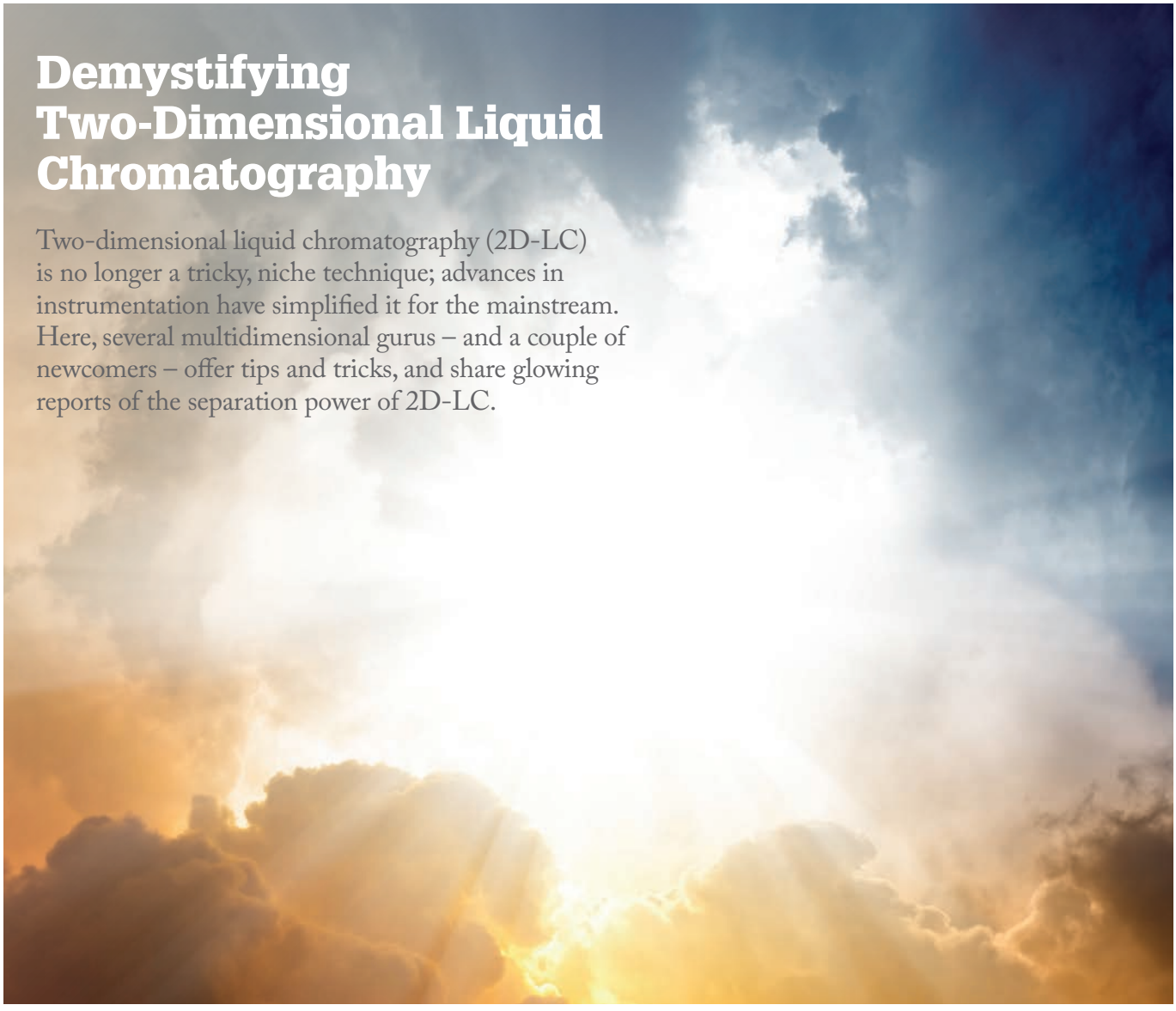
the Analytical Scientist

Demystifying Two-Dimensional Liquid Chromatography

Two-dimensional liquid chromatography (2D-LC) is no longer a tricky, niche technique; advances in instrumentation have simplified it for the mainstream. Here, several multidimensional gurus – and a couple of newcomers – offer tips and tricks, and share glowing reports of the separation power of 2D-LC.



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Introducing 2D-LC and the Demystification Process

In early 2014, Agilent Technologies approached us with a clear objective: to show how simple and robust two-dimensional liquid chromatography has become.

"Demystifying 2D-LC" was born. And so too was "The Analytical Scientist × Agilent Technologies" collaboration – and very fruitful it has been.

We've had the great pleasure of working with some of biggest names in the field, covering several key application areas. The RIC's Pat Sandra and Gerd Vanhoenacker begin with "Embracing the Second Dimension", which provides essential background theory and introduces 2D-LC as the answer to the demand for higher peak capacity in complex samples. "The New 2D-LC Kids on the Block", meanwhile, features two scientists who have more recently embraced the second dimension, openly asking them about their experiences. Other authors include Genentech's Cadapakam J. Venkatramani and Oliver Schmitz from the University Duisberg-Essen.

To really send the message home, a further two authors – Koen Sandra and Dwight Stoll – recently delivered a webinar to delve even deeper into certain aspects of practical 2D-LC and answered your questions live. You can access the on-demand version here: tas.txp.to/1114/2DLCwebinar

Both Agilent and The Analytical Scientist consider this compendium to be the beginning of the discussion on 2D-LC – not the end. We look forward to receiving your questions and feedback.

Rich Whitworth
Editor

Contents

Embracing the Second Dimension

Moving from 1D to 2D liquid chromatography is a big step towards the high peak capacities demanded by complex sample analysis.

By Pat Sandra and Gerd Vanhoenacker

In the past, the separation performance of a chromatographic system was described in terms of column efficiency (N). In liquid chromatography (LC), this value depends on the particle size (d_p) and on the column length (L). Using porous particles, $N=L/2d_p$. In 2006, superficially porous particles were re-introduced and the experimental plate number with state-of-the-art instrumentation approached $L/1.5d_p$ because of the fast mass transfer in the thin porous shell. Also around a decade ago, tremendous improvements were made in column technology with sub-2 µm porous particles. These, along with improved LC instrumentation that could withstand pressures of up to 1200 bar, opened new possibilities in terms of speed and resolution to LC practitioners.

For today's complex analyses, plate count is not an effective measure of performance; a better – and now well-accepted – alternative is peak capacity, n_c. Introduced by Giddings in 1967 (1), n_c is the maximum number of peaks that can fit side-by-side between the first and last peak of interest with a fixed resolution (normally 1). Originally introduced for isocratic separations, Horvath and Lipsky (2) were the first to realize that much higher peak capacities can be attained by gradient elution in LC or temperature programming in GC. The equation generally used to calculate the peak capacity in gradient elution is $n_c = 1 + t_g/W$ (3) in which t_g is the gradient run time and W is the average peak width (4 s).

Before the introduction of high-pressure instrumentation and small particles, peak capacities up to 200 could be obtained in conventional unidimensional LC (1D-LC). Presently, with sub-2 µm porous particles (or superficially porous particles), peak capacities of 570 in 50 min and up to 850 in 180 min have been reported (4). The peak capacity productivity (peaks/min) of a column can be optimized by fine-tuning the gradient time and/or flow to the complexity of the sample.

It is, however, wishful thinking that such peak capacities are sufficient to separate the very complex mixtures that make up biological, food, environmental or natural product samples. Peak capacity should significantly outstrip the number of components in a sample; statistical theory of peak overlap (5) tells us that peak resolution is severely compromised when the number of components present in the sample exceeds 37 percent of the peak capacity. Indeed, to resolve 98 percent of randomly distributed sample components, peak capacity should exceed the number of components by a factor 100 (6). This means that an n_c value of 10,000 (corresponding to around $1x10^8$ theoretical) is needed to "chromatographically" resolve a sample containing 100 components! Fortunately, chromatography is not the sole contributor to unraveling sample complexity; the selectivity capacity of contemporary mass spectrometers (MS) substantially lowers the separation-side

2D-LC and Me

Dwight Stoll has been working in 2D-LC since 2000. Here, he talks about the benefits of what is a core technology in his research.

How did you get into 2D-LC and why?

In 2000, I was working with Peter Carr who had been doing a lot of work on increasing the speed of HPLC separations. We decided to apply our expertise in performing fast separations to the second dimension; instead of 5–10 hour run times, we were aiming for 30 minutes without losing performance.

How was 2D-LC back then?

Pretty much everyone was making their own systems and writing their own software. Historically, the availability of robust 2D-LC systems has been a huge barrier – but that barrier is being torn down with a growing number of "off-the-shelf" solutions, which will undoubtedly change the perception and use of this powerful technique.

bar for many applications. However, even with the most powerful MS instruments, maximizing the resolution at the front-end is important, and in QA/QC laboratories where high peak capacities are often needed but mass spectrometers are not yet established, it is essential. One straightforward approach to increasing n_c is multidimensional LC, especially bidimensional or 2D-LC.

Explaining 2D-LC

On-line 2D-LC can be divided into "heart-cutting" and comprehensive approaches (see "2D-LC 101"). Heart-cutting 2D-LC resolves components within a selected retention time window, while in comprehensive 2D-LC (my focus here) the entire sample is subjected to two separations. Actually, the first example of comprehensive chromatography was 70 years ago, in the separation of amino acids by paper chromatography (PC) (7): after developing in one direction with solvent A, the paper strip was turned 90 degrees and developed for a second time with solvent B. Given the static nature of the PC procedure, this was straight-forward.

In comprehensive 2D-LC, two columns are connected in series via a switching valve (modulator) and small-volume fractions from the first column effluent are collected and injected on the second column in multiple repeated alternating cycles. A standard comprehensive 2D-LC modulator is a ten-port switching valve with two collecting loops. Introduction of a successive fraction (loop 2) onto the secondary column can only be done when the previous fraction (loop one) elutes completely from the column. No

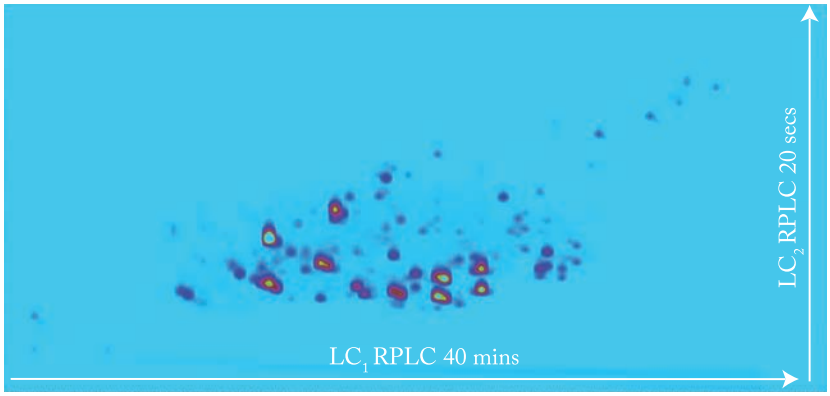


Figure 1. RPLC x RPLC 2D plot of polyphenols in green citrus tea recorded by UV at 320 nm. The first column is a C18 with a mobile phase gradient from 0.1% formic acid in water to methanol. The second column is a Phenyl-Hexyl with a mobile phase gradient from 0.1% formic acid in water to acetonitrile.

What are the main main benefits?

The three biggest broad benefits are:

i) The brute force of the 2D-LC technique in terms of separation power and the information that can be produced.
ii) The increased confidence in data in terms of hidden peaks – that's why heart-cutting 2D-LC is rapidly becoming more popular.
iii) The potential for throughput gains, for example, by taking a 30 min 1D separation, compressing it to 10 min, and regaining lost resolution by bolting on a second dimension.

