separate individual components in mixtures in just one simple measurement.

As an example, we demonstrate MRR's ability to fully characterize crude reaction mixture species present in a continuous pharmaceutical reactor, in both offline and online regimes. The superior specificity and resolving power of MRR enables simultaneous quantitative characterization of all components in this mixture, including their corresponding stereoisomers, without any chromatographic separation or chemometrics. In addition, due to a direct and unequivocal relationship between the MRR spectral features and structural parameters of a molecule, it was possible to ID and quantitate all the detected crude reaction mixture constituents without measuring reference standard spectra for pure chemicals.

INTRODUCTION TO MRR

Molecular Rotational Resonance (MRR) Spectroscopy is an emerging technology that is online-capable, information-rich, highly sensitive, extremely selective, relatively easy to use and simple to interpret. MRR utilizes electromagnetic radiation at millimeter or microwave wavelengths to directly measure the mass and charge distributions of molecules.

The ability of MRR spectroscopy to elucidate 3D-geometry of molecules has been known for decades **[1]**. However, until recently, data acquisition times were slow: up to dozens of hours or even days. Recent engineering breakthroughs in highspeed digital electronics combined with innovative "chirped-pulse" Fourier-transform MRR approach enabled dramatic sensitivity improvements **[2-7]**. The typical measurement times for commercial MRR instruments are a minute to a few hours for broadband measurements, and a few seconds to minutes for targeted "narrow-band" measurements.

Demonstrated process and/or lab-based applications of MRR include but are not limited to the following:

- VOC analysis **[3, 4]**
- Trace gas analysis: emissions, impurities, isotopologues **[5, 6]**
- Residual solvents **[7]**
- Time-resolved chemical reaction dynamics and kinetics **[8]**
- **Chirality and/or stereospecificity**: (stereo)isomers, enantiomers and/or conformers **[2, 9-12]**

WHY MRR FOR COMPLEX CHEMICAL MIXTURE ANALYSIS?

As a spectroscopy technique, MRR has several advantages over chromatography. These advantages include: ***fast analysis; online capability***; ***fast and straightforward method development; little or no sample preparation*****;** and ***essentially no consumables***.

Additional MRR advantages over most other spectroscopic techniques are summarized below:

- **Low Detection Limits for Spectroscopy:** 0.01 – 50 ppm (sample-dependent)
- **Unmatched resolving power and chemical selectivity**
    - Virtually no overlap or interferences between spectra of different species. Can resolve even "hard-to-resolve" or "hard-to-separate" individual analytes:
        - No chemical separation is needed for analysis
        - Can analyze dozens of analytes at once
- **High 3D-structural, stereochemical, and isotopologues specificity**
    - Sensitive to even subtle changes in mass and/or charge distributions to resolve:
        - Individual (stereo)isomers, conformers, enantiomers, and/or isotopologues
- **Information-Rich:**
    - >100 well resolved bands are typically available for analysis of each chemical
- **Easy to ID and Quantitate: NO CHEMOMETRICS REQUIRED**
    - Spectral band parameters are unequivocally related to 3D-structural parameters of a molecule. Thus, easy to ID.
    - Intensities of the spectral features are directly proportional to concentrations. Thus, easy to quantitate.
    - Large number of spectral bands and extremely high resolving power of MRR virtually eliminate any potential for overlap between spectral features of different analytes. Thus, no chemometrics is required for analysis.
    - **ID without Measuring Pure Reference Standards Possible** MRR spectral features are directly and unequivocally related to structural parameters of a molecule. Standard quantum chemistry methods are sufficient to unambiguously find the matching chemical structure, without measuring pure reference standards.

All the above mentioned capabilities of broadband MRR spectroscopy are directly demonstrated in the "Analysis Example" section of this white paper to enable quantitative chemical and/or stereochemical

analysis of individual components of crude reaction mixture sample that was taken directly from a continuous pharmaceutical reactor.

MRR MEASUREMENTS

Crude reaction mixture samples with unknown individual constituents can be analyzed using a BrightSpec's Chiral***MRR*** instrument **[12]**. As a first step, a high signal-to-noise broadband MRR spectrum of a mixture can be collected to enable ID and/or assignments of individual mixture constituents (please see "Analysis Example"). Once all analytes are identified and their analysis peaks selected, the instrument can be run in online-capable targeted mode that enables analysis times of seconds to minutes.

Figure 1*. A picture of a commercial* Chiral***MRR*** *Instrument (BrightSpec, Inc., Charlottesville, VA)*

Samples can be analyzed 'as is', without any preliminary sample preparation. In addition, an automated multi-stage heating process can be setup using BrightSpec's Edgar software to efficiently remove solvents prior to analysis and/or measure highly volatile analytes prior to less volatile analytes, if desired.

ANALYSIS EXAMPLE

We provide an example of a real chemical mixture analysis and explain how MRR was able to ID and quantitate all individual species in a crude reaction mixture without chemical separation, without use of chemometrics, and without reference standards of pure individual chemicals.

