analytical chemistry

Review

Cite This: Anal. Chem. 2019, 91, 240−263

pubs.acs.org/ac

Recent Developments in Two-Dimensional Liquid Chromatography: Fundamental Improvements for Practical Applications

Bob W. J. Pirok,*[,†,‡] Dwight R. Stoll,[§] and Peter J. Schoenmakers[†]

[†]University of Amsterdam, van 't Hoff Institute for Molecular Sciences, Analytical-Chemistry Group, Science Park 904, 1098 XH Amsterdam, The Netherlands

[‡]TI-COAST, Science Park 904, 1098 XH Amsterdam, The Netherlands

[§]Department of Chemistry, Gustavus Adolphus College, Saint Peter, Minnesota 56082, United States

CONTENTS

Liquid chromatography (LC) is an incredibly successful analytical separation tool. Its versatility is unprecedented because of the many different separation modes (reversed-phase LC, ion-exchange chromatography, size-exclusion chromatography, etc.) and because almost all samples can be dissolved in some kind of solvent, ranging from water to organic solvents to strong acids or bases. Conditions (mobile and stationary phases, additives, pH, temperatures, etc.) can be found to separate almost all pairs of analytes. For example, LC is immensely successful in the separation of enantiomers. Good selectivities can be accompanied by high efficiencies in a very short time, using contemporary ultrahigh-performance liquid chromatography (UHPLC) instrumentation and (short) columns packed with sub-2-μm particles. However, LC cannot deliver very high efficiencies in a short time. Unlike other techniques, such as gas chromatography (GC) or capillary electrophoresis (CE), plate counts exceeding 100 000 are not routinely obtained in LC. As a result, LC cannot easily deal with complex mixtures that contain more than a few dozen analytes. While the selectivity between any pair of analytes can be maximized, these peaks may then start to overlap with other relevant analytes or with matrix compounds. There simply is not enough room in LC chromatograms to separate very many compounds that behave "statistically",[1] and the attainable peak capacity does not suffice to separate complex samples. As a rule of thumb, LC offers a high probability of success for separating samples containing 10 or 20 components in 1 or 2 h or up to 50 components in about 10 h.[2,3]

When dealing with complex samples, comprehensive two-dimensional liquid chromatography (LC × LC) is an attractive approach (Figure 1). Peak capacities of several thousands[4−7] can be achieved, and 10 000 is within reasonable reach.[8] For high-resolution separations, LC × LC is also much faster, with a peak-production rate (peak capacity divided by the analysis time) of about 1 peak per second, as compared to 1 peak per minute for typical high-resolution one-dimensional LC (1D-LC). The "room" in the chromatogram created by the much-enhanced peak capacity creates the possibility to fully employ two different selectivities. Groups (or "classes") of analytes can be very efficiently separated from each other,[9−11] provided that the selectivities (retention mechanisms) employed in the two dimensions are very different. In the most favorable case, in which the retention times in the two dimensions are completely independent, we speak of orthogonal separations. When separation is obtained using two very different retention mechanisms, the uncertainty of peak assignment[12] can be dramatically reduced. Because of the diverse selectivities, high degrees of orthogonality can be achieved in combination with mass spectrometry (LC × LC-MS). In comparison, the combination of ion-mobility spectrometry (IMS) and MS is very fast but very much less orthogonal.[13]

Of course, there are some caveats. LC × LC separations typically take longer than 1D-LC separations (analysis times of 30 min to several hours are common). Successive dilution of the sample during two separations may result in decreased detection sensitivity and compatibility issues may arise, because without further intervention (e.g., "active modulation", see section Modulation below) the first-dimension (^{1}D) effluent is the second-dimension (^{2}D) injection solvent. Also, dedicated data-analysis and visualization software is needed. Finally, method development is arguably more complex and potentially (much) more time-consuming in LC × LC than in 1D-LC. These obstacles to successful implementation and

