MULTI-DIMENSIONAL CHROMATOGRAPHY

29TH OCTOBER 2019

ChromSoc
The Chromatographic Society - Founded 1956

Jealott's Hill International Research Centre

syngenta

Jealott's Hill International Research Centre
Bracknell, Berkshire
RG42 6EX, United Kingdom

A one-day symposium that explored the latest techniques in multi-dimensional chromatography.

Leading specialists from industry and academia explained how they apply the latest 2-D techniques to achieve very difficult separations in the liquid, supercritical fluid, and gas phases.

Instrumentation and consumables were displayed in a comprehensive supporting exhibition from relevant manufacturers.

EXHIBITING AND LECTURING COMPANIES

syngenta

SHIMADZU
Excellence in Science

NOVARTIS

SepSolve
Analytical

Agilent Technologies
Innovating the HP Way

anatune
Collaborate • Innovate • Automate

VWR
We Enable Science

rssl
science with service

BioPharma

HiChrom

crawfordscientific

ThermoFisher
SCIENTIFIC

DAICEL

SCIEX
The Power of Precision

ATG Scientific Ltd

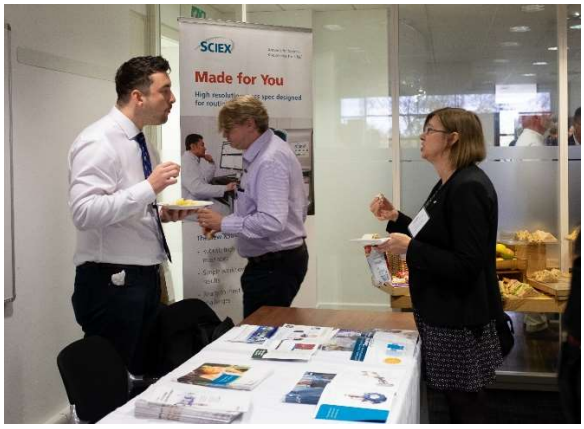
representing IonBench, MS Noise, Reichert Inc.

and other specialist manufacturers

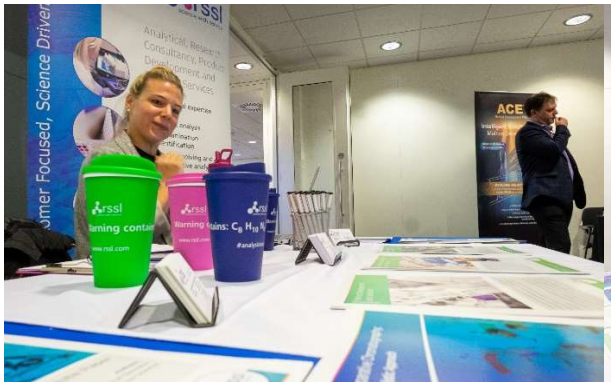
DR. RAYMOND WONG {SHIMADZU}

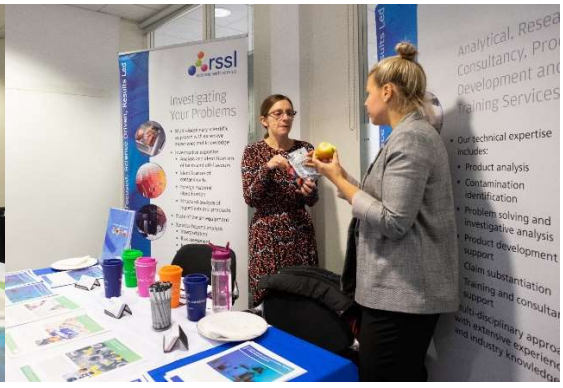
AN ATTENTIVE AUDIENCE IN A FULL LECTURE THEATRE

A FULL LECTURE THEATRE



SCIEX EXHIBITS





RSSL EXHIBITS

SCIENTIFIC REPORT FOR PUBLICATION IN CHROMCOM

An overview of the importance and utility of multi-dimensional separation methods.

Multidimensional Liquid Chromatography: a growing addition to separation science technology.

By Christina Jayne Vanhinsbergh, PhD researcher in 2D-LC, University of Sheffield.

Many industries rely heavily on liquid chromatographic separations for the analysis of molecular products, active pharmaceutical ingredients, reaction components and toxic substances. Improvements in stationary phase chemistry, along with reduction in particle size has helped to increase selectivity and peak capacity dramatically over the past decade.

A drawback for many separations, however, is that molecular species analysed today are structurally and physiochemically closely related. This, along with biased separation mechanisms, can lead to co-elution or reduced resolution of analytes.

Multidimensional chromatography couples orthogonal modes of separations to overcome challenging analyses. Orthogonal separations can pre-treat samples, simplify complex chromatography, as well as resolve co-eluting species. The technique is developing into both comprehensive and selective approaches, suited to the analytical drivers of separation.

Multidimensional liquid chromatography also has the potential for reducing reliance on technologies requiring highly skilled analysts, such as mass spectrometry - as separation of critical species reduces the requirement for targeted mass detection over a peak. In workflows where mass spectrometry is utilised, increases in peak capacity and resolution can improve quantitative analysis.

Multidimensional liquid chromatography presents challenges to the analyst, which require a detailed understanding of the mobile and stationary phase chemistries, potential analyte interactions and what factors can detrimentally effect separation (such as miscibility of mobile phases, dilution and

sensitivity). The challenges described, indicate that optimisation of the technique must be approached with due diligence to achieve the benefits of peak capacities ranging into the thousands. Multidimensional liquid chromatography could be applied for food, cosmetic, pharmaceutical, chemical and environmental analysis. A search for available literature documents evidence of its growing use in aforementioned industries, as is the growing number of chromatographic conferences dedicating presentation space to researchers in the field

---

"GC^2 Made simple - Comprehensive two-dimensional GC using a Thermoelectrically cooled modulator
Kathy Ridgway ANATUNE
Kathy Ridgeway unfortuately was unable to present her lecture.

