



**SMASH 2009**

## **Conference Program**

September 20<sup>th</sup>-23<sup>rd</sup>, 2009  
Chamonix, France

# SMASH 2009 NMR Conference

Dear SMASH 2009 Attendees,

Welcome to the 2009 SMASH NMR Conference!!

After the success of the 2007 meeting, SMASH has returned to Chamonix and the picturesque Mont Blanc scenery. As before the conference will take advantage of the variety of hotels and restaurants within Chamonix which, as you will see from the enclosed map, are all within close proximity to one another.

This year's program contains an exciting array of oral sessions and workshops, all of which are to be held in the Majestic Conference Center. Oral session topics include: Metabolomics, Multidimensional NMR of Small Molecules, Advances in NMR Analysis, Exotica, Human Health and Nutrition, Sensitivity Enhancement and Novel Developments in NMR, Latest Movements in Solids and the Student/Post-Doctoral Session.

As in the past, the program also includes four workshops. Monday's workshop topics include Hyphenation and Automation in the NMR Lab and Careers in NMR. The workshops for Tuesday deal with the latest applications and developments in pulse programming, and the latest advances in methods and tools for Metabolomics.

In addition, we will be treated to an after-dinner talk on Monday night entitled 'Serendipity in 40 years of NMR' by keynote speaker Prof. David Davies from Birkbeck College, University of London, UK.

On behalf of the entire SMASH Organizational Committee, we wish to thank you for your continued interest in, and support of, the SMASH NMR Conference.

We hope you enjoy this year's conference!!

Sincerely,

Jules Griffin & Andrew Nicholls  
Co-Chairs, SMASH 2009 NMR Conference

# SMASH 2009 NMR Conference Program

## Sunday September 20<sup>th</sup>

- 4:30 PM - 6:00 PM      **Registration, Le Majestic Centre de Congrès**  
6:00 PM - 8:00 PM      **Dinner, Le Vista, Hotel Alpina**  
8:00 PM -11:00 PM      **Mixer, Le Vista, Hotel Alpina**

## Monday September 21<sup>st</sup>

- 8:15 AM - 8:30 AM      **Opening Remarks**  
8:30 AM - 10:00 AM      [Multidimensional NMR of Small Molecules](#)  
Chair: Tim Claridge, University of Oxford, UK  
[Applications of Spectral Aliasing to the Study of Complex Mixtures Using 2D HSQC Spectra](#)  
Damien Jeannerat, University of Geneva, Switzerland  
[Solution State Crystallography - A New Way to Look at Correlation Spectra and NOEs](#)  
Craig Butts, University of Bristol, UK  
[Applications of 2D NMR Methods to Structure Elucidation in Drug Development](#)  
Ben Bardsley, GlaxoSmithKline, UK
- 10:00 AM - 10:30 AM      **Break**  
10:30 AM - 12:00 PM      [Latest Movements in Solids](#)  
Chair: Bénédicte Elena, Ecole Normale Supérieure de Lyon, France  
[Probing Hydrogen Bonding in Organic Solids: Applications to NMR Crystallography and Pharmaceuticals](#)  
Steven P. Brown, University of Warwick, UK  
[Molecular Dynamics in Solids: A Combined Analysis of Several Relaxation Times and Spectral Properties](#)  
Marco Geppi, University of Pisa, Italy  
[MAS NMR on the Tetrapyrrole Chromophore of the Photoreceptor Phytochrome](#)  
Thierry Rohmer, University of Leiden, The Netherlands
- 12:00 PM - 1:30 PM      **Lunch**  
1:30 PM - 3:00 PM      [Student and Post-Doctoral Session](#)  
Helen Atherton, University of Oxford, UK  
[Metabolic Markers of Combined Pesticide Exposure](#)  
Oliver Jones, University of Cambridge, UK  
[New Methods for NMR Crystallography of Powdered Solids](#)  
Elodie Salager, University of Lyon, France  
[Solvent Relaxation as a Tool to Screen Small Molecule-Metalloprotein Interactions – A Case Study Using Human Prolyl Hydroxylase Domain 2](#)  
Ivan K.H. Leung, University of Oxford, UK
- 3:00 PM - 3:30 PM      **Break**  
3:30 PM - 5:00 PM      **Workshops (Concurrent)**

*I. Careers in NMR*

Jules Griffin, University of Cambridge, UK

*II. Hyphenation and Automation in the NMR Lab*

Ron Crouch, Varian Inc., USA

- 5:00 PM - 6:00 PM      **Free Time**  
6:00 PM - 6:30 PM      **Social Gathering**  
6:30 PM - 9:00 PM      **Dinner, Le Vista, Hotel Alpina**

After Dinner Speaker: David Davies  
University of London  
*Serendipity in 40 years of NMR*

- 9:00 PM - 11:00 PM      **Mixer, Le Vista, Hotel Alpina**

**Tuesday September 22<sup>nd</sup>**

- 8:30 AM - 10:00 AM      [Advances in NMR Analysis](#)  
Chair: Gerhard Wagner, Harvard Medical School, USA

[New 2D and 3D DOSY Techniques and Applications](#)

Gareth Morris, University of Manchester, UK

[Application of Covariance NMR to the Analysis and Molecular Identification of Chemical Mixtures](#)

Rafael Bruschweiler, Florida State University, USA

[Efficient Detection and Characterization of Protein-ligand Interactions in H<sub>2</sub>O](#)

Alvar Gossert, Novartis, Basel, Switzerland

- 10:00 AM - 10:30 AM      **Break**

- 10:30 AM - 12:00 PM      [Exotica](#)  
Chair: Duncan Farrant, GSK, UK

[Spectral Editing of Organic Mixtures into Pure Compounds using NMR and Ultra Viscous Solvents](#)

André Simpson, University of Toronto, Canada

[NMR with Small Magnets](#)

Bernhard Blümich, Institut für Technische und Makromolekulare Chemie, Germany

[Reversible Interactions with para-Hydrogen Enhance NMR Sensitivity by Polarization Transfer](#)

Simon Duckett, University of York, UK

- 12:00 PM - 1:30 PM      **Lunch, Le Vista, Hotel Alpina**

- 1:30 PM - 3:00 PM      [Human Health and Nutrition](#)  
Chair: Jules Griffin, University of Cambridge, UK

[Assessment of Nutritional Interventions on Health by NMR-Metabolomics](#)

Xavier Correig Blanchar, Universitat Rovira i Virgili, Spain

[Nutritional Influences on the Metabolic Profile, Chances and Pitfalls](#)

Manfred Spraul, Bruker BioSpin GmbH, Germany

[Metabolic Health Assessment by Nutritional Metabonomics](#)

Serge Rezzi, Nestle Research, Switzerland

- 3:00 PM - 3:30 PM      **Break**

3:30 PM - 5:00 PM      **Workshops (Concurrent)**

*I. Metabolomics*

Hector Keun, Imperial College London, UK

*II. Pulse Programming*

Ulrich Gunther, Birmingham University, UK

5:00 PM - 6:00 PM      **Free Time**

6:00 PM - 7:30 PM      **Dinner, Restaurant La Calèche**

8:00 PM - 10:00 PM    [Poster Session & Mixer, Le Majestic](#)

10:00 PM - 11:00 PM   **Mixer, Le Majestic**

## **Wednesday September 23<sup>rd</sup>**

8:30 AM - 10:00 AM    [Metabolomics](#)

Chair: Jean Charles Portais, INSA-Toulouse, France

[Identification of Biomarkers for Toxicity by NMR-Based Metabonomics](#)

Nelly Aranibar, Bristol-Myers Squibb, USA

[Metabolic Profiling Techniques to Assess and Manage Quality in Fruit Production](#)

Dominique Rollin, INRA Bordeaux, France

[NMR-based Fluxomics: Current Challenges and Perspectives](#)

Stéphane Massou, INSA Toulouse, France

10:00 AM - 10:30 AM   **Break**

10:30 AM - 12:00 PM   [Sensitivity Enhancement and Novel Developments in NMR](#)

Chair: Adrian Davis, Pfizer, UK

[Industrial Process Control Employing NMR/MRI Based Sensors](#)

Michael J. McCarthy, University of California-Davis, USA

[Ultra-fast and Ultra-sensitive 2D NMR of Hyperpolarized Solutions](#)

Patrick Giraudeau, Weizmann Institute, Israel

[High-Field in-situ DNP – Applications from Small Molecules to Large Biomolecules](#)

Thorsten Maly, MIT, USA

12:00 PM - 12:15 PM   **Closing Remarks**

12:15 PM -              **Box Lunches**

# SMASH 2009 NMR Conference

## Acknowledgements

The SMASH 2009 Conference gratefully acknowledges the support provided by the following companies.

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# **SMASH 2009 NMR Conference Committee**

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## **Poster Session**

Helen Atherton  
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Monday, September 21<sup>st</sup>

8:30 AM - 10:00 AM

**Multidimensional NMR of Small Molecules**

Chair: Tim Claridge

Speakers:

Damien Jeannerat

University of Geneva, Switerland

Craig Butts

University of Bristol, UK

Ben Bardsley

GlaxoSmithKline, UK

# Applications of Spectral Aliasing to the Study of Complex Mixtures Using 2D HSQC Spectra

Damien Jeannerat<sup>1</sup>, Bruno Vitorge<sup>1</sup>, Mohammadali Foroozandeh<sup>1</sup>, Rupali Shivapurkar<sup>1</sup> and Jean-Marc Nuzillard<sup>2</sup>

1. Département de Chimie Organique, Université de Genève, Geneva, Switzerland
2. Institut de Chimie Moléculaire de Reims, Université de Reims Champagne–Ardenne, Reims, France

Spectral aliasing is a “fast method” allowing one to quickly reach a high-resolution in indirectly detected dimensions of 2D and other multidimensional NMR experiments. For protein NMR, the potential of reduction of the number of time increments is between three and eight in each dimension, but it reaches one to two orders of magnitudes in the carbon dimension of small molecules.[1]

Spectral aliasing is based on a reduction of the spectral width which increases the time increment so that fine differences in chemical shifts can be easily observed. The Nyquist condition being violated the methods have to provide a solution to eliminate the ambiguities in the chemical shifts. We will review the different approaches discussed in the literature of small molecules, including a one designed to be readily applicable by any chemist using standard equipment and experiments.[2] We will also discuss the possibility to extend spectral aliasing to 3D experiments, envisage the combinations of spectral aliasing with other “fast methods” and say a few words about the problem of signal quantification in 2D spectra.

Broadly speaking, spectral aliasing extends to complex mixture: the determination of chemical structure by taking advantage of quickly recorded 2D HSQC-TOCSY and HSQC-NOESY spectra[3], the measurement of diffusion coefficients using a new Constant-Time HSQC pulse sequence,[4] and the determination of kinetic parameters using series of HSQC spectra[5].

The development of an automatic titration system makes it possible to record series of HSQC spectra to determine binding affinities, pKa, etc.

1. Jeannerat, D, *J. Magn. Res.*, 186(1), 112-122, 2007.
2. Vitorge, B., Bieri, S., Humam, M., Christen, P., Hostettmann, K., Muñoz, O., Loss, S. and Jeannerat, D., *Chem. Commun.*, 950-952, 2009
3. Muri, D., Corminboeuf, C., Carreira E. M. and Jeannerat, D., *Magn. Reson in Chem.* In press., 2009
4. Vitorge, Bruno and Jeannerat, Damien, *Analytical Chemistry*, 78(15), 5601-5606, 2006.
5. Gasparini, G., Vitorge, B., Scrimin, P., Jeannerat, D., and Prins, L. *J. Chem. Commun.*, 26, 3034-3036, 2008.

# **Solution State Crystallography – A New Way to Look at Correlation Spectra and NOEs**

**Craig P. Butts** and Jeremy N. Harvey

School of Chemistry, University of Bristol, Bristol, UK

A de novo quantitative molecular structure elucidation in solution is demonstrated using data derived only from NMR spectroscopy. A distance-geometry approach is used whereby internuclear distances, assessed with surprising accuracy, are employed to generate both bonding and conformational information. Remarkably this technique does not rely on interpretation of the traditional semi-qualitative information such as chemical shift or coupling constants. This solution-state technique complements the high precision and intermolecular information available from X-ray crystallography, with structure elucidation in a more convenient state of matter.

# **Applications of 2D NMR Methods to Structure Elucidation in Drug Development**

**Ben Bardsley**

Chemical Development, GlaxoSmithKline R&D, Stevenage, UK

Structure elucidation is an important part of drug development due to the need to identify low level impurities and degradants of drug substances in line with regulatory (ICH) guidelines and to ensure patient safety through having a full knowledge of the components present in a drug product. In addition, the application of a Quality by Design approach to drug manufacture means that understanding the structures of impurities during the development phase can play an important role in the development of robust, manufacturing processes which are fit for use on the large scales required for commercial drug products.

This presentation focusses on the importance of certainty in structure elucidation arising from the above factors and gives examples of some structures with unusual function groups and cross-links where the careful use and selection of 2D NMR experiments was required to eliminate alternative structures and provide the required structural certainty to allow project progression. The interpretation of 2D spectra of non-ideal samples is also discussed and the ways in which specific data can be extracted and used to enable the resolution of manufacturing problems.

Monday, September 21<sup>st</sup>  
10:30 AM - 12:00 PM

**Latest Movements in Solids**  
Chair: Bénédicte Elena

Speakers:

Steven P. Brown  
University of Warwick, UK

Marco Geppi  
University of Pisa, Italy

Thierry Rohmer  
University of Leiden, The Netherlands

# Probing Hydrogen Bonding in Organic Solids: Applications to NMR Crystallography and Pharmaceuticals

S. P. Brown

Department of Physics, University of Warwick, Coventry CV4 7AL, UK

Solid-state MAS NMR experiments utilising recently developed methodology such as homonuclear  $^1\text{H}$  decoupling and through-bond J correlation are yielding new insight into hydrogen bonding interactions that control the packing of organic molecules into three-dimensional structures.

Specifically, the  $^1\text{H}$  chemical shift is a sensitive indicator of hydrogen-bonding strength and aromatic  $\pi$ - $\pi$  interactions, with high-resolution  $^1\text{H}$  double-quantum (DQ) experiments enabling the identification of proton-proton proximities [1], so as, for example, to distinguish between a hydrate and an anhydrate form of an active pharmaceutical ingredient in a tablet formulation [2]. Interestingly, a combined experimental and computational approach reveals changes in the  $^1\text{H}$  chemical shift of  $> 2$  ppm due to weak hydrogen bonding, e.g.,  $\text{CH}\dots\text{O}$  [3,4].

Complementary information is yielded from  $\text{NH}\dots\text{N}$  and  $\text{NH}\dots\text{O}$  hydrogen-bond mediated  $^2\text{h}J_{\text{NN}}$  and  $^2\text{h}J_{\text{NO}}$  couplings [5,6]: hydrogen-bonding partners can be unambiguously identified in refocused INADEQUATE spectra, while hydrogen-bonding strength can be quantified by the measurement of the J couplings in spin-echo experiments. Specifically, different intermolecular hydrogen-bonding arrangements as well as their strength have been determined in synthetically modified guanosines, so as to identify different modes of self-assembly, namely quartet or ribbon formation [5,7].

1. S. P. Brown, *Prog. Nucl. Magn. Reson. Spectrosc.*, 2007, 50, 199-251.
2. J. M. Griffin, D. R. Martin and S. P. Brown, *Angew. Chem. Int. Ed. Engl.*, 2007, 46, 8036-8038.
3. J. R. Yates, T. N. Pham, C. J. Pickard, F. Mauri, A. M. Amado, A. M. Gil and S. P. Brown, *J. Am. Chem. Soc.*, 2005, 127, 10216-10220..
4. A. C. Uldry, J. M. Griffin, J. R. Yates, M. Perez-Torralba, M. D. S. Maria, A. L. Webber, M. L. L. Beaumont, A. Samoson, R. M. Claramunt, C. J. Pickard and S. P. Brown, *J. Am. Chem. Soc.*, 2008, 130, 945-954.
5. T. N. Pham, J. M. Griffin, S. Masiero, S. Lena, G. Gottarelli, P. Hodgkinson, C. Filip and S. P. Brown, *Phys. Chem. Chem. Phys.*, 2007, 9, 3416-3423.
6. I. Hung, A. C. Uldry, J. Becker-Baldus, A. L. Webber, A. Wong, M. E. Smith, S. A. Joyce, J. R. Yates, C. J. Pickard, R. Dupree and S. P. Brown, *J. Am. Chem. Soc.*, 2009, 131, 1820-1834.
7. T. N. Pham, S. Masiero, G. Gottarelli and S. P. Brown, *J. Am. Chem. Soc.*, 2005, 127, 16018-16019.

# Molecular Dynamics in Solids: A Combined Analysis of Several Relaxation Times and Spectral Properties

Marco Geppi, Elisa Carignani, Silvia Borsacchi, Giulia Mollica

Dipartimento di Chimica e Chimica Industriale, Università di Pisa, via Risorgimento 35, 56126  
Pisa, Italy

The molecular dynamics of a solid drug strongly affects its pharmaceutical properties and other important characteristics, such as solid state degradations. Understanding the mobility of groups in solid drugs can also lead to a deeper knowledge of the forces responsible for conformational interconversions and the factors responsible for solid state reactions [1]. Moreover, molecular dynamics plays an important role in drug-excipient interactions, in turn significantly affecting the drug release properties.

To this regard, Solid State Nuclear Magnetic Resonance (SSNMR) is certainly one of the most powerful techniques to investigate molecular dynamics of drugs, since it offers several approaches to study molecular dynamics in a wide range of frequencies [2].

A characterization is presented of the dynamic properties of two forms of ibuprofen, acid (IBU-A) and sodium salt (IBU-S), obtained through SSNMR techniques.

A full assignment of  $^{13}\text{C}$  resonances and preliminary qualitative information about some motional processes were previously achieved in our research group [3]. In the present work we performed  $^{13}\text{C}$  and  $^1\text{H}$  longitudinal relaxation time (T1) measurements to investigate fast motional processes, with characteristic frequencies of the order of MHz;  $^{13}\text{C}$  and  $^1\text{H}$  longitudinal relaxation time in rotating frame (T1 $\rho$ ) measurements and  $^{13}\text{C}$  chemical shift anisotropy line shape analysis, in order to investigate the intermediate motional region (frequencies of the order of kHz), while insights about the slow motional regime (frequencies of the order of 1 kHz or less) could be obtained by looking at exchange processes occurring in the  $^{13}\text{C}$  high-resolution spectra. Combined analysis of all the data provided either qualitative or quantitative information about the motions of the various molecular fragments (phenyl ring, methyl groups, aliphatic chains).