Special Issue: Fundamental and Applied Reviews in Analytical Chemistry 2019

Published: November 1, 2018

DOI: 10.1021/acs.analchem.8b04841
Anal. Chem. 2019, 91, 240−263

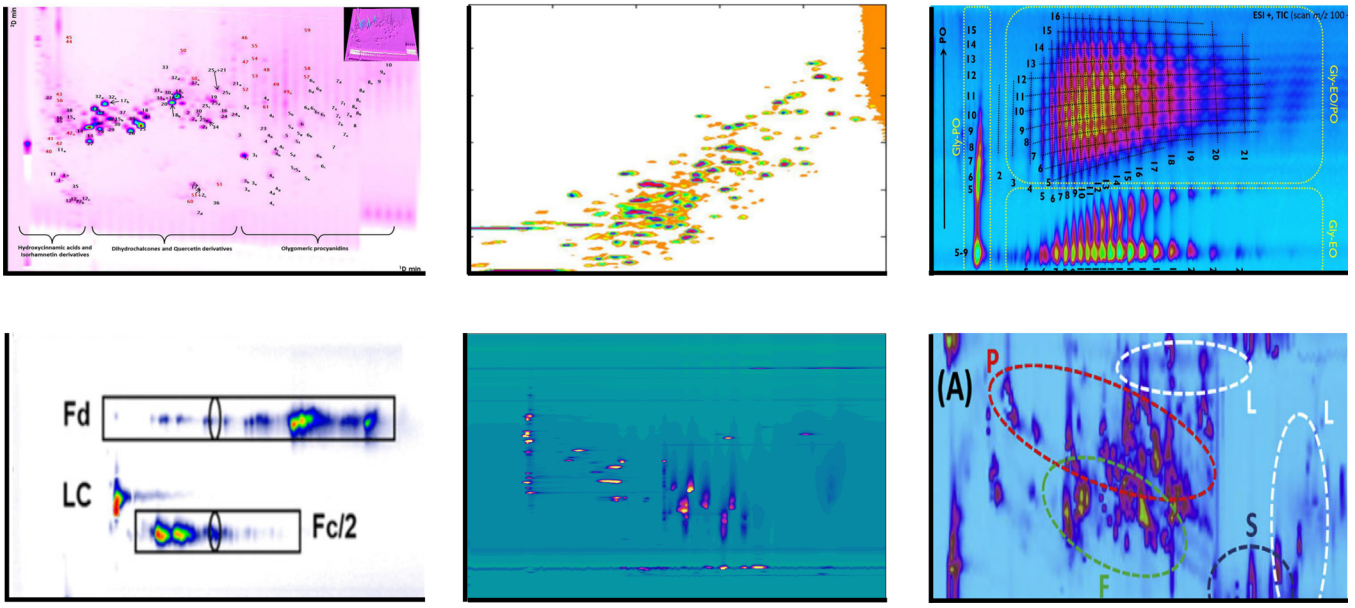

Figure 1. Examples of separations by 2D-LC. Top-left, HILIC × RPLC of polyphenols in apple extract;[6] top-middle, RPLC × RPLC of tryptic digest of three proteins;[14] top-right, HILIC × RPLC of polyether polyols;[9] bottom-left, HILIC × RPLC of therapeutic antibodies subunits;[15] bottom-middle, SAX × IP-RPLC of aged, synthetic dyes;[5] bottom-right, RPLC × RPLC of TCM Dengzhan Shengmai.[16] See respective papers for details. Top-left figure reproduced from Development of an improved online comprehensive hydrophilic interaction chromatography × reversed-phase ultrahigh-pressure liquid chromatography platform for complex multiclass polyphenolic sample analysis, Sommella, E.; Ismail, O. H.; Pagano, F.; Pepe, G.; Ostacolo, C.; Mazzoccanti, G.; Russo, M.; Novellino, E.; Gasparrini, F.; Campiglia, P. *J. Sep. Sci.*, Vol. *40*, Issue 10 (ref 6). Copyright 2017 Wiley. Top-middle figure reprinted from *J. Chromatogr. A, 1498*, Sarrut, M.; Rouvière, F.; Heinisch, S., Theoretical and Experimental Comparison of One Dimensional versus Online Comprehensive Two Dimensional Liquid Chromatography for Optimized Sub-Hour Separations of Complex Peptide Samples, pp. 183−195 (ref 14). Copyright (2017), with permission from Elsevier. Top-right figure reprinted from *J. Chromatogr. A, 1569*, Groeneveld, G.; Dunkle, M.N.; Rinken, M.; Gargano, A.F.G.; de Niet, A.; Pursch, M.; Mes, E.P.C.; Schoenmakers, P.J., Characterization of complex polyether polyols using comprehensive two-dimensional liquid chromatography hyphenated to high-resolution mass spectrometry, pp. 128−138 (ref 9). Copyright (2018), with permission from Elsevier. Bottom-left figure reproduced from Stoll, D. R.; Harmes, D. C.; Staples, G. O.; Potter, O. G.; Dammann, C. T.; Guillarme, D.; Beck, A. *Anal. Chem.* **2018**, *90* (9), 5923−5929 (ref 15). Copyright 2018 American Chemical Society. Bottom-middle figure reprinted from *J. Chromatogr. A, 1436*, Pirok, B. W. J.; Knip, J.; van Bommel, M. R.; Schoenmakers, P. J., Characterization of synthetic dyes by comprehensive two-dimensional liquid chromatography combining ion-exchange chromatography and fast ion-pair reversed-phase chromatography, pp. 141−146 (ref 5). Copyright (2016), with permission from Elsevier. Bottom-right figure reprinted from *J. Chromatogr. A, 1517*, Sheng, N.; Zheng, H.; Xiao, Y.; Wang, Z.; Li, M.; Zhang, J., Chiral Separation and Chemical Profile of Dengzhan Shengmai by Integrating Comprehensive with Multiple Heart-Cutting Two-Dimensional Liquid Chromatography Coupled with Quadrupole Time-of-Flight Mass Spectrometry, pp. 97−107 (ref 16). Copyright (2017), with permission from Elsevier.

application of LC × LC techniques and possible remedies have been discussed in detail elsewhere.[17]

To separate a single peak from a complex matrix or to reduce assignment uncertainty for a specific peak the separation of a single fraction of the ^{1}D effluent in a second dimension suffices. Indeed, such "heart-cut" two-dimensional LC approaches (which we denote with a hyphen, LC-LC) are very powerful tools for obtaining single, pure peaks or for establishing the purity of a specific compound.[18−20] In LC × LC all fractions are subjected to two different separations to obtain a comprehensive characterization of the sample. The middle ground is occupied by multiple-heart-cut techniques, in which a number of fractions from across the chromatogram are subjected to the second dimension, and "selective-comprehensive" two-dimensional LC, in which one or more bunches of successive ^{1}D fractions are subjected to the second dimension. The main strengths and weaknesses of heart-cut LC-LC are summarized in the SWOT analysis presented as Table 1. The corresponding analysis for LC × LC is presented as Table 2.

The astounding separation power offered by LC-LC and LC × LC entices chromatographers to overcome the challenges mentioned above, and 2D-LC methods are increasingly being implemented in a variety of application areas traditionally served by 1D-LC.

In this review, we consider some 160 applications of two-dimensional LC (2D-LC) techniques that have been published in 2016, 2017, or 2018. Progress in methodology in the same period is discussed in detail. Older publications are cited when needed to provide a foundation for the discussion. While an inventory is made of all applications, we will focus on online application of 2D-LC in our discussions. Much technical progress has been focused on the interface (modulator) between the two separation dimensions. These smart modulation techniques, such as stationary-phase assisted modulation and active solvent modulation, are discussed in detail in the Modulation section. Finally, significant attention will be paid to emerging strategies for developing 2D-LC methods in the Method Development and Optimization Strategies section. For a detailed treatment of the fundamental principles and successful implementation of 2D-LC techniques, readers are referred to useful guides published elsewhere.[2,23,24] Following the accepted nomenclature for comprehensive two-dimensional separations,[25] we refer to the respective dimensions with a prefix, i.e., ^{1}D refers to the first dimension and ^{2}D to the second dimension.

DOI: 10.1021/acs.analchem.8b04841
Anal. Chem. 2019, 91, 240−263

Table 1. SWOT Analysis of Heart-Cut Two-Dimensional Liquid Chromatography (LC-LC)

strengths	weaknesses
• Very high resolving power	• Somewhat increased conceptual and instrumental complexity
• Added selectivity from second ("orthogonal") dimension	• Analysis time is increased (especially when multiple fractions are selected for analysis in the second dimension)
• Choice from many different retention mechanisms	• Possibly reduced detection sensitivity[a]
• Enhanced purification or purity assessment of target analytes	• Phase-system incompatibility issues[a]
• Preparative separations possible	• Method development is relatively straightforward
• Greatly reduced uncertainty of peak assignments (in comparison with 1D-LC)	
• Readily combined with MS and MS/MS techniques	
opportunities	**threats**
• Rigorous assessment of peak purity is tantamount in (bio-) pharmaceutical industries	• For qualitative analysis high-resolution hyphenated techniques (LC-MS or LC-MS/MS) are usually preferred
• "Spatial" comprehensive two-dimensional (and three-dimensional) LC[21]	

[a]These issues may (largely) be addressed by incorporating active-modulation techniques (see section Modulation below).

