Meeting Review: The Chromatographic Society Spring Symposium: Analysis of Polar Molecules, AstraZeneca, Macclesfield, 21st and 22nd May 2014

A two day symposium focusing on the Analysis of Polar Molecules at AstraZeneca's Development and Manufacturing site at Macclesfield.The programme covered a diverse range of polar analyte classes and proposed multiple approaches towards the successful analysis of these types of molecules.

This meeting, which was organised by The Chromatographic Society in collaboration with the Society of the Chemical Industry (SCI), brought together renowned external speakers and delegates from academia, pharma and scientific instrument/technology companies alongside AstraZeneca colleagues spanning the Discovery, Development, and Operations divisions. It was attended by over 100 delegates and boasted around 20 vendors (Table 1) representing 12 companies (including the principal meeting sponsors Shimadzu) presenting the latest from their product portfolios making for a vibrant and informative atmosphere.

Polar molecules are not well retained on the most common hydrophobic liquid chromatography column types. This typically leads to poor chromatographic selectivity and resolution - and ultimately leads to drug and related impurity/degradent quantification issues. Accordingly the focus of the symposium was on the analysis of small polar molecules, with also a session on larger highly polar molecules.

Dr Paul Ferguson (AstraZeneca) opened the meeting and welcomed the audience on behalf of The Chromatographic Society and SCI. He also warmly thanked the vendors who provided financial sponsorship and support for the meeting. Paul then introduced Dr Simon Hartas of AstraZeneca who welcomed the audience on behalf of the hosts and provided a potted history of the local area and the development of AstraZeneca at the Macclesfield site.

The opening plenary lectures, chaired by Dr George Okafo (GSK and CSI), featuring analysis of polar chiral molecules were delivered by Professors Apryll Stalcup (Dublin City University, Ireland) and Wolfgang Lindner (University of Vienna, Austria). Professor Stalcup introduced her presentation, 'Implications of Chiral Separations for the Separation of Polar Organics' by advertising the International Symposium on Chromatography (ISC) which will be held in Cork, Ireland in 2016. This major international symposium, which she is co-chairing will be supported by The Chromatographic Society.

Company	Presenter and Title of presentation	Key products discussed
ARC Sciences	Simon Lambert - Rapid LC-MS of Un-derivatised Amino Acids and Dipeptides	Imtakt Scherzo SM-C18 and Intra-da amino acid columns
ATG Scientific	No presentation	
Chiral Technologies Europe	Dr Brian Freer - Enantiomer Separation of Polar Compounds by Chiral Chromatography	ChiralPak QN-AX, ChiralPak QD-AX, CrownPak CR-I, ChiralPak ZWIX columns
Crawford Scientific	Tony Taylor - A novel workflow solution for Antibobody Glycan Profiling	ThermoFisher Scientific GlycanPac AXH-1, AXR-1, Accucore Amide HILIC columns
Fortis Technologies	Mark Woodruff - Othoganol Selectivity to Solve Problems with Polars in Chromatography?	C18, H2o, diphenyl, HILIC, Speed-Core HILIC column
HiChrom	Gemma Lo - Using Silica Hydride technology to Break Traditional Limits in HPLC	MicroSolv Cogent Diamond Hydride, Cogent bidenentate C18, Cogent UDC-Cholesterol columns
International Lab Mate	No presentation	
Merck Millipore	Dr Rod McIlwrick - Further advances in ZIC-HILIC technologies and the use of ZIC-cHILIC columns in HPLC and LC-MS analysis of polar hydrophilic compounds	Sequant ZIC-HILIC & ZIC c-HILIC columns
Romil	No presentation	
Shimadzu (Principal Sponsor)	Dr Chris Titman - Traditional Approaches to Normal Phase Chromatography, how innovative software and innovative HPLC combinations can increase throughput	Nexera UHPLC LabSolutions and Open Solution software
Thermo-Fisher Scientific	Professor Tony Edge – The use of mixed mode ion exchange columns for the retention and separation of polar molecules	Acclaim mixed mode phases (WAX-1 and WCX-1), Trinity and Surfactant plus columns
VWR	See Merck Millipore presentation	
Waters	Dr Rob Frost - Approaches for Successful Polar Compound Retention Using Reversed Phase and HILIC	Acquity BEH Amide/ XBridge Amide, Acquity BEH HILIC/ XBridge HILIC, Atlantis HILIC, Cortecs HILIC columns

Table 1. Vendor organisations in attendance at the 'Analysis of Polar Molecules' meeting (listed in alphabetical order) and their presentation titles.