"The latest innovations in multi-dimensional GC software: What can ChromSpace do for you?"
Dr Laura McGregor Product Marketing Manager, SepSolve Analytical Ltd

SepSolve Analytical are instrument and solutions providers who are part of the Schauenburg Analytics group alongside Markes International
Whilst Markes are known for their world leading thermal desorption technology and sample introduction techniques, SepSolve are more concerned with the separation, detection and data analysis. They work closely together and are often utilise Markes TD or Centri systems for solutions.

ChromSpace is a software platform that can offer both instrument control (for a selection of GC systems, as well as data processing. It is also available as a standalone processing software tool. It has been designed for GCxGC data processing but has not been limited to only GCxGC data – 1D GC files can also benefit from the workflows and features of ChromSpace. ChromSpace is also compatible with third-party data – not just data generated by SepSolve's hardware solutions – so you can benefit from the wide range of qualitative, quantitative and comparative analysis features for a wide range of datafile formats. It is easy to use ChromSpace across multiple systems or sites, by introducing network licencing to allow a single dongle to be connected to your company's server and then users can sign-in to ChromSpace without the need for an activation dongle on their own PC. Additional licences can be added

easily and updates can be done through a remote connection by the support team.

What is 2D GCxGC and how can it give an extra level of information?
In a conventional GC separation – you choose a column with the stationary phase that's needed to separate your compounds of interest.
But, if it's a complex sample then there will often be co-elution of compounds – regardless of the mechanism you choose to separate them by – for example by their size or by their shape.

There's always a compromise. However, in GCxGC – you have the ability to separate the components using a second mechanism – so for example – both size & shape can be used, thus giving improved separation of the mixture
The software takes the modulated sub-peaks from the linear data and stacks them side by side to construct 3D peaks – these are typically visualised with a more pleasing colour gradient applied to them in the form of a surface chart.
The peaks can also be viewed from the top (in a bird's eye view) as a colour plot, where individual spots represent the peaks and a colour gradient shows peak intensity.

ChromSpace has been designed for GCxGC data processing but it's not limited to only GCxGC data – 1D GC files can also benefit from the workflows and features of ChromSpace. It's not just for hardware supplied in SepSolve solutions – such as the BenchTOF mas spectrometers – we can also use ChromSpace on a range of different data file formats– including mass spectrometers ranging from simple single quadrupoles, to time-of-flight and high-resolution systems.

It can also process data from a wide range of single-channel detectors – including FID, ECD and SCD – and they have already provided a number of full GCxGC solutions using these types of detector. LCxLC data can be processed too.
When opening a datafile we can see the many file formats supported by the software for both 1D GC and GCxGC. When we open a file for the first time it opens in the linear (unfolded view) but then we can simply click to switch to the colour plot.
Modulation period can be changed easily without having to re-import the file and the phase shift can be changed to alter the display to the user preference and help to display wrapped peaks.

The main view in ChromSpace shows that the colour plot is linked to the true chromatography, with linear cross-sections displayed in panels. The view is entirely flexible, with panes that can be collapsed/expanded or undocked and moved to a second monitor (if available) and the cross-sections can be hidden if not required.

The colour settings can be set to normalise to the largest peak when you zoom in on an area so you don't have to re-colourise to see trace peaks.

We can even create 3D surface charts of the TIC and the new EIC chromatogram. These are all dynamically linked so that when we zoom in on one, all others are updated too. We can even view the 3D charts stacked together by dragging and dropping them into the same window.
Instead of typing out multiple EICs every time – they can be saved as named sets for huge time savings.

ChromSpace isn't just for GCxGC we can also open 1D GC data.

A GC-TOF MS data file (using BenchTOF MS) for a flavoured e-cigarette liquid was shown – but it could be any of the file formats mentioned previously – .D, .raw, .cdf etc. The file is opened in the same way as GCxGC data and benefits from the same features. When we integrate – the settings are specific to 1D data – none of the extra features for GCxGC are shown.

We can choose to open multiple files too in the same window for quick visual comparisons. Here we can use stencil regions – drawn on top of the chromatogram – to classify the different groups and get area % values.
Once the quant method is saved, we can use it to run a batch sequence
The results can then be opened in our results browser – here we can see all the quant info at-a-glance – with target compounds on the right, the datafiles we've processed in the top panel, the Cal curve in the middle panel and the graphics showing the target peak for that sample and its spectrum at the bottom.
The flagging system shows any issues with the calibration – for example, if a peak had not passed the set qualifier ratios (or any of the suitability parameters that had been used) it would be flagged by a red cross. We have green ticks for indicating passes.
We can add in extra samples from this window and we can export all results to csv or to LIMS, or as a formal report.

Five different crude oil samples were run by GCxGC-TOF MS.

A stencil was created to allow target chemical classes to be compared in ChromCompare. ChromCompare does pairwise comparisons of datafiles and gives a match factor to describe the similarity/difference between datafiles (the higher the match factor, the more similar the samples (values between 1-1000)).
We also looked at a cannabis extract and attempted to identify PCBs using a more complex scripting filter.