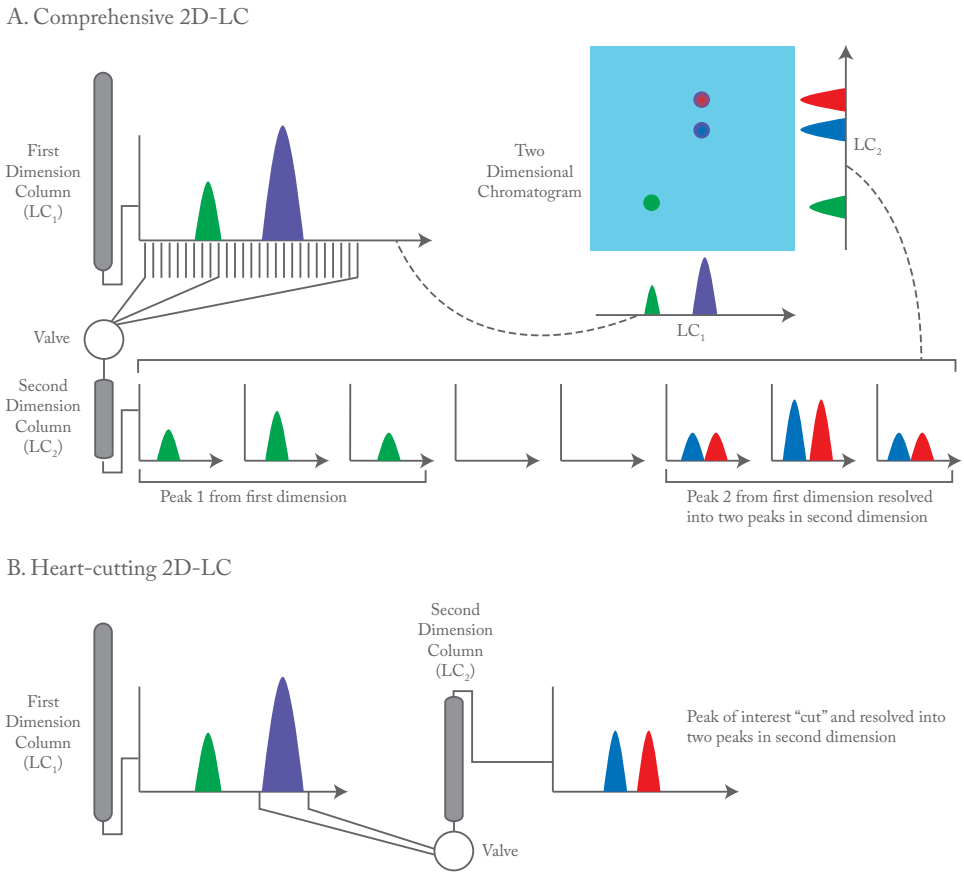
The final point is one that I'm very interested in. And while converting existing methods may seem irksome (every separation is different, after all), the potential in future method development is very clear. Instead of being satisfied with 30 min 1D runs, method development could be approached by starting with an end point in mind and pouring in separation power to gain the best efficiency.

Isn't 2D-LC difficult?

Actually, even before commercial instruments became available, 2D-LC wasn't exactly difficult, but it did take time and a certain amount of expertise and experience to know how to get the best out of it. Separation scientists in pharma and other big industries don't have time to "play around".

Much of our focus is on comprehensive 2D-LC where you're trying to get a global profile of what's in your sample. Heart-cutting separation work has been easier to do for a while, but there does seem to be a renaissance of late

2D-LC 101



In comprehensive 2D-LC, the complete effluent from the first column is transferred to the second column with a switching valve. The valve transfers the eluent in small portions that are analyzed with fast gradients of 20-30 seconds. After data acquisition, the partial chromatograms of both dimensions are aligned. In heart-cutting 2D-LC, only selected parts of the first column's effluent are transferred to the second column. By cutting out a peak from the heart of the separation, it can be analyzed with higher separation efficiency on the second column. The run time of the second dimension analysis is usually longer than the collection time from the first dimension.

– the pharma industry is picking it up pretty quickly. Invariably, as big vendors offer the technology, it opens it up to a whole new segment of the community.

What advances facilitated "easy 2D-LC"?

The inability to do really fast gradient elutions in the second dimension was one big challenge in the past, but advances in pump technology have taken us down from gradient delay volumes of 1 ml to 100 µl. That was a big milestone. The development time that has been put into the software has also dramatically improved the user experience.

At what point does 2D-LC become the go-to technique?

Ten years ago, it was clear to me (and everyone else) that 2D-LC was complicated enough that if its performance wasn't clearly superior to the 1D case, the effort could not be justified. What we now know through simulations and experiments is that the "crossover time" – that is, the separation runtime at which 2D becomes superior to 1D – is about 10-15 minutes (1). That provided us with great motivation to stick with it; there are so many potential applications where you can clearly get a far superior separation in the same runtime.

Are we at a tipping point for 2D-LC adoption?

Increasing awareness, availability of vendor solutions and an evolving recognition that we're at the wall with 1D are all coming together to make 2D

restrictions are made on the analysis time on the first column but the analysis time (including regeneration time after the gradient) on the second column should be equal to or smaller than the modulation period. In an ideal comprehensive 2D-LC combination, the total peak capacity is that of the first column multiplied by that of the second column: $n_c t = n_c 1 \times n_c 2$. Theoretically, this means that by coupling a column of $n_c 1$ 500 (high resolution) with one of $n_c 2$ 20 (high speed), $n_c t$ is 10,000. However, the experimental $n_c t$ is lower than the theoretical one as the 2D space can never be fully covered. Note that in comprehensive 2D-LC, the total analysis time is only slightly higher (typically 1 min) than the analysis time on the first column.

Optimum occupancy of the 2D space occurs when the separation mechanisms of the two dimensions have distinct retention profiles. The higher the orthogonality, the closer we get to theoretical peak capacity. Examples of high orthogonality include combining normal phase LC (NPLC) with reversed phase LC (RPLC); hydrophilic interaction liquid chromatography (HILIC) with RPLC; size exclusion chromatography (SEC) with RPLC; ion exchange chromatography (IEC) with RPLC; and even supercritical fluid chromatography (SFC) with RPLC. Surprisingly, although an RPLC × RPLC combination is, by definition, of low orthogonality, it provides interesting options for certain applications, such as peptide analysis at different pHs in the two dimensions (8). Moreover, in RPLC × RPLC, solvent incompatibility is not an issue and very robust methods, applicable in a QA/QC environment, can be developed as illustrated in Figure 1.

Comprehensive 2D-LC is mature enough to be very generally applicable, even in a routine environment; this is especially true with the use of robust instrumentation that offers both comprehensive and heart-cutting modes.

Pat Sandra and Gerd Vanhoenacker are at the Research Institute for Chromatography, Kortrijk, Belgium.

Further reading

"Comprehensive Chromatography in Combination with Mass Spectrometry", Editor L. Mondello, (John Wiley & Sons Hoboken , NJ, USA, 2011).
Agilent Technologies 2D-LC
Agilent Technologies Application Notes: tas.txp.to/0314/2D-LC

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that much more attractive. I recently had an email from a big pharma company saying, "We know we need more than 1D – please help us!" Put another way, when Genentech (for example) is able to contact Agilent (for example) with a 2D-LC part number, you know the landscape has changed dramatically!

Where do you foresee the biggest uptake?

Well, there are a number of groups in the polymer analysis community that have been using 2D-LC for a relatively long time and doing a lot of good work. They certainly deserve credit for their efforts in the field – presumably that will continue.

I think from now on, the biopharmaceutical industry is likely to make the biggest splash in terms of uptake because they have the most to gain. It's also a relatively easy place to start as they can benefit from lessons learned in proteomics, where the first separation is performed with ion exchange and the second with reversed phase. That's a really nice combination. The rumblings I've heard is that it is taking off like a rocket.

Dwight Stoll is assistant professor of analytical chemistry at Gustavus Adolphus College, MN, USA.

Reference

1. *D. R. Stoll, X. Wang, and P. W. Carr, "Comparison of the Practical Resolving Power of One- and Two-Dimensional High-Performance Liquid Chromatography Analysis of Metabolomic Samples", Anal. Chem. 80, 268–278 (2008).*

Exploring Chinese Medicine with 2D-LC

Two-dimensional liquid chromatography certainly sounds impressive and produces some pretty pictures, but is it essential in the analysis of complex samples? Here, we show that 2D-LC offers both style and substance.

By Oliver Schmitz and Duxin Li

Chinese medicine is an ancient art. The earliest existing Chinese text on medicine dates back to the second or first century BC – the Huang Di Nei Jing. Typically, in early Chinese medicine, several plants, each with hundreds or even thousands of compounds, are formed into a single drug formulation. Today, much has remained the same – the normal formulation of a Chinese herbal medicine (CHM) is extremely complex. Product control that is comparable to Western medicine is a huge challenge. CHM has, unsurprisingly, attracted a lot of attention throughout the world as our search for more effective medicines delves ever deeper. It follows that researchers are interested in the chemical constituents of Chinese herbs and the origins of their pharmacological and thus therapeutic activities.

Essential separations

We've probably all heard someone say that because the number of labs with high-resolution mass spectrometry (HRMS) is increasing, less and less chromatography will be required ahead of detection. For the non-chromatographers out there, that might even sound quite tempting. However, in reality, it's a statement that does not really make much sense: if all components of a sample are injected into the ion source at the same time and if a large percentage of those compounds are ionized, then – in a complex sample like Chinese herbs – several thousands of radical cations will be formed. And in an atmospheric-pressure ion source, such as ESI or APCI, all of these resulting radical cations can react or interact, each encountering approximately 20,000 collisions from the point of ionization to the entrance of the MS. The result is potential ion suppression and/or formation of artefacts. That's a problem. The solution? The addition of a high performance chromatographic platform in front of the MS! Such a combination is (and may always be) the gold standard, even with HRMS.

Comprehensive 2D-LC – or LCxLC – offers the high performance separation desirable for complex samples. Indeed, our group specialises in the use of comprehensive techniques to analyze various herbs in CHM. More non-polar species are analyzed using GCxGC-MS, whereas more polar compounds are analyzed using LCxLC-MS (see Figure 1).

Of course, as with any analytical technique, there are advantages and disadvantages of LCxLC, which are dependent on the application. For us, when compared with one-dimensional LC, the disadvantages of LCxLC could be summed up as potentially lower sensitivity with MS-detection and the more complex method development (1). However, the advantages are clear: much higher peak capacity (as noted in the first article on page 4) and the ability to produce contour plots that display peak intensity as a function of the retention times in the first and second dimensions – these are excellent for fingerprint-style analysis.

A shift in gear

So, how can we further optimize LCxLC separations? The answer is in the gradient programs used (see Figure 2). Our system allows the use of a constantly shifted gradient in the second dimension, which uses a narrower range of mobile phase composition than the full gradient program but continuously shifts the concentration range according to retention. The shift gradient is really a combination of a parallel gradient and a full gradient; the lower concentration range enables the retention of weakly retained fractions, while the higher concentration range is sufficient to elute strongly retained fractions, as with a parallel gradient. The shift gradient offers bandwidth suppression but also reduces the probability of "wrap-around" behavior, just like a full gradient.

In a correlated RPLCxRPLC system, the early-eluted analytes in the first dimension will have a weak retention in the second dimension; the

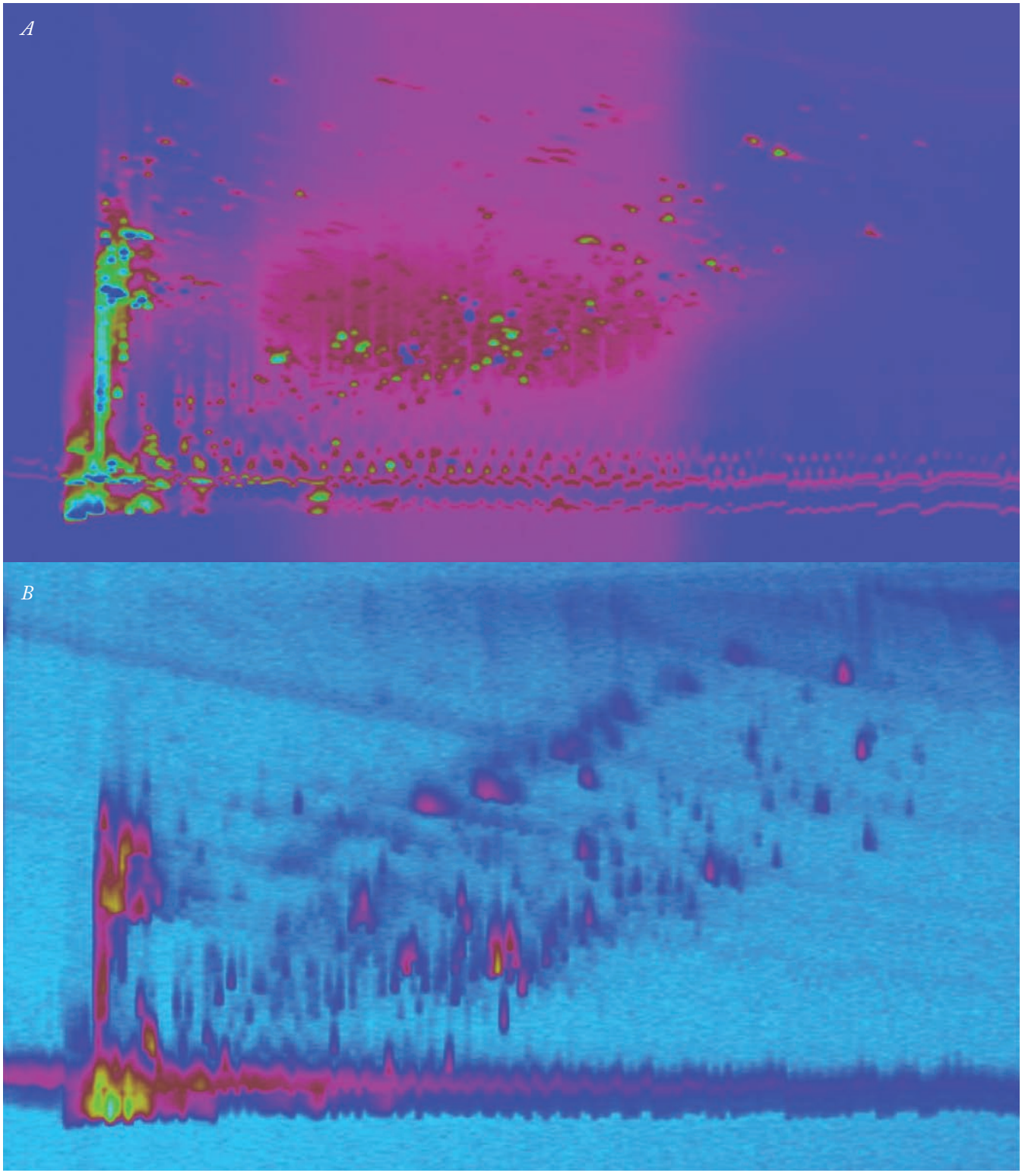

Figure 1. LCxLC analysis of an aqueous extract of two herbs, *Scutellaria barbata* and *Oldenlandia diffusa* (A: diode array detector, B: ESI-qTOF-MS).

analytes eluted in the middle of the first separation will be eluted in the middle of second dimension; and the late-eluted analytes in the first dimension will have a strong retention in the second dimension. And because the shift gradient runs in a continuous way, the cluster information in real world samples is preserved.