She introduced some of her early work on retention modelling [1] based on the 'slot model' of interaction on polymeric phases for polyaromatic hydrocarbons. Professor Stalcup then moved on to provide an introduction to the retention mechanism of hydrophilic liquid chromatography (HILIC) and chiral selectors for enantiomeric separations, particularly regarding her work with cyclodextrins [2]. Professor Stalcup then moved on to discuss her work on modelling retention for polar and ionisable molecules using linear solvation energy relationships (LSER) with a modified function to account for the degree of ionisation of an analyte with impressive accuracy on stationary phases containing embedded charge moieties [3].

The next speaker, Professor Wolfgang Lindner, opened his presentation, 'Enantioseparation of highly polar and ampholytic compounds: a challenge to be mastered', by discussing the thermodynamic equations related to chiral recognition mechanisms and the free energies of interaction for each of the different intermolecular forces involved. This led onto his group's development of chiral ion-exchange phases (Chiralpak QN/QD) and how resolution could often be reversed under the same conditions using the opposite quasi-enantiomer based on cinchona carbamate-derived anion exchangers and the separation of chiral bases on chiral sulfonic acid based stationary phases [4]. Professor Lindner then went onto discuss zwitterionic stationary phases (Chiralpak 'Zwix' phases - Figure 1) which possess very high specificity for zwitterionic compounds such as amino acids (with CAD detection [5]). Temperature effects on these phases were shown to obey van't Hoff kinetics. He also discussed at length how the bulk solvent (protic versus aprotic solvents i.e. methanol and acetonitrile) impacted upon retention in a HILIC-like fashion.

The symposium also included sessions dedicated to specific technique approaches for the analysis of polar molecules. For example, a session on super-critical fluid chromatography (SFC) included presentations from Dr Caroline West (University of O'rleans, France), Dr Jenny Kingston (Novartis, UK) and Dr Andy Poulton (AstraZeneca, UK). The speakers focussed on both the theoretical molecular diversity of compounds that can be analysed by this approach through to the application of this technology in Discovery and Development environments.

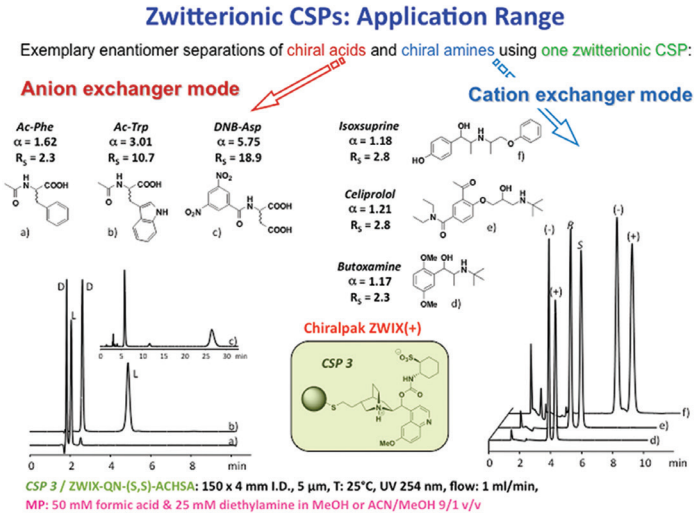

Figure 1. Exemplary enantiomer separations of chiral acids and chiral amines using a zwitterionic CSP as presented in Professor Wolfgang Lindner's lecture on 'Enantioseparation of highly polar and ampholytic compounds: a challenge to be mastered'. Reproduced with permission.
Conditions: ZWIX(+) CSP is based on quinine (QN) and (S,S)-ACHSA: 150 x 4 mm i.d., 5µm, Temper ature: 25°C, Flow rate: 1ml/min UV @254nm, Mobile phase: 50mM HCOOH & 25mM DEA in MeOH (left), ACN/MeOH 9/1 v/v (right).