Table 2. SWOT Analysis of Comprehensive Two-Dimensional Liquid Chromatography (LC × LC)

strengths	weaknesses
• High peak capacities (1 000−10 000) routinely possible	• Added conceptual and instrumental complexity
• High peak-production rates (typically 1 peak per second)	• Rather long analysis times (typically 30 min−2 h)
• Choice from many different retention mechanisms	• Possibly reduced detection sensitivity[b]
• Added selectivity from second ("orthogonal") dimension	• Phase-system incompatibility issues[b]
• Structured, readily interpretable chromatograms[a]	• Data-analysis software needed
• "Group-type" separations of classes of analytes	• Difficult and time-consuming method development[c]
• Readily combined with MS and MS/MS techniques	
• Greatly reduced uncertainty of peak assignments (in comparison with 1D-LC)	
opportunities	**threats**
• Increased need for detailed characterization of complex samples from many fields[d]	• High-resolution hyphenated techniques (LC-MS, LC-IMS-MS, IMS-MS) may compete for certain applications[e]
• "Spatial" comprehensive two-dimensional (and three-dimensional) LC[21]	

[a]In the case of low sample dimensionality.[22] [b]These issues may (largely) be addressed by incorporating active-modulation techniques (see section Modulation below). [c]This may be overcome by using advanced method-development software. [d]See Table 3 for an overview of these. [e]IMS = ion-mobility spectrometry.

MODULATION

Incompatibility. At the heart of any 2D-LC setup is the modulation interface, which has the function of transferring fractions of the first-dimension (^{1}D) effluent to the second-dimension (^{2}D) column. The most common tool for fraction transfer is a 2-position 8- or 10-port valve equipped with two identical storage loops, which "passively" (see below) sample the ^{1}D separation. Alternatingly, one loop samples the ^{1}D effluent, while the contents of the other loop are injected into the ^{2}D column.

Using the setup of Figure 2, the ^{1}D effluent is the injection solvent of the second dimension. This may give rise to "incompatibility issues". Regardless of the mode of 2D-LC operation (LC-LC or LC × LC), the order in which the two separation methods are combined is extremely important. The pursuit of maximal orthogonality between two separation dimensions with respect to the sample dimensions often culminates in a challenge to combine two incompatible solvent systems, with the ^{1}D effluent detrimentally affecting the ^{2}D separation.

An extreme case arises when the two mobile phases are completely immiscible, but more-subtle incompatibilities often occur. For example, a significant difference in viscosity between the two solvent systems may result in flow instabilities. At the interface of the two solvents, a low viscosity ^{2}D mobile phase can penetrate a high-viscosity injection plug in finger-shaped cones as it percolates through the porous medium of the ^{2}D column. This effect is known as viscous fingering and potentially results in peak deformation and even peak splitting.[28,29] However, it seems that this is really only a serious issue in cases of extreme viscosity contrast between the mobile phases used in the two dimensions.[30] In our experience, other factors such as a solvent strength mismatch between the two dimensions can be far more problematic in most applications.[31]

A significant difference in solvent strength may also result in peak deformation or peak splitting. If the ^{1}D effluent is a relatively strong injection solvent in comparison with the ^{2}D eluent, the intended retention mechanism may be disturbed as the analytes are not strongly retained by the stationary phase in the strong injection solvent. A classic example of this problem is the combination of organic size-exclusion chromatography (SEC) and reversed-phase LC (RPLC), where the fully organic ^{1}D effluent can prevent hydrophobic analytes from being retained by the hydrophobic stationary phase. Within the injection plug, size-exclusion conditions prevail, and larger analytes will move faster than the average velocity of mobile-phase molecules. Ahead of the plug, the eluent is weak, causing analytes to slow down and be caught up by the strong-solvent plug. Conversely, analytes at the rear of the plug will disperse in the weak ^{2}D eluent and be retained by adsorption effects in the SEC separation. This effect is known as breakthrough, with an unretained ("breakthrough") peak eluting with the dead volume and a typically much smaller "real" peak at the normal location.[32] However, the potential combination of RPLC and SEC in reverse order may also lead to problems because the aqueous RPLC effluent may induce undesired adsorption effects.[33] Components of the ^{1}D effluent matrix may potentially complicate detection after the second dimension as well. One example of such detector incompatibility is the use of salts in the ^{1}D separation with MS or ELSD detection of the ^{2}D, where the salt from the ^{1}D elutes as a concentrated band into the detector. Finally, the ^{1}D effluent may lead to column degradation in the second dimension. For example, an ion-chromatography ^{1}D separation with a sodium-hydroxide mobile phase may not be combined with a conventional ^{2}D reversed-phase separation because silica-based ODS columns usually degrade rapidly in basic eluents. The frequent switching

DOI: 10.1021/acs.analchem.8b04841
Anal. Chem. 2019, 91, 240−263

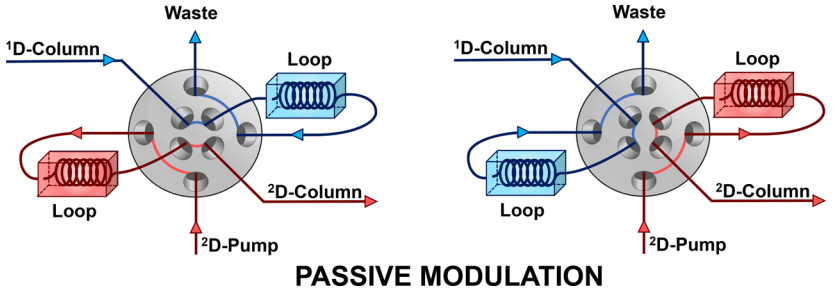

Figure 2. Generic scheme of a loop-based, passive-modulation interface for use in 2D-LC. As the ^{1}D effluent is sampled by one loop, the contents of the other loop are, without any further modification, injected into the second dimension. In the configuration shown, the loops are filled and emptied in opposite directions ("backflush" mode). The scheme shows an 8-port valve, but a 10-port valve can also be used.[26,27] In (multiple) heart-cut 2D-LC, the loops can be replaced by "decks" each containing an array of sampling loops to allow storage of more than one fraction.