To Summarise, ChromSpace provides:
Intuitive processing for both GC and GC×GC data
Fast data navigation with customisable, dynamically-linked charts, tables and spectra
Greater flexibility through multi-vendor compatibility – import various datafile formats.
Fast and easy quant workflows – for single-channel and MS (EIC) data.
ChromCompare for simple & objective comparisons.
A retention indexing scheme for increased confidence in identification.
Simple scripts for fast and confident identification of target compounds/classes.
Network licensing to unify lab processing easily.

"Development of Two-Dimensional Supercritical Fluid Chromatography for highly polar aqueous samples" Raymond Wong, Shimadzu

SUMMARY
Raymond Wong took on an interesting self-imposed challenge to explore the use of 2D SFC chromatography for separation of polar compounds.
Conditions were explored to retain, pre-concentrate and solvent-switch highly POLAR aqueous samples for online SFC analysis

A somewhat different approach strategy was explained and pilot trial analyses reported. The presentation was intended to stimulate interest with a view to perhaps collaborate on new ideas.
Dr. Wong started by explaining the principles of SFC.
The properties of supercritical fluids were explained and their unique selectivity and retentivity of SFC compared with LC in a multi-residue pesticide screen application.

Access to an extremely broad mix of stationary phases to analyse a spectrum of both hydrophilic and hydrophobic compounds is required to offer

Improved selectivity and hence improved resolution.

CHALLENGES

Polar compounds prefer an aqueous medium to remain soluble.
However, unfortunately Supercritical fluid CO2 is not very miscible with water
In order to Analyse aqueous samples Wong utilises
1D: Online retention, pre-concentration, solvent switch and elution
2D: SFC analysis
This approach was demonstrated to separate vitamin B3 from other polar molecules.
LOADING, ELUTION, ANALYSIS AND 1ST DIMENSION, HEART CUT,
A methodology for water soluble vitamin analysis, e.g. Niacin (Vitamin B3)
Niacin may help lower cholesterol, ease arthritis and boost brain function
Separation of niacin from ascorbic acid and succinic acid was shown.

Other polar compounds investigated by this methodology were#
#

Compound
3-Nitrophenylhydrazine
Adenosine triphosphate
Ascorbic acid
GABA
Glyphosate
Inosine 5'-monophosphate
L-Asparagine
Nicotinic acid (Niacin)
Salicylic acid
Succinic acid

The separated compounds were shown in a table with the corresponding separation conditions and resulting chromatograms.
Wong's lecture was inspiring and showed the challenges in using a multidimensional approach to SFC .

"Multi-dimensional liquid chromatography: from small molecules to large particles and from method development to data analysis" - Bob Pirok (Van 't Hoff Institute for Molecular Sciences University of Amsterdam)

Dr. Pirok provided an overview of the current state of two-dimensional liquid chromatography. Through application of two-dimensional LC, high peak capacities may be obtained. Both in heart-cut mode as well as comprehensive mode new information may be obtained by combining two orthogonal (sufficiently different, non-correlated) separation mechanisms. Pirok then proceeded to identify four challenges/opportunities also referred to in his presentation as "encumbrances".

Analytical: One critical challenge is the limited sensitivity due to the additional dilution of the analyte matrix by using a second separation dimension. The MS now must work harder to detect something. In addition, solvents used in the first dimension may not be entirely compatible in the second dimension. Active modulation techniques, such as stationary-phase-assisted modulation (SPAM) or active-solvent modulation (ASM) have recently been introduced to (partially) circumvent these issues.

Data: With the introduction of two-dimensional LC, nowadays often coupled with high-resolution MS, the size of datasets grows rapidly indeed. From a chemometric point of view we thus must start to think how to rapidly and efficiently extract the useful section that we want. Pirok warned that our community should stop for a moment with instrumental and chromatographic development and stop coupling always the most powerful detector to the separation system. Datasets are already too complex; this is where we need to focus now.

Instrument: As we increase the complexity of our chromatographic system, we also require more time to develop the method. This is often experienced as a burden in industry as a 2D-LC method may easily take months to develop, in contrast to the weeks for 1D-LC. He showed that here computer-aided method development may offer refuge. Recent publications have applied this and shown its feasibility.

Sample: Current 2D-LC systems use the modulation interface to simply transfer the analyte-containing effluent from one dimension to another. But what if we use this time to our advantage and carry out a reaction? He showed that in Amsterdam they work on light-induced, enzymatic, chemical

and microbial degradation techniques to be used as “reaction modulators” within 2D-LC systems.

In the MANIAC project, completely different and (seemingly) incompatible separation mechanisms are compared into a single highly efficient and extensively optimized instrument. Hence the name “Making Analytically Incompatible Approaches Compatible”. In MANIAC, various chemical, physical and microbial processes are integrated with (multi-dimensional) separation systems.
Amongst the investigated applications is the characterization of complex polymeric nanoparticles encountered in coating formulations and drug-delivery systems. These complex samples feature a multitude of sample dimensions, such as the particle-size distribution, the surface composition and charge, and the molecular weight and chemical composition of the constituting molecules including its active-ingredient if applicable. A successful technique for the separation of complex mixtures is comprehensive two-dimensional liquid chromatography (LC×LC).
Comprehensive two-dimensional liquid chromatography is indispensable for the separation of complex mixtures. In principle, the development of an LC×LC method requires establishing two separation dimensions with vastly different (“orthogonal”) selectivities. However, with the advent of state-of-the-art instrumentation for LC×LC, the number of options to realize and optimize LC×LC separations is increasing dramatically. Advanced modulation interfaces have significantly reduced the threats of solvent incompatibility and limited detector sensitivity in the comprehensive mode (LC×LC). However, these developments are accompanied by an increase in the complexity of the system and, thus, the time required for method development.