Undoubtedly, the shift gradient increases the separation power in the second dimension significantly as shown in Figures 3 and 4 (2).

2D-LC or not 2D-LC, that is the question

Should you be leveraging the power of 2D-LC? If you want increased separation of complex samples, then absolutely! Non-targeted analyses, such as identification of disease biomarkers, have become much more powerful using LCxLC-MS. And we are only at the beginning. The next step is LCxLC-IMS-qTOF-MS. Some of the first investigations of this sort are being done in my lab right now…

Oliver Schmitz is a professor of applied analytical chemistry and Duxin Li is a post-doc at University Duisberg-Essen, Germany.

References

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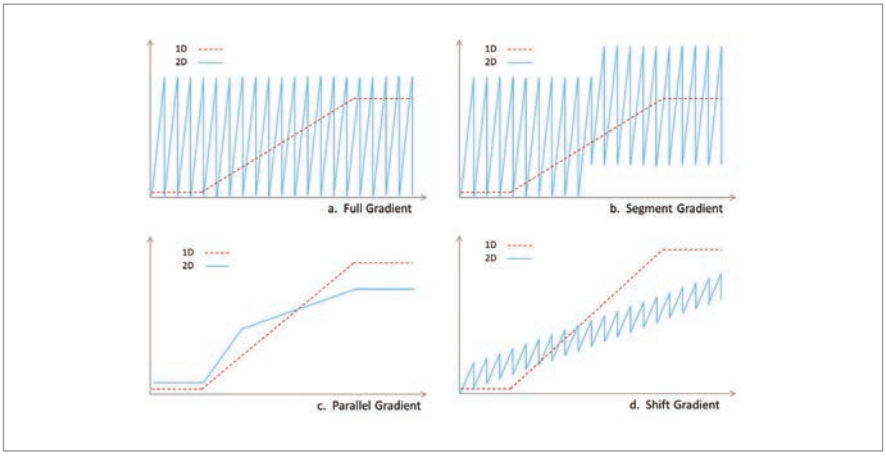


Figure 2. Graphical representation of different gradient programs used in 2D-LC.

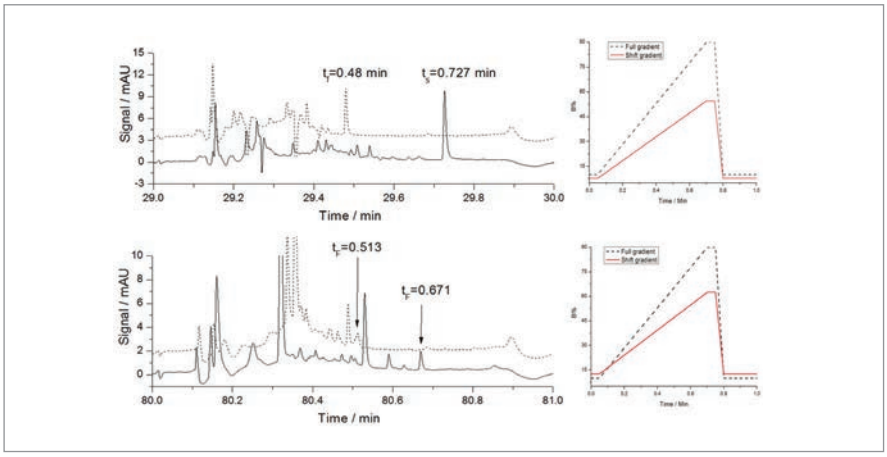


Figure 3. Comparison of full gradient (dotted line) and shift gradient (solid line) in fractions 29 (upper chromatogram) and 80 (lower chromatogram) of an LCxLC analysis from an aqueous extract of *Hedyotis diffusa* and *Oldenlandia diffusa*, with corresponding gradient programs on the right.

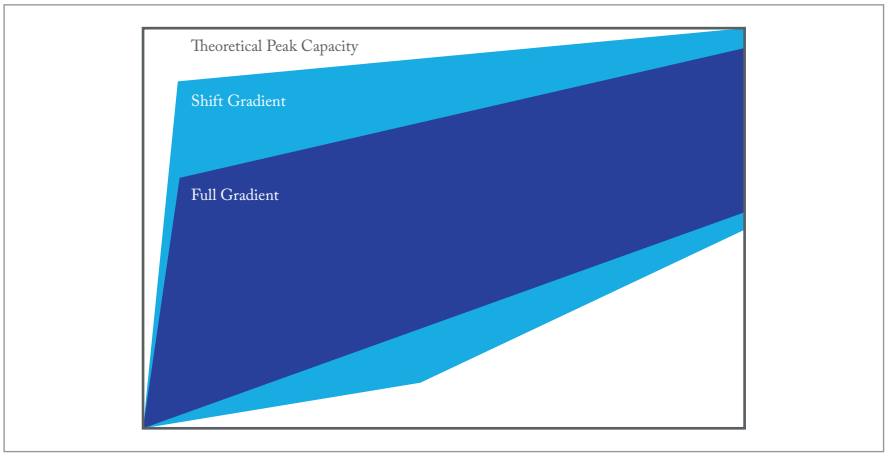


Figure 4. Comparison of peak distribution area of full and shift gradients (adapted from reference 3).

Comprehensive 2D-LC Analysis of Chinese Herbal Medicine

Achieving the highest chromatographic resolution using the Agilent 1290 Infinity 2D-LC solution.

Sonja Krieger and Jens Trafkowski

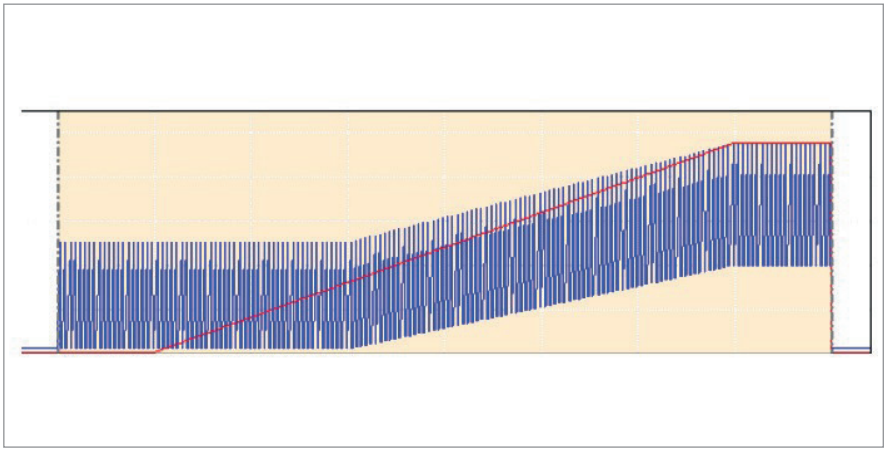


Figure 1. A complex gradient designed for the second-dimension separation of components of a decoction from mori ramulus.

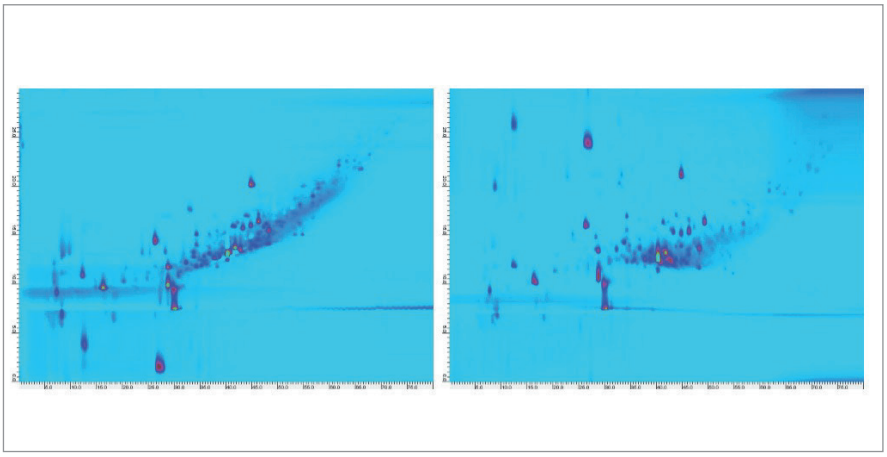


Figure 2. Comprehensive 2D-LC analysis of a decoction from mori ramulus; (A) using repeating second-dimension gradients from 5 to 95 % acetonitrile; (B) using the complex second-dimension gradient with detection at 254 nm.

Introduction

Chinese herbal medicine (CHM) is one aspect of traditional Chinese medicine (TCM) and uses single plants or preparations of several plants. The effectiveness of CHM depends on the synergistic effects of multiple components in the plants. The plants used in CHM present extremely complex samples. Therefore, comprehensive two-dimensional liquid chromatography (comprehensive 2D-LC) is the method of choice for their analysis.

Results and Discussion

For comprehensive 2D-LC analysis, samples of the plants were prepared as decoctions. It can be expected that decoctions of plants used in CHM contain a range of very polar compounds besides many less polar compounds. For this reason, an Agilent ZORBAX SB-Aq column was chosen for the first-dimension separation because it retains hydrophilic compounds and can be run under 100 % aqueous conditions. Further, methanol showed better separation for the ingredients of mori ramulus, as well as adding 0.1% formic acid to both eluents. The gradient was run from 0 to 95 % methanol over a duration of 80 minutes.

To choose the right column for the second dimension, several columns were tested whereby an Agilent ZORBAX Bonus-RP column provided the best separation for the fast gradients. Selectivity differences between the first and second dimension separation were achieved by using acetonitrile as eluent and by adding 0.1 % formic acid to both eluents. Besides this better orthogonality of both dimensions, using acetonitrile was also advantageous because of the lower backpressure generated compared to methanol. Hence, higher flow rates could be deployed in the second dimension.

To enlarge the accessible two-dimensional separation space, a shifted gradient was applied in the second dimension as shown in Figure 1. The highly orthogonal separation is shown in Figure 2.

Conclusion

Comprehensive 2D-LC is ideally suited for the analysis of herbal formulations, especially when combining two RP phases in the two separation dimensions.

For the full solution, visit: tas.txp.to/2DLC/herb

Two-dimensional Bioanalysis

Bringing biopharmaceuticals and biosimilars to market with 2D-LC.

By Koen Sandra, Gerd Vanhoenacker and Pat Sandra

In recent years, the top ten pharmaceuticals sales list has been extensively populated with protein therapeutics, such as monoclonal antibodies and recombinant proteins, that are used to treat various life-threatening diseases, including cancer and autoimmune diseases. A number of these blockbuster biopharmaceuticals are coming off patent in the near future, a fact that has resulted in an explosion of activity in the biosimilar or "biogeneric" market.

Irrespective of whether a company is developing innovative biopharmaceuticals or copycat biosimilars, a detailed characterization must be performed. Indeed, product characteristics need to be very closely monitored prior to clinical or commercial release. With complexity far exceeding that of small molecule drugs, the characterization of protein biopharmaceuticals represents significant analytical challenges, typically involving a wide range of analytical techniques and methodologies (1).

Peptide mapping is a commonly used characterization methodology as it provides great detail of the molecule under investigation. Let's take a monoclonal antibody of 150 kDa as an example; a trypsin digestion will generate over 100 peptides all with varying physicochemical properties over a wide dynamic concentration range. The complexity associated with these digests demands the very best in terms of separation power. Compared with one-dimensional separations (1D-LC), two-dimensional LC – and especially comprehensive 2D-LC (LC × LC) – drastically increases peak capacity as long as the two dimensions are orthogonal and the separation obtained in the first dimension is maintained upon transfer to the second dimension.