Dr West's presentation on 'Where are the polarity limits for SFC?' started with an excellent overview of the technique and what exactly 'polarity' is and how it relates to linear solvent energy relationships using Abrahams descriptors [6]. She noted that contrary to popular belief, all stationary phases available to LC may be employed in SFC, and also additional SFC specific phases are available (and highlighted their characterisation [7,8]). She discussed how mobile phase density (and thus solvating power) of the fluid can be varied continuously by changing pressure and temperature up to a certain point, but highly polar molecules require a co-solvent (Figure 2). Dr West emphasised that CO_2 is miscible with all solvents, even water in small proportions allowing a wide variety of solvent polarities to be analysed, comparing SFC with HILIC separations for the same analyte types [9]. For analytes requiring methanol as a co-modifier for elution, Dr West explained how methanol can react with CO_2 under pressure to form methylcarbonic acid which has an apparent pKa of 4-5 and how this allows favourable analysis of acidic species without the use of an additive. She also indicated that amine additives may react with CO_2 to form carbamic acid which may potentially ion pair with positively charged analytes. She concluded her presentation with a discussion on the use of water as a mobile phase additive and how this can have a dramatic effect on the elution power of the mobile phase, even up to 30% (v/v) fractions (in combination with IPA which allows this volume fraction of water to be employed) for the elution of highly polar acids [10].

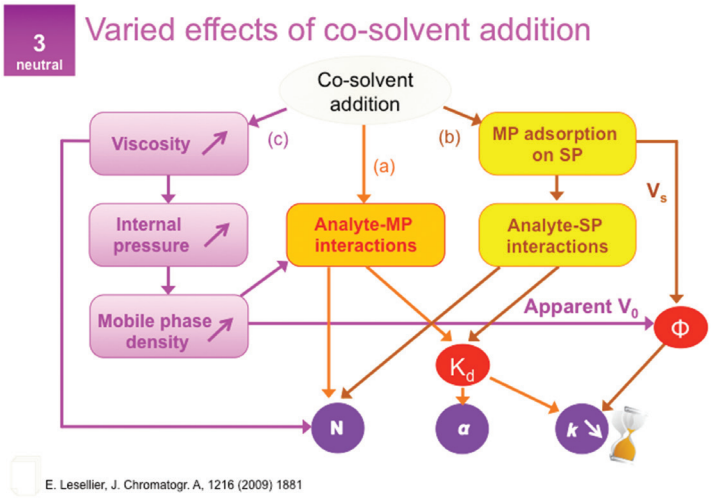

Figure 2. Effects of co-solvent addition in SFC for neutral ionisable molecules as described by Dr West in her lecture. Reproduced with permission.

The following presentation by Dr Jenny Kingston was more industrial applications orientated. Her experience was that SFC can provide similar performance to reversed-phase UHPLC with similar analysis times for the same sample set (different selectivity), but with a more diverse range of stationary phase selectivities. At Novartis, SFC is used as the primary technique for preparative chromatography as post-column solvent evaporation is easier. The stationary phases used in their generic screen are silica, amino, diol, DEAP and 2-EP (the latter now being replaced with a cyano phase) and have a customised algorithm for eluting the analyte of interest at the apex of a programmed gradient for optimal peak shape in preparative separations. The presentation was interspersed with examples illustrating the applicability of SFC for a range of differing polarity molecules. Dr Kingston also discussed the addition of small fractions of water to aid the elution of highly polar molecules such as nucleosides and nucleotides.

In the final presentation of the session entitled 'Adventures of an SFC - Applications to Process Development', Dr Andy Poulton described the use of SFC for chiral and achiral drug development activities. The first examples he demonstrated involved the coupling of chiral columns for the separation of chiral and achiral molecules in a particular drug sample and an achiral and chiral column for similar purposes. Dr Poulton reasoned that while SFC is very versatile, it is still a complementary technique to LC. However, his final example illustrated how SFC can be used for achiral analysis with increased resolution to a similar LC separation (2-EP column in SFC versus a Hypercarb column required for retention in HPLC). In his conclusions, he also noted the utility of adding water to the mobile phase for very polar analytes and also commented on the ability to easily transfer methods between Agilent Aurora and Waters UPC_2 SFC systems.