We recently demonstrated a proof-of-principle MANIAC system, which combined a separation of particles in aqueous hydrodynamic chromatography with a fast separation of the constituting polymers by organic size-exclusion chromatography. The developed method featured a novel implementation of intermediate sample transformation and now has been expanded to allow even hydrophilic and charged particles to be modulated. In addition, we focus on the use of capillary and chip-based microreactors to improve the applicability and flexibility of the overall potential of LC×LC separations towards drug-delivery particles.
In this presentation, these and other developed methods for large and small molecules will be shown.

Moreover, great attention was given to the feasibility of 2D-LC as a technique in the industrial routine lab. The development and application of method-development and data-analysis tools to facilitate easy and flexible use of 2D-LC was demonstrated.

"2D-LC in Pharmaceutical R&D. Applications beyond increasing peak capacity"

Adrian Clarke, TRD and CHAD Analytical Network Leader
Novartis Pharmaceuticals, Basel, Switzerland

Abstract

Conventional liquid chromatography (1D-LC) is not always capable of effectively resolving complex samples. This limitation is not solely due to the lack of column efficiency, but is pre-dominantly due to insufficient chromatography selectivity and the need to separate the analytes of interest by utilising orthogonal retention mechanisms. These limitations are the main reason, two-dimensional liquid chromatography (2D-LC) is continuing to attract much interest for its markedly higher resolving power compared to 1-D separations. This presentation will discuss the benefits of 2D-LC, and show examples of multiple applications of (multiple heart-cutting) 2D-LC in pharmaceutical analysis. The presentation will demonstrate 2D-LC is not only beneficial to increase peak capacity, but also to support process and product development and investigations. Applications include: tracking of the various peaks of interest across 2 different methods (e.g. method transfer from an MS incompatible buffer to an MS compatible buffer). A second application using 2D-LC-SPE-MS for on-line enrichment of impurities peaks to facilitate improved impurity identification. The third application supports method development by confirming peak purity using orthogonal stationary phases in the 1st and 2nd dimensions and can be was used to investigate mass balance. Further applications demonstrate the use for the analysis complex pharmaceutical molecules and drug delivery systems (e.g. new modalities). The talk will finish with a summary on the challenges and future outlook for 2D-LC in the regulated pharmaceutical industry.

Dr. Clarke started his lecture by rhetorically asking the following question;

<u>Why bother with 2D-LC</u>?

WHAT IS IN THE CHROMATOGRAPHER'S TOOLBOX?

We need a wide range of chromatographic modes/techniques & detectors to cover a diverse range Pharmaceutical substances & products.

• Method orthogonality ensures confidence in methods and products.

• RP LC-UV is valuable, but other modes & 2D LC, SFC, MS & universal detectors are beneficial.

There is a theoretical motivation for 2D separations.

• 2D LC = higher cost & complexity, (in)compatibility, time consuming, unproven performance, robustness and possibly regulatory acceptance (?).

• Limitations of 1D-LC, since 2D offers more resolving power (+ different modes of 2D-LC, MS etc)

2D LC: Offers improved quality & understanding.

• Recent improvements in commercial 2D instrumentation are now available

– Now commercial state-of-the-art equipment is used in Pharma Industry.

– Previous practical problems now appear to have solutions, have become robust & are no longer "academic".

– Not yet a routine QC technique though: data analysis is not as simple as conventional LC; peak volume (not area), requires special software, and expertise needed.

– But evaluations in Novartis show analysts can learn and use it with training.

• The 2D-LC is a novel "state of the art" technology, & a key strategic tool to support development projects (non-GMP).

– Support analytical method, process and product development.

– Method development finalisation (peak purity confirmation, e.g. stability indicating methods).

– Peak alignment/tracking between 2 methods.

– DS-DP method alignment.

– or MS method transfers (MS unfriendly MPs → MS unfriendly MPs)

– Detailed product characterisation: selection of starting material suppliers/qualities

– Investigational work

– Complex molecules

– mass balance issues

– Impurity/degradant identification

Method development: 2D LC

• Many challenges and (interlinked) considerations...

• Which modes to employ (solvent and retention compatibility)?

• Isocratic vs gradient & gradient range(s) in 2nd Dimension

• Short gradient better than Isocratic in 2nd Dimension
• So far we haven't used focused gradients
• Which column dimension in 2nd D?
• Overload issues frequently observed with lower i.d. columns (e.g. 2.1 mm)
• Peak fronting/splitting
• Improved by at column dilution, active solvent modulation (ASM) etc...
• Which Stationary phase?
• "Orthogonal" separations RP-RP are 1st choice
• Base 2D column on the physicochemical properties & nature of the analyte(s)
• Base on potential interactions of your compounds, then choose 2nd D with different interaction mechanisms
• simplified if 2nd D column is more retentive than 1st D
• Use column (selectivity) databases to compare columns selectivity
• Euerby Petersson-extended Tanaka: https://www.acdlabs.com/resources/freeware/colsel/
• FS factor of columns ("orthogonal" when > 50): http://www.hplccolumns.org

• Other parameters (temperature, flow cell volumes etc.