1. S.R. Byrn, W. Xu, A.W. Newman, *Adv. Drug Deliver. Rev.* 48 (2001) 115-136.
2. M. Geppi, G. Mollica, S. Borsacchi, C.A. Veracini, *Appl. Spectrosc. Rev.* 43 (2008) 202-302.
3. M. Geppi, S. Guccione, G. Mollica, R. Pignatello, C.A. Veracini, *Pharm. Res.* 22 (2005) 1544-1555.

# MAS NMR on the Tetrapyrrole Chromophore of the Photoreceptor Phytochrome

Thierry Rohmer<sup>1</sup>, Christian Bongards<sup>2</sup>, Jon Hughes<sup>3</sup>, Wolfgang Gärtner<sup>2</sup>, and Jörg Matysik<sup>1</sup>

1. Leiden Institute of Chemistry, Leiden University, The Netherlands
2. Max-Planck-Institut für Bioorganische Chemie, Mülheim, Germany
3. Pflanzenphysiologie, Justus-Liebig-Universität Giessen, Germany

Phytochromes compose a family of red-/far red light sensitive photoreceptor found in plants, cyanobacteria, bacteria, and fungi [1]. Their photochemically-active chromophore is an open-chain tetrapyrrole, which triggers the conversion between two thermally parent states, called Pr and Pfr. In the Pr ground state, the chromophore adopts a ZZZssa geometry at the methine bridges [2] and absorbs red light ( $\lambda_{\text{max}} \sim 660$  nm) triggering a Z-to-E photoisomerization of the C15=C16 double bond and thus converting the protein into the far-red light absorbing Pfr state ( $\lambda_{\text{max}} \sim 700$  nm) [3].

The tetrapyrrole chromophore has been studied in both thermally ground states Pr and Pfr by <sup>1</sup>H, <sup>13</sup>C and <sup>15</sup>N CP/MAS NMR. Differences in <sup>13</sup>C Chemical shift reflect changes of the electronic structure of the cofactor at the atomic level and its interactions with the chromophore-binding pocket. The Pfr state is characterized by a strengthened hydrogen bond at the ring D carbonyl. The red shift observed in the Pfr state is explained by the increasing length of the conjugation network in this state. The enhanced conjugation within the  $\pi$ -system stabilizes the more tensed chromophore in the Pfr state [4].

The photochemically induced back-reaction has been followed at low temperature by MAS NMR spectroscopy, allowing the two intermediates, Lumi-F and Meta-F, to be trapped. MAS NMR reveals that the mechanical process of bond rotation around the C15 methine bridge occurs in two distinguishable steps: while in Lumi-F the double bond is photoisomerized, a single bond rotation occurs during the transformation to Meta-F. The structural analysis shows that the chromophore does not undergo full double-/single bond rotations but adopts a distorted conformation in Lumi-F. Meta-F shows features already similar to Pr, however, the final hydrogen bonding interactions at the ring D nitrogen are still to be formed. A mechanical model for the signal transduction and the photo-triggered back-reaction is presented.

1. Rockwell NC, Su YS, Lagarias JC, *Annu Rev Plant Biol*, 57, 837-858, 2006.
2. Essen LO, Mailliet J, and Hughes J, *Proc Natl Acad Sci USA*, 105(38), 14709–14714, 2008.
3. Rüdiger W, Thümmeler F, Cmiel E, and Schneider S, *Proc Natl Acad Sci USA*, 80(20), 6244-6248, 1983.
4. Rohmer T, Lang C, Hughes J, Essen LO, Gärtner W, and Matysik J, *Proc Natl Acad Sci USA*, 105(40), 15229–15234, 2008.

Monday, September 21<sup>st</sup>

1:30 PM - 3:00 PM

**Student and Post-Doctoral Session**

Chair: Helen Atherton

Speakers:

Oliver Jones

University of Cambridge, UK

Elodie Salager

University of Lyon, France

Ivan K.H. Leung

University of Oxford, UK

# Metabolic Markers of Combined Pesticide Exposure

**Oliver A.H. Jones**<sup>1</sup>, Sara Long<sup>2</sup>, Claus Svendsen<sup>3</sup>, Richard F. Shore<sup>2</sup>, Anthony Turk<sup>2</sup>, Hazel Turk<sup>2</sup>, Julian Griffin<sup>1</sup> and Steven Murfitt<sup>1</sup>

1. Department of Biochemistry, University of Cambridge, Cambridge, UK

2. Centre for Ecology & Hydrology, Lancaster, UK

3. Centre for Ecology & Hydrology, Wallingford, UK

In this project, funded by the UK Food Standards Agency, we are using high resolution <sup>1</sup>H Nuclear Magnetic Resonance (NMR) spectroscopy of urinary metabolites to assess the effects of pesticides (alone and in combination) on systemic metabolism in a laboratory model, the male Fischer F344 rats (*Rattus norvegicus*). We aim to identify compound-specific and general health biomarkers following exposure to four pesticides with similar and different modes of action: the benzimidazole fungicides carbendazim (CBZ) and thiabendazole (TBZ) and the bipyridylium herbicides chlormequat (CMQ) and mepiquat (MPQ).

Initially, separate acute exposure experiments were conducted for each pesticide to identify metabolic markers. Metabolite profiles were obtained using <sup>1</sup>H NMR spectroscopy of urine collected before (0-24 hours pre dose) and 0-8, 8-24 and 96-120 hours after dosing. There were clear metabolic changes associated with acute doses of 50 mg/kg bw for CMQ and MPQ, at 100 mg/kg bw for CBZ, and at 40 mg/kg bw for TBZ. Responses were detected within 0-8 hr and 8-24 hr for CMQ and MPQ, respectively, and at 96-120 hr in the case of CBZ and TBZ. Each of the four compounds produced specific changes and several were common between pesticide classes, but none were consistent across all four compounds.

The second phase of the project involved 29 day repeated-dose studies to determine if chronic and acute biomarker responses matched. Metabolic profiles did indeed reflect those following acute exposure responses and remained constant over time and were robustly modelled using the pattern recognition technique partial least squares (PLS).

Three binary repeated dose experiments were then performed over 25 days. The first two involved pesticides with similar modes of action (TBZ & CBZ; CMQ & MPQ) and the third used compounds (TBZ and MPQ) with dissimilar modes of action. A quaternary mixture experiment was performed.

The results of this work indicate that it is possible to identify metabolomic biomarkers of effect to pesticides and use these biomarkers to test the validity of current reference models for mixtures. The detailed modeling of these interactions is on-going, but currently demonstrates that similar modes of action are not truly additive in terms of their induced response at a metabolic level. Our results also suggest that, for these compounds at least, the UK acceptable daily intake (ADI) of the compounds studied (but not the NOEL) is sufficiently precautionary to avoid effects occurring when there is simultaneous exposure to a number of other compounds.

# New Methods for NMR Crystallography of Powdered Solids

**Elodie Salager**<sup>1</sup>, Robin S. Stein<sup>1,2</sup>, Graeme M. Day<sup>3</sup>, Jonathan R. Yates<sup>4</sup>, Chris J. Pickard<sup>5</sup>,  
Bénédicte Elena<sup>1</sup>, and Lyndon Emsley<sup>1</sup>

1. Universite de Lyon, Centre de RMN a tres hauts champs, Villeurbanne, France
2. Bruker BioSpin, Coventry, UK
3. Department of Chemistry, University of Cambridge, Cambridge, UK
4. Department of Materials, University of Oxford, Oxford, UK
5. Department of Physics and Astronomy, University College London, London, UK

Structural characterization of powdered samples at natural abundance, without the need for isotopic enrichment, is essential in many areas of chemistry. As protons are widely present in most organic solids, <sup>1</sup>H solid-state NMR data are of special importance. Pharmaceutical drugs are a particularly interesting target for characterization using NMR of powders, as they have to be characterized while minimizing the risk of polymorphic change.

A new protocol for NMR crystallography of powders has been recently developed. Structural information contained in proton-proton two-dimensional spin-diffusion experiments is extracted using a rate-matrix analysis model and combined with molecular modeling to determine the crystalline structure of the powder. The structure is then refined using state-of-the-art periodic DFT (CASTEP) and chemical shift measurements are compared to calculated chemical shifts in order to validate the resulting structure. The final structure is identical to the known crystal structure within the single crystal X-ray diffraction error.

In parallel to powder NMR crystallography, sophisticated structure prediction techniques have been developed. These calculations provide a small ensemble of potential structures, which might contain the correct structure. We show that information contained either in proton-proton spin-diffusion data or in the chemical shifts is sufficient to determine the real structure from among the ensemble of predicted structures in the case of thymol, a small drug molecule.

# Solvent Relaxation as a Tool to Screen Small Molecule-Metalloprotein Interactions - A Case Study Using Human Prolyl Hydroxylase Domain 2

Ivanhoe K. H. Leung, Emily Flashman, Kar Kheng Yeoh, Christopher J. Schofield and Timothy D. W. Claridge

University of Oxford, Department of Chemistry, Oxford, United Kingdom

NMR spectroscopy is a valuable screening tool for the binding of small molecules with proteins. However, most methods are limited by ligand binding kinetics, and many require relatively large amounts of small molecules or isotopically labelled proteins. For instance saturation transfer difference (STD) requires a large excess of ligand molecules, which is not always desirable and may encourage non-specific binding [1]. Transferred NOE (tr-NOE) requires a delicate ligand-protein ratio, and is more difficult to apply to systems such as peptide-protein interactions [2]. Furthermore, chemical shift perturbation using  $^1\text{H}$ - $^{15}\text{N}$  HSQC requires access to  $^{15}\text{N}$  labelled proteins, and is more difficult to apply in systems larger than 25 kDa [3]. In an alternative approach explored here, water molecules may also be used as reporters of such ligand binding events. In the presence of a paramagnetic metalloprotein, the relaxation rates of water molecules are enhanced and such effects are measurable by NMR [4]. Changes in the relaxation rates of bulk water upon ligand binding at the paramagnetic centre are therefore indicative of these binding events and are able to probe these both qualitatively and quantitatively. Using the catalytic domain of human Prolyl Hydroxylase Domain 2 (a  $\text{Fe}^{2+}/2$ -oxoglutarate (2OG) dependent enzyme involved in the body's response to hypoxia), we describe the feasibility of screening binding activities that would otherwise be challenging to detect using other NMR techniques. A set of inhibitors have been studied and our results are consistent with previous biophysical studies [5], thus proving the applicability of this technique for ligand screening at relatively high magnetic fields and when using conventional NMR instrumentation. PHD2 forms a ternary complex with the co-factor 2OG and its peptide substrates and we also demonstrate the method is sensitive to multiple binding events. Such information may be useful in the study of enzyme mechanism and function.

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3. Kay, L. E.; Gardner, K. H.; *Curr. Opin. Struct. Biol.*, 7, 722, 1997.
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Tuesday, September 22<sup>nd</sup>

8:30 AM - 10:00 AM

**Advances in NMR Analysis**

Chair: Gerhard Wagner

Speakers:

Gareth Morris

University of Manchester, UK

Rafael Bruschweiler

Florida State University, USA

Alvar Gossert

Novartis, Basel, Switzerland

# New 2D and 3D DOSY Techniques and Applications

Gareth A Morris

School of Chemistry, University of Manchester, UK

Diffusion-ordered NMR spectroscopy (DOSY) can be a very powerful tool for mixture analysis, but to be effective it requires both that the NMR spectrum of the mixture be well-resolved and that its component species have different rates of diffusion. New pulse sequences, data processing methods, and physical interventions that seek to relax or circumvent these limitations will be described.

Major improvements in diffusion resolution, including separations previously impossible with DOSY, can be obtained by manipulating the solvent matrix within which solute molecules diffuse - matrix-assisted DOSY. For example, an aqueous mixture of isomers, all of which diffuse at the same rate, can be completely resolved by adding a simple surfactant, because of the different extents to which the surfactant micelles bind the different isomers [1]. A similar separation can be achieved in hydrophobic solvents using reverse micelles.

An appealingly simple and effective way to improve resolution is to generate pure shift spectra by suppressing the effects of homonuclear couplings. Ignored when first proposed [2,3], such methods have recently been revived. Although the Zangger-Sterk [3-5], BIRD [2,6], anti-z-COSY [7] and phase-sensitive 2DJ spectroscopy [8] methods seem very different, they all share a common strategy of splitting the sample into active and passive subsets of spins, and all have considerable potential in DOSY.

The requirement for resolved NMR signals can also be relaxed if the covariance between different signals from the same species is exploited. Multivariate statistical methods such as CORE [9] and SCORE [10] allow heavily overlapped spectra to be resolved in diffusion-weighted experiments, while PARAFAC can give a completely model-free decomposition of trilinear data, for example in analysing diffusion-weighted time series of spectra to investigate chemical kinetics [11].

Illustrative examples will include studies of the solubilisation of oligosaccharide hydrolysis kinetics [11], antimalarial drugs [12], and proton exchange [13].

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# Application of Covariance NMR to the Analysis and Molecular Identification of Chemical Mixtures

Rafael Brüschweiler, Fengli Zhang, Steven L. Robinette, and Lei Bruschweiler-Li

Department of Chemistry and Biochemistry and National High Magnetic Field Laboratory,  
Florida State University, Tallahassee, FL 32306, U.S.A.

Elucidation of the chemical composition of complex biological samples is a main focus of systems biology and metabolomics. NMR spectroscopy has a unique potential for this task. In cases where the analysis of 1D spectra is ambiguous, efficient and reliable 2D methods are required. We have explored the use of covariance NMR[1,2] for this task, which provides high-resolution along both dimensions requiring only a small number of increments[3]. Homonuclear and heteronuclear covariance TOCSY spectra[4] can be deconvoluted into 1D spectra of individual molecules or spin systems (DemixC)[5], which can be efficiently screened against a NMR spectral database for compound identification[6]. Based on these principles, we have set up a public suite of web server for complex mixture analysis by NMR, COLMAR at <http://spinportal.magnet.fsu.edu>, which currently consists of 3 web servers that perform covariance processing, DemixC, and database query and which will be demonstrated using different examples.

1. R. Brüschweiler and F. Zhang, Covariance nuclear magnetic resonance spectroscopy. *J. Chem. Phys.* 120, 5253-5260 (2004).
2. F. Zhang and R. Brüschweiler, Indirect covariance NMR spectroscopy. *J. Am. Chem. Soc.* 126, 13180-13181 (2004).
3. Y. Chen, F. Zhang, W. Bermel, and R. Brüschweiler, Enhanced covariance spectroscopy from minimal datasets, *J. Am. Chem. Soc.* 128, 15564-15565 (2006).
4. F. Zhang, L. Bruschweiler-Li, S.L. Robinette, and R. Brüschweiler, Self-consistent metabolic mixture analysis by heteronuclear NMR. Application to a human cancer cell line, *Anal. Chem.* 80, 7549-7553 (2008).
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6. S. L. Robinette, F. Zhang, L. Bruschweiler-Li, and R. Brüschweiler, Web Server Based Complex Mixture Analysis by NMR, *Anal. Chem.* 80, 3606-3611 (2008).

# Efficient Detection and Characterization of Protein-ligand Interactions in H<sub>2</sub>O

Alvar D. Gossert, Chrystèle Henry, Marcel J. J. Blommers, Wolfgang Jahnke  
& César Fernández

Novartis Institutes for Biomedical Research, Novartis Pharma AG, CH-4002, Basel,  
Switzerland

Due to its ability to reliably detect binding of ligands to proteins in solution, NMR is a well established technique in contemporary drug research. Here, an improved ligand screening experiment will be presented, the PO-WaterLOGSY. It allows testing low molecular weight compounds for binding to a target protein with up to 5 fold higher time efficiency when compared to the standard WaterLOGSY. This improvement is based on rational optimization of the water handling, which allows shortening delays considerably and leaving away every second inter-scan delay. The measurement time per sample has been reduced from 30 minutes to typically 6 minutes, thereby significantly increasing the throughput of our NMR screening facilities.

Compounds that have been identified to bind by e.g. the PO-WaterLOGSY are then further characterized. Structural information on the compound-protein interaction is gained using chemical shift mapping or inter-molecular NOEs. To obtain the necessary assignments of protein backbone and side chain resonances, we use an automated approach based on projection spectroscopy (APSY) with deuterated proteins.

Tuesday, September 22<sup>nd</sup>

10:30 AM - 12:00 PM

**Exotica**

Chair: Duncan Farrant

Speakers:

André Simpson

University of Toronto, Canada

Bernhard Blümich

Institut für Technische und Makromolekulare  
Chemie, Germany

Simon Duckett

University of York, UK

# Spectral Editing of Organic Mixtures into Pure Components Using NMR Spectroscopy and Ultra-viscous Solvents

André J. Simpson, Gwen Woods and Omid Mehrzad

The Department of Chemistry, University of Toronto, 1265 Military Trail, Toronto, ON, M1C 1A4, Canada

A general technique is described that permits the extraction of a complete  $^1\text{H}$  NMR spectrum for components in organo-soluble mixtures[1]. The approach should find a wide range of applications considering that pure component spectra can be generated without the need for physical separation. This technique is especially significant for synthetic organic chemistry and the pharmaceutical industry due to the potential to isolate a product spectrum even in the presence of overlapping starting materials, byproducts, or degradation products. For organo-soluble mixtures a viscous oil-based solvent system that can be temperature-manipulated from essentially a solid at one extreme to a freely flowing liquid at the other is employed. The system contains no protons and is miscible with common organic solvents. Through careful control of the temperature and thus solvent viscosity, the behavior of small molecules moves from the positive to the extreme of the negative NOE regime. Under such conditions, all protons in a molecule correlate with all other protons as propagation by spin diffusion becomes highly efficient, behavior normally only observed with rigid macromolecules in conventional solvents. Therefore, as long as a single proton (or carbon signal in hybrid experiments) is resolved for a component in a mixture, the entire proton spectrum for that molecule can be cleanly extracted from a 2D NOESY spectrum (or from selective 1D NOE-based analogues). A solvent system compatible with aqueous systems will also be introduced. For both aqueous and organo-soluble mixtures preliminary results are highly encouraging, indicating that the approach may be feasible for a wide range of molecules and mixtures and is highly complimentary to more conventional separation approaches based on chromatography.