Another themed session was on LC approaches for the analysis of polar molecules. Dr Fiona Harvey-Doyle (Pfizer, UK) gave a fascinating insight into how electrochemical reaction cells could be used in the analysis of potential (polar) drug degradent species formed through on-line electrochemical cells and how this could be applied to the preparative isolation of small amounts of oxidised or reduced pharmaceutical material for method development activities. The use of an Antec ROXY electrochemical system allows the degradation of materials in a highly clean manner without chemical or enzymatic agents [11]. This was exemplified using glucose (which can be used as an excipient in drug formulations) which can degrade to 5-hydroxymethylfurfural (a potential genotoxic impurity) on heating. These degraded solutions were analysed using ion exchange (IC) chromatography with pulsed ampometric detection (PAD). Dr Harvey-Doyle then moved on to discuss a range of approaches for analysing polar molecules including HILIC, CE-MS and mixed-mode stationary phases [13].

Dr James Heaton (University of the West of England, UK) then presented his work on the 'Investigation into the Efficiency and Optimal Operating Conditions of Hydrophilic Interaction Compared with Reversed-phase Liquid Chromatography'. He showed that for the same analyte with the same retention factor (nortriptyline) under reversed-phase (RP) and HILIC that the system back pressure was much lower for HILIC. Comparison of nortriptyline through van Deemter plots showed similar performance in both HILIC and RP with the same Zorbax substrate (but silica and C18 for HILIC and RP respectively), but the van Deemter B term observed in HILIC was significantly lower - which could be attributed to diffusivity of the analyte in the less viscous mobile phase. He then went on to discuss the need to determine the effective diffusion coefficient to help normalise the data to make a more objective comparison. These measurements were achieved via a peak parking method approach using a novel dual column set-up. This reduced pressure perturbations and led to more precise measurements of the diffusion constant.

Dr Heaton then went on to discuss the influence of surface diffusion (the molecular migration of adsorbate molecules in the vicinity of the surface while remaining under an adsorbed state) which is thought to be significant in axial diffusion behaviour [14]. He then talked about particle morphology and if it had an influence on axial diffusion in HILIC when surface diffusion is limited for sub 2-micron particles (including fused core particles). He identified that the b term was found to be comparable on all phases illustrating that morphology doesn't influence analyte diffusion behaviour in HILIC. He completed his presentation by illustrating the dramatic changes in peak shape and retention encountered in HILIC due to variation of buffer concentration, even over a relatively small range of 1-8 mM. With neutral analytes, an increase in buffer concentration resulted in increased retention which was thought to be due to an increased water layer thickness, but with bases decreased retention is observed and this was postulated to be due to buffer ion competition.

The final presentation in the session was given by Tim Underwood (GSK, UK) who discussed his group's strategy for the rapid analysis of polar molecules synthesised in Discovery at GSK, Stevenage – 'Generic Approaches to the Analysis and Purification of Small, Highly Polar Molecules'. Their eight open-access analytical reversed phase UHPLC instruments at Stevenage perform around 16,000 individual analyses every month with a 95% success rate, providing the time for the analysts to focus on more difficult separation challenges. Equally, their generic six open-access mass-directed preparative instruments perform around 900 individual purifications every month. For analytes that fall outside traditional RP analysis, alternative approaches to analysis and purification of molecules are used. These include HILIC, aqueous normal phase (ANP) chromatography with a Diamond Hydride phase, mixed mode phases (Primesep 200 column) in RP and ion-pairing systems. The mixed mode column and ANP system are also available in preparative scale. SFC is also utilised using a generic system of a Luna HILIC (diol) column and MeOH modifier containing 20mM ammonium formate

The final themed session addressed the analysis of oligonucleotides. These highly polar, high molecular weight molecules are being investigated across a wide range of therapeutic areas by many pharmaceutical companies.

First up, Dr George Okafo (GSK, UK) who is heavily involved with GSK's oligonucleotide development strategy, gave an excellent overview of the major classes of oligonucleotides, their mechanism of action and the known compounds in development. He went on to describe the analytical challenges with these molecules. Not only are they highly polar, they are difficult to synthesise and impurities proliferate with each phosphoramidite added (amino acid subunit). The techniques utilised to analyse these types of molecules include:

- Reverse Phase Ion Pairing UPLC (RP-IP-UPLC)
- Anion Exchange HPLC (AEX)
- Size Exclusion HPLC (SEC)
- Capillary Gel Electrophoresis (CGE)
- 1D NMR spectroscopy (1H & 31P)