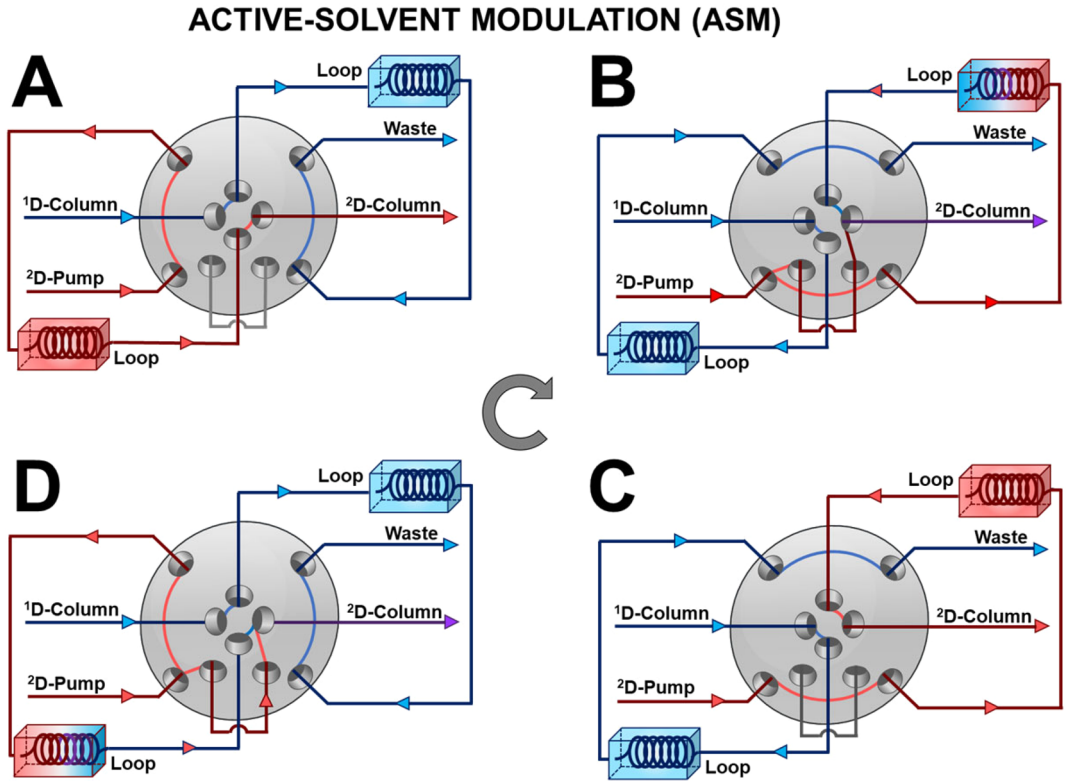

Figure 3. Illustration of the function of Active Solvent Modulation (ASM) for coupling the two dimensions of separation in a 2D-LC system. This valve has eight ports and four positions. Postions A and C are functionally identical to those of a conventional 8- or 10-port two position valve (e.g., see Figure 2). In positions B and D, however, part of the flow from the ^{2}D pump is split and travels through the bypass capillary. This portion of the flow joins the stream of fluid exiting the sample loop before the mixture leaves the valve and enters the ^{2}D column. In this way, this split part of the ^{2}D flow acts as a diluent for the ^{1}D effluent fraction injected into the ^{2}D column.

of the modulation valve may also affect the lifetime of the ^{2}D column. The modulation valve may be constructed so as to minimize the pressure pulses that accompany valve switching.[34]

It is not surprising that much research in the 2D-LC community is devoted to overcoming these incompatibility issues. Generally, this is addressed by making modifications to the modulation process. When some type of action is taken between the two dimensions, other than just "passively" collecting fractions of the ^{1}D effluent as shown in Figure 2, we speak of *active modulation*. Chromatographers attempt to adjust the ^{1}D effluent matrix to prevent incompatibility issues. In some cases, the application of active-modulation techniques offers opportunities to significantly improve the ^{2}D separation, rather than affecting it negatively. Importantly, active modulation may also result in a concentration of the analyte band prior to injection in the second dimension, thus enhancing the detection sensitivity.

Active-Solvent Modulation (ASM). In 2017, Stoll and co-workers introduced an active-modulation approach, which they refer to as Active Solvent Modulation (ASM).[35] The concept behind this approach is illustrated schematically in Figure 3. In this case, two ports are added to a typical valve used for 2D-LC (see Figure 2), along with a bypass capillary. Also, two rotational positions are added to the two normally used with a conventional valve. Two of the positions (A and C) resemble those shown in Figure 2, where all of the ^{1}D effluent passes through one of the loops and all of the ^{2}D mobile phase passes through the other loop, displacing previously collected ^{1}D effluent into the ^{2}D column. In the

DOI: 10.1021/acs.analchem.8b04841
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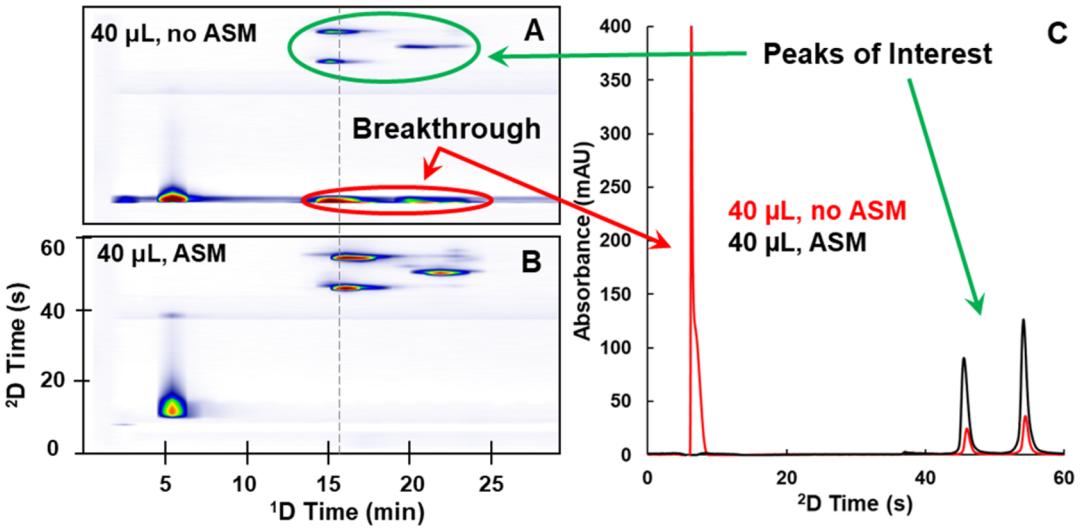

Figure 4. Comparison of two 2D and ^{2}D chromatograms for LC × LC separations of mAb fragments with (B) and without ASM (A). First dimension separations are in the HILIC mode with about 70% ACN in the eluting mobile phase for the peaks of interest, and ^{2}D separations are in the RP mode with about 25% ACN in the starting mobile phase. In each case, 40 μL of ^{1}D effluent is ultimately transferred to the ^{2}D separation. In case B, the sample is diluted 1:2 with water-rich diluent, such that the total volume injected in each ^{2}D cycle is 120 μL. The ^{2}D chromatograms in panel C are extracted from the 2D chromatograms on the left at the position of the gray dashed line. Reproduced from Stoll, D. R.; Harmes, D. C.; Staples, G. O.; Potter, O. G.; Dammann, C. T.; Guillarme, D.; Beck, A. *Anal. Chem.* **2018**, *90* (9), 5923−5929 (ref15). Copyright 2018 American Chemical Society.