1. Simpson, A. J., Woods, G. and Mehrzad O. *Anal. Chem.* 2008, 80, 186-194.

# NMR with small magnets

**Bernhard Blümich**, Juan Perlo, Ernesto Danieli and Federico Casanova

RWTH Aachen University, Institut für Technische und Makromolekulare Chemie, D-52056  
Aachen, Germany

NMR with small magnets derives from mobile NMR. The original intention was to do NMR at the site of interest by bringing the instrument to the object. As the object is often too large to fit inside a magnet, stray-field NMR with instruments like the NMR-MOUSE became an attractive option for materials testing in terms of relaxation and diffusion measurements. Like magnetic fields inside a magnet, stray fields can be shimmed to excellent linearity and homogeneity in a limited region outside the magnet. Even chemical-shift resolved spectra can be acquired in this way from a solution-containing flask on top of a permanent magnet. Such magnets are built from permanent-magnet blocks, with field variations of several percent between similar blocks. We have learned to build open stray-field sensors and closed Halbach magnets from such imperfect magnet blocks with regions of magnetic field sufficiently homogeneous to measure single-shot NMR spectra at about 30 MHz with 0.16 ppm resolution. Such a Halbach magnet is not much bigger than a coffee cup and accommodates conventional 5 mm sample tubes. This technology offers exciting possibilities for small-molecule NMR where sensitivity is less of an issue than for large molecules. The state of the art of small-magnet NMR is reported together with experimental data.

# Reversible Interactions with para-Hydrogen Enhance NMR Sensitivity by Polarization Transfer

Simon B Duckett<sup>1</sup> and Gary GR Green<sup>2</sup>

1. Department of Chemistry, University of York, Heslington York. YO10 5DD
2. York Neuroimaging Centre, The Biocentre York Science Park, University of York, Heslington, York, YO10 5DG

The sensitivity of both nuclear magnetic resonance spectroscopy and magnetic resonance imaging is low because the strength of the detected signal depends on the small population difference between magnetic spin states. Hyperpolarization methods can be used to increase this difference and thereby enhance signal strength. This has been achieved previously by incorporating the molecular spin singlet para-hydrogen into hydrogenation reaction products. We show here that a suitable metal template can facilitate the reversible interaction of para-hydrogen with an organic substrate such that greater than 5,000-fold increases in proton, and substantial increases in carbon, and nitrogen signal strengths, are seen for the magnetic signature of the substrate without its hydrogenation. These signals are then employed in traditional high resolution NMR and MRI data collections.

Some of these results have been communicated (1).

1. Paul I. P. Elliott, Simon B. Duckett, Gary G. R. Green, Iman G. Khazal, Joaquín López-Serrano and David C. Williamson. *Science* 2009, 323, 1709-1711.

Tuesday, September 22<sup>nd</sup>

1:30 PM - 3:00 PM

**Human Health and Nutrition**

Chair: Jules Griffin

Speakers:

Xavier Correig Blanchar  
Universitat Rovira i Virgili, Spain

Manfred Spraul  
Bruker BioSpin GmbH, Germany

Serge Rezzi  
Nestle Research, Switzerland

# Assessment of Nutritional Interventions on Health by NMR-Metabolomics

Miguel A. Rodríguez, Maria Vinaixa, Cinta Blade, Jesus Brezmes, Nicolau Cañellas and **Xavier Correig**

Metabolomics Platform, IISPV, Universitat Rovira i Virgili (URV), CIBER de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Spain.

Metabolomics is a new emerging omic science in systems biology that is aimed to decipher the metabolic profile in complex systems through the combination of data-rich analytical techniques, such as NMR, and multivariate data analysis.

Nutrition science is nowadays one of the hottest research areas in biomedicine and biochemistry, provided that obesity and metabolic diseases (i.e. diabetes) has a very high (and increasing) prevalence in occidental societies. One of the problems in nutrition science is the assessment of the health effects of a certain nutritional intervention on an individual or on a population. This is very challenging because the metabolic response of a body to a certain aliment or diet is in general weak and varies from one person to other person (i.e. responders or non responders). Moreover, the phenotypes between individuals before the intervention are very different. Is in this context that metabolomics could represent a valuable tool in order to assess the effects of a diet on health [1], because it allows the measurement of a high number of metabolites in blood or urine samples cheaply and quickly. The new multivariable algorithms like O-PLS and multilevel PCA, which aims to cancel the biological variability between phenotypes are key factor in this studies.

The talk will focus on some nutritional studies performed by the Metabolomics Platform, in collaboration with different clinical and biochemistry groups. The Metabolomics Platform [2] was born as a joint research facility created by URV (Universitat Rovira I Virgili, Tarragona, Spain) and CIBERDEM (the Spanish Research Institute on Diabetes and Metabolic Diseases). This Platform is meant to provide a high quality service in the field of metabolomics for leading research groups in biomedicine. The Platform not only acts as a NMR measurement facility but to offer consulting, covering the process from the experimental design to data analysis.

In particular, three experiments will be mentioned in the speech:

- Study of a fiber diet effects on the cardiovascular risk in humans by NMR metabolomics fingerprinting
- Metabolomic assessment of metabolic effects of the administration of procyanidines in rats fed with hyper caloric diets.
- NMR profiling study of liver steatosis progression in LDL  $-/-$  mice under different diets

1. Favé, Gaëlle et al, Genes Nutr, Volume: 4, Issue: 2, Date: 2009 Jun , Pages: 135-41
2. <http://www.metabolomicsplatform.com>

# **Nutritional Influences on the Metabolic Profile, Chances and Pitfalls**

**M.Spraul, H.Schäfer, E.Humpfer and B.Schütz**

Bruker BioSpin GmbH Germany

Metabolic Fingerprinting and Profiling has become a standard tool in pharmaceutical drug toxicity screening, where major effects can be often observed, based on the dose of a toxic substance. In the meantime, this NMR approach finds more and more applications in clinical research, nutritional health and food quality control. While for example the search for inborn errors of metabolism often is enabled by high concentrations of certain biomarkers, this is much less the case in many other clinical applications and especially in food related response of the metabolic profile. What needs to be observed in this case, are small changes of many metabolites at the same time and only rarely the situation is clarified by a new biomarker. Also when the task is to observe early stages of disease development, subtle changes must be analyzed. Therefore the reproducibility within and across different laboratories becomes vital. It is demonstrated, that NMR allows excellent reproducibility of certain requirements are fulfilled: Sample Preparation, Transfer to the instrument, Measurement and Processing have to be performed under completely defined protocols, instruments have to be calibrated against temperature, presaturation strength and pulses. Once such a procedure is established, samples with minimal deviations due to disease development or nutritional changes can be successfully subjected to statistical analysis. It is also shown, how invariant personalized metabolic profiles can be used to detect effects of nutrition. Besides statistical routines, targeted screening is important to observe variations of individual compound concentration changes due to nutritional changes or disease development and progression. In vast mixtures like urine, it is dangerous to quantify only based on 1D-spectra. The use of rapid J-Resolved spectra acquired together with a 1D-spectrum can substantially improve compound identification and quantification. Examples based on nutritional changes are shown, using the procedures explained.

# Metabolic Health Assessment by Nutritional Metabonomics

F.P. Martin, S. Collino, F. Arce Vera, S. Kochhar, **S. Rezzi**

Nestlé Research Center, Switzerland  
BioAnalytical Science Department  
Metabonomics & Biomarkers Group

Nutrition research has emerged in the last century from understanding and combining nutritional features of ingredients and foods as a mean of nourishment to the general populations. Since the last decades, modern nutrition research has evolved towards providing additional health and wellness benefits through the delivery of nutritionally enhanced foods with bioactive nutrients. This also prompts scientists to be able to decipher how the changes in environmental conditions and lifestyle influence human genetics, physiology and predisposition towards developing modern diseases, such as obesity. Therefore, understanding how these interactions determine diverse physiological processes and the outcome of nutritional interventions is one of the greatest challenges of modern nutrition. In this context, the development of reliable analytical approaches to assess bioefficacy of nutrients has become essential to understand modes of action at the metabolic level. In this, metabonomics has rapidly evolved from a clinical and toxicological diagnostic tool into a powerful approach to aid tailoring future nutritional programs for health maintenance and disease prevention. Nutritional metabonomics is an efficient approach to measure multi-parametric metabolic responses of living systems to specific dietary stimuli. Metabolic profiling techniques are mainly based on proton nuclear magnetic resonance ( $^1\text{H}$  NMR) spectroscopy and mass spectrometry (MS) applied to provide a comprehensive analysis of hundreds of metabolites opening therefore a window into the regulation of biochemical pathways at the system level. In this lecture, recent applications of NMR spectroscopy in Nutritional Metabonomics will be introduced with special emphasis on gut metabolic health and microbiota interactions.

Wednesday September 23<sup>rd</sup>  
8:30 AM - 10:30 AM

**Metabolomics**

Chair: Jean Charles Portais

Speakers:

Nelly Aranibar  
Bristol-Myers Squibb, USA

Dominique Rollin  
INRA Bordeaux, France

Stéphane Massou  
INSA Toulouse, France

# Identification of Biomarkers for Toxicity by NMR-Based Metabonomics

**Nelly Aranibar**, Karl-Heinz Ott, Vasanthi Bhaskaran, Lindsay Tomlinson, Mark Tirmenstein,  
Jeff Vassallo, Lloyd Lecureux, Joseph Horvath, Lois Lehman-McKeeman, Greg Cosma

Bristol-Myers Squibb  
R & D

We present applications of NMR-based metabonomics in the identification of excreted metabolic biomarkers of toxicity. In the instance of a drug-related muscle toxicity, metabonomics findings led to hypothesis generation and confirmation for the molecular mechanism of the toxic insult. In a second example, urinary biomarkers were identified which led to the monitoring of P450 enzyme induction.

In the instance of a drug-related accumulation of lipids and associated necrosis in dog muscle, the appearance of lipid vacuoles in muscle tissue was correlated to the urinary excretion of methyl succinic acid, ethyl malonic acid, and other metabolites related to the catabolism of short chain fatty acids. In the second example, agents such as phenobarbital and diallylsulfide, which induce numerous xenobiotic metabolizing enzymes through activation of the constitutive androstane receptor (CAR), resulted in changes to the urinary metabonomic profile, with the major changes being a marked increase in the excretion of gulonic and ascorbic acids, thus demonstrating alterations in vitamin C metabolism. Moreover, transcriptional changes in enzymes regulating ascorbic acid biosynthesis and reutilization were identified, providing further support to the metabonomics findings. In the presented cases, the detection of unique endogenous metabolites not normally found in biofluids, elucidated specific metabolic pathways that were modulated by the test agent and helped identify enzymes whose function had been altered.

The examples cited demonstrate how the metabonomics paradigm allows converting the information made available by the simultaneous quantification of many metabolites in a single sample into biochemical and physiological knowledge of mechanisms of toxicity. Metabonomics can be applied in an investigative manner to generate mechanistic hypotheses, by relating the changes in the composition of biofluids to metabolic pathways and, subsequently, to identify small molecule biomarkers.

# Metabolic Profiling Techniques to Assess and Manage Quality in Fruit Production

Benoît Biais<sup>1</sup>, Catherine Deborde<sup>1,2</sup>, Mickael Maucourt<sup>1,2</sup>, Stéphane Bernillon<sup>1,2</sup>, Cécile Cabasson<sup>1</sup>, Yaakov Tadmor<sup>3</sup>, Joseph Burger<sup>3</sup>, Arthur A. Schaffer<sup>4</sup>, J. William Allwood<sup>5</sup>, Royston Goodacre<sup>5</sup>, Annick Moing<sup>1,2</sup>, **Dominique Rolin**<sup>1</sup>

1. UMR Fruit Biology Bordeaux, France
2. Metabolome-Fluxome Facility of Functional Genomics Centre Bordeaux, France
3. Department of Vegetable Research, Newe Ya'ar, Israel
4. Department of Vegetable Research, Volcani Center, Israel
5. School of Chemistry, The University of Manchester, UK

Fruits, our major source of vitamins and anti-oxidants, represent a market of dozens of billions of € per year. However, fruit production is relentlessly challenged by environmental hazards, but also by changes in the consumer's demand for taste and nutritional value, thus requiring constant efforts to breed new varieties, but also improve cultural practices. The Metabolome-Fluxome Facility of the Functional Genomics Centre Bordeaux (<http://www.pgfb.u-bordeaux2.fr/>) provides targeted or untargeted metabolite profiling of plant extracts using LC-QTOF-MS, HPLC or <sup>1</sup>H/<sup>13</sup>C-NMR, quantification of metabolic fluxes using <sup>1</sup>H/<sup>13</sup>C-NMR for labelling measurements or in situ analysis using in vivo NMR. It is more particularly involved in developing techniques and savoir-faire that allow quantification of metabolic fluxes and metabolome studies. This will be illustrated through the description of a set of representative studies on melon (*Cucumis melo L.*). This set of studies was carried out in the context of the European project 'META-PHOR' which aims at developing a high throughput metabolite-based phenotyping platform using several metabolomics strategies for the targeted improvement of plant breeding, engineering and processing strategies of food products with enhanced nutritional quality. This part of the project focused on the major metabolites of melon fruit which are related with melon taste. We used quantitative <sup>1</sup>H-NMR profiling of hydro-ethanolic or methanolic extracts and multivariate analyses to characterize sample similarities or differences and highlight the metabolites discriminating culture conditions or years, genotypes and fruit tissues. In order to link environmental conditions and cultural practices with melon composition, several genotypes grown under different conditions were analyzed by <sup>1</sup>H-NMR spectroscopy providing targeted or untargeted metabolite profiling. Two commercial varieties of melon (subsp. *cantalupensis* and *inodorous*) cultivated in greenhouse or field in Israel showed different metabolite patterns in flesh at maturity for fruit grown in greenhouse compared with open field. For melons cultivated in field in France, fruit profiling of one *cantalupensis* variety, with three different dates of sowing/harvest, highlighted differences in metabolite levels between the three growth periods (including sugars, organic acids and amino acids). Profiling of two commercial varieties of melon (*cantalupensis*) cultivated in field in France over three years showed that cultivar differences depended on the year. To help geneticists and breeders, the same strategy was used to characterize the fruit composition of flesh and rind of about 50 genotypes representing a broad range of the genetic variability in *C. melo*. To investigate the spatial changes of metabolism in melon fruit, a metabolomics approach using <sup>1</sup>H-NMR and GC-MS profiling of primary metabolites (Biais et al. 2009a) and quantification of adenine nucleotides with luciferin bioluminescence was employed (Biais et al. 2009b). Several metabolites and adenylate energy charge gradients were observed indicating metabolic acclimation to hypoxia in the fruit centre. This short presentation confirms the

potential of <sup>1</sup>H-NMR spectroscopy as a diagnostic tool helping to probe the operation of metabolic pathways in plant ecophysiology and physiology and as a phenotyping tool in genetics.

1. Biais B., Allwood J. W., Deborde C., Xu Y., Maucourt M., Beauvoit B., Dunn W., Jacob D., Goodacre R., Rolin D., Moing A. (2009a) *Anal. Chem.* 81: 2884-2894.
2. Biais B., Beauvoit B., Allwood J.W., Deborde C., Maucourt M., Goodacre R., Rolin D., Moing A. (2009b) *J Plant Physiol* (in press)

# NMR-based Fluxomics: Current Challenges and Perspectives

Stéphane Massou, Fabien Letisse and Jean-Charles Portais

INSA, Laboratoire d'Ingénierie des Systèmes Biologiques et des Procédés, UMR INSA/CNRS  
5504/INRA 792, Toulouse, France

In living systems, metabolism is governed by a complex network of chemical reactions organized in metabolic pathways. To measure metabolic fluxes in these pathways, NMR combined with  $^{13}\text{C}$ -labeling strategies has been increasingly used over the past two decades. Metabolic fluxes are estimated from the distribution of label within metabolites extracted from biological material (cells, tissues or organisms) fed with  $^{13}\text{C}$ -labeled substrate(s). NMR is unique in providing direct access to the amount of  $^{13}\text{C}$  atom incorporated in individual carbon positions of metabolites. This makes NMR a reliable technique for monitoring the fate of specifically labeled substrates – e.g.  $[1-^{13}\text{C}]$ glucose – in biological systems, but also for resolving the distribution of positional isotopologues in experiments carried out with uniformly labeled substrates – e.g.  $[U-^{13}\text{C}]$ glucose –.

Considering the number of possible isotopologues for a given compound ( $2^n$ , n: carbon number), the analysis of the labeling pattern of metabolites is complex. Indeed, each isotopologue has a specific spin system and therefore the resulting NMR spectra are different. Moreover, with the current trend to investigate metabolic networks of increasing size and complexity, there is a need to increase the number of metabolites for which the isotopic information can be resolved. Consequently, various NMR methods - including 2D-NMR experiments - that provide accurate quantitative measurements of the isotopologue distributions have been developed.

In this presentation, a survey of the 1D and 2D-NMR methods available for the measurement of isotopologues will be presented, and their relevance will be discussed. A strategy based on the combination of 2D-HSQC[1 and the recently developed 2D ZQF-TOCSY[2,3] will be presented that allows the application of isotopologues approaches to any combinations of specifically- and uniformly- labeled substrate. Such combination of NMR experiments provides also a basis for extending the size of the metabolic networks that can be investigated and for examining non steady-state conditions.

1. Szyperski T, Eur J Biochem., 232(2), 433-448, 1995
2. Massou S, Nicolas C, Letisse F, Portais JC, Phytochemistry, 68(16-18), 2330-2340, 2007
3. Massou S, Nicolas C, Letisse F, Portais JC, Metab. Eng.;9(3), 252-257, 2007

Wednesday September 23<sup>rd</sup>  
10:30 AM - 12:00 PM

**Sensitivity Enhancement and Novel  
Developments in NMR**

Chair: Adrian Davis

Speakers:

Michael J. McCarthy  
University of California-Davis, USA

Patrick Giraudeau  
Weizmann Institute, Israel

Thorsten Maly  
MIT, USA

# **Industrial Process Control Employing NMR/MRI Based Sensors**

**Michael J. McCarthy**

Department of Food Science and Technology, University of California, Davis, CA 95616 USA

Nuclear magnetic resonance and magnetic resonance imaging provide complementary measurements of sample properties when compared with other experimental techniques. NMR/MRI based analyses have been historically limited to laboratory settings and only recently have begun to be utilized in remote geographical and industrial settings. The advances permitting NMR/MRI to be utilized in these new settings are an interplay between hardware and software driven by economic potential. These advances in hardware/software will be described by examples of the implementation of MRI for sorting of fruit in a packing house (detection of seeds in citrus and peelability of processing tomatoes). The extension of this equipment/methods to pharmaceutical, chemical and consumer products will be discussed.