SUMMARY

2D LC is an extremely useful tool in Pharmaceutical R&D and is here to stay: it offers multiple benefits & application areas
– 2D LC can provide additional information not provided by conventional approaches (e.g. detect additional impurities)
– Multiple application areas:
– Method quality (peak purity), Characterisation or materials
– Method comparison/peak alignment, salt swapping /desalination for MS
– Technology, software and workflows and "know how" are improving, quantification is now possible
– Still questions about or challenges:
– cost, ease of use & need for expertise
– CDS control (MS may need 2 PCs), need further development of user-friendly chromatographic software
– Simple general method development concepts and covering more chromatographic space
– Challenges matching different modes, mobile phases and pHs and column dimensions.
– modulation techniques are improving the situation
– similar challenges for trapping, elution for desalination/enrichment
– more orthogonal modes and techniques needed (inc. 2D SFC X LC)

– Robustness: regulated QC applications to date are limited, particularly in small molecule analysis
– Increased use in Pharma will be driven by the need (complex molecules/products/stages)
– analogous to Biopharma (4D-LC/MS), includes Q.

Dr. Clarke illustrated many of the points in his arguments for employing 2D LC with numerous examples and chromatograms.

"2D-LC coupled with radio-detection: applications for chiral agrochemical analysis" - Dr Mark Garrod (Syngenta, Jealott's Hill, UK)

Evaluation of 2D-LC for Product Metabolism and Analytical Sciences (PMAS).
PMAS are required to track the degradation of active ingredients in various environmental test systems for regulatory purposes.
Often trace levels of compounds need to be analysed in complex mixtures for both quantitative and qualitative purposes.
A range of environmental matrices was tested, each with diverse properties.
Products were radiolabelled in metabolically stable parts of the molecule so that degradation could be tracked quantitatively.
E.g. HPLC with radio detection (β-ram, to detect beta particle radiation) was the primary analytical tool for the majority of PMAS studies, as this is a powerful tool for separating degradation products in low level biological samples and accurate quantitation of each peak.
Heart-cutting 2D-LC allowed for more advanced separations such as:
Chiral chromatography, polar metabolite analysis, peak purity and LCMS.
Despite definite advantages there are drawbacks to using radiolabelled test substances that make 2D-LC challenging: Comprehensive 2D-LC is not currently feasible.
A residence time of β-ram needs to be at least 6 seconds for accurate quantitation (>time = <LOD). Columns with larger internal diameter are used (usually 4.6 mm) in order to allow enough time to capture enough data points across each peak without altering the standard β-ram setup. Peak width tends to be between 0.5 and 1 minute.
Methods tend to be longer, reversed phase, generic gradient elution (typically 1 hour).
As complete metabolic profiles are not known until the end of studies, gradients are often developed before many analytes are known.
Large heart-cuts are needed.
Specialised software availability and compatibility were discussed.
A range of testing was proposed to scope feasibility

In order to see if 2D-LC would be compatible with PMAS some initial testing was conducted on a system which was already available in collaboration with Rianne Van Outersterp (MSc student):

2D-LC can give adequate separations and as a minimum will solve immediate needs for high throughput chiral analysis of samples.
Consideration is needed for method compatibility but the testing conducted showed surprising robustness to heart-cut size. Basic method development may be required to optimise methods.
Useful information on system configuration and setup was gained by having the opportunity to run some basic tests.

Benefits:
Previously fraction collection, re-concentration, confirmation of correct peak collection and finally chiral analysis would be required – at least 2 to 3 days/sample (best case). All 2D analysis above was conducted in 3 days.
If methods are available it is straightforward to heart-cut a different peak.
Majority of endogenous material is not transferred to a second gradient – extended column life.

Learnings:
Endogenous material can affect some elution times significantly. Crop samples eluted approximately 1 minute later than all other samples.
Software isn't very intuitive and requires some patience.

Conclusions:
In the examples presented similar size (by time) heart cuts were taken but as the flow rate was half the original gradient, half the amount of material was heart cut. Smaller loop sizes could be investigated to further improve 2D chromatography.
Transferring methods to faster more UHPLC style gradients can improve throughput and 2D chromatography but there is a need to be cautious of 1st dimension results.
Gradients can be manipulated to obtain better peaks for heart cutting – this could be especially beneficial for polar regions.
We are looking for further chiral methods to set up and run – high throughput so ideal for early stage scoping projects where multiple samples can be run.
Chiral metabolites – New guidance on chiral metabolites just released indicating the same principles apply as for parent. Implications – far more chiral separations will need to be run.
Further investigation into loop size and first dimension gradient adjustments.

Investigate using for polar material compound producing a large level of polar multiple components can be heart-cut onto a polar method.
LCMS separations – both for better initial ionisation conditions and a two method clean-up step.

Acknowledgements:
Simon Perry, Kevan Richardson, Sarah Webb, Aniko Kende, Camilla Black and Pablo Navarro at Syngenta
Rainne E. van Outersterp, Radbound University

"Development of a screening method for peak purity assessment by 2D-LC". - Jeorg Weber (Syngenta, Münchwilen, Switzerland)

Overview
Why employ 2D-LC?
Peak purity assessment of LC methods which are relevant for product registration (→ authorities) usually performed with LC/MS
The limits of LC/MS: co-eluting isomers, small peaks co-eluting with active ingredient.
2D-LC might be complementary to LC/MS
LC application to be tested is set up in 1D

Combination of achiral and chiral analysis
chiral purity of selected peaks

2D-LC system configuration
Agilent Infinity II 2D-LC System

Configuration of the 1D UHPLC:
quaternary pump
autosampler with integrated column thermostat
multiple wavelength detector (MWD)

Configuration of the 2D UHPLC:
binary pump (→ low dwell volume)
multicolumn thermostat (max. 8 columns)
diode array detector (DAD) with 60 mm flow-cell
(→ high sensitivity)

2D Interface:

two 6-position selector valves which are connected to a 2-position interface valve
each selector valve bears a cluster of six 40 µl sampling loops
up to 10 cuts can be stored at a time!
upgraded with an active solvent modulation valve (ASM valve)

Development of a 2D-LC screening method to assess peak purity of reversed phase LC applications
Several questions at the beginning:
Which LC separation mode in 2D?
Which stationary phases and column dimension in 2D?
Which mobile phase in 2D?
Which method parameters in 2D?