Overview of Different Separation Methods

Separation Method (denaturing)	Try first for separations of:	Pros & Cons	Comments
Anion Exchange (AEX)	• PO impurities • impurities of different charge	High capacity scalable to preparative scale	Elution order often dependent on base composition
Ion Pair-Reverse Phase HPLC (IP-RP-LC)	• Impurities containing (lipophilic) modifications • Shortmers • Longmers	Very high resolution possible	Compatible with ESI-MS
Capillary Gel Electrophoresis (CGE)	• Shortmers • Longmers	Very High resolution possible	Used as an orthogonal test Not compatible with MS

Separation Method (non-denaturing)	Try first for separations of:	Pros & Cons	Comments
AEX	• Multimers, Duplexes and Monomers	High capacity scalable to preparative scale	Elution order often dependent on base composition
IP-RP-LC	• Duplexes and Monomers Duplex variants	Very high resolution possible	Compatible with ESI-MS
Size Exclusion Chromatography (SEC)	• Multimers, Duplexes and Monomers • unstructured monomers from hairpin structures	Physiological buffer conditions possible Low capacity	Elution according to hydrodynamic size

Figure 3. Chromatographic approaches for the separation of oligonucleotides as presented by Dr Okafo. Reproduced with permission.

and have various pros and cons associated with them (Figure 3). Dr Okafo highlighted these approaches with a range of examples e.g. see reference [15]. He concluded his talk by highlighting a key text in this area for those interested in learning more about this complex area of analysis [16].

On the same theme, Laurent Joron (Dow Chemical, France) concentrated on the use of LC polymeric stationary phases. He opened by discussing the synthesis of divinylbenzene polymeric stationary phases and their physical characteristics, particularly their wide pH stability. The ability to synthesise these materials in a simple manner and a range of formats was discussed e.g. different structural properties such as surface area, porosity, swellability etc and different selectivities such as ion-exchange, affinity or simple hydrophobic interactions. When used for purification, these phases have very high capacities with Amberchrom CG161 a popular choice for small molecule purification and Amberchrom XT for polypeptides and molecules of similar size.

Also presenting in this session was Dr Elena Bichenkova (University of Manchester, UK) who gave an eloquent account of the use of LC-NMR for analysing these oligonucleotide molecules and synthetic analogues. She highlighted the principal issues using NMR to analyse these molecules which were (i) the large number of protons, some of which display a close similarity in chemical shifts, (ii) the molecules often produce highly overlapping resonance regions in the 1H NMR spectra, and (iii) the interpretation of spectra can be a challenging task, especially without implementing 2D NMR techniques. However, more recently the potential of HPLC-NMR for the characterisation of oligonucleotides has been extended by the development of powerful solvent suppression techniques and the use of homo- and heteronuclear 2D correlation NMR techniques. The use of COSY (COrrelated SpectroscopY – see Figure 4) and NOESY (Nuclear Overhauser Enhancement SpectroscopY) are the most common ways to elucidate oligonucleotide structure. Some of the challenges associated with LC-NMR are that proton carrying solvents of the mobile phase generate strong resonance signals and the cost of fully deuterated solvents is expensive (except for D2O). To compensate for this, strong signals of the mobile phase can be selectively suppressed by pulse sequences (including multiple solvent suppression). NMR pulse sequences are also available for the selective suppression of water signals without considerable distortion of other spectral regions.