# Ultra-fast, Ultra-sensitive 2D NMR Spectroscopy of Hyperpolarized Solutions

Patrick Giraudeau<sup>1,2</sup>, Yoav Shrot<sup>1</sup>, Mor Mishkovsky<sup>1</sup> and Lucio Frydman<sup>1</sup>

1. Chemical Physics Dept., Weizmann Institute of Science, Rehovot, Israel
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NMR is a powerful analytical tool for elucidating the structure of complex biomolecular and pharmaceutical compounds in liquids. It is, however, a notoriously insensitive method, requiring relatively large amounts of sample and/or long acquisition times. This is largely due to the low levels of thermal polarization achievable under conventional conditions. Recent developments have made it possible to reach polarization levels that exceed these thermal equilibrium values by several orders of magnitude. One of the most promising and generally applicable techniques to achieve this hyperpolarization is based on *ex situ* dynamic nuclear polarization (DNP), which leads to signal-to-noise enhancements of several tens of thousands compared to conventional NMR [1]. In this experiment the hyperpolarization is carried out outside the NMR magnet, in a cryogenic setting. The technique then involves an irreversible melting and transfer of the sample to the NMR spectrometer. Because of ensuing T1 limitations, DNP-NMR experiments are best suited to the acquisition of a single or at most a small number of free induction decays. This makes *ex situ* DNP ill-suited for collecting an array of transients involving complex pulse sequences, of the kind needed in 2D NMR.

In order to deal with this drawback, it was recently suggested to combine DNP with spatially-encoded ultrafast 2D NMR methods, capable of yielding 2D NMR spectra in a single-scan [2]. Particularly promising are inverse-detected heteronuclear methods exploiting the hyperpolarization of slow-relaxing nuclei, which is well preserved during the sample transfer from the polarizer to the NMR spectrometer, with the higher sensitivity of <sup>1</sup>H detection [3]. Using this approach, we show that 2D heteronuclear NMR spectra of small-molecule mixtures can be recorded at very low concentrations and in one scan: 2D <sup>13</sup>C-<sup>1</sup>H HMBC spectra were thus obtained within 200 ms, on a 0.1 mM mixture of aromatic compounds at natural abundance.

A main limitation of these ultrafast <sup>13</sup>C-<sup>1</sup>H 2D DNP-NMR experiments, rests in their inability to cover a spectral range higher than a ten or so ppms. This is a consequence of the strong gradients ( $\approx 100$  G/cm) that would otherwise be required to cover the large bandwidths demanded by broadband <sup>13</sup>C experiments. To deal with this problem we discuss a new spatial/spectral encoding strategy, involving use of selective excitation pulses and of suitably chosen gradients, to fold <sup>13</sup>C resonances into the desired spectral windows. This new spectral-spatial approach makes it possible to obtain, in a single scan, multiple 2D heteronuclear spectra of different spectral regions and involving different kinds of correlations. Applications of these principles to the single-shot acquisition of HMBC and HSQC 2D spectra on  $\approx 1$  mM mixtures of terpenes, characterizing at natural abundance sites that are spread over nearly 100 ppm bandwidths, will also be presented.

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# High-Field in-situ DNP – Applications from Small Molecules to Large Biomolecules

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In-situ high-field dynamic nuclear polarization (DNP) is a method that can enhance the detected signal intensities in liquid- or solid-state NMR experiments by up to two orders of magnitude. During a DNP experiment, the large thermal polarization of a paramagnetic polarizing agent, such as a stable free radical, is transferred to the nuclei of interest by microwave irradiation of the sample at the electron paramagnetic resonance (EPR) [1,2]. This enhanced nuclear polarization is of considerable interest in a variety of applications ranging from particle physics to structural biology and clinical imaging.

In in-situ DNP the polarization and observation of the NMR signals are performed in the same magnet. This is in contrast to ex-situ DNP experiments such as the “dissolution experiment” where the sample is dissolved and transferred to another spectrometer. This has many advantages, the main being that the polarization process can be repeated and conventional multi-dimensional experiments are easily performed. Furthermore, in-situ DNP can be applied to solution-state DNP by using the temperature-jump method (TJ-DNP), developed in our laboratory [3].

Here we will focus on experimental results, in particular on (a) new polarizing agents for DNP to optimize the DNP process, (b) the application of in-situ high-field solid-state DNP to distance measurements in small molecules and large biomolecules such as amyloid fibrils (c) application of TJ-DNP to obtain DNP-enhanced liquid-state NMR spectra.

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Tuesday, September 22<sup>nd</sup> 8:00 PM - 10:00 PM

Poster Session

Chairs:

Helen Atherton

Daneen Hadden

Andrew Nicholls

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# 1 Residual Dipolar Couplings in Short Peptidic Foldamers: A Flexible Tool for Rigid Systems

Markus Schmid<sup>1</sup>, Matthias Fleischmann<sup>1</sup>, Valerio D'Elia<sup>1</sup>,  
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Conformational preferences crucially influence the applicability of organic compounds in medicinal chemistry and organocatalysis. In this context, derivatives of the unnatural *cis*- $\beta$ -aminocyclopropanecarboxylic acid ( $\beta$ -ACC) have been shown to stabilize secondary structure elements even in short peptide sequences [1] while conferring affinity and selectivity to NPY receptor ligands [2] and integrin ligands[3]. In addition, tripeptides based on  $\beta$ -ACC and proline residues can yield high selectivities as organocatalysts in asymmetric inter- and intramolecular aldol reactions[4].

Here we present conformational studies on a  $\beta$ -ACC containing tripeptide in order to elucidate the induction of conformational preferences in short linear peptides by  $\beta$ -ACC. For our combined NMR and restrained Molecular Dynamics (MD) investigations, we applied residual dipolar couplings (RDCs), measured in a stretched PDMS/ $\text{CDCl}_3$  gel[5], as a further source of structure information besides the well-established NMR structural parameters, NOE enhancements and J couplings.

We could show that RDCs can be employed to select appropriate MD force field parameters for the rigid unnatural amino acid  $\beta$ -ACC, that they provide conformational information on the peptide backbone as  $\beta$ -ACC can be used as a molecular alignment probe and that they allow for the detection of conformational preferences in the proline side chains. Our results reveal that, consisting of only three amino acid residues, the peptide investigated shows surprisingly little conformational flexibility[6].

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# 2 Atropisomerism: Quirality, Energy Restrictions and Dynamic Effects on NMR Chemical Shifts

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The atropisomerism is a particular isomerism phenomena consequence of the special structural arrangements such as the restricted intramolecular motion. Steric overlap, dipolar interactions and resonance in pi-systems and electron pi-pairs systems are the more important structural features related to restricted motion around single bonds, and influence the energy surface which can show high energy barriers. Under these conditions, the collisional temperature is not sufficient to promote the free rotation.

In this work several molecular systems with restricted rotation around R-CO-NH-R' and R-CO-NR'-R'' bonds, and its effects on the chemical shifts tensors (CST) were analyzed. The energy implications of restricted rotations on the NMR chemical shifts were examined in terms of: molecular energy, Boltzmann populations distribution analysis, tunneling effect on the nitrogen quirality inversion, CO-NH electron hyperconjugation, orbital contributions over specific single nucleus chemical shifts tensors using natural bonding orbital analysis (NBO), and changes in atom hybridization as a consequence of free rotation and electron hyperconjugation.

The molecular electronic calculus was carried out at Density Functional Theory level of theory, using a B3LYP functional and a 6-311+G(2d,p) basis set. The rotational surface was calculated in a stepwise scan of 10 degrees. After optimizing geometries and taking into account the nitrogen chirality changes, the chemical shift tensors (CST) were calculated using GIAO method and natural bonding orbital analysis (NBO), implemented on Gaussian03 software. Having taken into account the analysis population, dynamic effects were considered and CSTs were calculated and compared with CSTs for two minimum energy conformations.

The theoretical characterization of the molecular energy surface and NMR experiments confirm the preliminary observations and support the existence of two minimum energy configurations at room temperature in the amide function. Orbital distribution on the nitrogen atom, in conjugated amidic bonds, is a combination of sp<sup>2</sup> and sp<sup>3</sup> hybridization, depending on the rotation angle, and its consequence is analyzed in terms of NMR chemical shifts.

# 3 H-Bond Network Elucidation of Guanidines via NMR

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Specific interactions of arginines are often the crucial factors in protein/ligand or RNA/protein recognition processes, based on the formation of multiple H-bonds. In the field of medicinal chemistry, also in the ligand design for the different Neuropeptide Y G-protein coupled receptors the arginine moieties and their H-bond networks are vitally important[1]. For example, NG-acylation of the NPY Y1 antagonist BIBP 3226 enhances receptor affinity.[2] As a model system, an artificial arginine receptor initially developed by T. Schrader [3], has been employed in our group for H-bond network elucidation of arginine derivatives by NMR[4].

Here we present the direct detection of H-bonds between individual acylguanidinium protons and the bisphosphonate groups of the receptor via  $^2\text{J}_{\text{H,P}}$  scalar couplings[5]. Expansion of these measurements onto a partially  $^{15}\text{N}$ -labelled arginine guest carrying an NG-propionyl substituent now yields a differentiated picture where the w-shaped end-on binding motif is retained despite the disappearance of strong  $\text{N}\alpha\text{-H}$  interactions with the bisphosphonate.  $^{15}\text{N}$ -labelling greatly simplifies assignment as well as enabling the detection of  $^3\text{J}_{\text{N,P}}$  scalar couplings for the first time in non-biomacromolecules leading ultimately to evidence about the spatial arrangement of the NH - OP H-bonds[5].

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# 4 Synthesis and NMR Investigations of C3-Symmetric $\alpha/\beta$ Hybrid Peptides Derived from Trimesic acid

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Self-assembly of various Trimesic acid (TMA) peptide derivatives have a strong tendency to aggregate in solution as well as in solid state.[1] In our present we report the self assembly of TMA peptides containing  $\alpha/\beta$  hybrid peptides[2] derived from Trimesic acid(TMA) as central core unit, TMA[ $\alpha$ ]3, TMA[ $\alpha$ - $\beta$ ]3, TMA[ $\alpha$ - $\beta$ - $\alpha$ ]3, where  $\alpha$  is DPhe/Val and  $\beta$  is homo-Gly. The CDCl<sub>3</sub> solution of these carboxamides with di and tripeptides showed that all the amide protons are hydrogen bonded. Their self assembly was revealed by sequential addition of DMSO-d<sub>6</sub>. The phenomenon is more pronounced in larger peptides, which also display nOes between the protons at the N-termini with that at C-termini indicate antiparallel arrangement of strands in solution. These nOes are absent in polar solvent DMSO-d<sub>6</sub> solution, implying lack of molecular association. Self assembly of these peptides is further supported by IR [1b, 3] TEM and MASS spectral studies.

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# 5 Novel Pure Shift DOSY techniques

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Diffusion-based NMR techniques for mixture analysis allow the spectrum of a mixture to be resolved into its individual components on the basis of differences in their diffusion coefficient. However, peak overlap in the NMR dimension, almost unavoidable in <sup>1</sup>H NMR, leads to distortions and artefacts, such as peaks appearing at compromise positions in the diffusion dimension. Signal overlap and its attendant problems can be greatly reduced by simplifying the proton spectrum to give a broadband homodecoupled or ‘pure shift’ spectrum.

Pure shift techniques based on homonuclear 2D J spectroscopy [1] have been available for many years, but are all more or less unsatisfactory. The properties of the phase-twist lineshape that is inherent to the technique [2] mean that it requires the use of severe weighting functions and absolute value display, yielding pure shift spectra with broad lines and distorted intensities.

The introduction of the Zangger-Sterk pulse sequence element [3] has allowed significant improvements. The combination of a selective pulse and a weak magnetic field gradient allows a subset of the spins to be treated as heteronuclei, allowing independent manipulation of active and passive spins. This has led the development of a number of absorption mode sequences for pure shift spectroscopy [3, 4] and 2D J spectroscopy [5]. Here the extension to pure shift DOSY is demonstrated.

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# 6 Isomer Resolution By Matrix-Assisted DOSY

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Diffusion-ordered NMR spectroscopy (DOSY) [1] is a very useful tool in the analysis of mixtures, separating the NMR signals of different components according to diffusion coefficient, but it relies on different species having sufficiently different hydrodynamic radii. The great majority of DOSY experiments to date have used solutions of mixtures in simple solvents, but there is considerable scope for manipulating relative diffusion coefficients by the use of modified solvents [2, 3, 4].

There is a close analogy here between DOSY and chromatography. Much of the richness of chromatography as an analytical tool stems from the control allowed by varying the nature of the stationary phase; the introduction of co-solvents and -solute, to create a complex matrix in which differential diffusion between solutes is measured, gives DOSY a comparable flexibility.

One simple and effective manipulation of the solution in DOSY is to use a surfactant co-solute above its critical micelle concentration [2, 4]. This allows species of very similar size, such as isomers, to be distinguished from one another by virtue of their differing degrees of interaction with the micellar structures formed. Excellent resolution of the proton spectra of isomers can be obtained by using common surfactants such as SDS in aqueous solution [4]. Good diffusion resolution is retained over a much wider range of concentrations of solute and surfactant than would be expected on the basis of a simple model of solute binding to micelles. The same principle may be exploited in non-aqueous solvents by using reversed micelles, such as those of AOT in chloroform [4]. Here good resolution is once again readily obtainable, with added flexibility afforded by the possibility of varying the water content of the reversed micelles.

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# **7** Quantitative Assessment of Surfactants in Complex Detergent Formulations by NMR

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Surfactants critically determine the performance of household detergents, and this necessitates methods for their identification and quantification. Quantitative assessment of surfactants is not trivial due to their strong tendency to form micelles and to interact with other species in detergent formulations. By taking recourse to adequate extraction procedures one can isolate surfactants from the full formulation. Subsequently, micelle formation can be suppressed by selecting an adequate solvent, thus opening up the possibility to use NMR for quantification of surfactant levels. By using standardized sample conditions, one can minimize NMR signal overlap and enhance spectral reproducibility. DFTMP was used as an internal standard as it was most compatible with sample (preparation) conditions. Thus a large range of surfactants and ethanol soluble actives could be quantified (0.1-100 w/w %) in a single NMR experiment in complex detergent formulations. For low-medium-high surfactant levels in liquids and powders formulations, within-laboratory relative standard deviations were 1-2% and 2-3%, respectively.

# 8 NMR-based Nutrikinetic Approach for the Assessment of Inter-individual Variation in the Bioconversion of Dietary Polyphenols in Humans

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So far, most nutritional intervention studies on dietary polyphenols are directly focused on pre-identified markers for intake, typically intact polyphenols and/or their conjugates. In recent years the role of colonic microbiota as a metabolically versatile biological digester of polyphenols has been recognized. Conventional metabonomic approaches are not adequate due to the complexity of co-metabome interactions between the host and gut microbiome and metabolic variations associated with lifestyle, gender, genotypes, and microbiome diversity. In-vitro gut models are not fraught by co-metabolome interactions and NMR metabolic profiles showed significant inter-individual variation in microbial conversion of polyphenols. In order to handle the multiple sources of biological variation in humans, we exploited the cross-over design of dietary polyphenol intervention trials by integrating these with multi-level data-analysis [1]. Thus a range of gut microbial bioconversion products in sub-mM concentration range were found by NMR to be significantly increased after bolus interventions with different dietary polyphenols sources. Subsequently, the (NMR) time courses of these discriminating metabolites in placebo and treatment period were simultaneously fitted with a one-compartment nutrikinetic model [2]. Variations in urinary excretion between the subjects were used to provide a phenotypic description of the test population.

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# 9 **Structural and Quantitative Characterization of Biologically Created Analytical Standards by NMR**

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<sup>2</sup>Bruker BioSpin Corp. Billerica, MA 01821, U.S.A.

In early discovery through development there are situations where having a qualified metabolite standard is advantageous; monitoring reactive metabolites, assessing pharmacological activity of metabolites or quantification of metabolites in a clinical setting. In many cases timely chemical synthesis of these metabolites is difficult and rate limiting. Alternatively, biological generation and isolation of metabolites in the low to mid nanomole range is eminently feasible. However, without accurate quantitative assessments of the purity and concentration of these isolates, their utility is limited. Definitive structural characterization of drug metabolites has long been the domain of one and two dimensional NMR. Additionally, there is a significant history of NMR as a quantitative technique. Only recently have these two concepts been merged to provide structural and quantitative information on biologically generated isolates.

Quantitation via NMR is generally performed with an internal standard (IStd), either through the addition of a separate chemical entity or using an artificially generated signal as an IStd. In cases where an IStd is used there are two common approaches, the addition of a known quantity of an additional compound or using the residual solvent line as an internal standard. Using an artificially generated signal has typically been done using the ERETIC method, where an IStd resonance is introduced into the spectra during acquisition using a signal generated on a free channel of the NMR spectrometer. Alternatively, a signal can be mathematically generated and inserted into a spectrum post acquisition. Once this signal is characterized in intensity it can be used as a quantitative reference. These methods, direct addition of an IStd with a known concentration of a compound and signal insertion with a mathematically generated signal were compared for precision, accuracy, specificity, stability and dynamic range.

# 10 New Basis Set for Calculations of $J_{PX}$ ( $X=C,H$ ) and Experimental Data

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There is no adequate basis set for estimation values of  $^1J_{PC}$  and  $^2J_{PH}$  couplings in the literature with accuracy. In this work, several density functionals and basis sets were employed and, also, some new basis sets were developed leading to calculated values closer to our experimental data. In the first step, some functionals as BLYP, B3LYP, B98, B971, B3PW91, BP86, PBEPBE and PBE1PBE were tested, as well as the IGLO-II and IGLO-III basis set, with geometries and couplings obtained at the same level (DFT/IGLO-II or III) or geometries at B3LYP/Aug-cc-pVDZ and couplings at DFT/IGLO-II or III levels of theory. The best results were obtained with B98 e B971 functionals, using the IGLO-III triple-zeta basis set [1]. To reduce the deviations from the experimental values, the basis set for the phosphorus atom was optimized, using B98 and the simplex routine [2], and an alkyl-phosphine molecule model. The property was adjusted with respect to energies ( $\Delta E$ ),  $J_s$  ( $J_{Av}$ ) and electric field vector ( $\epsilon T$ ) to give an answer  $A$ :  $A = \Delta E + J_{Av} + \epsilon T$ . This new basis set (IGLO-III-J) for the P atom led to an improvement in the calculated values for alkyl- and aryl-phosphines, which encouraged an attempt to optimize the IGLO-III basis set for C and H atoms, but without significant changes in the results. The use of this new set and B98 level of theory led to results far better than the ones obtained with other triple-zeta basis (aug-pcJ-3, sadlej-pVTZ, aug-cc-pVTZ) [3,4], for the alkyl- and arylphosphines. However, the results for some chloro- and oxygen-containing phosphines were still not close to the experimental values. The reoptimized basis sets for Cl and O atoms, together with IGLO-III-J for P yielded to the smallest difference between  $^1J_{PC}$  and  $^2J_{PH}$  calculated and experimental values ever described in the literature. This remaining small difference can be attributed to the inappropriate description of the Cl and O atoms by IGLO-III basis sets. The  $^1J_{PC}$  and  $^2J_{PH}$  experimental coupling constants were taken from the  $^{13}C$ ,  $^{31}P$  and  $^1H$  NMR spectra recorded in a BRUKER DPX-250 and are accurate to 0.1 Hz (average value).