Which separation mode in 2D?
Reversed phase (RP) has important advantages:
Variety of LC columns with very different stationary phases available on the market
Combination of RP-RP ensures compatibility of 1D and 2D eluents
RP allows changes of the pH of the eluents
→ different ionization states of the analytes
→ different selectivity
Which stationary phases in 2D?
by means of a column selectivity chart provided by Waters Corporation 8 different stationary phases have been selected
Criteria for selection:
different ligands
different polarity
max. operating temperature ≥ 45°C
max. pressure ≥ 1000 bar
beneficial: extended pH range

Selection of 8 different stationary phases:

Which column dimension in 2D?

Requirements for short 2D run-times:
Low column dead volume (→ short equilibration time)
High linear velocity of the mobile phase (→ fast chromatography)
Low flow rates (→ reduce solvent consumption)
Low peak volume (→ high sensitivity)

Which mobile phase in 2D?

Two pairs of eluents have been selected:
different modifiers
different pH (acidic ↔ alkaline)
pre-mixed eluents (→ smooth baseline)

2D-LC operation mode?

Example of a 2D-LC analysis
Artefacts in 2D-LC
Active solvent modulation (ASM)
Summary

"The Investigation of 2D Separation Techniques for the Analysis of Agrochemical Formulations and Co-formulants" - Pablo Navarro (Syngenta, Jealott's Hill, UK)

Dr. Navarro discussed the following:

- What are Agrochemical Formulations Made from?
- 2D separations
- 2D-LC
- Examples of 2D-LC Separations
- Ion Mobility Mass Spectrometry
- Examples of Ion Mobility Mass Spectrometry
- Benefits and Downsides of Offline 2D Separations
- Offline 2D Separations - Proof of Concept Project
    - LC and SFC Screening and Optimisation
    - Use of Different 2nd Dimension Techniques
- Conclusions

He started by sharing some of the 2D LC separations carried out by students working under Prof. Peter Schoenmakers at the University of Amsterdam for Syngenta.
The main part of the talk was about using offline 2D separations – HPLC in the 1st and then another separation in the second following fraction collection: HPLC, LC-MS, GC, NMR and SFC. A proof of concept was carried out to screen a number of columns, optimise the separation and then determine the amount

of material recovered and the number of injections required to have enough for a proton NMR for difenoconazole diastereomeric pairs.

"Development of two-dimensional high-performance liquid-chromatography for the separation and characterisation of therapeutic oligonucleotides and associated manufacturing impurities" - Christina J Vanhinsbergh (Uni Sheffield)

-The pharmaceutical landscape is predominated by two mountains: small molecules and biological therapeutics. Oligonucleotide therapeutics align with elements of both but not conclusively enough to be regulated under either. Like biological pharmaceuticals, oligonucleotides are complex polymers, with many chemical moieties contributing to their chemistry. Like small molecule drugs, oligonucleotides are manufactured synthetically and have relatively low molecular weights.

-There is an argument that oligonucleotide therapeutics are fast becoming their own entity due to recent successes in clinical trials and increases in regulatory registration.
This is a result of improved efficacy and lower toxicity due to advances in chemistry. There are 8 registered oligonucleotide therapeutics on the market, which is estimated to reach a net worth of $2.5 billion by 2022.
The term oligonucleotide therapeutic encompasses many different types of nucleic acid drugs. Types are defined by molecule structure and function. Manufacturing is a synthetic cyclic process of the addition of nucleotides to a growing chain. For oligonucleotide therapeutic batches, approximately 20% of manufactured nucleic acid consists as impurities of failed elongations, aberrant longmers and structures closely resembling the full-length product (such as isomers and abasics).
-Chemical modification enhances drug efficacy, improves nuclease resistance and reduces renal clearance.
-Additional impurities are derived from increasing structural complexity, such as the chemical modification of the molecule. This introduces failed modification impurities.
-The oligonucleotide can also be conjugated to a delivery vehicle, such as GalNac moieties. This introduces failed conjugation impurities.
-The analysis of oligonucleotide therapeutics is a multifaceted approach to ensure drug substance and drug product integrity. Liquid chromatography is a strong contributor to enable separation of mixtures from manufactured batches.

-My work at the University of Sheffield focused on separation of manufacturing impurities- these are oligonucleotides that are very similar to the target molecule, also known as the full-length product.
-The challenges of one-dimensional liquid chromatography analysis are that these closely related species make the synthesis mixture complex. This complexity results in low chromatographic selectivity. Secondary interactions are often observed, especially with modified phosphorothioate back bones, which results in low efficiency and some impurities can co-elute under the full-length product peak.

-Biased separation mechanisms can limit chromatographic capacity. In addition, detection by mass spectrometry is not compatible with salt containing mobile phases used in some modes of chromatography.

-Oligonucleotides are commonly purified and analysed by reversed phase ion-pair and anion exchange chromatography. Size exclusion and HILIC are also applicable modes of LC for oligo analysis.
-Optimisation of various modes of chromatography involved understanding the retention mechanisms of oligonucleotides in each mode, and then manipulating parameters to effect these retention mechanisms.