Crude Reaction Mixture Sample. A crude reaction mixture sample of an antimalarial drug was taken by a customer directly from a continuous synthesis reactor (followed by solvent removal) and submitted to BrightSpec 'as is'. As described by the customer, at least seven components could be present in the provided crude reaction mixture sample: unreacted reagent (one stereoisomer), product (desired diastereomer), the undesired product's epimer (undesired diastereomer), and four stereoisomers of an undesired overreduction byproduct.

The customer's need was to identify and quantitate the individual components present in the crude reaction mixture including their individual stereoisomers.

Identification of a Crude Mixture Individual Components without Reference Standards. None of the pure individual constituents nor their corresponding reference standard spectra were available for MRR analysis. Due to a direct and unequivocal relationship between MRR spectral features and 3D-structural parameters of molecules, it was possible to easily calculate accurate theoretical MRR spectra for seven molecular candidates from the list, and then use them instead of measured references.

Figure 2 shows a measured broadband MRR spectrum of the antimalarial drug crude reaction mixture (top spectrum, black). BrightSpec's analysis identified that the crude reaction mixture is dominated by only four (out of at least seven possible) major constituents: a) The desired product (blue); b) the undesired product's epimer (green); c) One of four stereoisomers of the undesired overreduction byproduct (red); and d) Unreacted reagent (brown). It should be noted that MRR showed no evidence of

either of three remaining stereoisomers of the overreduction product, nor of any other detectable species.

The extracted MRR spectra of the individual reaction constituents were added to BrightSpec's reference library. These spectra can then be used to enable automated ID of the reaction mixture components using a conventional spectral library match approach, and/or online process control applications.

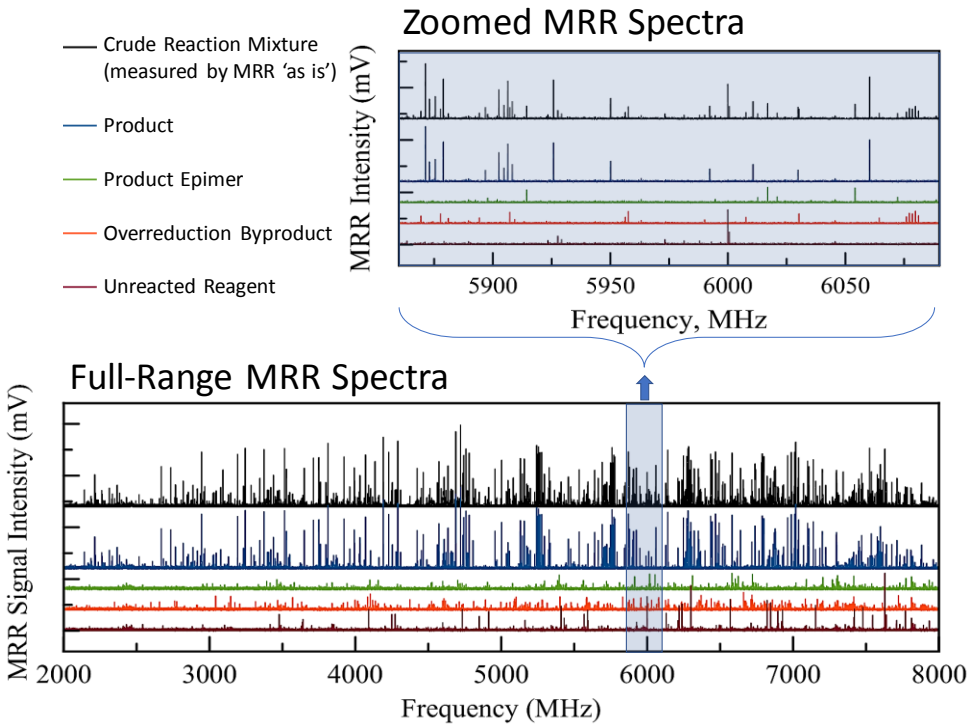

***Figure 2**. Decomposition of the antimalarial drug crude reaction mixture broadband MRR spectrum into its individual components without chemical separation, chemometrics, and/or use of reference standards. Bottom plot shows the broadband MRR spectra (full range). The inset (top plot) shows a zoomed portion of the MRR spectra to directly demonstrate that the spectrum of every component is highly resolved and unique with little or no overlap between the spectral features of the individual components. Please see text for detail.*

*Spectra legends (from Top to Bottom). **Black**: Measured MRR spectrum of the crude reaction mixture sample 'as is'. **Blue**: Extracted spectrum of the desired product. **Green**: Extracted spectrum of the undesired product's epimer (unwanted diastereomer). **Red**: Extracted spectrum of the unwanted overreduction byproduct. **Brown**: Extracted spectrum of the unreacted reagent.*

Quantitation of Individual Components in a Crude Reaction Mixture without Reference Standards. Measured MRR band intensities are directly proportional to concentrations. Thus, once individual components of a chemical mixture are identified, the relative spectral band intensities of individual chemical mixture components can be directly related to their relative concentrations in the mixture.

Table 1 shows the percentages for all of four major components detected in the antimalarial drug crude reaction mixture sample. As expected, we found that the mixture is dominated by a desired product at ~56.5% level. We also found that the crude reaction mixture contains ~14.6% of the unreacted reagent, as well as about 28.9% of undesired products or byproducts: ~19.8% of the overreduction byproduct, and ~9.1% of the undesired product's epimer. 3D-structures and concentrations of all detected species were reported to the customer to enable further process improvements.