Orthogonal combinations for 2D-LC based peptide mapping include strong cation exchange and reversed phase LC (SCX × RPLC); hydrophilic interaction chromatography and reversed-phase LC (HILIC × RPLC); and reversed-phase LC and reversed-phase LC (RPLC × RPLC) with different pH levels in the two dimensions (2-4). The highest orthogonality is obtained with SCX × RPLC and HILIC × RPLC, because the separation mechanisms are completely different. However, RPLC × RPLC is particularly interesting; excellent solvent compatibility in both dimensions makes it the most rugged, but it still offers very high peak capacity (driven by high plate numbers in the individual dimensions) and great orthogonality, which is due to the zwitterionic nature of peptides, such that major selectivity differences are achieved when performing separations on RPLC at pH extremes.

Figure 1 shows the 2D-LC tryptic peptide maps of two production batches of the monoclonal antibody trastuzumab using RPLC × RPLC. Trastuzumab has been marketed as Herceptin since 1998, and is still being used in the treatment of HER 2 positive breast cancer. The peptide map provides a wealth of information that allows identity and purity to be assessed. Trastuzumab's identity is spread over 62 tryptic peptides (20 light chain and 42 heavy chain) of which the majority is baseline resolved. Several of these tryptic peptides contain amino acids that are prone to modifications, such as deamidation, isomerization, oxidation, and so on. Such product related impurities can have an influence on both the safety and efficacy of the product and need to be closely monitored.

The benefits of 2D-LC technology with respect to such modifications are highlighted in Figure 2, which shows a zoomed view of the 2D-LC peptide map of a non-stressed and pH stressed originator. A deamidation event is substantially increased upon stressing trastuzumab at pH 9 for three days. This modification, which occurs on an asparagine located within the third tryptic peptide of the light chain, is already observed at around 10 percent in the non-stressed sample. Peak identity was obtained using MS and modification sites were revealed by MS/MS.

The precision associated with the RPLC × RPLC methodology combined with UV-detection (retention time RSD < 0.2% and area RSD < 5% for n=5) makes this very a powerful approach to demonstrate comparability between production batches, as demonstrated in Figure 1, and between originator biopharmaceuticals and biosimilars.

So, the big question is: where in the biopharmaceutical development

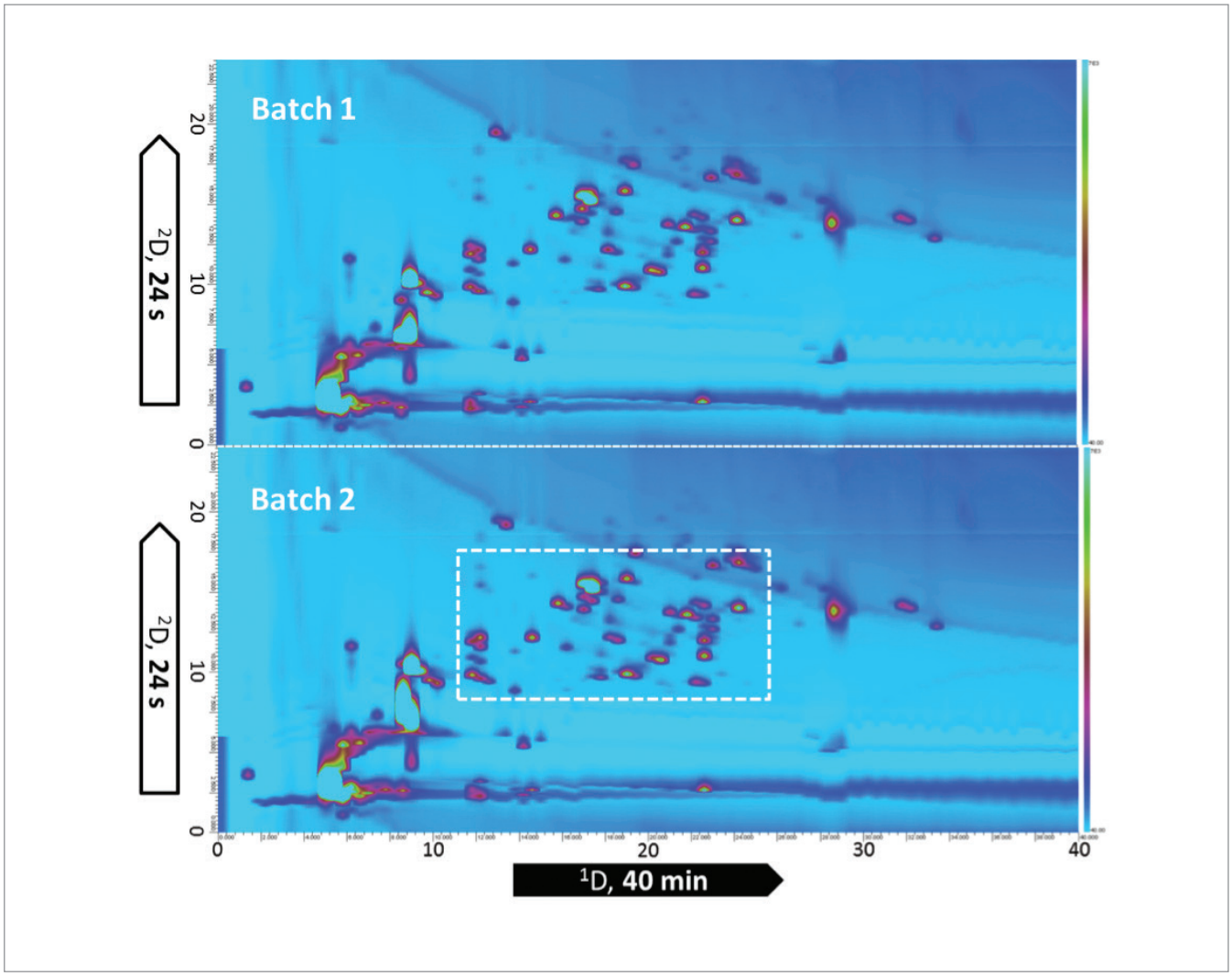


Figure 1. 2D-LC peptide map of two Herceptin production batches. First and second dimension separation consist of reversed-phase LC operated at high and low pH, respectively. Fractions were transferred from one dimension to the other using a dual-loop interface. UV detection was performed at 214 nm.

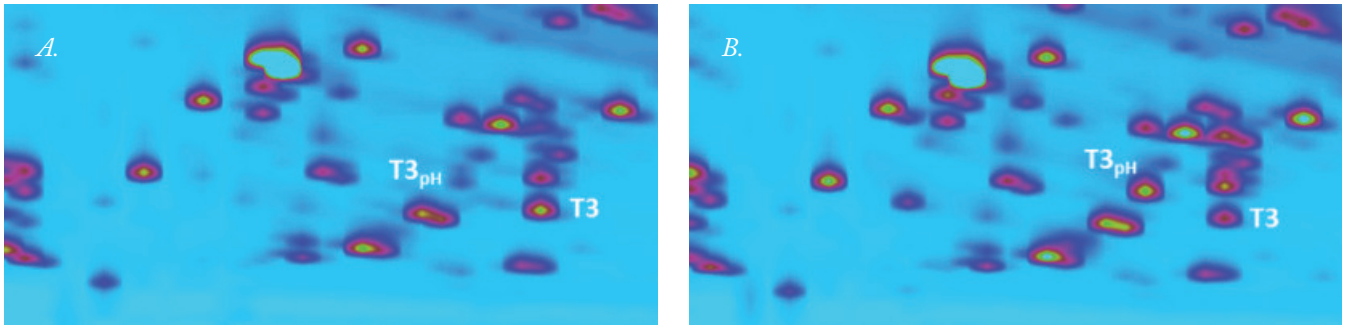
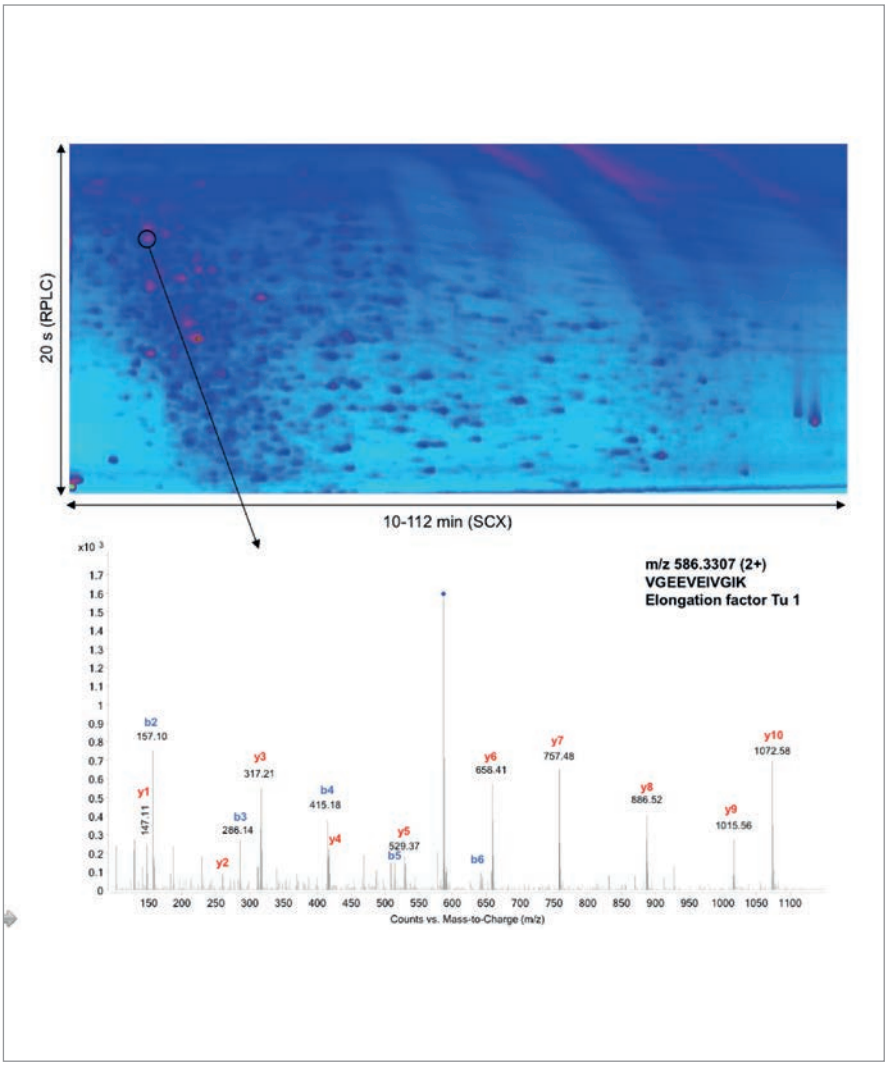


Figure 2. Zoomed view in the 2D-LC peptide maps of a non-stressed (A) and pH stressed (B) Herceptin production batch demonstrating asparagine deamidation in the light chain of the monoclonal antibody. T3 refers to the third tryptic peptide counting from the N-terminal side of the light chain. $T3_{pH}$ refers to the deamidated variant.

Beyond Biopharmaceutical Analysis: 2D-LC Based Peptide Mapping

By Koen Sandra, Gerd Vanhoenacker and Pat Sandra

Two-dimensional liquid chromatography (2D-LC) based peptide mapping finds applications beyond biopharmaceutical analysis as illustrated by the SCX × RPLC separation of a tryptic digest of a total E. coli lysate. Peak capacity is increased tremendously compared to 1D-LC. The high orthogonality between the two dimensions results in a remarkable separation and the hyphenation to a fast Q-TOF MS system enables elucidation of peptide and protein identities on the observed spots.



pipeline do we see the true benefit of 2D-LC? It's fair to say that in early stage development, one dimensional-LC-MS is certainly an option; RPLC columns that provide peak capacities well in excess of 500 are widely available nowadays – when used in combination with high-resolution mass spectrometry, a powerful characterization engine emerges. However, as we move along the development pipeline and enter the clinical or commercial release phases, MS preferably has to be replaced by UV detection and the enhanced resolution offered by 2D-LC comes into its own. Certainly, the appearance of commercial instrumentation represents a major step towards more widespread use of 2D-LC in biopharmaceutical analysis.

Koen Sandra is R&D Director, Gerd Vanhoenacker is LC Product Specialist/Manager, and Pat Sandra is Founder and President, all at the Research Institute for Chromatography, Kortrijk, Belgium.

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Analysis of Monoclonal Antibody Digests with the Agilent 1290 Infinity 2D-LC Solution

Two-dimensional liquid chromatography: HILIC x RPLC-MS.

Gerd Vanhoenacker, Koen Sandra, Isabel Vandenheede, Frank David, Pat Sandra, and Udo Huber.