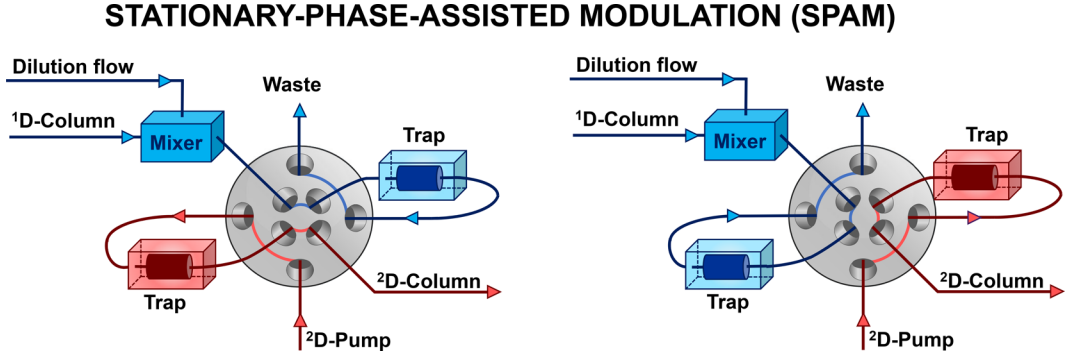

Figure 5. Schematic of the two positions of a stationary-phase-assisted modulation (SPAM) interface. Rather than using large storage loops, analytes are effectively filtered out of the ^{1}D effluent using low-volume trapping columns. Optionally, the ^{1}D column effluent may be diluted using a weak eluent to facilitate retention on the traps. Moreover, the waste line may be equipped with a detector to monitor premature elution from the traps during method development. (Multiple) heart-cut 2D-LC setups are possible as long as the multiple traps are identical.

two additional positions (B and D), the flow from the ^{2}D pump is split into two parts, one that goes through the loop and one that bypasses the loop, such that the ^{2}D eluent acts as a diluent for the fraction of ^{1}D effluent. The benefit of this approach is best appreciated through chromatographic examples. In some of their most recent work, Stoll and co-workers[15] have used ASM to effectively couple HILIC and RP separations of proteins in an online LC × LC format, which is otherwise quite difficult because of the solvent-strength mismatch between the conditions typically used for these two separation modes. Figure 4 shows a comparison of 2D and ^{2}D chromatograms obtained for a partially digested monoclonal-antibody sample, with and without implementation of ASM. The left two panels (A and B) show the 2D chromatograms that are obtained with and without the use of ASM. In panel A the peaks circled in red are due to breakthrough of protein analytes. These proteins elute around the dead time of each ^{2}D separation because of the solvent-strength mismatch. On the other hand, when ASM is used, as shown in panel B, no breakthrough is observed at all, even though the same volume of ^{1}D effluent is ultimately injected into the ^{2}D column in the two cases. This is because in the case of ASM the ^{1}D effluent fraction is diluted 1:2 with water-rich diluent, which lowers the sample concentration of ACN to about 23%, below the starting point in the ^{2}D RP gradient (about 25%). Panel C offers a more focused view of the quality of ^{2}D separations in the two cases.

In addition to the HILIC × RP separation of proteins described above, ASM has been implemented for separations of peptides by mLC-LC with RP separations in both dimensions,[35] quantitative determination of target molecules in polymer matrixes,[36] and the separation of water- and fat-soluble vitamins by sLC × LC using HILIC and RP separations.[37]

As ASM is a relatively new approach, a lot remains to be learned about how to efficiently optimize 2D-LC separations. Some of the early papers contain guidance about the effects of different method parameters relevant to ASM. Additionally,

DOI: 10.1021/acs.analchem.8b04841
Anal. Chem. 2019, 91, 240−263

Stoll and co-workers have developed numerical simulation methods that can be used to both make predictions of the effects of different ASM-related method parameters, such as dilution factor and injection volume, on retention and peak width and to visualize what happens inside the ^{2}D column under these conditions.[31,38]

Stationary-Phase-Assisted Modulation (SPAM). Another increasingly popular active-modulation strategy relies on the use of low-volume trapping (or "enrichment") columns, often referred to simply as "traps", rather than large storage loops (Figure 5). Demonstrated first in LC × LC by Vonk et al.,[39] this technique has been referred to as stationary-phase-assisted modulation (SPAM)[39] or as focusing modulation.[40] Typically, guard columns containing a stationary phase similar to that of the ^{2}D column are used as trapping columns.

As the ^{1}D effluent is sampled by the modulator (Figure 5), it is envisaged that the analytes are retained by the stationary phase in the traps, whereas the ^{1}D solvent system passes unretained and leaves the chromatographic system. Upon switching of the valve, the ^{2}D mobile phase (gradient program) elutes the trapped analytes as sharp, concentrated bands and introduces these into the second-dimension column. To facilitate sufficient retention on the traps, the ^{1}D effluent flow may be diluted (optionally inserting a mixer) to significantly lower the elution strength of the ^{1}D effluent prior to entering the trap.

The advantages of SPAM include (i) reducing solvent incompatibility issues, thanks to removal of most of the ^{1}D mobile phase,[2,40] (ii) improvement of detection sensitivity as a result of analyte focusing on the trapping cartridges,[4,41,42] (iii) a decrease in ^{2}D injection volumes which allows the use of short ^{2}D columns without a loss in efficiency, significantly reducing the total analysis time.[4,42]

However, the use of SPAM is not without disadvantages. It is imperative that all analytes from the ^{1}D fraction are sufficiently retained (i.e., during the full duration of the modulation) to avoid loss of analytes (incomplete recovery) and to prevent discrimination effects. This can be challenging if the various analytes have vastly different chemical properties and a dilution solvent may not always ensure complete trapping of all analytes. In addition, the trapping columns themselves potentially reduce the overall robustness of the system. It is imperative that both traps are identical (and share the same history) to avoid different ("asymmetric") performance of alternating modulations.[43]

After struggling with the low loadability of nanoscale ^{1}D and microscale ^{2}D separations, Vonk et al. first implemented SPAM in an LC × LC workflow for the SCX × RPLC−HRMS characterization of peptides.[39] The use of the active-modulation technique allowed the authors to use a high-loadability ^{1}D column in combination with a nanoscale ^{2}D column and splitless hyphenation with a high-resolution MS instrument. The authors noted that these developments opened the possibility of online SCX × RPLC−HRMS separations to replace the currently dominant off-line SCX prefractionation followed by LC-MS. Gargano and co-workers recently developed a nanoflow LC × LC system for the separation of intact proteins.[44] The combination of silica-based weak cation exchanger run in HILIC mode and RPLC through a SPAM interface followed by HR-MS yielded a highly orthogonal system, targeting a separation based on charge and hydrophilicity in the first dimension and hydrophobicity in the second-dimension. The method allowed the authors to identify twice as many histone proteoforms compared to previous methods.