Table 1. *Quantitation of individual components in the antimalarial drug crude reaction mixture*

DETECTED COMPONENT	MEASURED PERCENTAGE
Product	56.5%
Unwanted Epimer of a Product	9.1%
Unwanted Overreduction Byproduct	19.8%
Unreacted Reagent	14.6%

IMPLICATIONS FOR ONLINE CHEMICAL ANALYSIS

Once the initial method development using a broadband mode is completed (Figure 2 example), the Chiral***MRR*** instrument can be run in online-capable targeted mode, with measurement times of seconds to minutes. Figure 3A shows BrightSpec EDGAR software interface developed for continuous monitoring. This interface enables an analyte peak(s) selection and a relevant control plot(s) generation.

Figures 3B and 3C directly compare online MRR and offline NMR data. MRR data points were recorded at the same times as NMR samples were taken out of the continuous pharmaceutical reactor. Conditions in the reactor were deliberately altered between measurements to produce changes in composition.

Relative concentrations of the reaction mixture species, determined by MRR and NMR, are in good agreement and consistent with the condition changes in the reactor. However, in contrast to MRR, the conventional NMR was not able to monitor the overreduction byproduct in this mixture. Conventional HPLC was also not able to adequately detect this overreduction product.

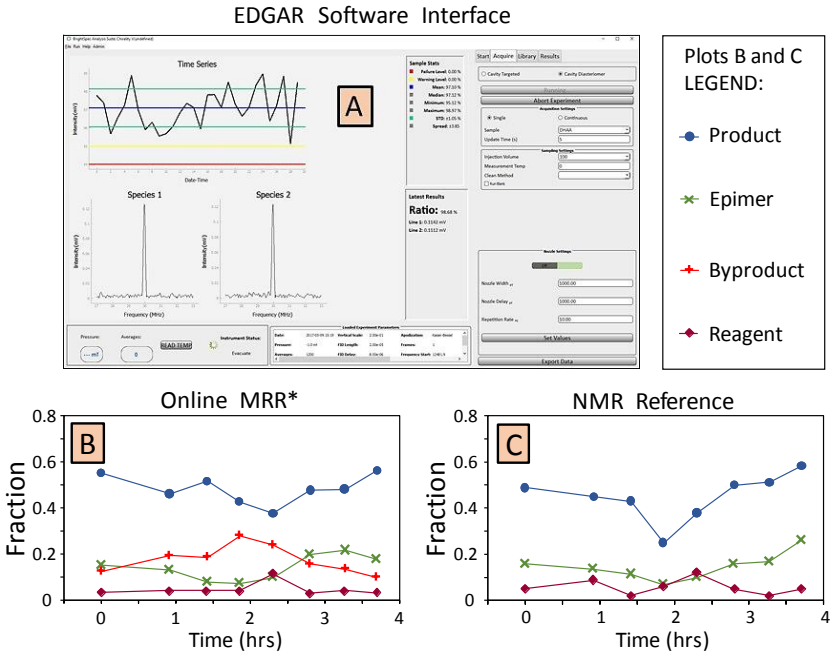

Figure 3*. Online monitoring of crude reaction mixture using BrightSpec's* Chiral***MRR*** *instrument.* ***A)*** *Screenshot of Brightspec's EDGAR software interface for continuous monitoring applications. Relevant control plots can be generated for selected analytes.* ***B)*** *and* ***C)*** *Comparison of* ***online MRR*** *and* ***offline NMR*** *data (please see text for detail). MRR is capable of direct monitoring of all crude reaction species, whereas conventional NMR cannot directly monitor the overreduction byproduct (red timeseries).*

** MRR data was offset to correct for differences in analyte vapor pressures.*

Thus, MRR is a powerful alternative to conventional analytical techniques such as NMR, GC, HPLC and LC-MS that are typically utilized to characterize individual species in reaction mixtures. In addition to MRR's sensitivity to even subtle changes in molecular structure, the MRR measurements are fast and simple, and both online and offline analysis options are readily available. In contrast to MRR, conventional analytical techniques are typically used offline, and it may take hours or even days to complete the analysis that can be accomplished by MRR in minutes.

SUMMARY

In this work, MRR spectroscopy was successfully utilized to identify and quantitate individual components of a continuous synthesis reaction of an antimalarial drug, both offline and online. MRR was able not only to resolve all the individual components of this chemical reaction including the associated reagents, intermediates, products and byproducts, but also enabled unambiguous identification and quantitation of their corresponding stereoisomers and/or conformers to directly monitor chiral purity of each analyte, which is of crucial importance for essentially any pharmaceutical and/or organic synthesis.

It was also demonstrated that in addition to the conventional approach that utilizes reference standards of pure individual analytes, MRR offers a simple and direct alternative to unequivocally ID and quantitate the individual chemical mixture constituents even if their corresponding reference standards and/or spectra are not available. Further, no chemical separation or chemometrics is required for analysis.

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