Introduction

Biopharmaceuticals such as therapeutic monoclonal antibodies (mAbs) are becoming increasingly important in the treatment of various diseases. Peptide mapping is a commonly used technique for their comprehensive characterization and purity determination. The complexity associated with mAb digests demands the highest separation power. Compared to one-dimensional separations, comprehensive two-dimensional LC (LC×LC) will drastically increase peak capacity.

Results and Discussion

This article describes the application of HILIC×RPLC to the analysis of tryptic digests of trastuzumab using the Agilent 1290 Infinity 2D-LC solution coupled to an Agilent 6530 Accurate-Mass Q-TOF LC/MS System. A combination of HILIC in the first dimension and RPLC in the second dimension should provide good orthogonality for compounds such as peptides in an LC×LC setup. This orthogonality and complementarity has been proven in various reports, but mostly using a stop-flow or an offline LC×LC approach. The LC×LC peptide map of trastuzumab is shown in Figure 1. The contour plot was generated with the MS total ion current data. The spots were identified by matching the experimentally acquired data on the theoretical trastuzumab sequence at high mass accuracy (< 5 ppm) using Agilent MassHunter Bioconfirm software. The resulting separation shows good orthogonality between both dimensions.

The applicability of the developed method was evaluated with stressed and non-stressed samples. Trastuzumab was subjected to forced degradation conditions prior to digestion. Comparing stressed with non-stressed data should reveal degradation products. Figure 2 shows the extracted ion plots for peptide T41 and its oxidation product. From the comparison of non-stressed and oxidized samples, it is clear that the non-stressed trastuzumab already contains small amounts of the oxidation product. The inserts in Figure 2 show the mass spectra.

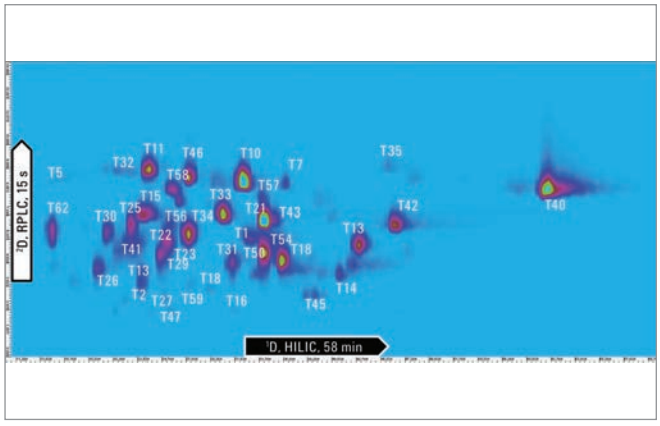

Figure 1. LCxLC contour plot for the analysis of a tryptic digest of trastuzumab.

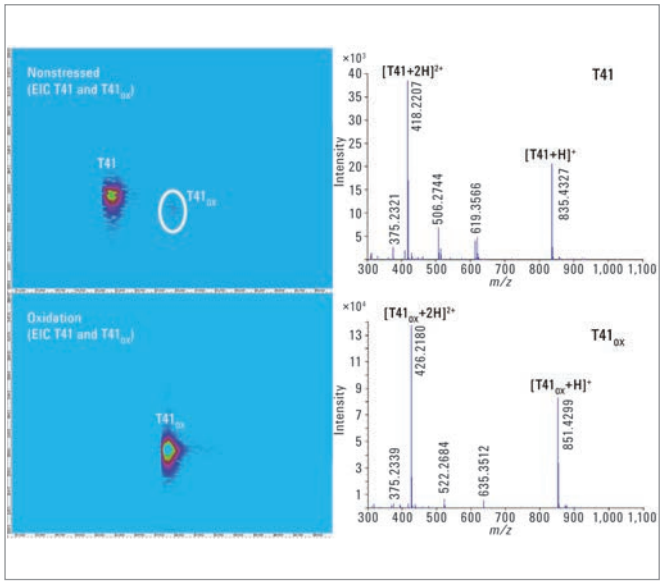

Figure 2. LCxLC extracted ion contour plot for the analysis of a tryptic digest of nonstressed and oxidatively stressed trastuzumab.

Conclusion

The Agilent 1290 Infinity 2D-LC solution coupled to an Agilent 6530 Accurate-Mass Q-TOF LC/MS system is ideal for the comprehensive analysis of monoclonal antibody digests. The possibilities of LC×LC coupled to a high-end mass spectrometer for detailed peptide mapping analyses are illustrated with some representative results on stressed and non-stressed trastuzumab digests.

For the full solution, visit: tas.txp.to/2DLC/antibody

Harnessing 2D-LC for Big Pharma

Over 10 years ago, the potential of two-dimensional liquid chromatography seemed obvious to me – so I built a system and never looked back. Now, a more mature 2D-LC is ready for a bigger role in pharmaceutical analysis.

By Cadapakam J. Venkatramani

At graduate school, I became fascinated by the research of the late professor John B. Phillips, who invented a comprehensive two-dimensional gas chromatographic (GC) system in early 1990. My two-dimensional journey began with me joining his group; I was extremely excited about the prospect of sampling entire primary column eluent into the secondary column for further separation using complementary phases. I devoted much of my graduate research to 2D-GC – in particular, separations of petroleum samples. A highlight was the resolution of over 4,000 sample components in a petroleum sample using comprehensive 2D-GC, which fully demonstrated the potential of the technique (1). In the late 1990s, I joined a pharmaceutical company and figured it would be the perfect place to extend 2D-GC concepts to liquid chromatography (LC). However, it wasn't until the early 2000s that an opportunity came my way and I actually got to pursue my 2D-LC goals; a case of right place, right time.

Homemade 2D-LC

Two-dimensional GC worked, so I knew that 2D-LC should too. The big question was, "how do I build a system?" There were no commercially available instruments, but luckily we had multiple HPLC systems sitting side by side, so it really was a matter of configuring them and designing a 2D-LC interface to transfer primary column eluent to the secondary column. To cut a long story very short, I put together a system using a 12 port dual position valve and published the work back in 2003 (2). The paper highlighted several different 2D-LC setups, including single and dual columns in parallel in the second dimension, multiple detectors – amongst other things. In fact, what we were doing back then is very similar to current research in multidimensional LC. I guess we were ahead of our time!

Of course, building your own system presents challenges. One of the problems was the noise generated by back and forth switching of the valve, which made it difficult to discern co-eluting impurities from noise spikes. I needed to fully synchronize the valve timing, which was no small feat given system constraints. I had to integrate a high-speed electronic timer that, once triggered, would automatically and reproducibly start the switching sequence every 30, 60, or 90 seconds as per project needs. To summarize, I had to take into account three main considerations: (i) how do I configure the two HPLC systems so that they can communicate, (ii) how do I successfully take a fraction from the primary column and focus it at the head of the secondary column for further separation, and (iii) how do I reduce the baseline noise created by valve switching. And that's before we even got any data.

Retrieving the two-dimensional data from the 2D-LC system was equally challenging. The HPLC systems gave a series of detector responses as a function of primary column retention time. The second dimension's retention time was a real missing link. This had to be manually recreated in Excel taking into consideration the data acquisition frequency and the switching frequency of the valve (3, 4). A 2D contour plot of a sample mixture made of acidic, basic, and neutral compounds resolved on mixed mode stationary phases in two-dimensions, acidic in primary and basic in secondary, is shown in Figure 1 (4). The sample components are separated into acidic and basic zones with the neutrals along the diagonal. The location of sample components in the two-dimensional plane reflects its chemical nature.

So, despite the challenges, we still got great data to demonstrate the concept, which made the extra efforts worthwhile. Funnily enough, we were using sub-2 µm columns even back then for some of our proprietary, unpublished work without realizing one day it would emerge into what is commonly known as sub-2 µm chromatography. We just knew we needed to use columns with small particle sizes (1.8 µm) for high-efficiency, high-speed separations.

In short, 2D-LC required significant creativity and hard work on our part in those early years but, in return, provided excellent rewards.

Taking 2D-LC to the next level

I was using my homebuilt system until about two years ago when Agilent introduced its own 2D-LC-MS model.

Now, researchers like myself don't need to worry about many of the problems we faced. 2D-LC has become a very seamless and intuitive process; it's no longer a research tool in the hands of few researchers but a commercial tool with repetitive gradient capability. Each gradient starts at progressively higher organic strength than the previous gradient, improving efficiency. Previously, I was forced to use shallow gradients in both dimensions, but in modern systems, repetitive gradient programming is almost unlimited,

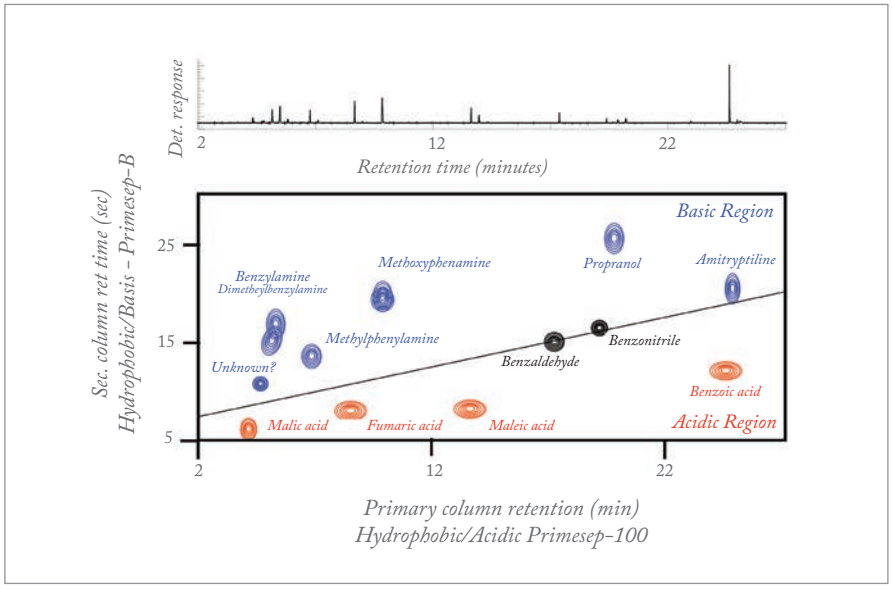

Figure 1. Complementary 2D-LC separation of a test mixture on a Primesep-100 column in the primary dimension and Primesep-B column in the secondary dimension. The one-dimensional chromatogram (top) was used in the generation of the two-dimensional contour plot. The primary column flow rate was 0.5 mL/min and the secondary column flow rate was 3.25 mL/min. The UV detection was at 215 nm.

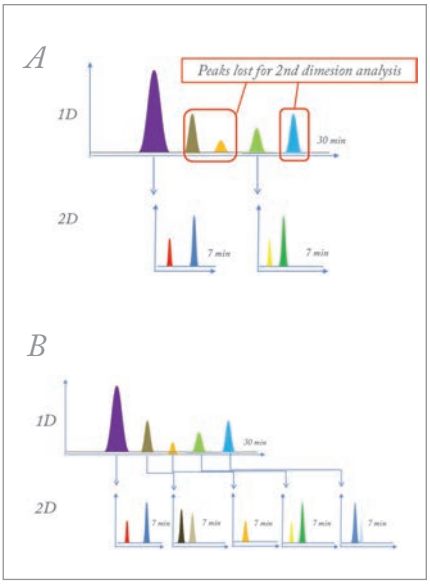

Figure 2. A. Standard heart-cutting 2D-LC. B. 2D-LC with peak parking solution for multiple heart-cutting, which allows the collection and storage of multiple fractions in 12 sample loops.