-Within the reverse-phase ion-pair modality, the negative charge of the oligonucleotide backbone is neutralised by the positive element of an alkyl amine ion pair reagent. The hydrophobic alkyl chains of the ion-pair reagent enable hydrophobic separation of oligonucleotides. Hydrophobicity increases with the length of the oligo, as the phosphorus bond between each nucleotide is what pairs with the alkyl amine.

-The ion-pair reagent itself forms a layer upon the stationary phase surface to dynamically exchange oligonucleotides. Secondary interactions between the bases of the oligonucleotide and the stationary phase are observed, with hydrophobicity increasing from cytosine> guanine> adenine> and thymine. These secondary interactions can be minimised with chromatographic parameter alterations and utilisation of an alkyl amine with increased hydrophobicity. Shown here is triethylammonium acetate and tributyl ammonium acetate to demonstrate increasing hydrophobicity of the ion pair reagent.
-Hexafluoroisopropanol has been shown to improve resolution by increasing stationary phase saturation with the ion pair reagent. HFIP desolubilises the ion pair reagent somewhat from the mobile phase, driving its partition towards the hydrophobic stationary phase.

-A more pronounced ion-pair reagent layer reduces secondary interactions and improves efficiency by promoting the dynamic ion exchange retention mechanism. This is especially observable when separating phosphorothioated oligonucleotides. Sequence based separation is still permissible with high concentrations of the ion-pair reagent.

-HFIP is also beneficial for MS analysis as it improves sensitivity by promoting ionisation efficiency.
-The negative charge of the oligonucleotide enables separation via strong anion exchange LC. A positively charged stationary phase electrostatically retains the oligonucleotide and separates oligos in a size-based fashion- reflective of their net negative charge.

-Increasing the ionic strength of the mobile phase, within a salt gradient, elutes oligos by counterion competitive inhibition of their adsorption. Secondary interactions are observed between the bases and the stationary phase, allowing sequence-based separations to occur. Sequence based separations are manipulated further by changing the mobile phase pH. At elevated pH, tautomeric guanine and thymine residues are ionised.
My research used a Thermo U3000 HPLC system equipped with dual pumps, multi-column switching valves and a fractionation valve.
2D-LC was plumbed in the fashion described here. Initially the sample is taken up by the needle and fills the sample loop.
-Unmodified oligonucleotide models were utilised for simplistic demonstration of the utility of 2D-LC for their analysis. Orthogonal workflows would aim to separate by size in one dimension and sequence in another.

-Shown here are co-eluting events in both ion-pair reversed-phase LC, using tributylammonium acetate, and strong anion exchange LC, using sodium perchlorate and elevated pH. Unmodified oligo's separated in a size basis in the IP-RP mode and more towards sequence in SAX mode.

-Notice that oligos that co-elute in one mode, separate in the other? A 2D-LC workflow was optimised to demonstrate that co-eluting events could be reduced.
-Co-eluants in the 2nd dimension strong anion exchange mode are first separated by size in the 1st dimension. This enables sequence-based separation to be performed orthogonally in a 2D-LC workflow.

-Oligos were mapped with reference separations to track elution events and confirmed by mass spectrometry analysis of the first-dimension fractions.
-Increasing chemical complexity results in lower selectivity, efficiency and resolution of oligonucleotides. We see here that a model oligonucleotide therapeutic and its associated manufacturing impurity co-elute in various modes of chromatography.

-If a specific analytical strategy was described, an optimised 2D-LC workflow would enable identification and characterisation of that impurity. We take the case study of the phosphodiester impurity of this oligonucleotide therapeutic, shown in the green trace. Its best selectivity from closely related molecules is observed within strong anion exchange. To improve resolution further, the N-1 and N-2 impurities could be removed from the separation space in a first dimension.

-HFIP buffered IP-RP, using triethylammonium acetate enabled size-based separation of these two impurities and so was applied as first dimension of a 2D-LC workflow.
-By simplifying the separation space, the phosphodiester impurity was resolved and identifiable with this approach. The n-x impurities were removed within the first fraction of the 1st dimension. The concentration of impurities was reduced from equimolar in retention order studies to 1.5% of the full-length product. This would more closely resemble a manufacturing batch.

-It is noticeably harder to resolve impurities with higher chemical complexity, in addition, selectivity within modalities is analyte specific. Chromatographic behaviour of this oligonucleotide therapeutic changed when its respective conjugated successor was analysed.

-Manipulating co-elution of n-x impurities within the main peak retention time is a slight verge from normal aims of chromatography, but successfully allowed resolution of a target species.

Lessons learned and Conclusion:

Chemical modification & OGN sequence affects Rs and α of impurities from full length OGN. α and Rs are OGN specific.
Analytical strategy must be well defined prior to 2D-LC optimisation.
2D-LC has been demonstrated for oligonucleotides of varying modification:
IP-RP > SAX for unmodified OGN's.
HFIP/IP-RP > IP-RP for phosphorothioated OGN's.

HFIP/IP-RP > SAX for OGN therapeutics.
Simplification of the separation space is enabled by 2D-LC.
2D-LC could be of best utility during development programmes (impurity monitoring).

"Capillary Electrophoresis hyphenated to mass spectrometry (CESI-MS) for the analysis of polar analytes such as pesticides and metabolites"
Karsten Hendriks, SCIEX

Specifically:
Residue analytical method for the determination of trifluoroacetic acid and difluoroacetic acid in plant matrices by CE-MS/MS

Background / Development
Why use CE-MS/MS in Residue Analysis?