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# 11 An Alternative Scheme for the Multiplexed Acquisition of 1D and 2D NMR Spectra

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One of the best known applications of selective radiofrequency pulses in liquid-state NMR spectroscopy is certainly the reduction of spectral dimensionality [1]. A full 2D NOESY spectrum can be replaced by a small number of 1D NOESY spectra that concentrate on the resonances of interest by means of their selective excitation. However, the sequential recording of 1D spectra does not provide the best possible signal to noise ratio than can be expected within a given spectrometer occupancy time.

This raw selective pulse approach can be improved by replacing  $n$  separate 1D acquisitions by  $n$  multiplexed acquisitions in which each desired 1D spectrum is present, but in variable proportions. The demultiplexing operation boils down to the computation of  $n$  linear combinations of the  $n$  acquired spectra and may result in an overall signal to noise improvement if the combination coefficients are adequately chosen. The most popular implementation of such a strategy is known as Hadamard NMR spectroscopy [2]. Its practical feasibility highly depends on the ability of simultaneously and selectively acting on individual resonances or on groups of resonances.

Our poster describes an alternative to Hadamard encoding that also provides amplitude modulated mixtures of individual spectra. The method is well adapted to the analysis of oligosaccharides and was used to assign the resonances of a chemically modified  $\beta$ -cyclodextrin [3]. The seven 1D-TOCSY, 2D-DQF-COSY and 2D-HSQC spectra of the seven spin systems of this compound are presented. In this particular case, the low dispersion of the chemical shifts of the anomeric protons would have imposed very long soft pulses (hundreds of ms) to correctly set up the Hadamard encoding scheme.

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# 12 On the Reliability of SOPPA(CCSD) and DFT Methods to Predict $^1J_{CF}$ in p-Fluorobenzenes

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Theoretical calculations of SSCCs in NMR, plays an important role in the experimental interpretation concerning molecular electronic structures [1]. Perturbative methods like SOPPA(CCSD) have shown a good reliability to predict SSCC involving F atoms [2]. However, it is well known that DFT-based approaches have failed to estimate SSCC involving at least one F atom [3]. The DFT accuracy goes from good to worse in the series H, C, N, O and F depending on the number of lone pairs at the coupling nucleus and therefore on the ability of the exchange-correlation functional to describe the spin-density perturbation [4]. In this work, we compare the reliability of the SOPPA(CCSD) and DFT methods to predict the SSCC  $^1J_{CF}$  in para-substituted fluorobenzenes (with nitro and amino groups) with experimental data and evaluate the probable source of errors of the DFT method with respect to SOPPA(CCSD). Both methods have shown similar overall performance for the  $^1J_{CF}$  total coupling trends in these three compounds. These  $^1J_{CF}$  are strongly dominated by FC term. The large discrepancy between the two methods comes from FC and PSO terms, which are evaluated using excited triplet and singlet states, respectively. In these particular systems, the DFT method did not describe correctly the excited states in comparison with SOPPA(CCSD). In the PPA framework, the focus is on the perturbation itself, which is described in terms of excitation and deexcitation operators [5,6]. Besides, the inclusion of dynamic electron correlation effects in calculations of SSCC [7], yields an improvement of the wave function description by SOPPA. It is also suggested [4] that an adequate exchange-correlation functional will improve the accuracy of a DFT approach for describing the behavior of the spin-density in such complex structures.

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# 13 Phenylephrine, Human Metabolite - Preparation, Isolation and Identification

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Phenylephrine is used as a substitution of abused pseudoephedrine in some medicines. The main human metabolite of phenylephrine is reported to be phenylephrine-O-sulphate[1]. There are two isomers of phenylephrine-O-sulphate and the aim of this work was to prepare and isolate both isomers. Their structures have been determined and distinguished by NMR and MS spectroscopy.

The required sulpho ester of phenylephrine (real metabolite) has been prepared by the reaction of phenylephrine phenolate anion with pyrosulphate in alcalic media. Its isomer has been prepared by reaction of phenylephrine with sulphur trioxide – pyridine complex. Isolation of regio isomers was achieved by preparative HPLC. Differences in NMR and MS spectra of isomers are shown and discussed.

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# 14 Spectroscopic Approaches for Reaction Optimisation and Scale Up

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NMR has an important role in monitoring reaction progression as it provides both quantitative data for kinetic investigation and structural information to elucidate reaction mechanism. Homogeneous reactions are generally conducted in conventional NMR tubes where experiments are recorded at regular time points. For more complex reaction conditions we, and others have demonstrated the use of on-line flow NMR to monitor reaction progression [1,2]. NMR is often used at AstraZeneca R&D Charnwood to identify potential reactive intermediates in a chemical step or confirm formation of intermediates in telescoped reactions. The non-invasive nature of the NMR experiment is well placed for the detection of intermediate species that may not be observed by other methods (e.g. sampling for HPLC) in this regard. An example of this will be presented to show the utility of NMR. AstraZeneca R&D uses several approaches including chromatography, vibrational spectroscopy, and NMR to assist Process Chemistry. The key to effective implementation is using the right tool at the right time, and to correlate information from one technique to the next. NMR monitoring, whilst of significant use when developing and optimising reaction conditions, does not translate easily to monitoring in a manufacturing environment. We therefore show how NMR can be correlated with other spectroscopic techniques such as Near InfraRed (NIR), which is more suited for use in a large scale synthesis. Collecting multiple datasets allows techniques such as NIR to be validated against the NMR data to create a robust monitoring method before this can be used and relied on for reactions performed at such scale.

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# 15 Solvent Relaxation as a Tool to Screen Small Molecule-metalloprotein Interactions - A Case Study Using Human Prolyl Hydroxylase Domain 2

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NMR spectroscopy is a valuable screening tool for the binding of small molecules with proteins. However, most methods are limited by ligand binding kinetics, and many require relatively large amounts of small molecules or isotopically labelled proteins. For instance saturation transfer difference (STD) requires a large excess of ligand molecules, which is not always desirable and may encourage non-specific binding [1]. Transferred NOE (tr-NOE) requires a delicate ligand-protein ratio, and is more difficult to apply to systems such as peptide-protein interactions [2]. Furthermore, chemical shift perturbation using  $^1\text{H}$ - $^{15}\text{N}$  HSQC requires access to  $^{15}\text{N}$  labelled proteins, and is more difficult to apply in systems larger than 25 kDa [3]. In an alternative approach explored here, water molecules may also be used as reporters of such ligand binding events. In the presence of a paramagnetic metalloprotein, the relaxation rates of water molecules are enhanced and such effects are measurable by NMR [4]. Changes in the relaxation rates of bulk water upon ligand binding at the paramagnetic centre are therefore indicative of these binding events and are able to probe these both qualitatively and quantitatively. Using the catalytic domain of human Prolyl Hydroxylase Domain 2 (a  $\text{Fe}^{2+}/2\text{-oxoglutarate}$  (2OG) dependent enzyme involved in the body's response to hypoxia), we describe the feasibility of screening binding activities that would otherwise be challenging to detect using other NMR techniques. A set of inhibitors have been studied and our results are consistent with previous biophysical studies [5], thus proving the applicability of this technique for ligand screening at relatively high magnetic fields and when using conventional NMR instrumentation. PHD2 forms a ternary complex with the co-factor 2OG and its peptide substrates and we also demonstrate the method is sensitive to multiple binding events. Such information may be useful in the study of enzyme mechanism and function.

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# 16 2D DOSY $^1\text{H}$ NMR for the Detection of Active Pharmaceutical Ingredients in Herbal Dietary Supplements

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This study presents an application of 2D Diffusion Ordered Spectroscopy (DOSY) and 3D DOSY-COSY  $^1\text{H}$  NMR for the analysis of herbal slimming products.

Over the last few decades, traditional herbal medicines have gained popularity worldwide as a source of complementary and alternative remedies. Given the medical and social impact of being overweight, more and more patients often turn to over-the-counter weight-loss products. The dietary ingredients in these products may include vitamins, minerals, herbs or other botanicals. However, common problems affecting the safety of herbal medicines include adulteration with conventional pharmaceuticals, which can lead to severe side effects.

Twenty herbal dietary supplements marketed for weight loss were analyzed with DOSY  $^1\text{H}$  NMR. The method allows a global analysis of the samples with detection of both active and inactive ingredients present in the complex matrix. Thirteen formulations were found to be adulterated with the anorexigen sibutramine. Five out of these thirteen herbal products contain likewise phenolphthalein known for its laxative effect. Some other active compounds were detected as synephrine, methyl synephrine, vitaberine (thiamine-O-isobutyryl disulphide), and caffeine. Furthermore, few other compounds were characterized in all formulations (sugars, vitamins...). A 3D DOSY-COSY  $^1\text{H}$  NMR experiment was carried out on an adulterated formulation. This experiment provides both virtual separation and structural information as it allows observing COSY subspectrum of each component of the complex mixture.

This study highlights the interest of using DOSY  $^1\text{H}$  NMR, a holistic method allowing to consider the drug preparation as a whole and to detect simultaneously all proton-containing compounds.

# 17 NMR Based Metabolomics for Biomarkers Identification in a Mouse Model of Alzheimer Disease

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Alzheimer's disease (AD) is one of the most important causes of progressive dementia in the elderly, characterized by a slow and progressive impairment of cognitive function. No definitive biomarkers are available for this neurodegenerative disease and this represents a major problem for the advance of efficient drug discovery programs.

A metabolomic approach based on <sup>1</sup>H Nuclear Magnetic Resonance spectroscopy (NMR) combined with multivariate statistical analysis has been used to identify metabolic perturbations in a mouse model of AD (AppSwe Tg2576). The metabolomic profiles of five brain regions (frontal cortex, rhinal cortex, hippocampus, midbrain, and cerebellum) at three different disease ages (3, 6 or 11 months) were compared in healthy and transgenic mice.

After a biphasic extraction of the cerebral tissue, <sup>1</sup>H NMR spectroscopy analysis was performed on the aqueous fraction. Twenty four metabolites were identified. NMR spectra were segmented by the bin area method and statistically analysed using univariate, bivariate (ANOVA, boxplot) and multivariate (PCA, PLS-DA, OPLS-DA) methods.

Taurine, creatine, gamma-aminobutyric acid and N-acetylaspartate are the discriminating metabolites between the brain regions in both control and transgenic animals whatever is the age of mice. The statistical analysis between control and AD disease transgenic mice leads to a good predictive model (i) for both frontal and rhinal cortex at the earliest stage of AD with taurine concentration increasing in transgenic mice; (ii) for rhinal cortex at 11 months with myo-inositol concentration decreasing in transgenic mice.

# 18 Mixture of $^{13}\text{C}$ Labelled Metabolic Tracers by DNP NMR: How Low Can We Go?

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Dynamic Nuclear Polarization (DNP) provides an unforeseen signal-to-noise ratio (SNR) enhancement for a host of nuclei (such as  $^{13}\text{C}$ ,  $^{15}\text{N}$ ,  $^{29}\text{Si}$ ,  $^{31}\text{P}$ ,  $^{89}\text{Y}$ ,  $^{109}\text{Ag}$ , etc.). HyperSense® is a commercially available automated stand-alone polarizer, which is used for polarizing samples to gain remarkable gain in SNR enhancement. Use of DNP technology in HyperSense® involves cooling a sample to  $<4\text{ K}$  in a strong magnetic field ( $B_0 = 3.35\text{ T}$ ) in the presence of a trityl radical. This process is followed by dissolution of the cold sample with hot solvent and quickly transferring the sample solution to the Nuclear Magnetic Resonance (NMR) spectrometer for data acquisition.

$^1\text{H}$  NMR spectroscopy has been extensively used in the field of metabolomics and for identification and elucidation of trace amounts of metabolites in biological samples. The profiling of trace amounts of certain metabolites, metabolic tracers, and biomarkers enable us to understand and/or predict the disease or the chance of occurrence of a certain disease. However, identification and determination of metabolic tracers by other low gamma NMR active nuclei ( $^{13}\text{C}$ ,  $^{15}\text{N}$ ) has been a long-standing problem because of their low natural abundance. In addition, the low concentration of metabolic tracers, metabolites, or biomarkers make detection of  $^{13}\text{C}$  and/or  $^{15}\text{N}$  NMR signals more difficult. In here, we report 1D  $^{13}\text{C}$  DNP-NMR studies of a mixture of 10  $^{13}\text{C}$  labeled metabolic tracers that are usually found in metabolic mixtures. Only 50 nmols of each of the 10  $^{13}\text{C}$  labeled metabolic tracers (pyruvic acid, urea, methanol, acetate, lactate, palmitic acid, aspartic acid, glycine, tryptophan, and asparagine) were used for polarization. Each of the metabolic tracers exhibited a remarkable SNR enhancement when polarized at  $\sim 1.4\text{ K}$  for 50 mins and in presence of a mixture of two radicals (OX63 and FINLAND) and MeOH-d4 and D2O as glassing agents. Dissolutions were performed with 80% MeOH and 20% EDTA-H2O buffer. In addition, microwave sweep profiles for each of the metabolic tracers were also obtained to determine the optimal irradiation frequency for each metabolite. This study showed that although the optimal frequency for the  $^{13}\text{C}$  labeled metabolic tracers were not identical, irradiation of the mixture at the optimal irradiation frequency of any one metabolic tracer resulted in SNR significant enhancement. The effect of radicals, glassing agents, and dissolution solvents on the polarization efficiency were also studied. This study shows the capability of DNP technology, and hence, HyperSense® in detecting very low concentration of metabolic tracers in a mixture. Such applications have a tremendous potential in the field of metabolomics and opens a new horizon in use of nuclei such as  $^{13}\text{C}$ ,  $^{15}\text{N}$ , etc in addition to the existing  $^1\text{H}$  NMR data.

# 19

## Pyridine-Pyridinium Tetrachlorogallate (III) [Py•••H– Py][GaCl<sub>4</sub>]: <sup>2h</sup>J<sub>NN</sub>, <sup>1H</sup>J<sub>NH</sub> and <sup>1</sup>J<sub>NH</sub> Coupling Constants Determination

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Following our research project on the study of hydrogen bonds in crystalline samples, [1-3] we present here our results on the determination of the <sup>2h</sup>J<sub>NN</sub>, <sup>1H</sup>J<sub>NH</sub> and <sup>1</sup>J<sub>NH</sub> coupling constants in the <sup>15</sup>N-labeled pyridine-pyridinium tetrachlorogallate [4].

The hydrogen-bond mediated J coupling, <sup>2h</sup>J<sub>(N1-H...N2)</sub>, has been identified by means of the two-dimensional refocused INADEQUATE experiment in which signals of the two J-coupled nuclei are observed. To extract the J coupling, a series of 1D CPMAS spin-echo experiments were recorded with different tau values and the integrated intensities in the frequency-domain spectra were fitted to equation: S(tau)= A cos(2piJ tau) exp(-2 tau /T2'). The observation, in the plot of experimental S(tau) against tau, of the first zero crossing at tau= 1/4J for N1 and N2 resonances, immediately gives a <sup>2h</sup>J<sub>NN</sub> of 10.6 and 9.8 Hz, respectively.

The calculated value at the B3LYP/6-311++G(d,p) theory level for <sup>2h</sup>J<sub>NN</sub> is 14.1 Hz, and the origin of the discrepancy is still under investigation. It can arise from the tetrachlorogallate anion effect that modifies the couplings, or due to the calculation level. At the same level theoretical values found for <sup>1H</sup>J<sub>NH</sub> and <sup>1</sup>J<sub>NH</sub> are of -3.5 Hz and -70 Hz, respectively.

We will also illustrate the presence of the <sup>1</sup>J<sub>NH</sub> coupling, and attempts to observe the small <sup>1H</sup>J<sub>NH</sub> coupling with acquisition of <sup>1</sup>H-<sup>15</sup>N 1D and 2D through-bond INEPT experiments, under efficient proton homonuclear dipolar decoupling schemes.

We are indebted to the EU-NMR InfraStructures (Contract # RII3-026145) for allowing us to perform the appropriate hardware and expertise. Thanks are also given to MEC of Spain for economic support (project number CTQ2007-62113).

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# 20 Metabolic Profiling of *Caenorhabditis elegans* by Whole Organism Nuclear Magnetic Resonance

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The interest in studying *Caenorhabditis elegans* nematode, first animal model whose genome was sequenced, is to monitor a wide range of biological processes and to characterize genetic mutations responsible for human diseases. However, many genetic mutations do not display any obvious morphological or behavioral phenotype under classical observations.

We develop, validate and apply a metabonomic protocol based on <sup>1</sup>H HRMAS NMR spectroscopy of intact *C. elegans* worms, and have shown its ability to investigate the metabolic signature induced by mutations of oxidative stress enzymes.[1] A metabonomic analysis, i.e. the hypothesis-free interpretation of biological NMR data by multivariate statistics, is particularly suited to understand pathophysiological perturbations in *C. elegans* mutants. We also demonstrate and characterize the robustness of our metabolic phenotyping method, discriminating wild-type N2 from mutant *sod-1(tm776)* animals, and we identify and quantify several confounding effects to establish guidelines to ensure optimal quality of the raw data across time and space.[2]

Finally, we present an algorithm based on the landscape of the covariance/correlation ratio of consecutive variables along the chemical shift axis to restore, prior to significance testing, the spectral dependency and recouple variables in clusters which correspond to physical, chemical, and biological entities: statistical recoupling of variables (SRV).[3] We show how this procedure improves the interpretability of latent variables for metabolic biomarker recovery.

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# 21 NMR and Computational Study of Conformational Behaviour of Medium-sized Ring Triones

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The oxidative fragmentation of bicyclic ring systems has proved to be robust method of accessing medium-sized rings [1]. Once formed, the presence in these systems of a number of functional groups in close proximity often result in the restricted interconversion of ring conformers leading in some cases, to atropisomerism [2]. In particular, the m-CPBA mediated cleavage of the central double bond in 1a to afford 2a has been reported [3].