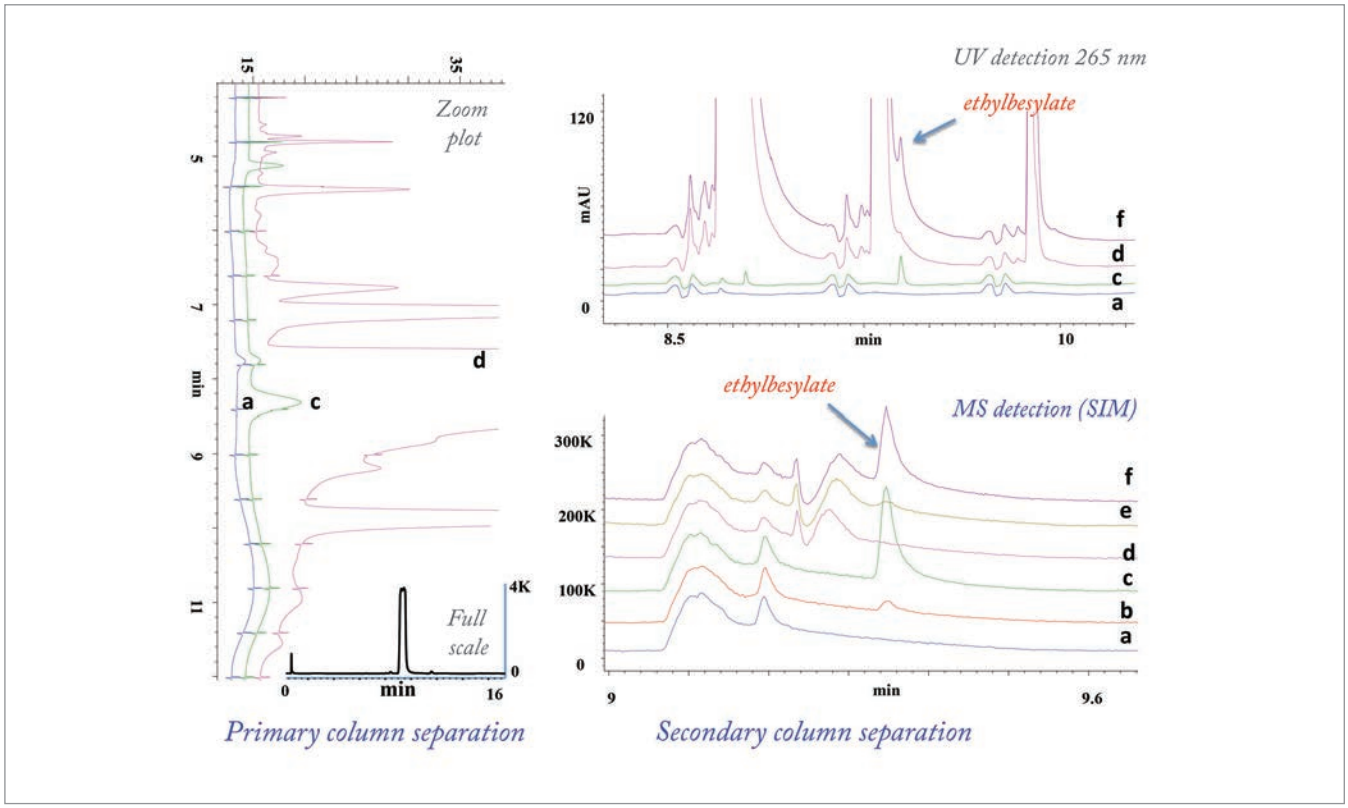

Figure 3. 2D-LC-MS separation of ethylbesylate, a genotoxic impurity, spiked into an active pharmaceutical ingredient (API). The plot to the left is the primary column separation on a C18 column (5 cm x 2.1 mm x 1.8 µm). The full-scale plot of the primary column separation is inset. The plots to the right are the secondary column separations of transferred fractions monitored using UV detection at 265 nm (top) and MS-SIM of ions characteristic ethylbesylate (M+1 ion, bottom). A phenylhexyl column (5 cm x 2.1 mm x 1.8 µm) was used in the secondary dimension.

Ethylbesylate is partially resolved in the secondary column with UV detection. The MS trace of unspiked and spiked samples at the bottom demonstrates the power of 2D-LC with MS detection. a: diluent blank, b: 0.5 ppm standard of ethylbesylate, c: 5 ppm of ethylbesylate standard, d: unspiked API, e: 0.5 ppm ethylbesylate spiked into API (10 mg/ml), f: 5 ppm ethylbesylate spiked into API (10 mg/ml).

Quick Tips for Getting into 2D-LC

1. *Know your sample.*
2. *Ask yourself, "what am I trying to achieve?" and "will 2D-LC help solve my problem?"*
3. *Review the literature. Find out what has been done before; do not reinvent the wheel.*
4. *Find out what instrumentation is commercially available; make things easy on yourself.*
5. *Experiment and have some fun playing around.*

which opens up a whole new range of applications.

In pharmaceutical analysis, we are particularly interested in resolving and identifying any trace co-eluting impurities in the midst of the main active pharmaceutical ingredient. But because chemical components that elute in the proximity of the main peak are often similar in structure (or isomeric), developing a specific and sensitive HPLC method can be difficult and conventional detection techniques like the diode array detectors (DAD) and MS have their limitations. Furthermore, the concentration levels of these impurities are often a few orders of magnitude lower. It's like trying to look at a main component peak the size of the tallest structure in the world (the Burj Khalifa in Dubai) whilst not wanting to miss an impurity the size of a pedestrian on the street beneath it. Using 2D-LC, we get a second chance to find any co-eluting components by using another mechanism of separation in the second dimension. That's a major advantage for the pharmaceutical industry.

In small molecule pharmaceutical science, impurity analysis confined to the proximity of main component does not warrant a fully comprehensive 2D-LC run. We tend to adopt a selective or pseudo comprehensive method (which is somewhat like extended heart-cutting). Currently, as we can only generate secondary chromatograms every 30 or 60 seconds, we slow the primary column flow rate (for example, from 1 ml/min to 0.05 ml/min) over the course of the main peak, which allows us to send more fractions to the secondary column. A recent innovation introduced at HPLC 2014 by Agilent is the peak parking solution for multiple heart-cutting, which allows the collection and storage of multiple fractions in 12 sample loops that can then be analyzed sequentially (see Figure 2). Using it, we should no longer need to slow the flow in the first dimension. I believe it will be of great value in the pharmaceutical industry. We are awaiting our demo version.

Another example of 2D-LC's potential in pharma is assessing MS incompatible methods for potential co-elution. You can take a small portion of the non-MS compatible mobile phase from the primary column and introduce it onto the secondary column for further separation without impacting the MS. I also see potential application of 2D-LC in the analysis of genotoxic impurities, which have significant bearing on patient health and must be quantified at very low levels. Application of 2D-LC-MS in the analysis of co-eluting genotoxic impurities is shown in Figure 3. Ethylbesylate, a genotoxic impurity co-eluting in the midst of API peak in the primary column, is partially resolved in the second dimension using UV detection. Use of specific, sensitive detector like MS enables the detection of the co-eluting impurity, which differs in concentration by more than four orders of magnitude, demonstrating the power of 2D-LC.

Achiral/chiral analysis is another area where 2D-LC could play a pivotal role, especially with increased numbers of chiral centers. We demonstrated the proof of concept in our earlier work on simultaneous, achiral/chiral analysis using 2D-LC (5).

In the future, I am positive that 2D-LC will be used to assess stability indicating methods for co-elution and as a feedback mechanism to optimize or improve routine methods. By embracing 2D-LC as a research tool earlier in development, I believe we can produce more robust stability indicating methods.

Given the advantages, I would not be surprised if 2D-LC starts to take center stage in the next 5-7 years – especially, if regulatory agencies take a firmer stance and scrutinize stability-indicating analytical methods for potential co-elusion.

Cadapakam J. Venkatramani is Senior Scientist at Small Molecules Pharmaceutical Science, Genentech Inc., San Francisco, CA, USA.

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Achiral-Chiral Heart-Cutting 2D-LC Analysis of Chiral Pharmaceutical Substances

Impurity analysis with simultaneous determination of enantiomeric composition using the Agilent 1290 Infinity 2D-LC Solution.

By Sonja Krieger and Udo Huber

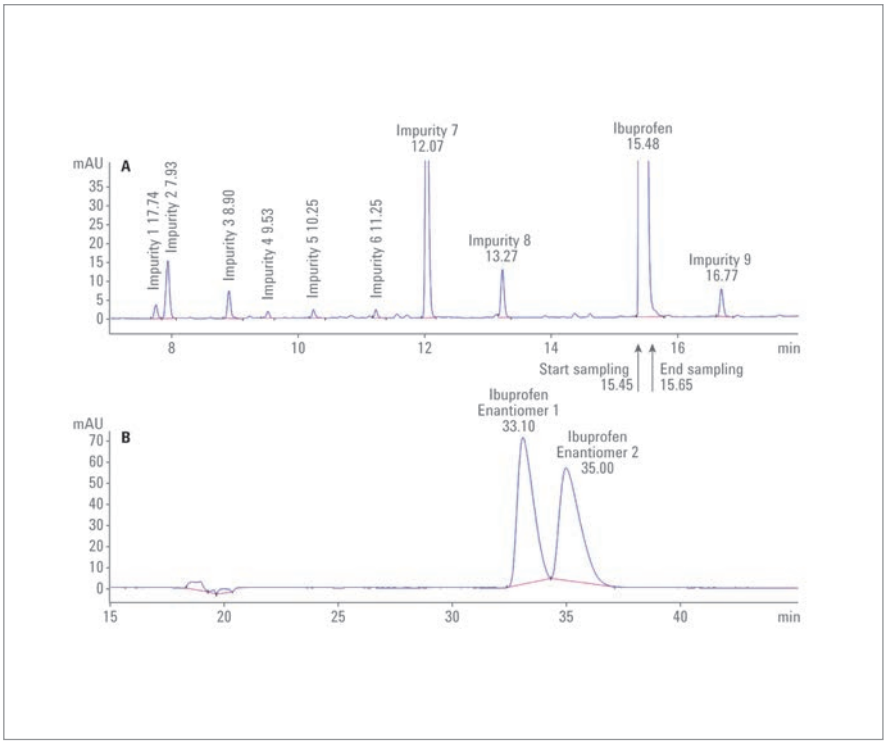


Figure 1. A. Separation of ibuprofen and impurities 1–9 on the first-dimension reversed phase column and B. heart-cutting of the ibuprofen peak and transfer to the second-dimension chiral column for separation of the enantiomers.

Introduction

According to ICH guideline Q3A (R2), impurities in new drug substances at levels of 0.05 % or above must be reported, and impurities at 0.1 % or above must be identified. Enantiomers of chiral drugs often show differences in pharmacokinetic behavior and pharmacological activity. One enantiomer might be pharmacologically active, while the other might be inactive, or even toxic. Therefore, the FDA has released guidance on the development of new stereo-isomeric drugs, demanding that the stereo-isomeric composition of a drug with a chiral center is known, and that specifications for the final product include assurance of purity from a stereochemical viewpoint.

Results and Discussion

The analysis of impurities contained in pharmaceutical substances can be accomplished by subjecting a concentrated solution of the substance to liquid chromatographic analysis. Impurities separated from the pharmaceutical substance are detected as small peaks next to a large peak originating from the main compound.

Racemic ibuprofen was chosen to prove the principle of the analysis of impurities in chiral pharmaceutical substances with simultaneous determination of the enantiomeric composition of the API. Figure 1A shows the chromatogram resulting from the first dimension reversed phase analysis of ibuprofen. Here, several impurities are separated from the main compound.

The effluent from the first-dimension column was sampled at 15.45 minutes with a loop fill time of 0.20 minutes to transfer the ibuprofen peak to the second-dimension chiral column and enable separation of the enantiomers. Figure 1 shows when the effluent of the first-dimension column was cut and transferred to the second-dimension column (A) and the separation of the ibuprofen enantiomers on the second-dimension chiral column (B). The ibuprofen enantiomers were separated with a resolution of Rs = 1.25 on the second-dimension chiral column.

Conclusion

This article demonstrates that the Agilent 1290 Infinity 2D-LC Solution is ideally suited for the analysis of impurities in chiral pharmaceutical substances and for the simultaneous determination of the enantiomeric composition of the API. In the first dimension, a reversed phase separation was used to separate achiral impurities from the API. A heart-cutting experiment was used to transfer the API to a second-dimension chiral column for determination of the enantiomeric composition.

For the full solution, visit: tas.txp.to/2DLC/chiral

The New 2D-LC Kids on the Block

A pair of relative newcomers to two-dimensional liquid chromatography relate their experiences to date and discuss how they plan to take advantage of the technique in the future.

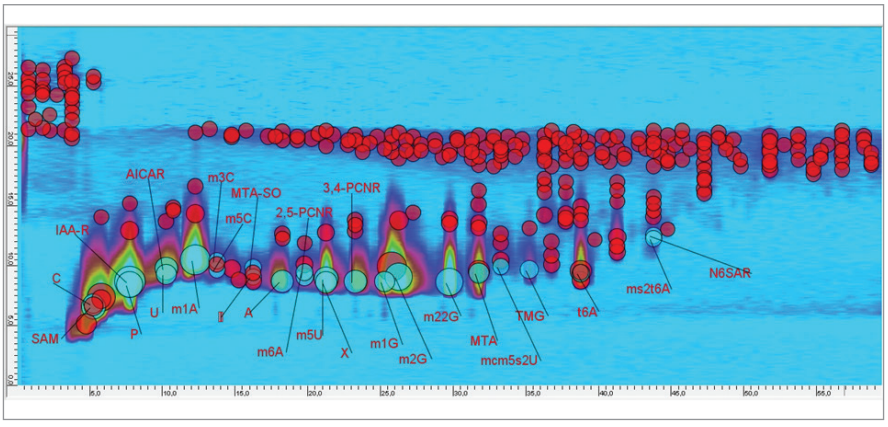


Figure 1. Detection of modified nucleoside in SPE-purified urinary samples by 2D-LC-QToF MS.

The metabolomics master

Bernd Kammerer heads the metabolomics facility at the Center for Biosystems Analysis (ZBSA), University of Freiburg, Germany, the purpose of which is to identify and quantify the full range of metabolites in biological systems. Bernd has broad scientific experience in metabolite and metabolome analysis, particularly with MS and NMR methods, and is familiar with bioinformatic methods for cluster analysis and metabolomic data mining.