The Method for TFA and DFA

Validation Results Summary
Successful Validation of plant residue method for TFA and DFA under GLP
LOQ @ 0.01 mg/kg in all matrices
Very good repeatability
SD/RSD within guideline specification (n = 5 per FL, two FL);
except TFA in straw @ 10-LOQ
TFA is present in almost all plant samples
Good/sufficient sensitivity and linearity for TFA and DFA
Very low amount injected (46 nL of a 1 - 2.5 µg/L sample @ LOQ)
Very good S/N ratios
None or very low matrix effects, but it affects migration times and peak shapes
Good robustness (450 injections, 260 with matrix samples)

CE-MS/MS in Residue Analysis – Summary
Advantages
Perfect matching between analyte and separation technique
Analytes are already in the right charge state for MS
Extremely low flow rates, ~10-30 nL/min
Reduced or no matrix effects!
Improves ESI process = higher sensitivity
Small inner diameter; high sensitivity in MS-detection
Sharper peak profile

No LC-like distribution between mobile and stationary phase, “plug-flow profile”.
Higher precision compared to LC-MS/MS method
Important in case of TFA residue in control subtraction
Very easy to handle applications
Only few parameters to change
Easy to mount the nano-spray ion source and CE probe
Instrument on a separate movable table
Enables Extract-and-shoot applications or reduced sample prep.
Different capillary types available; modifier kits
Peak evaluation in standard LCMS software e.g. Analyst® or Multiquant®

Disadvantages
Lower sensitivity compared to LC-MS/MS (~ factor 10); but…
LC-MS/MS: 0.02 – 10 µg/L, LOQ = 0.1 µg/L; inj. Vol. 10 µL = 2 pg
CE-MS/MS: 0.2 – 100 µg/L, LOQ = 1 – 2.5 µg/L; inj. Vol. 46 nL = 46 - 115 fg
In total factor 17 - 43 more abs. sensitivity
Additional software for instrument control
Extra qualification for users
In this example to mount the nano-spray ion source the mass spec has to be vented as a different l orifice and curtain plate was required.
Stable isotope-labelled internal standards are strongly recommended
Strong migration time shifts depending on matrix or even samples

CE-MS/MS - Outlook / Next Steps
Analysis of field residue studies (rotational crops) with new CE-MS/MS method
Set-up of a generic application
Test end-capped capillary (no EOF)
Test other separation buffer / analyte combinations
Switch from voltage-fixed to current-fixed application (migration time shift)?
Explore more data in matrix
Test many more compounds/matrix combinations
By cooperation foster use of CE-MS/MS in authority labs
Enforcement methods

Conclusions
Get a robust, reliable and accepted tool in hand complementary to LC-MS/MS standard for difficult/ionic target analytes

“Novel Applications in Enantioseparations”

Brian Freer,
DAICEL; HICHROM, VWR International Limited
CHIRAL TECHNOLOGIES EUROPE
Parc d'Innovation
160, Bd Gonthier d'Andernach, CS 80140, 67404 ILLKIRCH CEDEX, France
Tel : +33 (0)3 88 79 52 00 Fax : +33 (0)3 88 66 71 66
www.chiraltech.com bfreer@cte.daicel.com

CTE gave a short presentation that discussed alternate uses of chiral phases in 1D and 2D for lab-on-a-chip as well as separation of peptides and oligonucleotide fractions. In addition, the talk proposed the use of novel Daicel achiral phases for 2D LC.

“A comparison of GC modulators. Dispelling the Myths”

Bryan White, GC product specialist AGILENT

Principles of GCxGC were explained.
GCxGC (Comprehensive Two-dimension Gas Chromatography)

All Material that enters the 1st dimension column passes through the 2nd dimension column to the same detector

Uses a Modulator to partition 1st Column Effluent as discrete plugs onto the 2nd dimension column
In GCxGC an orthogonal separation occurs when the separation mechanisms of the two columns are independent of each other

Overview

Direct flow modulator:
Poor resolution, not indicated for complex matrices but suitable for groups analysis.
Very robust and simple, ideal for routine work.
Reverse flow modulator:
Good chromatographic resolution, excellent robustness and repeatability.
Rather complex, suitable with skilled operator and no need for frequent set-up changes.
Suitable for accessible analysis of targets, also in complex matrices.
Thermal modulator:
Best resolution and sensitivity, flexible configuration.
Repeatability is satisfactory but not as good as for flow modulation.

To be preferred for the profiling of unknowns and for multiple applications/research.
There is no “best GC×GC modulator”, each approach excels in some aspects.

“Recent 2D applications developed on the Vanquish Duo LC system”
Norman Ramsey, Thermo Fisher Scientific
Use the Thermo Scientific™ Vanquish™ Duo UHPLC Systems to support four workflows (Dual LC, Tandem LC or LC-MS, Transcend Duo LX-2 and Inverse Gradient) by combining two flow paths in one integrated UHPLC solution. These workflows improve your productivity by saving time, reducing cost per sample, increasing capacity without added bench, and using your resources more efficiently. Vanquish Duo UHPLC Systems expand the benefits of the Vanquish platform without compromising performance, robustness, or ease-of-use.

“Summing Up, Thanks and Farewell”
Dr. Chris Bevan, The Chromatographic Society

All the presentations in our one-day symposium on the leading edge of multi-dimensional chromatographic methodologies and applications were excellent and demonstrated the utility and power of these techniques. Many of our presenters had already adopted the techniques in their everyday analytical work and showed us how useful these are becoming.

Complete PowerPoint Slide sets for most of the presentations are available from:
The Chromatographic Society as they are too voluminous to reproduce herein.

The ChromSoc is indebted to Syngenta, our host company, for allowing us to use their superb facilities and benefit from their expertise in the presentations and exhibition. We thank all those presenters and exhibitors for supporting this symposium.