Recently, Sommella et al. combined HILIC and RPLC for the separation of polyphenols using trapping columns.[6] The authors compared the performance of the SPAM approach with passive modulation and concluded that with SPAM higher peak capacities and sensitivities could be obtained. In addition, the use of trapping columns allowed the authors to circumvent the requirement to use microbore columns in the first dimension. Toro-Uribe and co-workers used SPAM in their HILIC × RPLC separation of procyanidins from green cocoa beans.[40] The authors diluted the ^{1}D effluent (20 μL min^{-1}) with 100 μL min^{-1} of weak ^{2}D eluent and observed improved resolving power and less solvent-mismatch issues as the ^{1}D effluent was removed by the SPAM process. That the enhancement of peak intensies can be significant is visible from the comparison shown in Figure 6. This work, by Baglai

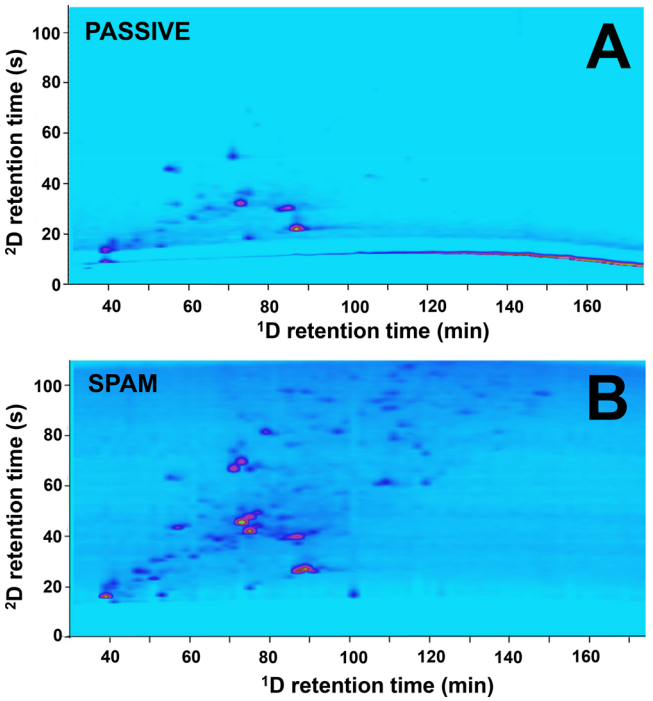

Figure 6. RPLC × RPLC-MS separation of steroids in bovine urine using (A) passive modulation and (B) stationary-phase assisted modulation (SPAM). 76 analytes could be detected using SPAM due to the improvement in S/N, relative to 36 using passive modulation.[41] Reprinted from *Anal. Chem. Acta, 1013*, Baglai, A.; Blokland, M.H.; Mol, H.G.J.; Gargano, A.F.G.; van der Wal, Sj.; Schoenmakers, P.J., Enhancing detectability of anabolic-steroid residues in bovine urine by actively modulated online comprehensive two-dimensional liquid chromatography−high-resolution mass spectrometry, pp. 87−97 (ref 41). Copyright (2018), with permission from Elsevier.

and co-workers,[41] utilized two identical cyano trap columns for improvement of the RPLC × RPLC-MS separation of steroids in bovine urine. As is also visible in Figure 6, a strong enhancement of peak intensities and signal-to-noise ratios was obtained by using SPAM relative to passive modulation. The authors reported an increase in S/N ratio by a factor of 7 and were able to detect 76 compounds using SPAM, relative to 36 for passive modulation.

Jakobsen et al. combined trapping-based active modulation with pulsed-elution of the ^{1}D to increase the flexibility of the

DOI: 10.1021/acs.analchem.8b04841
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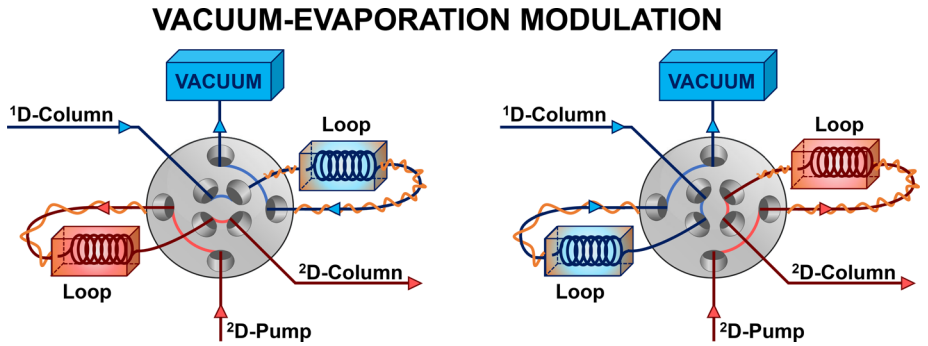

Figure 7. Schematic of a vacuum-evaporation modulation (VEM) interface. ^{1}D column effluent is thought to be rapidly evaporated due to the combination of supplied heat and applied vacuum, so that the analytes are deposited in the loop. Upon switching, the ^{2}D eluent redissolves the analytes for introduction into the ^{2}D column. Based on the work of ref 54.