Both compounds 1a and 2a are of interest from a stereochemical perspective. It was shown that 1a possesses a stereogenic axis due to restricted rotation about N(12)-C(1') bond and isolation of short-lived enantiomers has been achieved by HPLC [4]. An extensive variable temperature NMR and computational study of 2a and the ring expanded analogs 2b and 2c showed that a second stereogenic axis associated with conformation of the medium sized ring is present, in addition to the restricted rotation about N(12)-C(1') bond [5]. Although, <sup>1</sup>H NMR spectra suggest that 2a-c undergo racemization in solution the mechanism of this process remained unclear.

Here we want to present more detailed understanding of the racemization mechanism of compounds 2a-c which has emerged from total lineshape analysis of variable temperature <sup>1</sup>H NMR spectra and theoretical calculation on RM1//B3LYP/6-31G\*\* level of theory.

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# 22 Gradient Enhanced Inverse Detection of the Heteronuclear Overhauser Effect between $^{183}\text{W}$ and $^1\text{H}$ in Tungstenocene Stannyl Complexes

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Recently we have started a relaxation behavior study of tungstenocene stannyl complexes of the form  $\text{Cp}_2\text{WHSnCltBu}_2$ ,  $\text{Cp}_2\text{WHSnClPh}_2$ , and  $\text{Cp}_2\text{WHSnPh}_3$ . We have been trying to observe the Nuclear Overhauser Effect between the tungsten and hydride bond. Although tungsten has a large theoretical NOE value of 12 [1], NOE detection of tungsten has been limited in low sensitivity nuclei such as tungsten. To our knowledge, heteronuclear NOE measurements of tungsten have only been reported by Benn, Brevard, Ruffinska, and Schroth [2] in 1987.

In order to overcome the lower sensitivity of the low gamma-nucleus, we have programmed a set of pulse programs that perform gradient enhanced inverse detection on  $^1\text{H}$ . Inverse detection of NOE is useful for our compounds since it takes advantage of the large scalar coupling between the hydride directly attached to the tungsten.

The first pulse program we wrote is used to determine the steady state NOE. Decoupling is applied to the proton channel to develop an NOE on tungsten. Once that is done, the magnetism is transferred from tungsten back to proton to be read. The transfer is done by an inverse INEPT sequence. By varying the length of the decoupler pulse, we see a trend in the NOE values. The second pulse program detects the transient state NOE. In this pulse program, a  $180^\circ$  pulse is placed on the proton and the system is allowed to relax a taumix time period, in order for the NOE to buildup. By varying taumix, we can make a NOE buildup curve to find the NOE value.

In both pulse programs, magnetism is transferred from tungsten back to proton in order to collect an FID on the proton channel. This process is done using an inverse INEPT sequence. To remove the magnetism generated from the inverse INEPT sequence in an NOE experiment, a corresponding experiment containing only the inverse INEPT sequence is done. The spectrum produced by the inverse INEPT sequence is then subtracted from the NOE spectrum. Thus, we get a spectrum that shows a pure NOE. Furthermore, application of matched gradient pulses destroys unwanted magnetization generated by some of the other pulses present in our program. In our poster, we desire to showcase our three pulse programs and present the work that was accomplished using these pulse programs our tungstenocene stannyl complexes.

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# 23 Establishing the Chirality of Acyclic Polyfunctional Compounds by NMR: The 1,2,3-Triols Case.

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Determination of the absolute configuration of chiral acyclic molecules such as alcohols, implies the derivatization of the compound with the two enantiomers of a chiral derivatizing agent. The formation of stable conformers in both derivatives produce differences in their  $^1\text{H}$  NMR spectra that can be correlated with the absolute configuration of the substrate.[1] This methodology has been applied to determine the absolute configuration of many monofunctional compounds such as alcohols, amines, thiols...[2]

In the case of linear polyfunctional compounds, such as diols, aminoalcohols and triols, the presence of several chiral units in the molecule makes these systems more complex than the monofunctional ones.[3] Thus, these derivatives should generate stable conformers with specific distribution of the anisotropic effects. The presence of these conformers induce differences in the proton chemical shifts of the substrate in both derivatives, that can be correlated with the absolute configuration of the polyfunctional compound.

We demonstrate that the absolute configuration of linear 1,2,3-triols can be easily assigned by the formation of their tris-MPA ester derivatives.[3] The generation of a well defined conformational equilibria allowed us to correlate the variations on the NMR spectra in both MPA derivatives with the absolute configuration of the triol by the use of a new set of NMR parameters.[4]

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# 24 Structure Determination of Small Molecules Using Residual Dipolar Couplings (RDCs)

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By introducing a small degree of alignment into a sample, anisotropic NMR parameters like residual chemical shift anisotropy, residual quadrupolar couplings, and residual dipolar couplings (RDCs) become observable.[1] Among these, RDCs have been intensively used in structure calculation and/or refinement of biomacromolecules as they allow the extraction of distance as well as orientational information from any two coupled spins.[2] After the development of appropriate alignment media, RDCs have also been used for the structure determination of small to medium-sized organic molecules in recent years.[3]

Here we present two examples for the application of RDCs in the field of small molecules. Configurational analysis has been achieved for a natural product isolated from aged beer. This formerly unknown major degradation product of a key bitter compound in hop products was found by standard NMR methods like COSY and HMBC to be a substituted tricyclic hydrocarbon. Its relative configuration could be solved by the analysis of one-bond  $^1\text{H}$ - $^{13}\text{C}$  and two-bond  $^1\text{H}$ - $^1\text{H}$  RDCs measured in a stretched poly (acrylonitrile) / DMSO gel.[4]

In a second example RDCs have been used to analyze an unknown reaction product. For this compound classical NMR methods (COSY, HMBC, ADEQUATE) failed to unambiguously solve the constitution of the molecule. By fitting RDCs measured in a polystyrene /  $\text{CDCl}_3$  gel[5] against all possible structures, the reaction product could be identified. This example shows that for small molecules RDCs can not only be used for the investigation of conformation and configuration, but even for the determination of a molecule's constitution.

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# 25 pH-responsive Lanthanide Probes for $^{19}\text{F}$ Magnetic Resonance Spectroscopy

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We describe the design and preparation of responsive probes for in-vivo spectroscopy. Our strategy is to use  $\text{CF}_3$  reporter groups attached to a stable complex containing a paramagnetic lanthanide ion. The lanthanide induced shift (LIS) depends strongly on the distance of the  $\text{CF}_3$  reporter group from the metal and its orientation with respect to the pseudo axis of symmetry of the complex. Therefore, relatively small changes in environment (for example protonation and de-protonation as a function of pH) can be amplified into relatively large changes in LIS.

The presence of the lanthanide ion also increases the rate of relaxation of the fluorine nuclei resulting in a net increase ( $\sim 10\times$ ) in signal acquired per unit time under optimized conditions (Ernst angle) compared with typical diamagnetic fluorine-containing molecules. The relaxation behaviour has been extensively studied by computational methods and the results allow detailed optimization of the complexes.

# 26 Quantitative $^1\text{H}$ NMR with Single External Standard and its Application to Solubility Test

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Determining the solubilised concentration of drugs for an in vivo study is an important aid to investigating its efficacy. In addition, the assessment of absolute purity or concentration of compounds has increased in importance recently as library screening has become the routine tool for drug discovery.

Method development for each compound, required for uv-vis HPLC or LC/MS techniques makes these two traditional purity assessment methods tedious and time consuming when dealing with structurally versatile compounds. On the other hand, quantitative  $^1\text{H}$ -NMR (qHNMR) has progressed dramatically in this decade with advances in stability, resolution and sensitivity of instrumentation. With a validated protocol, it is possible to achieve around 1% precision and accuracy.[1] The unique requisite of NMR, i.e. that the area of each peak is directly proportional to the number of the corresponding nuclei, suggests qHNMR as the best method for the quantification of compounds. Response factor is not dependent on molecular structure and hence method development is not necessary for individual compounds.[2]

In this study, we developed and evaluated a quantitative  $^1\text{H}$  NMR method for open access. The method was designed to use one single external standard as calibrant to measure the concentration of small molecules seen in drug discovery process. Among 15 commercial compounds we tested, 70% samples had less than 5% relative error with the maximum being 10% and 83% samples had less than 2% relative standard deviation with the maximum being 2.5%. In a comparison measurement by HPLC-UV, a similar accuracy and precision were obtained for these samples. As an example of its application, the solubility of small molecules using in fragment based drug discovery were measured using this method.

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# 27

## Identification of Drug Substance Impurities by LC-NMR. A case study: Vestipitant.

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High-performance liquid chromatography coupled with NMR spectroscopy (LC-NMR) is a key-tool in pharmaceutical industry for structure elucidation of impurities in drug substance batches. LC-NMR rapidly provides detailed structural information on small quantities of substances, which can be resolved by analytical HPLC, significantly reducing the time needed for isolating purified samples while providing the requisite sensitivity [1].

The application of LC-NMR for the successful identification of drug substance impurities present in manufactured batches of Vestipitant will be presented. Vestipitant is a novel NK-1 antagonist currently being developed to both tackle Central Nervous System (CNS) diseases and relieve chemotherapy-induced nausea and vomiting (CINV) symptoms [2].

LC-NMR, as well as LC-MS, were performed on a mother liquor sample of Vestipitant, which was enriched in the impurities present in very low amount in the final drug substance. These techniques provided a fast and effective solution to the identification of the main impurities in Vestipitant batches.

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# 28 **Alleviating the Reference Problem in Quantitative NMR**

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For structural investigations, integral intensities only need to be measured with accuracies of around 10% in order to gain knowledge of the number of protons present in different environments. However many studies have shown that, with due care, integrals can be reliably measured with better than 1% accuracies and hence precise and accurate quantitative analytical studies are also possible. Such studies can be very useful in for instance metabolite/natural product quantification and purity analysis of pharmaceuticals.

Our experience of qNMR is that whilst controlling instrument parameters to ensure quantitative responses is not a problem, working with reference materials as internal standards is tiresome. In this poster we discuss two approaches to removing internal reference materials that make qNMR more convenient and accessible.

# 29 <sup>1</sup>H NMR Metabolomics of Earthworm Responses to Organic Chemical Exposure in Soil

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<sup>1</sup>H NMR metabolomics of earthworm tissues may provide insight into any metabolic changes that may occur during exposure to sub-lethal and bioavailable concentrations of environmental contaminants. We have examined the response of *Eisenia Fetida*, the recommended earthworm species for ecotoxicity tests, to phenanthrene (a model polycyclic aromatic hydrocarbon) exposure in soil. We studied both short-term and long-term responses to varying concentrations of phenanthrene. Earthworm tissues were extracted into a D<sub>2</sub>O-based buffer and analyzed by <sup>1</sup>H NMR (using PURGE for water suppression) and data were analyzed with multivariate statistics (AMIX). Our first study showed that PCA separation and clustering of exposed earthworms was observed for the higher phenanthrene concentrations when compared to control earthworms. PC1 loadings plots showed that alanine, leucine, lysine and valine were responsible for the majority of the observed variation. Furthermore, amino acid concentrations were observed to increase with increasing phenanthrene concentration suggesting a strong link between metabolomic responses and soil phenanthrene concentrations after only 48 hours of exposure. Maltose was also identified in loadings plots but was also found to be variable amongst the control group. PLS regression of earthworm responses as compared to both total and bioavailable phenanthrene revealed strong, positive correlations suggesting that <sup>1</sup>H NMR metabolomics may be a potential tool for measuring bioavailability directly. *E. fetida* responses to phenanthrene exposure over a 30 day period showed a correlation between amino acid levels and the concentration of phenanthrene in soil. Earthworm responses were still detectible even when the bioavailable phenanthrene concentration diminished. These studies demonstrate the potential for <sup>1</sup>H NMR metabolomics to further the understanding of *E. fetida* ecotoxicity to sub-lethal concentrations of organic chemicals in soil. Future research will focus on testing the inherent variability of observable <sup>1</sup>H NMR metabolites in *E. fetida* and the response of *E. fetida* in different soil types.

# 30 **New Insights into Epothilone-tubulin Interactions. First Determination of the Conformation of Epothilone B Bound to Dimeric Tubulin**

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During the last years, great efforts have been made to obtain the bioactive conformation of epothilones. The solid state (deduced by either X-ray or solid state NMR) structure of free epothilone B is known,[1, 2] as well as its conformations in organic solvents.[3] However, its tubulin bound bioactive conformation is still a controversial issue. The first epothilone tubulin-bound conformation was determined by solution state NMR, using an unpolymerized soluble form of  $\alpha\beta$  tubulin complexed to epothilone A.[4] Almost immediately, the tubulin/epothilone A complex was also studied through electron-crystallography, using tubulin sheets.[5] The conformation of the tubulin-bound epothilone was strikingly different in the two studies, suggesting the need for further investigation. As this discrepancy may reflect the dependence of the epothilone binding mode on the tubulin polymerization state, or the presence of different binding sites in the protein, further studies are required. The aim of the present work is to determine the conformation of epothilones A and B bound to dimeric tubulin in solution by NMR to gain insight into the epothilone-tubulin recognition processes.

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# 31 $^1\text{H}$ , $^{13}\text{C}$ , $^{15}\text{N}$ and $^{14}\text{N}$ NMR Structural Characterization of Polynitrogen Polycycles

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Conjugated polynitrogen polycycles are interesting intermediates for organic synthesis.

i) Tetrazine-based structures represent a source of new materials bearing specific properties (1,2). Potential applications of such compounds are related to the synthesis of new optically and electro-active polymers devoted, for example, to sensor applications or to the development of functionalized selective resin extractants for water treatment.

ii) Mesomeric heteropentalenes betaines are three fused-cycle compounds characterized by the presence of two nitrogen atoms at the bridge positions (3). These triazapentalene derivatives have been the subject of extensive investigation and, due to their electronic structure, particular focus has been directed to their participation in dipolar cycloadditions. These different nitrogen-rich assemblies also represent a basis for the development of a new class of insensitive energetic materials.

We present here the  $^1\text{H}$ ,  $^{13}\text{C}$ ,  $^{15}\text{N}$  and  $^{14}\text{N}$  structural characterization of these compounds including small molecules and polymer materials. Depending on the number and nature of hydrogen atoms, the available quantity of sample or its solubility, the presence of H-X couplings, different NMR methods were used : in solution we performed direct  $^{13}\text{C}$ ,  $^{15}\text{N}$ ,  $^{14}\text{N}$  measurements and inverse detection HSQC sequences using different delays ; in solid state, we recorded high resolution  $^1\text{H}$  MAS spectra with a CPMG sequence synchronized with sample rotation, and  $^{13}\text{C}$ ,  $^{15}\text{N}$  CP/MAS spectra using different spinning speeds. We reported, apparently for the first time, nitrogen NMR data and coupling information on such systems. Inter-ring long range correlations across five bonds with  $^{15}\text{N}$  ( $^5J_{\text{HN}}$ ) and up to seven bonds with  $^{13}\text{C}$  ( $^6J_{\text{HC}}$  and  $^7J_{\text{HC}}$ ) were detected in HSQC experiments of heteropentalene derivatives. An increase in magnitude of the remote couplings was observed when incorporating an electron-withdrawing substituent such as  $\text{NO}_2$ . This effect was accompanied by a deshielding of nearby protons, carbons and all nitrogen atoms of the structure, including ones situated in other cycles.

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# 32 An NMR Study on Anion-induced Conformational Changes in Indole-based Receptors

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Anion receptor chemistry continues to be an area of intense research activity [1-5]. The vivid growth in this area of supramolecular chemistry has been stimulated by many roles played by anions. Indole derivatives are found in several enzymes in the sulfate binding proteins and in active site of haloalkane dehalogenase. NH protons may act as hydrogen bond donors and their ability to partake in hydrogen bonds is regulated by the choice of substituents on the heterocyclic scaffold. In this study we focused on bis-amido and mono-amido-mono-urea 2,7-functionalized indoles which showed remarkable new binding properties upon functionalization [6].

The conformational analysis of four indole receptors was performed by a combination of NMR spectroscopy and quantum chemical calculations showing excellent agreement [7]. All receptors exhibit conformational preorganization in acetone solutions, where anti-anti orientation across C2–C2 $\alpha$  and C7–N7 $\alpha$  bonds is highly predominant. In addition, anion-receptor interactions have been assessed through  $^1\text{H}$  and  $^{15}\text{N}$  chemical shift changes. Anion properties affect the anion-receptor interactions and different ways of bindings have been observed. NOE enhancements in the presence of anions revealed that anion-receptor complexes favor the syn-syn conformation of the C2 and C7 substituents.

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# 33 From FID to Structure in 30 Minutes?

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Structure elucidation of impurities, degradants and active pharmaceutical ingredients is essential for delivering new drugs to the market. It is often a laborious process, involving significant acquisition, processing and analysis time.

We present the combined use of high mass sensitivity NMR micro coils [1] [2], computer assisted structure elucidation [3] and SmartFormula3D [4] for fast impurity identification, structure elucidation of challenging molecules, structure confirmation and automatic assignment tool.

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# 34 Quantitative NMR Spectroscopy: Applications in Drug Discovery

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Although chromatographic methods are still first choice as they are well established and documented in analytical standards, pharmacopoeia and drug master files, quantitative NMR (qNMR) methods have gained increasing acceptance and widespread use in recent years.

NMR spectroscopy can be considered as primary relative (ratio) quantification method, as the ratio of substances in a mixture can be determined directly from the NMR spectrum. Absolute amounts of substances have long been determined using internal or external reference substances; however that implies contamination of the analyte or cumbersome sample preparation, respectively. The introduction of ERETIC [1] (electronic reference to access in-vivo concentrations) techniques resolved these issues, however, required extra hardware components.

Using techniques such as PULCON [2] (pulse length based concentration determination) that are related to the principle of reciprocity, absolute, unknown concentrations of a sample can, for a given coil, be determined based on the known concentration of a unrelated reference sample.

The poster will present applications of the PULCON technique to different analytical or physico-chemical sets of problems that frequently recur during the lead optimization stages.

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# 35 Anomeric Discrimination on Ligand Recognition by Lectins – A <sup>19</sup>F-STD NMR Study

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Saturation transfer difference (STD) NMR is a spectroscopic technique that allows the fast and efficient screening of compound mixtures in the presence of a receptor [1]. By the strategic employment of novel <sup>19</sup>F detected STD-NMR techniques [2], the values of affinity – with anomeric discrimination - of a lectin towards a monosaccharide ligand is herein shown for the first time. It was possible to identify the active species of 2-FDM bound to conA, as well as obtain individual affinity information on each anomer.

ConA is considered a proper system model as it is known to have a highly specific recognition for  $\alpha$ -D-glucose and  $\beta$ -D-mannose [3], manifesting a patent discernible behavior when interacting with one or the other anomer.