What are the specific analytical needs of metabolomics?

Well, the complexity and high dynamic range of metabolite concentrations pose tough challenges for qualitative and quantitative analysis. Typically, the target compounds are small molecules with masses ranging between 100 and 1000 Da. These are studies in a wide variety of biological matrices, such as cell culture samples, urine and blood. On the one hand, you need precise MS analysis; on the other hand, a highly effective chromatographic separation is indispensible.

How has your view of 2D-LC changed over the last decade?

Ten years ago, 2D-LC was accomplished mainly by coupling the first and second dimensions offline. This provided fantastic separation power but it was very time-consuming, something that has been solved by modern 2D-LC systems. Today, it is possible to perform comprehensive 2D-LC without loss of time and with fewer potential sources of error.

What problem were you addressing when you first considered 2D-LC?

A major research project for us is the identification of a metabolic signature for early detection of human breast cancer. To do this, we are analyzing different biological samples with a high degree of complexity. Our target substances – modified nucleosides and ribosyl derivatives – differ only slightly in terms of their chemical structure and, therefore, retention in LC. Consequently, we decided to lift the chromatography to the next level, that is, to two-dimensional separation.

Metabolomics generally deals with complex matrices containing several hundred compounds, so separation power is essential. Insufficient separation can lead to the formation of highly reactive radical cations that interact with each other in the ion source (ESI or APCI) before entering the MS, causing ion suppression and artefacts. Since we are often looking to distinguish between two different biological states, it is important to improve the (semi-)quantitative analysis of target compounds, something that has particular relevance to ion suppression. An additional advantage of increased separation power is the possibility of determining previously hidden metabolites.

What were your expectations of 2D-LC?

Two things really. The structural class we deal with is differentially modified nucleosides, and we expected to achieve a better differentiation between isomers and nucleosides that could hardly be separated at all using a single column. And we were hoping for purification of peaks in the second dimension separation to help limit background noise.

What has been your experience so far?

We have just started with the new technique and are in the process of method optimization. Choosing the right separation mechanism is the first step of method development. Column dimensions, solvents and elution gradients all offer many possibilities to improve your analysis and different combinations of column materials lead to striking differences in orthogonality, retention and peak capacity. Our 2D-LC can be used as a standalone solution with a diode array detector, but it can also be coupled to different mass spectrometers. This high degree of functionality and flexibility is important for studies of complex biochemical pathways.

Currently, we are looking forward to the first comprehensive measurements from a large batch of real life samples. The first results look very promising (see Figure 1).

Do you anticipate 2D-LC being adopted more fully in metabolomics?

Yes. The importance of comprehensive 2D-LC will grow rapidly because of its universal applicability. I expect that 2D-LC in combination with different ion sources and MS solutions will become essential in applications of analytical chemistry, especially in research that must cope with complex matrices and/or complex analyte spectra.

How will you use 2D-LC in the near future?

We are planning to combine our 2D-LC solution with different mass spectrometers to address a range of sample and chromatographic challenges. Optimization of the chromatographic methods for the particular challenge at hand is clearly important here. In particular, for the separation of structural isomers, which occur frequently in modified nucleosides, I can see 2D-LC opening up new vistas.

The impurity analyst

Ole Gron has been in the pharmaceutical industry for over 10 years after finding his way into separation science via spectroscopy. Ole now works out of Vertex's San Diego R&D site in the analytical development department, which offers support functions from the lead generation stage of drug discovery right through to clinical trials.

How long have you been using 2D-LC?
We've been evaluating the technique for two years to see if it's something that Vertex wants to adopt at a larger scale.

What specific challenge prompted you to look at 2D-LC?
To be honest, when I first heard about 2D-LC it sounded like an interesting novelty, but I didn't see a real need in my environment – after all, we don't suffer from the overcrowded chromatograms seen in other fields. However, commercialization made us consider it more seriously, as it meant that we could test the technique without wasting time building a system that would be robust enough. Our major need is in impurity analysis; although our chromatograms are not overcrowded, we can have structurally related impurities that co-elute. We often run two separate but orthogonal LC methods to give us increased confidence; basically, we want to see as much as we can, as early as we can. We wondered if we could couple those two 1D runs into a single 2D-LC method.

How easy or hard have you found 2D-LC?
Of course, there is a learning curve – and there are a number of parameters that need to be considered carefully. However, after getting used to the setup, running the system is relatively easy. More importantly, I feel that I can trust a commercial system to provide the same result time after time. Robustness is important.

How are you using your 2D-LC system now?
I've been using multiple heartcuts to assess each impurity peak in my first dimension separation. Right now, I've got my system set up to store each impurity using 'peak parking', which gives me longer run time in the second dimension.

How fast do you think 2D-LC will be adopted by the pharmaceutical industry?
I don't think we'll see an explosive uptake; the pharmaceutical industry is pretty conservative when it comes to adopting new techniques, so it will take time. However, it's hard to go back once you've tried something superior. I've been assessing 2D-LC in a number of different applications and I can imagine wider adoption within the next year or two at Vertex.

If you are also a newcomer to 2D-LC, why not learn from the masters by watching the on-demand webinar presented by Koen Sandra and Dwight Stoll at: tas.txp.to/1114/2DLCwebinar

Multiple Heart-Cutting and Comprehensive 2D-LC Analysis of Bio-pharmaceuticals

Online 2D-LC analysis of complex N-Glycans for highest resolution using the Agilent 1290 Infinity 2D-LC solution.

Sonja Schneider, Edgar Naegele, Jens Trafkowski, and Sonja Krieger

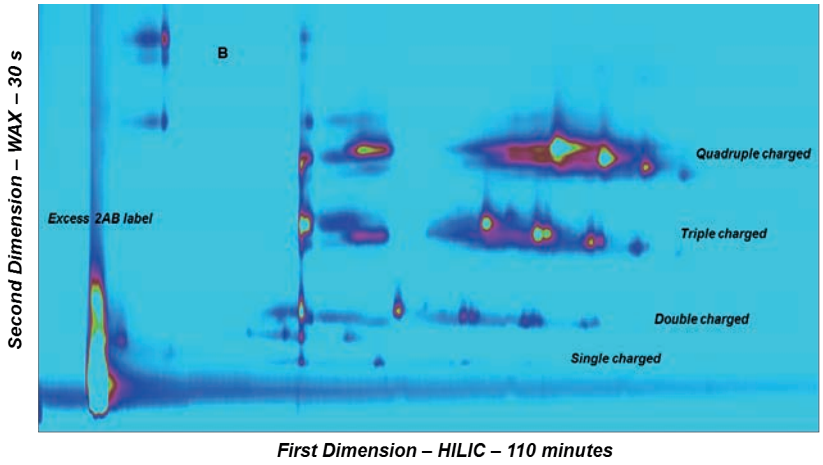


Figure 1. Comprehensive HILIC/WAX 2D-LC separation of fetuin (A) and EPO (B), showing highly orthogonal separation. The ion exchange chromatography in the second dimension reveals the charge pattern of the glycans.

Introduction

Erythropoietin (EPO) is a 30400 dalton (Da) glycoprotein hormone that regulates the production of red blood cells (erythropoiesis). The glycosylation of EPO is highly variable because it contains multiple glycosylation sites, each of which can have a wide variety of glycan structures. This results in a huge complexity of glycan structures that is referred to as microheterogeneity. Recombinant human EPO (rhEPO) has proven to be highly efficient in the treatment of different diseases such as anemias associated with cancer, chronic renal failure and HIV infection. Detailed characterization of the glycan profile of biopharmaceuticals is a regulatory requirement as differences in glycosylation can affect both the pharmacodynamics and pharmacokinetic behavior in the human body. Hence, it is necessary to develop advanced analytical technologies for efficient and detailed glycosylation analysis.

Results and Discussion

The method of choice for standard HPLC analysis of released glycans is typically hydrophilic interaction chromatography (HILIC) after labelling with 2-aminobenzamide (2AB) for sensitive fluorescence detection. Whereas HILIC efficiently separates glycans according to hydrodynamic radius, it is insufficient to fully resolve the complex mixture of branched glycan structures that are present in samples such as EPO. Fortunately, weak/strong anion exchange chromatography (WAX/SAX) provides a highly orthogonal separation that depends on the number and arrangement of acidic monosaccharides in the glycan. A combination of WAX/SAX and HILIC has a huge potential to enhance peak capacity in two-dimensional liquid chromatography (2D-LC) due to the highly orthogonal nature of these two separation techniques.

The Agilent 1290 Infinity 2D-LC solution enables online 2D-LC workflows for either comprehensive or (multiple) heart-cutting analysis. Comprehensive 2D-LC analysis, using two sample loops connected through a 2-position/-4-port duo valve, captures all peaks from the first dimension. If higher resolution is desired in the second dimension, the 1290 Infinity multiple heart-cutting 2D-LC solution provides more flexibility, for example, for applying longer cycle times or using longer columns.

The 2D separation provides high peak capacity and resolution, and many of the co-eluting peaks from the HILIC dimension are well separated by WAX. The second dimension separation groups glycans by their charge. The neutral glycans, which elute immediately with the injection peak, are shortly followed by the singly charged glycans. More clearly separated, the double, triple, quadruple and a few quintuple (fetuin) charged glycans elute with increasing salt gradient in the second dimension. EPO isoforms are classified according to their net charge (epoetin alpha, beta, and so on). This setup enables simultaneous charge profiling in combination with a well resolved glycan peak pattern. The perfect orthogonality of both

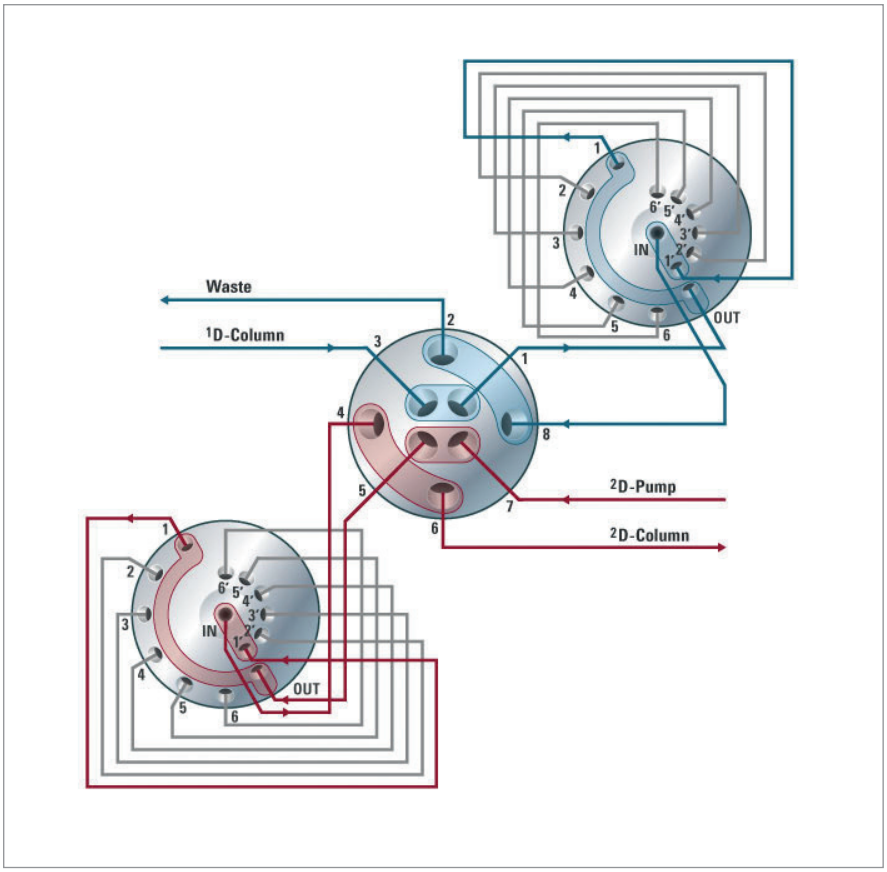


Figure 2. Plumbing diagram of the 2-position/-4-port duo valve in combination with two 6-position/-14-port valves with twelve pre-installed 40 µL loops.