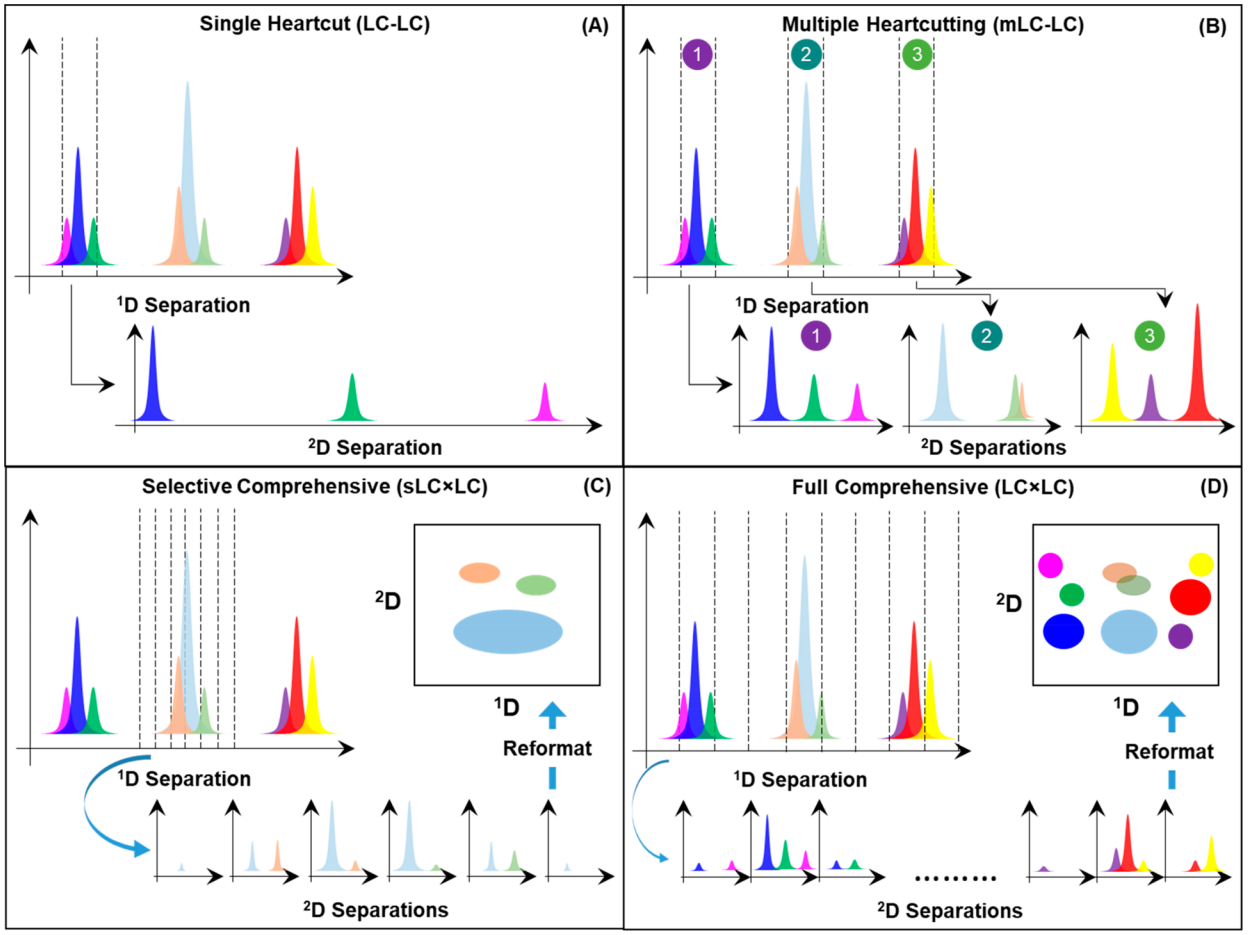

Figure 8. Illustration of four different modes of 2D-LC separation. For a more thorough explanation of the similarities and differences between these different modes of 2D separation see ref61.

2D-LC separation.[42] Pulsed elution utilizes pulses of strong eluent rather than a continuous linear gradient with increasing levels of strong eluent. Using this elution technique, the authors reported the ability to control the elution of analytes in a sufficient number of modulations to prevent loss of ^{1}D peak capacity as a result of undersampling. As the time between pulses could be increased from 1 up to at least 10 min, the ^{2}D analysis was not time-constrained and could be tailored toward increasing the 2D peak capacity ($^{2}n_{c}$). By combining this strategy with SPAM, the authors furthermore reported a reduction of the additional band broadening that would occur in the ^{2}D separation in case of poor refocusing. The concept was demonstrated in practice by a separation of a fraction of vacuum gas oil.

The potential of actively modulating the ^{1}D effluent is not necessarily restricted to overcoming solvent-compatibility issues. Modulation may also target the analytes. Pirok and co-workers combined an aqueous hydrodynamic chromatography (HDC) separation of a polymeric nanoparticle dispersion with a separation of the constituent polymers using organic SEC.[43] The authors first achieved online sample transformation by diluting the aqueous ^{1}D effluent with

DOI: 10.1021/acs.analchem.8b04841
Anal. Chem. 2019, 91, 240−263

tetrahydrofuran in an Agilent Jet Weaver V35 mixer. The composition of THF and aqueous buffer was fine-tuned to destabilize the particles so as to obtain the constituting polymers, while preserving a sufficiently large fraction of buffer to facilitate retention on the trapping columns in the modulator. The THF used in the ^{2}D SEC separation finally eluted the polymers for analysis. A second diode-array detector was used to monitor the modulation effluent for eventual premature elution of trapped polymers.

SPAM is also frequently applied in heart-cut 2D-LC separations.[45−48] Chen et al. used SPAM in their heart-cut SCX-RPLC-MS/MS separation of tobacco-specific N-nitrosamines in cigarette smoke.[46] Trapping was facilitated by diluting the ^{1}D effluent with a weak-eluent and by adjusting the pH.

The implementation of SPAM in 2D-LC is far from optimized, and its effectiveness can be further improved by miniaturizing the trap volumes,[39] enhancing the robustness of the traps,[43] and by developing micromixers to combine two solvent systems rapidly and effectively.[49]

Other modulation strategies. Aside from ASM and SPAM, other active-modulation techniques have also been introduced, although none of these is currently widely applied. Recent examples are evaporative membrane modulation[50] and longitudinal on-column thermal modulation.[51] A completely different approach that was recently proposed is fractionized stacking and sampling, where the ^{1}D effluent is split in a number of segments prior to introduction to the second-dimension column.[52] The last approach that we will discuss is vacuum-evaporation modulation (VEM), which was developed by the group of Guan.[53] The setup is illustrated in Figure 7. It was mainly devised to overcome incompatibility problems encountered during the combination of normal-phase LC (NPLC) and RPLC separations. Briefly, the ^{1}D effluent is passed through a heated loop (Figure 7, left, blue) which in turn is routed to a vacuum outlet. The strong NPLC solvents are envisaged to evaporate rapidly as a result of the increased temperature and reduced pressure and the (nonvolatile) analytes are thought to be deposited on the wall of the loop. Upon switching (Figure 7, right, red) the ^{2}D eluent is introduced, which, with the help of the heat, quickly redissolves the analytes so that they are introduced into the ^{2}D column. Since its introduction, the VEM interface was used in a number of applications in noncomprehensive mode, including the analysis of Traditional Chinese Medicines (TCM)[54] and, more recently, the analysis of lipid species in human plasma[55] and mouse serum.[56] While the potential of VEM has been clearly demonstrated, studies in its fundamental principles and limitations are required, including (i) the required volatility of the to-be-evaporated solvent, (ii) the extent of accidental loss of volatile analytes, and (iii) the rate of dissolution for large analytes that are difficult to dissolve. Data demonstrating to what degree different analytes are deposited on the loops during vacuum evaporation is needed to provide insight in these above fundamental limitations.