Furthermore, we are able to discuss the influence of the temperature on the recognition process and its remarkable effect on con A's anomeric specificity.

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# 36 Applications of Reduced-Dimensionality NMR Methods for Elucidation of Complex Mixture Composition

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Elucidation of metabolite compositions of biological samples constitutes a first step in metabolomics, towards better understanding of biochemical processes in living systems. NMR spectroscopy was established as a key technique in this approach, by providing rich information data. Identification of mixture compositions, in a general case comprising a few hundred metabolites, requires unambiguous signal assignment, that could be achieved by increasing dimension of experiments to resolve spectral overlap. However, in a biological context, natural abundance acquisition of  $^1\text{H}$  and  $^{13}\text{C}$  NMR data limits the use of conventional high-dimensional experiments (3D, 4D, ...) that would require unrealistically long acquisition times.

Recently, the concept of reduced-dimensionality NMR spectroscopy has seen a strong revival (1,2,3) based on alternative schemes of indirect dimension exploration for multidimensional experiments, with main applications in the field of protein NMR spectroscopy. Here, we intend to recruit projection NMR techniques for metabolite studies, giving us access to high-dimensional correlations in order to capture essential information necessary to resolve overlaps in a mixture, in a reasonable amount of time.

Proof of principle for this approach has been obtained for a mixture made of ornithine, putrescine and arginine where putrescine resonances are superimposed on the other molecule signals in classical 2D  $^1\text{H}$ - $^1\text{H}$  TOCSY spectrum and 2D  $^1\text{H}$ - $^{13}\text{C}$  HSQC spectrum, making it indistinguishable. Acquisition of a targeted 2D projection of a 3D  $^1\text{H}$ - $^{13}\text{C}$  TOCSY-HSQC spectrum (for the given example defined by an angle equal to  $+30^\circ$  with respect to the  $^1\text{H}$ - $^1\text{H}$  plane), allowed the elucidation of this mixture composition by resolving peak overlaps.

Further efforts are carried out to optimize low-dimensional projection scheme of high-dimensional NMR experiments to resolve ambiguities for different biological complex mixtures, recovering more information from these thousand compound mixtures of medical interest.

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# 37

## In situ NMR Monitoring of a Ru-catalyzed Ring Closing Metathesis Reaction: Mechanistic Clues Toward Yield Optimization

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In recent years olefin metathesis has provided a popular means to synthesize large ring systems via ruthenium catalyzed ring closure (ring closing metathesis; RCM) of linear diene substrates [1]. In industrial scale settings however the application of this reaction type poses significant challenges. For instance, the reaction typically has to take place in high dilution to avoid competitive polymerization reactions, which results in tremendous production costs and excessive waste volumes.

We currently apply a ring closing metathesis step in the synthesis process of a macrocyclic compound featuring a 14-membered ring containing two amide functions. The initial cyclisation reaction (using a first generation Ru catalyst) suffered from high dilution, low yield, side product formation and high catalyst load. By rational substrate modification and the use of alternative ruthenium catalysts we aimed for a more practical process.

As revealed by in situ <sup>1</sup>H NMR spectroscopy, we report a dramatic switch of the RCM initiation site with the use of a modified substrate [2] as well as with the switch from 1st to 2nd generation catalyst. These mechanistic changes can be correlated with RCM reaction efficiencies, providing a rationale for improved reaction performance.

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# 38 In-vivo 2D-TR-NOE Interrogates and Ranks Ligand-receptor Interactions in Living Cells

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Integrins, the major class of heterodimeric transmembrane glycoprotein receptors, and the membrane-spanning surface protein aminopeptidase N (CD13), play a pivotal role in tumour growth and metastatic spread. Recently it has been demonstrated that deamidation of the NGR sequence gives rise to isoDGR, a new  $\alpha V\beta 3$ -binding motif [1]. which is a competitive antagonist of  $\alpha V\beta 3$ -ligand RGD. No competition is observed with cyclo-peptides containing DGR or NGR sequences, thus implying a precise stereospecific recognition at the basis of isoDGR interaction with  $\alpha V\beta 3$  [2]. We designed and synthesized a small library of pentacyclic-peptides whose conformational properties were first analysed by classical solution NMR methods, refined by MetaDynamics (MtD) and finally docked onto the  $\alpha V\beta 3$  crystallographic structure using HADDOCK-2.0.

Here we show that it is possible to apply 2D-TR-NOE techniques directly on human cancer cells to prove selective binding of anti-angiogenic ligands to structurally characterised and uncharacterised receptors, such as  $\alpha V\beta 3$  and APN (CD13), respectively. Using 2D-TR-NOE experiments we investigated the binding of these cyclopeptides onto 2 human cancer cell lines differently expressing  $\alpha V\beta 3$  and CD13, including a melanoma (MSR3:  $\alpha V\beta 3$ +CD13- cells) and a non-small lung carcinoma (MR300:  $\alpha V\beta 3$ +CD13+) cell line, which display different phenotypes for CD13 and integrins. Small amount of receptors are needed to prove binding (in the picomolar range), as it is sufficient that the receptor is detectable by FACS analysis. The method allows using different cell lines, with different receptors, which can be also silenced with siRNA techniques to prove recognition specificity. Finally, 2D-TR-NOESY can be applied in living tumour cells to screen varying ligands to the same receptor, performing competition experiments thus defining an affinity ranking of different ligands in a physiological context.

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# 39 Open-access Software and Databases to Help Metabolites Recognition in Metabonomics: The User's Point of View

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Nowadays, metabolomics is underpinned by a number of freely and commercially available databases and automated approaches for metabolite identification and structure elucidation. Here we present a protocol based on open-source databases and software aiming to help the spectroscopist in the characterization and identification of molecules inside a complex mixtures of compounds. Our protocol is divided in two steps: in the first step we scan unknown peaks deriving from <sup>1</sup>H-<sup>1</sup>H-TOCSY and <sup>1</sup>H-<sup>13</sup>C-HSQC NMR spectra, from three different cell culture media, against public databases, in order to rapidly screen and identify organic molecules. Three different databases were used (the Biological Magnetic Resonance Data Bank, the Humane Metabolome Database and the NMR metabolomics database of Linkoping)[1] which are integrated and accessible at different stages from two tested open-access software/web portal (MetaboMiner[2] and Colmar [3]).

Results are then used in a second step, where a CCPN project is built[4]. 2D-NMR spectra of standard compounds as identified in the first step, can be free downloaded from public databases and included in a CCPN project. Hence spectra acquired on an unknown mixture can be easily compared, superimposed and assigned on the basis of standard spectra.

Once unknown peaks of culture media are identified on the bases of the standard compounds, CCPN can easily transfer these assignments to spectra obtained from the same culture media after incubation with a certain cell line. In this way it is possible: (1) to have an overview of the similarity and differences of spectra coming from different culture media samples; (2) to have an overview of the amount of the unassigned peaks; (3) to have a detailed peak list of the unambiguous and ambiguous assigned peaks, as far as the unassigned peak list. These peak lists could also be checked or screened versus other public databases or could be the starting point for a de novo metabolite identification.

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2. <http://wishart.biology.ualberta.ca/metabominer/index.html>
3. <http://spinportal.magnet.fsu.edu/>
4. <http://www.ccpn.ac.uk/ccpn>

# 40 Isomerization, Tautomerization of N-containing Heterocycles Studied by Multinuclear NMR Spectroscopy

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Synthetic approaches aiming at the preparation of linearly fused triazolo- and tetrazolo-isoquinolines with valuable biological activity have been published by us [1]. Recently we reported that the reaction of 2,3-diaminoisoquinolinium salt with aldehydes could follow two different pathways, i.e. in addition to the triazolo derivatives isoquinolyhydrazones may be formed [2].

In this presentation the detailed description of two systems, one consisting of ring-chain tautomers (tetrazole-azide), the other consisting of E/Z isomers (hydrazones) is given. Structure elucidation was performed using multinuclear one and two-dimensional NMR experiments. <sup>1</sup>H and <sup>13</sup>C data were completed with <sup>15</sup>N chemical shifts, enabling the total assignment of the compounds. The <sup>15</sup>N signals were determined either from <sup>1</sup>H-<sup>15</sup>N gHMBC spectra or from direct <sup>15</sup>N measurements of <sup>15</sup>N-labelled derivatives.

By measuring solid-state <sup>13</sup>C and <sup>15</sup>N CP-MAS spectra the tautomeric/isomeric composition of the synthesized solid samples was determined. Differences of the tautomeric/isomeric ratios in solution and in the solid phase are discussed in the context of the energetic behavior of the reactions.

The financial support of GVOP-3.2.1-2004-04-0210/3.0 and GVOP-3.2.1-2004-04-0311/3.0 are gratefully acknowledged.

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# 41 Statistical Comparison of $^{13}\text{C}$ Chemical Shift Predictions Performed by Empirical and Quantum-Mechanical Methods

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Over the last decade, many works devoted to  $^{13}\text{C}$  NMR chemical shift prediction of organic molecules using GIAO approximation of the DFT approach were published. As far as we know, the results of quantum-mechanical (QM) shift predictions published in manifold journals have not been generalized yet, and computational errors were not compared with those intrinsic to empirical methods based on HOSE code and neural net (NN) algorithms. Meanwhile, these empirical methods are constantly improved and some examples of their applications in parallel with QM methods have been published [1].

In this study, we extracted from literature the data of the QM  $^{13}\text{C}$  chemical shift predictions for 205 structures (totaling > 2500 shifts) and compared the results with ours obtained for the same structures using HOSE code and NN approaches. Approximately one half of all structures contained  $\leq 10$  carbon atoms, and  $\sim 85\%$  of structures contained no more than 20 carbon atoms.

Structures of this test set were input into ACD/CNMR Predictor, and  $^{13}\text{C}$  chemical shifts were generated based on the HOSE codes and NN. These results were directly compared to the experimental values as well as the QM-calculated  $^{13}\text{C}$  chemicals shifts presented in corresponding articles. Mean absolute errors (MAE), maximum absolute errors, and standard deviations (SD) were estimated for all three examined methods. We found that the overwhelming majority of MAEs calculated by all three methods were less than 4–5 ppm. The average MAE values are equal to 1.51, 1.91, and 3.29 ppm for HOSE, NN, and QM based methods correspondingly. The major finding was that, in general, both the HOSE code and NN approaches vastly outperformed QM-based methods on the average MAE values and the maximum absolute error.

The aim of this poster is to illustrate that while QM calculations can allow one to predict  $^{13}\text{C}$  chemical shifts accurately, this method does not provide any advantages relative to the empirical methods investigated herein. The latter remain irreplaceable for the molecular structure elucidation by CASE systems.

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# 42 Low Temperature NMR Spectroscopic Investigation of Self-assembled Systems: Differential Line Broadening Effects in NMR of Small Molecules

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Extensive line broadening effects in chemical exchange processes often hamper the routine room temperature NMR study of self-assembled systems where intramolecular conformational flexibility and intermolecular interactions (among monomeric, dimeric etc. species) are equally present. In order to understand such chemically important multi-site exchange processes and to obtain relevant structural information it is essential to reach the slow-exchange regime by lowering the sample temperature in a suitable solvent at high magnetic field. We have experienced that this strategy often results in significantly increased solvent viscosity and therefore slow molecular tumbling even for small organic molecules (<1kDa).

In this poster we demonstrate that low temperature NMR of self-assembled systems shows analogies to the spectroscopy of large biomolecules. The magnetic field, solvent and temperature dependence of differential line broadening effects are presented for two examples: 1. a helically distorted gold(I) macrocycle derived from xantphos [1]; 2. a thiourea modified 9-epi-cinchona organocatalyst [2]. Both compounds are significant in metal-based and metal-free catalysis. The extensive self-association of the cinchona catalyst allowed us to investigate the regime of slow molecular tumbling ( $\tau_c=335$ ns), negative  $^1\text{H}$ - $^1\text{H}$  NOE enhancements and differential line broadening of  $^1\text{H}$ - $^{15}\text{N}$  doublets (TROSY) even for small molecules. The scope of the research is to determine the effective molecular sizes in symmetrically self-assembled systems by describing the relaxation properties of their  $^1\text{H}$ ,  $^{13}\text{C}$ ,  $^{15}\text{N}$  and  $^{31}\text{P}$  nuclei.

The support of the Hungarian GVOP-3.2.1.-2004-04-0210/3.0 project, OTKA K69086 and K68498 grants are gratefully acknowledged.

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# 43 Study on Fully Automated Structure Verification With $^1\text{H}$ NMR on Ligands

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A study of a fully automatic structure verification of small molecules by one dimensional  $^1\text{H}$ -NMR is given. The data were obtained by using a library of about 2000 substances (~250u) measured in phosphate buffered saline with 3% D<sub>2</sub>O with five concentrations at 700MHz.

For a sufficient high quality structure verification most spectra are verified by an expert group within the NMR lab of the Merck KGaA. The automatic structure verification process has to meet the same standards. Particularly, this required a minimized false positive rate and a qualified rejection of false structure suggestions. An automatic rejection with a simple "No - wrong structure" is not sufficient within the analytic workflow. That is why the software usually generates two answers "OK" and "I don't know". At the moment a qualified rejection statement can only be delivered by the NMR expert group.

This study predicts the spectral properties using PERCH ([www.perchsolutions.com](http://www.perchsolutions.com)) and their ACA (Automated Consistency Analysis) tool to evaluate the consistency between predictions and measured spectra. Typically a cross correlation is used to determine a false positive rate. With the amount of data used here this approach is not feasible (eg. one verification lasts 1 to 10 minutes on an actual cpu). To create false positives the predicted spectral information is transformed into a graph based on shift, coupling constants and spin systems. For each spectrum/structure combination (true positive) a set of similar structures (false positives) were extracted with a graph similarity algorithm. Therefore three results can be observed: 'true positive rate', 'false positive rate' and whether the true structure has the best similarity value compared with the whole set for that specific spectrum or not. An overview regarding the predominant factors for the negative responses ("I don't know") is presented.

# **44** Improved Mass Sensitivity For Mass Limited Samples in 1 mm Tubes Measured in 5 mm Probes by Optimized Sample Preparation

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Mass sensitivity for mass limited samples in NMR tubes may be increased by optimizing the sample volume while taking field homogeneity into account.

When using a viscous solvent as dimethylsulfoxide in 1 mm tubes, it is possible to concentrate the sample in the active part of the receiver coil by embedding with pure identical solvent and still get good shimming results. This can give an initial increase in mass sensitivity of up to 50 % (3  $\mu$ L sample, 9  $\mu$ L embedding solvent, 12  $\mu$ L in total), which slowly decreases because of diffusion. This offers an alternative to use of Shigemi tubes or ELISE NMR [1]. By starting a thorough NMR investigation with the least sensitive experiment using embedding, considerable experiment time can be saved.

Examples and practical aspects of the sample preparation will be shown.

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# 45 Investigation of Standardized *Ginkgo biloba* Preparations by <sup>1</sup>H NMR-based Metabolomics and HPLC-PDA-MS-SPE-NMR

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Commercial preparations of *Ginkgo biloba* are very complex mixtures prepared from raw leaf extracts by a series of extraction and prepurification steps. The pharmacological activity is attributed to a number of flavonoid glycosides and unique terpene trilactones (TTL's), with largely uncharacterized pharmacological profiles on targets involved in neurological disorders. It is therefore important to complement existing targeted analytical methods for analysis of *Ginkgo biloba* preparations with non-selective methods for their comprehensive and global characterization.

In this work, <sup>1</sup>H NMR-based metabolomics and hyphenation of high-performance liquid chromatography, photo-diode array detection, mass spectrometry, solid-phase extraction, and nuclear magnetic resonance spectroscopy, (HPLC-PDA-MS-SPE-NMR) were used for investigation of 16 commercially available preparations of standardized *Ginkgo biloba* extracts, originating from Denmark, Italy, Sweden, and United Kingdom.

The results demonstrates that <sup>1</sup>H NMR-based metabolomics is an attractive method for comprehensive characterization of the global composition of standardized *Ginkgo biloba* preparations. <sup>1</sup>H NMR spectroscopy is intrinsically non-selective and information-rich, and PCA analysis of <sup>1</sup>H NMR spectra therefore enabled simultaneous assessment of TTL's and flavonoid glycosides. The method also allowed identification of preparations fortified with quercetin, simultaneous generation of relative distribution data for TTL's, which disclosed cases of non-conformity to standard ratio between ginkgolides and bilobalide, and detection of the potentially harmful 15:2-anacardic acid. In addition, the analysis demonstrated the presence of constituents that are usually not considered in the standardization protocols (shikimic acid and 3,4-dihydroxy benzoic acid) as well as excipients (glycerol and citric acid).

All these features are of likely importance in relation to consumer experience with *Ginkgo* products as well as for pharmacological or clinical studies using *Ginkgo* extracts, and the present study shows that <sup>1</sup>H NMR-based metabolomics is an attractive method for analysis of *Ginkgo* extracts.

# 46 Quantitative Analysis of Trace Level Impurities by NMR

Ian Jones and Andrew Phillips

AstraZeneca

NMR is a well-established quantitative technique for looking at relatively small amounts of material – for example residual solvents within an active pharmaceutical ingredient down to levels of 0.1% w/w.

Recently there has been considerable concern from pharmaceutical regulatory agencies over the control of potential genotoxic impurities (PGI) in medicinal products. The Threshold of Toxicological Concern (TTC) for PGIs in commercial products is 1.5 micrograms/day, or single-figure ppm with respect to a typical drug substance. Consequently, methods for the measurement of impurities in the single-ppm range are required – presenting a significant analytical challenge.

Traditionally techniques that have been used to perform such analysis are GC/MS, HPLC/MS and headspace GC in combination with extraction and/or derivatisation.

NMR has not been used because of its inherent lack of sensitivity. However, with the use of new technology and experimental developments we have recently shown that NMR can detect down to these very low levels – often with significant advantages over other techniques in terms of method development, sample preparation and experiment time.

This poster will focus on these developments and a number of real applications.

# 47

## Dynamic NMR Studies of the Hindered Amide Bond Rotation of Enediamides

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Enediamides[1] are important peptidomimetic scaffolds since they may themselves act as  $\gamma$ -turn mimics, and are intermediates to the synthesis of potent  $\beta$ -turn mimics. These novel heterocycles display a range of interesting chemistry based on the unusual nature of the double bond.[2]

An unusual feature of the NMR spectra of these enediamides is the very pronounced rotameric peaks, arising from restricted rotation about the exocyclic amide bond. The activation parameters for a series of these enediamides have been investigated using full lineshape analysis and selective inversion recovery NMR experiments over a range of temperatures and different magnetic fields. The conformation of the rotamers has been determined using selective-1D and 2D NOESY NMR methods.