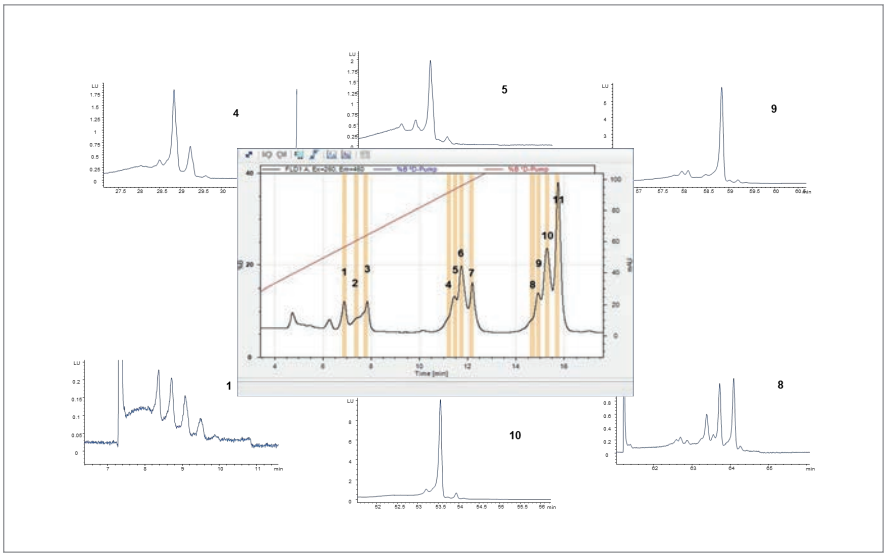


Figure 3. Six examples for the high resolving power of the multiple heart-cutting setup, resolving up to eight peaks under the area that is marked with number 8.

separation mechanisms is shown in the comprehensive 2D-LC chromatogram (Figure 1). This approach offers the possibility to screen and get an overview of the whole sample, which can be used for fingerprinting analysis.

To achieve highest resolution, the new multiple heart-cutting 2D-LC approach was developed. In this setup the two sampling loops from the comprehensive 2D-LC setup are exchanged by two 6-position/14-port column selection valves, which are equipped with six sample loops (Figure 2).

Multiple heart-cutting 2D-LC also delivers the highest resolution in the second dimension by allowing longer cycle times or columns. This additional flexibility enables the use of HILIC in the second dimension for the current application, which is difficult to achieve in comprehensive 2D-LC due to the ultrashort run times but with the longer re-equilibration times required for HILIC columns. A gradient time of 3.5 minutes was used with a re-equilibration time of 1.4 minutes. The glycans were retained on the short HILIC column and good 2D resolution was achieved (Figure. 3). Six examples are shown to demonstrate the resolving power of the HILIC separation within the multiple heart-cutting setup (peaks 1, 4, 5, 8, 9, and 10). Under most of the peaks, which show one major peak in the first dimension, several underlying peaks were detected and resolved, for example, peak 8 with at least eight peaks in the second dimension.

Conclusion

The combination of both comprehensive and multiple heart-cutting 2D-LC, performed on a single instrument, delivers an ideal tool for a comprehensive analysis of glycoproteins.

For the full solution, visit: tas.txp.to/2DLC/glycan

Making 2D-LC Mainstream

Two-dimensional liquid chromatography is not a new technique – but it's only relatively recently that commercial systems have added usability, reliability and reproducibility to 2D-LC's formidable separation power. The result? 2D-LC is now more accessible to a much wider audience. Here, Michael Frank and Jens Trafkowski share Agilent's commitment to the technique to date and offer a glimpse into the future.

Michael Frank is Senior Director of global marketing for the liquid phase separations division, which includes analytical systems and capillary electrophoresis. Before Michael joined Agilent 10 years ago, he was Director of Analytics at Graffinity Pharmaceuticals (later renamed to Santhera). He originally trained as an inorganic chemist at the University of Heidelberg and holds a PhD in chemistry.

Jens Trafkowski is Global Product Manager for the liquid phase separations division and took over responsibility for Agilent's 1290 Infinity 2D-LC solution in 2012. Before joining Agilent in 2011, Jens gained over six years' experience as an application specialist, offering training and technical support for LC–MS systems. Jens gained his PhD from the Institute of Legal Medicine in Bonn with work on LC–MS/MS applications in forensic and clinical toxicology.

Michael, could you please provide a brief overview of Agilent's LC strategy?

Michael Frank: Agilent Technologies offers a broad range of analytical LC systems to address the needs of different customers – from routine systems right up to ultra-high performing systems for researchers at the cutting-edge of the field.

Looking at the upper end of our portfolio, our clear intention is to offer the highest performance possible. The main drive is efficiency, which we split into three areas:

Analytical efficiency – the customer must be provided with the best possible data to solve complex analytical problems.

Instrument efficiency – the operator should be able to easily interact with the system, for example, to quickly create new methods.

Laboratory efficiency – the system should offer backwards compatibility, be easily integrated into the existing lab environment, help to reduce overall operating costs, and so on.

As noted above, an important part of our strategy from the beginning has been backwards compatibility, meaning that all new systems must – first and foremost – be able to match previous systems. We've spent heavily on R&D to deliver on this promise.

And where does 2D-LC fit into the mix?

MF: In our view, two-dimensional LC offers the highest peak capacity and resolution possible – so it immediately lends itself to complex samples. There are a number of ways to increase resolution and peak capacity. The first is to reduce the particle size. That philosophy gave us UHPLC, and we've certainly moved into that space. But smaller particles increase system backpressure and the actual gain in peak capacity is small. With 2D-LC, the gains are not measured in percentage points but rather by factors of two or three.

We've invested a great deal to drive 2D-LC forward from its position as an experimental "homemade" technique by focusing on robust, commercial systems that can be operated by those with even basic LC experience. We launched 2D-LC as a technology in 2012, so we've had a chance to see our system being used in productive environments, for example, in method development labs in big pharma – very far away from the its beginnings as a niche technological curiosity.

A number of companies have already published scientific papers using 2D-LC (several of which are referenced throughout this supplement) – this is an excellent sign that 2D-LC has been accepted as a valid technology for the mainstream market – and that's really where we want to go.

Jens Trafkowski: 2D-LC fits perfectly into our strategy as it adds to the existing portfolio by extending our customers' ability to solve complex problems. By fully commercializing the technique, we can offer unprecedented ease of use and reduce the risk of making mistakes. And because we've done a lot of work in the background, creating methods and protocols, users are able to achieve results right out of the box – and, in the meantime, they can read the 2D-LC Primer we have created to understand the theory behind

the technique. We think this approach offers the best possible introduction to a technique that – once conquered – adds a fantastic, flexible and formidable new technique to the analytical toolbox.

How important is collaborative development?

MF: From the early stages of development we have enjoyed very close collaborations with several 2D-LC thought leaders in both academia and industry. They have scrutinized our systems and provided excellent input, which has enabled us to increase usability and improve performance.

Our current collaborations have taken us away from the original thought leaders and experts, and into laboratories with much less multidimensional experience – this is another very good sign for us as a manufacturer. Since 2012, we've made several improvements, for example, we most recently commercialized multiple heart-cutting 2D-LC (see Figure 2, page 17) as a complete kit for users – something that is especially useful in the pharmaceutical industry.

What would you say to those with limited need for 2D-LC?

MF: That's a good question. If users mainly want to do 1D LC, but sometimes require the power of 2D-LC, the switchable 1D – 2D configuration fully optimizes the use of the instruments, meaning you don't end up with a 2D-LC system sitting in the corner gathering dust. Offering such flexibility fits very nicely with our philosophy about lab efficiency.

You already have the "2D-LC Primer" (see inside back cover) – how else are you engaging your customers from an educational perspective?

MF: We've been very active at conferences with technical seminars dedicated to offering a re-education on 2D-LC. But I think our fast-growing library of application notes from us and our customers gives insight not only into how 2D-LC works and in what areas, but also how flexible the technique can be depending on the objective – heart-cutting, multiple-heart-cutting, comprehensive – each one offers unique benefits by using the extra peak capacity in a different way. In many cases, we have already developed the solutions that prospective 2D-LC users will need.

JT: We really want users to get the best possible performance out of our systems – knowledge and education is key to that. With my background as an application specialist, I truly understand that once people know more about the technique and gain more confidence, the outcome will be much improved. We're on a constant mission to make that the case.

What future 2D-LC developments can we expect to see?

MF: Though I can't disclose too many details about future plans (I'm sure our competitors would love that!), I can clearly state that we have a number of firm objectives – one of the biggest is to make it even easier to set up 2D-LC methods. Our ambition is to remove all complexity so that people may approach 2D-LC without trepidation – just as they would with any other LC method. Advancing data analysis capabilities is also important in that regard.

JT: It's clear that we're not at the end of technical development. There are lots of ideas and possibilities on the table – some of which have been provided by our customers. Development is – and should always be – an ongoing process. We are the technology leaders and we want to maintain that position.

Where do you expect to see the biggest uptake?

MF: Two-dimensional LC really comes into its own with complex samples – I see no limitations in terms of market space in that regard. We have companies looking for allergens in processed food, for example. Others are finding uses in the hydrocarbon processing industry. Biopharmaceuticals go hand in hand with complex samples, and there are also applications in small molecule analysis in terms of impurity analysis and method development. Immediately, you can see that there is a really broad application range – further applications will come online as customers grow in confidence and see success in other areas.

Right now, 2D-LC has crossed over the threshold of 'early adopters,' and moved into applications where the need for separation power is greatest. We expect the uptake of 2D-LC to closely mirror the story of UHPLC; ten years ago, UHPLC was predominantly used in R&D departments in companies and academics, but now it has spread into common usage.

A good indication that 2D-LC has a great future is the fact that other analytical system manufacturers are also starting to move into the space. That's confirmation for us that we made the right decision to invest in the technology so early on. We have the great advantage of being first to market – and we'll not be giving that up.

JT: Certainly, as 2D-LC proves itself time and time again in multiple application areas, people will recognize the benefits and new applications will line up. We can't predict all of these, but I don't see them being limited to certain field. From bioanlysis to food and everything in between, 2D-LC will take root.

A final word of encouragement?

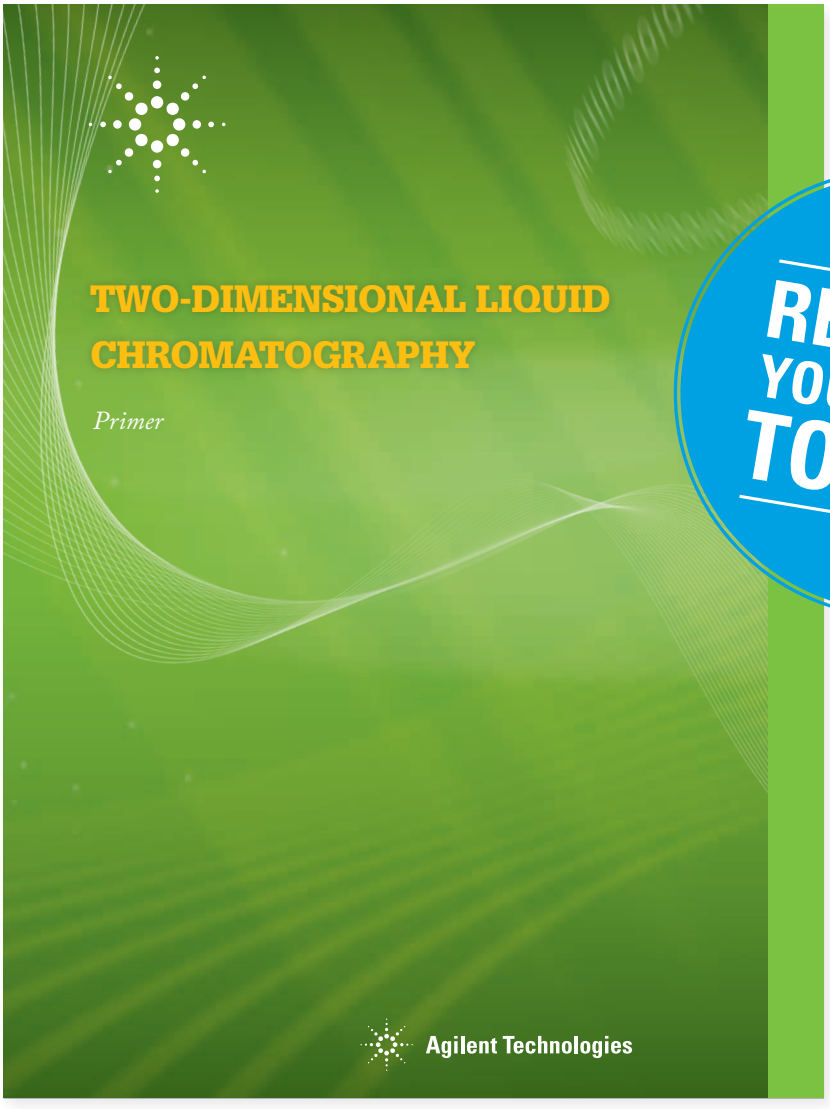
JT: Chromatographers don't want to miss a single peak, but they also want reliable, reproducible systems – and that's exactly what we offer.

MF: For those who have heard of 2D-LC and have some interest, I would say: do not be afraid! It's a great tool that can help you solve your analytical problems faster than ever before. It's only one small step from 1D-LC to 2D-LC.

Get Your Free 2D-LC Primer

Two-Dimensional Liquid Chromatography

Professor Dr. Peter W. Carr, University of Minnesota, Minneapolis, MN, USA
Professor Dr. Dwight Stoll, Gustavus Adolphus College, St. Peter, MN, USA



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