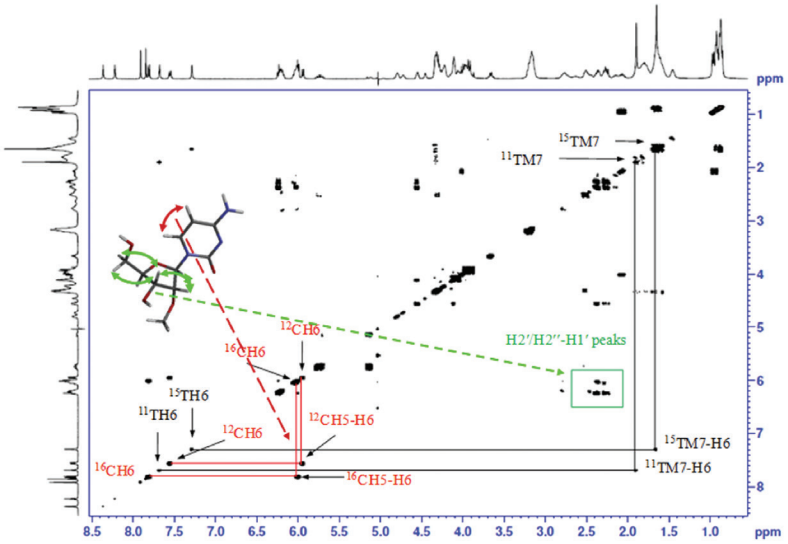

Figure 4. An example COSY spectra of an oligonucleotide analogue as presented by Dr Bichenkova. The structure shown on the spectra represents only one building block (i.e. cytidine) of the entire sequence and not the whole oligonucleotide conjugate. Reproduced with permission.

The final presentation in the session was given by Dr Cari Sanger-van de Griend (Kantisto, The Netherlands). This was on the application of capillary electrophoresis (CE) in the pharmaceutical industry. Dr Sanger-van de Griend introduced the basic theory and practice regarding CE and then discussed common application areas such as counter-ion quantification, micellar electrokinetic chromatography (MEKC) for the analysis of combination drug formulations [17], chiral enantiomeric excess determination [18] and pKa determination. She then went on to highlight the various modes of this technique which may be used with biomolecules e.g. capillary gel electrophoresis (CGE).

The invited speaker programme was interspersed with presentations from a number of vendor companies. Their presentations (Table 1) mainly covered the use of different liquid chromatographic column technologies for analysing polar molecules. The vendors were also present in the exhibition space where there representatives were on hand to discuss their latest product ranges and offer technical advice.

The meeting received excellent feedback from delegates, presenters and vendors. The success of the meeting illustrated the interest in this area of research and the meeting helped provide many solutions to the challenges of analysing polar molecules. The Chromatographic Society Spring Symposium will focus on the analysis of biopharmaceuticals next year and will be held at the AZ MedImmune site in Cambridge on the 12th-13th May 2015.

Figure 5. Delegates conversing during the interval between scientific presentations

Figure 6. Delegates and presenters socialising at the symposium dinner where International Labmate sponsored the pre-dinner drinks.

References

1. A.M. Stalcup, D.E Martire & S.A. Wise, J Chromatogr. A 442 (1988) 1

2. A.M. Stalcup & K.H. Gahm, Anal. Chem., 68 (1996) 1369

3 P R. Fields, Y. Sun & A.M. Stalcup, J. Chromatogr. A 1218 (2011) 467

4. C.V. Hoffmann, M. Laemmerhofer, W. Lindner J. Chromatogr. A 1161 (2007) 242

5. A. Gargano, M. Kohout, P. Macíková, M. Lämmerhofer, W. Lindner Anal Bioanal Chem (2013)

6. M.H. Abraham et al., J. Org. Chem., 65 (2000) 7114

7. C. West & E. Lesellier, J. Chromatogr. A, 1191 (2008) 21

8. C. West & E. Lesellier, J. Chemometrics, 26 (2012) 52

9. A. dos Santos Pereira et al., J. Sep. Sci. 33 (2010) 834

10. C.H. Arnaud, Chem. & Eng. News 92 (2014) 10

11. A. Baumann & U. Karst, Expert. Opin. Drug Metab. Toxicol. 6 (2010) 6

12. Z. Jiang, N.W. Smith, P.D. Ferguson & M.R. Taylor, J. Sep. Sci. 32 (2009) 2544

13. A. A. Kazarian, M.R. Taylor, P.R. Haddad,. P.N.Nesterenko, P. Brett Analytica Chimica Acta 803 (2013) 143

14. K. Miyabe & G. Guiochon. J. Chromatogr. A 1217 (2010) 1713

15. S. Li, D-D. Lu, Y-L. Zhang & S-Q. Wang, Chromatographia 72 (2010) 215

16. G. Okafo, D. Elder and M. Webb (Eds) Analysis of Oligonucleotides and Their Related Substances (2013) ILM Publications, St Albans.

17. K. Persson Stubberud and O. Åström, J. Chromatogr. A 826 (1998) 95

18. C.E. Sänger – van de Griend et al., J. Pharm. Biomed. Anal. 41 (2006) 77