Understanding the conformational nature of these compounds is important if we are to use them in synthesis, since the rotamers, if they are separated by a high rotational barrier, must be treated as diastereomers, and thus may react differently in any downstream reactions.

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# 48

## A Robust NMR Tool for Drugs Fingerprinting

Yann Prigent<sup>1,2</sup>, Marina Gaillard<sup>1</sup>, Redouane Hajar<sup>1</sup>, Marie-Aude Coutouly<sup>1</sup>, Olivier Assemat<sup>1</sup> and Francis Taulelle<sup>3</sup>

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The quality of medicines becomes increasingly difficult to ensure, due to the modifications of the drugs market (generics drugs, counterfeit...). To this end, it is necessary to develop new tools.

In addition to traditional techniques, such as <sup>13</sup>C cross polarization magic angle spinning (CPMAS) which are used to identify active molecules or excipients and their crystalline state [1], a new tool has been developed: TOSY [2] (rate of relaxation Ordered Spectroscopy). This experiment based on the evolutions of relaxation times, provides a fingerprint of a given formulation as well as valuable information regarding interactions between all the drug components. The 2D NMR experiment TOSY displays the traditional spectrum in the direct dimension and the distribution of relaxation rates in the indirect dimension. It provides a picture of materials properties, separating the chemical and physical identifications, without performing a mixture separation.

In contrast to the classical relaxation analysis using exponential fitting, no hypothesis is made here on the dispersity or the number of species present in the formulation, thanks to maximum entropy processing [3]. This ensures a more robust method.

Tests have been run on different galenic formulations. With these illustrations, one can note that TOSY is also very sensitive to the manufacturing process of the tablets.

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3. M.A. Delsuc and T.E. Malliavin, *Anal. Chem.*, 1998, 70, 2146-2148

# 49 Spectral Realignment of PFG NMR Diffusion Data

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The DOSY experiment, by separating on two spectral axes the chemical shift information and the translational diffusion information, is a powerful analytical tool for solution analysis.

The accuracy of the diffusion coefficient values, as well as the quality of the separation of overlapping signals in the diffusion dimension are strongly dependant on the acquisition conditions and the overall system stability. In fact, perturbations which are almost invisible in the direct dimension lead to poor quality DOSY spectra.

A new method, called SOPHORA (“SOPHisticated Optimization by spectral ReAlignment”) [1], is used to correct for spectrometer imperfections. This method works as follows : first, a peak picking is done on a small spectral region (containing one signal) of each 1D row. The chemical shift of this signal in the first row is used later on as the reference. Then, for each row, the shift from the reference is estimated. Finally, this shift is corrected by shifting the whole spectrum, using the method described in [2] for shearing. Results obtained on various complex oligosaccharides mixtures will be presented, showing significant improvement of the resolution and the separation in the diffusion dimension.

SOPHORA is available as a macro running under the NMRnotebook<sup>TM</sup> software [3].

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# 50 **Rapid identification of Bioactive Constituents from *Cautleya gracilis* by LC-SPE-NMR**

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*Cautleya gracilis*, also named “hardly shaped ginger”, is an Asian Zingiberaceae which is known as a decorative plant in house and garden but it has never been phytochemically investigated. While screening for plants with anticancer effects, we found a dichloromethane extract of its roots significantly active in ex vivo pediatric patient leukemia / neuroblastoma cell lines and a medullary thyroid carcinoma cell line [1].

Our aim was a target oriented time and cost saving identification of the bioactive principles. Besides utilizing HPLC-DAD-MS methods our approach focused on using the powerful technique of LC-SPE-NMR [2,3]. Due to the complexity of the crude extract, rapid and simple activity-guided fractionation had to be performed. We ended up with a series of active samples containing only a small number of HPLC-DAD-MS detectable compounds. LC-SPE-NMR analysis of these fractions resulted in the identification of eight novel labdane type diterpenes, the diterpene zerumine and a cucurmine derivative. After finalizing the 1D- and 2D-NMR analyses the compounds were collected by elution from the flow probe head to evaluate their apoptosis inducing bioactivity in leukemia and neuroblastoma cell lines. The labdane derivative 6-oxolabda-7,11-14-triene-16-oic acid lactone, showed the best activity with an IC<sub>50</sub> of approximately 25 µg/ml (0.06 µM) after 24 h.

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# 51 **A Tool for Molecular Mass Measurement by means of Diffusion-Ordered Spectroscopy**

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Recent work published by Augé et al. [1] investigated the power law relating molecular mass of different molecular families with diffusion coefficient (equation 1).

$$D \propto M^{-\alpha} \quad (1)$$

By bringing theoretical evidence that  $\alpha$  is a measure of the fractal dimension  $dF$  of the molecular family under study (equation 2), this work allowed to propose a general theory applicable to a wide range of sizes and molecular types.

$$\alpha \approx 1/dF \quad (2)$$

Molecular mass estimation of species in solution being derived from equations 1 and 2, one can see that direct molecular mass determination without need for prior calibration curve is obtainable by diffusion measurement. A useful tool [2] based on the software implementation (NMRnotebookTM [3]) of this result is used here for molecular mass estimation of oligosaccharides mixtures and polymer mass distribution determination.

1. S. Augé, P.O. Schmit, C.A. Crutchfield, M.T. Islam, D.J. Harris, E. Durand, M. Clemancey, A.A. Quoineaud, J.M. Lancelin, Y. Prigent, F. Taulelle, and M.A Delsuc J. Phys. Chem. B, 2009, 113 (7), 1914-1918
2. O. Assémat, M.A. Coutouly, R. Hajjar, and M.A. Delsuc, submitted.
3. NMRTEC, <http://www.nmrtec.com/software/nmrnotebook.html>

# 52 The Absolute Configuration Challenge Overcome for Aldehyde and Ketone Cyanohydrins by NMR

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Optically active cyanohydrins are natural products and versatile chiral building-blocks in organic synthesis.[1] They are mainly useful in the preparation of hydroxy carbonyl compounds and amino alcohols, among others. Moreover, the presence of the cyano group in the aldehyde and ketone cyanohydrin structures turns them into special cases of secondary and tertiary alcohols. For that reasons, reliable methods which allow the assignment of their absolute configurations become desirable.

Herein we present methods for determining the absolute configuration of cyanohydrins from aldehydes[2] and from ketones[3] based on the preparation of their 2-methoxy 2-phenyl acetic acid (MPA) derivatives that yield both diastomers. The resulting conformational preference causes a shielding effect over one cyanohydrin substituent in each derivative, while the other substituent remains unaffected. The differences observed in the NMR spectra of both derivatives can be correlated with the absolute configuration.

1. (a) Gregory, R.J.H. *Chem. Rev.* 1999, 99, 3649. (b) Brunel, J-M.; Holmes, I.P. *Angew. Chem. Int. Ed.* 2004, 43, 2752.
2. Louzao, I.; Seco, J.M.; Quiñoá, E.; Riguera, R. *Chem. Commun.* 2006, 1422.
3. Louzao, I.; García, R.; Seco, J.M.; Quiñoá, E.; Riguera, R. *Org. Lett.* 2009, 11, 53.

# 53 NMR Investigation of Complex Natural Mixtures in Arctic Sediments

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Naturally occurring Organic Matter (OM) which is ubiquitous in the environment is a complex, heterogeneous mixture of organic molecules derived from microbes, fungi, plant litter and their degradation products. This large carbon pool is nearly three times the mass of all aboveground life [1]. To date Arctic OM has not been studied to the same extent as those in temperate regions, yet the northern ecosystems are estimated to contain 25–33% of the world's stored carbon, 12% of which is stored in the tundra regions alone [2]. Due to ongoing climate change, there is the potential of tremendous alteration of the large accumulation of Arctic OM; therefore knowledge of its structural components is critical for the assessment of its future vulnerability. Here we explore both solution-state and solid-state NMR approaches to assess the structural components present in Arctic OM. Solid-state (500 MHz) <sup>13</sup>C cross polarization – magic angle spinning (CP-MAS) NMR yielded bulk characteristics regarding the abundances of functional groups and carbon bonds in the whole unaltered samples whereas solution-state NMR provided insight into the individual components present in Arctic OM such as proteins, lignin, lipids, and carbohydrates. In addition, solution-state NMR allowed for some individual structures to be identified in the sediment extracts. Collectively, solid and solution-state NMR techniques represent a powerful combination providing both a comprehensive overview and structural detail in natural Arctic OM samples. Insights gained from this study will provide a deeper understanding of how Arctic OM varies both temporally/spatially and will be used to predict how it will respond to climate change.

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2. Billings, W.D. *Quat. Sci. Rev.* 1987, 6, 165.

# 54 Characterisation and Identification of the Cediranib N<sup>+</sup>-Glucuronide Metabolite

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Cediranib (4-[(4-fluoro-2-methyl-<sup>1</sup>H-indol-5-yl)oxy]-6-methoxy-7-[3-(1-pyrrolidinyl)propoxy]quinazoline; RECENTIN™), a vascular endothelial growth factor (VEGF) tyrosine kinase inhibitor (TKI) of all three VEGF receptors, is currently in phase III clinical trials for the first-line treatment of colorectal cancer and the treatment of recurrent glioblastoma.

During its clinical development a N-glucuronide metabolite was identified as a major circulating metabolite. Given the possibility of four sites for the conjugation of the glucuronic acid moiety, determination of the location of the conjugation site on cediranib was warranted. A small quantity of the N glucuronide metabolite of cediranib was initially generated using recombinant human uridine glucuronosyltransferase 1A4 (UGT1A4) enzymes. The metabolite generated was characterised and identified by HPLC-UV and mass spectrometric (HPLC-MSn) detection and by <sup>1</sup>H NMR spectroscopy. The N-glucuronide metabolite generated in the UGT1A4 scale-up experiment was confirmed by HPLC-MSn to have the same retention time, molecular mass and mass fragmentation data as the metabolite generated in a human liver microsomal incubate sample.

<sup>1</sup>H NMR spectroscopy clearly showed the characteristic anomeric doublet at approximately 4.7 ppm. Selective Rotating frame Overhauser Effect Spectroscopy (ROESY) experiments, irradiating the anomeric proton, showed no enhancement in the aromatic region of the spectrum, but enhancement in the aliphatic region. The identity of these enhanced signals was confirmed by a 2D <sup>1</sup>H-<sup>1</sup>H Correlation Spectroscopy (COSY) experiment.

In conclusion, the site of glucuronidation on cediranib was confirmed to be on the pyrrolidine nitrogen, suggesting that cediranib N<sup>+</sup> glucuronide formation is only likely in human and higher primates (great apes). Significant species differences exist in the N<sup>+</sup>-glucuronidation of cyclic tertiary amines and this metabolite has not been detected in rat or cynomolgus monkey.

# A New Combination of Aliased Spectra for Unambiguous Determination of Chemical Shifts in 2D Heteronuclear Experiments

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Decreasing the spectral width in the carbon dimension of 2D heteronuclear experiments results in aliasing [1]. The obvious advantage is an increased resolution and, in many cases, a reduction of signal overlap. In spite of the resolution enhancement in aliased spectra the carbon chemical shifts are ambiguous because of the violation of the Nyquist condition. We recently introduced the idea of using 10-ppm spectral window to facilitate the determination of carbon chemical shifts in aliased spectra [2]. In these spectra, most of the digits of the chemical shifts are directly readable on the carbon chemical shift scale. An ambiguity remains for the first digits because of the  $\pm n \cdot 10$  ambiguity. One therefore needs a complementary spectrum to determine the value of the integer  $n$  and complete the chemical shifts. Although a full spectrum can provide the necessary information, large differences in scale and resolution makes it non-ideal. We developed two solutions to this problem. The first consists in coding the missing information using selective pulses to generate a set of four 10-ppm spectra in which the signs of the signals code the value of  $n$ . The second solution consists in recording a single aliased spectrum with a slightly different scale and resolution. In the present work we recorded a pair of HSQC spectra with 10 and 10.1 ppm carbon windows. When printing the spectra in a proper manner, each signals appear twice with a distance equal to  $n \cdot 0.1$  ppm. The value of  $n$  can therefore be readily obtained. We believe such pair of spectra to be the best available method to increase spectral resolution with the minimal inconvenience to determine the true chemical shifts.

1. Damien Jeannerat, *Magn. Reson. Chem.* 2000; 38: 415–422
2. Vitorge et al. *Chem. Commun.*, 2009, 950–952

# 56 **Determination of pKa's in Complex Mixtures Based on Aliased HSQC Spectra Using NMR Titration**

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We developed an automatic titration system allowing chemists to study changes in chemical shifts upon addition of a titrant. Depending on the application, these changes can be translated into pKa's, binding constants, etc. In order to illustrate how complex mixtures can be studied using this method, we followed signals in 2D HSQC spectra instead of simple 1D  $^1\text{H}$  spectra so that signal overlap can be resolved in the carbon dimension. Additionally this avoids second-order effects. The HSQC spectra were acquired with 10.00 ppm windows in the carbon dimension in order to reach a high resolution with less time increments. This allowed us to measure carbon chemical shifts changes with high precision and accuracy in 30-minute experiments making it possible to run a full titration overnight. We illustrate this method to determine the pKa's in a mixture of carboxylic acids.

# 57 **A Region-Selective HSQC Experiment Designed for Use in an Open-Access NMR Laboratory**

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Our group maintains an open-access NMR laboratory that is used by medicinal chemists. We offer a variety of one- and two-dimensional experiments, both to meet the needs of the chemists and to provide our own group with non-routine experiments that can be set up to run overnight. All experiments run under automation. We have selected robust experiments that perform reliably when run under automation. The latest addition to the collection is a region-selective HSQC pulse sequence. By limiting the sweep width in the carbon dimension, the experiment provides better carbon resolution and shorter acquisition times than the standard experiment.

Our new region-selective HSQC experiment is based in part on a sensitivity-enhanced sequence that our group published in 2008.[1] The new experiment uses frequency-swept pulses, offering nearly perfect inversion and refocusing over a range of power levels. We have run the experiment on a variety of samples in different NMR solvents. In our experience, the experiment is well suited for automation, offering good performance without the need for sample-to-sample calibration or probe tuning.

1. T. D. Spitzer, R. D. Rutkowske, and G. F. Dorsey, Jr., "Frequency-Swept HSQC Sequences for High-Throughput NMR Analysis," *Magn. Reson. Chem.*, 2008, 46, 564

# 58

## <sup>13</sup>C Solid-State NMR Chromatography: <sup>1</sup>H T1 Relaxation Ordered Spectroscopy Under Magic Angle Spinning

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A simple method to separate the <sup>13</sup>C NMR spectra of solid mixtures is introduced. The <sup>1</sup>H longitudinal (T1) relaxation time is used to separate the overlapping <sup>13</sup>C chemical shift spectra of solid mixtures via an inverse Laplace transform of the relaxation dimension. The resulting 2D spectrum of the mixture contains separate <sup>13</sup>C spectra for each component of the mixture that are identical to <sup>13</sup>C spectra of the isolated materials. The separation is based on the equalization of <sup>1</sup>H T1 values in a single domain by rapid <sup>1</sup>H spin diffusion and on the <sup>1</sup>H T1 value differences between different domains. The method is demonstrated on a mixture of two disaccharides and on a commercial drug containing several compounds.

# 59

## Phenolic Database of Experimental and Predicted $^1\text{H}$ -NMR Spectra

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NMR spectroscopy is the most selective technique for identification of unknown metabolites. Compared to MS, NMR suffers from relative low sensitivity especially of two-dimensional NMR-experiments needed for unique identification. This identification process can be accelerated when MS (MS/MS) and high quality  $^1\text{H}$ -NMR data are combined.  $^1\text{H}$ -NMR chemical shifts and  $^1\text{H}$ - $^1\text{H}$  couplings are very sensitive to changes in the 3D structure and the chemical environment. Here we present a database of experimental and predicted phenolic compounds based on high quality NMR data acquired under very well controlled identical conditions. The predicted proton spectra were generated from 3D chemical structures using the PERCH NMR Software trained on a set of experimental spectra. The structures were first optimized in three-dimensional space, and then the conformational space was mapped using Monte-Carlo/Molecular Dynamic analysis, and solvent effects were also taken into account. Equivalent and nonequivalent protons were automatically differentiated based on the symmetry of the molecule. By incorporating the stereochemistry, intra-molecular interactions, and solvent effects into the mathematical model, chemical shifts and couplings can be predicted with much greater accuracy. The predictive model will be used to extend the database with predicted  $^1\text{H}$ -NMR spectra of a vast number of phenolic compounds available from public resources. The  $^1\text{H}$ -NMR spectra and 3D Mol files are automatically annotated confirming the accepted in the literature atom labels. The experimental NMR data were completely analyzed, which makes the prediction very reliable and the database so valuable. A molecule is considered as a match if the mass is within 1-2 ppm deviation from the obtained from MS (and/or MS/MS) accurate mass and the predicted  $^1\text{H}$ -NMR spectrum correctly matches the  $^1\text{H}$  NMR experimental spectrum. Automated fitting of experimental and theoretical  $^1\text{H}$  NMR data is provided by PERCH NMR Software. Text searching, spectrum and structure querying, and data browsing tools are also provided.

# 60 NMR Spectroscopic Quantification of Free Formaldehyde in Aqueous Glues and Paint Resins

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The quantitative determination of free formaldehyde in industrial products (glues, resins, biocides, textile additives etc.) bases on chemical transformations like (redox)-titrations or derivatization with chromophors followed by photometric quantification. Such formaldehyde-consuming analytical methods usually result in too high findings as the "free" formaldehyde is always in equilibrium with the "bound" one (N-methylols, oligo-formaldehydes).

An appropriate method to investigate such products "tel quel" is the quantitative NMR spectroscopy with internal standard. By means of two examples, a coating resin etherified with methanol and an aqueous melamin-formaldehyde-glue the poster demonstrates how free formaldehyde can be quantified simply and safely in the <1%-region.

# 61

## Strong Coupling Effects in INADEQUATE

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The INADEQUATE experiment is enjoying revived interest, due to the fact that it can be done on a modern spectrometer with 10-20 mg of sample. The standard experiment on well-separated carbon lines is straightforward, but complications will occur. In a complex molecule, ambiguous carbons may well be close in chemical shift, leading to strong coupling effects. As well, long-range couplings may create unexpected correlations between carbons that are not directly bonded. There is already some discussion of these points in the literature, but we present a comprehensive picture of all of these unusual features, both from an experimental and a theoretical approach.