METHOD DEVELOPMENT AND OPTIMIZATION STRATEGIES

Since the introduction of single heart-cut (LC-LC)[57] and comprehensive (LC × LC)[58] 2D-LC separations in the late 1970s as online, automated methods, these two modes of 2D separation have been the dominant implementation of the method. However, in the past 10 years or so we have seen the development of hybrid modes of 2D separation that combine features of both heart-cutting and comprehensive 2D separations. Figure 8 illustrates the differences between the conventional single heart-cut and comprehensive modes, and the two hybrid modes multiple heart-cutting (mLC-LC)[59] and selective comprehensive (sLC × LC)[60] 2D separation.

A thorough discussion of the similarities and differences between these different modes of 2D separation was presented recently by Stoll and co-workers[61] and will not be repeated here. One of the most practically important virtues of the mLC-LC implementation as it is drawn in Figure 8B relative to the traditional single heart-cut approach is that the processes of sampling the ^{1}D separation and separating previously collected fractions of ^{1}D effluent in the second dimension are executed in parallel. This provides tremendous flexibility to the user during method development, particularly with respect to increasing ^{2}D analysis times in the interest of improving the resolving power that each ^{2}D separation contributes to the overall 2D separation. Nevertheless, one of the disadvantages of the heart-cutting approach, whether single or multiple heart-cuts are made, is that analytes that had been resolved by the ^{1}D separation can be mixed back together if the fractions collected are larger (in time or volume) than the volume of the peaks as they exit the ^{1}D column. This problem is referred to as "undersampling".[62] Readers interested in more detail on this issue are referred to a number of previous articles that address the problem thoroughly.[24,63] The desire to avoid or at least minimize the effect of this problem gives rise to sLC × LC, as illustrated in Figure 8C. Here, regions of interest in the ^{1}D separation are first finely sampled to avoid remixing of previously separated analytes. These fractions are temporarily stored in an array of sampling loops or traps prior to subsequent further separation in the second dimension. The best answer to the question, which mode of 2D separation should I use?, is entirely application dependent but depends strongly on the number of target analytes of interest in a particular assay. Methods with a small number of targets are well served by single heart-cut methods, whereas applications in which the entire sample is of interest, such as "-omics" types of applications, benefit most from fully comprehensive 2D separations. The mLC-LC and sLC × LC modes are typically most suitable for applications involving some 10−30 target compounds.

Method Development for Noncomprehensive Modes. When developing a single heart-cut application (i.e., Figure 8A), two very important questions encountered in method development are (1) What volume of ^{1}D effluent will be transferred to the second dimension? and (2) What will be the dimensions of and operating conditions for the ^{2}D column? In applications where the analyte of interest is abundant and detection sensitivity is not a primary concern, the user has a lot of flexibility. In these cases, one should minimize the volume of ^{1}D effluent transferred to minimize the risk of undersampling the first-dimension separation and to avoid compromising the separation performance of the ^{2}D column. However, when the analyte of interest is present at a low concentration, we must consider ways to transfer large volumes of ^{1}D effluent to the ^{2}D column, while minimizing the elution peak volume of the analyte from the ^{2}D column. In this context, the active modulation strategies discussed above may be very useful because they enable transfer of relatively large volumes of ^{1}D

DOI: 10.1021/acs.analchem.8b04841
Anal. Chem. 2019, 91, 240−263

Table 3. Overview of 2D-LC Applications from 2016 until October 2018[a]

application	mode			^{1}D	^{2}D	detection	remarks	ref
Biopharma								
Antibody-drug conjugates	N	-	P	SEC	RPLC	UV−vis		95
Antibody-drug conjugates	N	-	P	RPLC	RPLC	UV−vis		96
Antibody-drug conjugates	N	-	P	SEC	RPLC	UV−vis		97
Antibody-drug conjugates	N	-	P	HIC	RPLC	MS		98
Antibody-drug conjugates	N	×	P	RPLC	RPLC	MS, UV−vis		99
	N	-	P	RPLC	RPLC			
Antibody-drug conjugates	N	×	P	HIC	RPLC	MS, UV−vis		100,101
Antibody-drug conjugates	N	×	P	HIC	SEC	MS		102
Antibody-drug conjugates (free species)	N	-	P	SEC	RPLC	UV−vis		95
Antibody-drug conjugate (free species)	N	-	P	MM	RPLC	MS		103
Biopharmaceuticals (impurities)	N	-	N	RPLC	RPLC	MS		104
Bovine insulin (degradants)	N	-	A	RPLC	RPLC	UV−vis		35
Monoclonal antibodies	N	-	P	Affinity	SEC	UV−vis		105
Monoclonal antibodies	N	-	P	WCX	SEC	UV−vis	mLC-LC	106
Monoclonal antibodies	N	×	P	SCX	RPLC	MS		107
Monoclonal antibodies	N	-	P	SCX	RPLC	MS		108
Oligonucleotides (impurities)	N	-	S	SEC	IP-RPLC	MS	Utilized a 50 mm × 4.6 mm C18 trap.	47
	N	×	S	RPLC	IP-RPLC			
	N	-	S	SAX	IP-RPLC			
	N	×	S	IP-RPLC	IP-RPLC			
Therapeutic antibodies	N	-	P	IEX	RPLC	MS		109
Therapeutic antibodies	N	×	A	HILIC	RPLC	MS		15
Environmental								
AChE inhib. in wastewater effluent	N	×	P	RPLC	RPLC	MS, UV−vis	^{2}D effluent fractionated and studied.	110
Emerging contaminants	N	×	P	RPLC	RPLC	MS	Column selection by orthogonality metrics.	86
Polycyclic aromatics	N	×	P	RPLC	RPLC	UV−vis	Theoretical gradient optimization.	111
Food								
Black chokeberries pomace	N	×	P	HILIC	RPLC	MS, UV−vis		112
Overripe fruits (carotenoids)	N	×	P	NPLC	RPLC	MS, UV−vis		113,114
Hop cones, pellet extracts	N	×	P	RPLC	RPLC	MS, UV−vis		115
	N	×	P	HILIC	RPLC			
Soybean (Kunitz Trypsin Inhibitor)	F	-	M	WAX	SEC	MS		116
Corn oil (Triacylglycerols)	N	×	P	AgLC	RPLC	MS	Compared data-analysis techniques.	117
Tea, Grape seed, red wine (extracts)	N	×	P	HILIC	RPLC	IMS-MS	Optimized LC × LC × IMS-MS separation.	81
Wine (polyphenols and contaminants)	N	×	P	RPLC	RPLC	MS, UV−vis		118
Metabolites								
Acyl-Coenzyme A (mouse liver)	N	-	P	RPLC	RPLC	MS	Two parallel ^{2}D columns.	119
Amino acids (of gramicidin, bacitracin)	N	×	P	Chiral	Chiral	UV−vis	Amino acids were derivatized.	120
Amino acids (tea, amino acids)	F	-	M	RPLC	Chiral	UV−vis		121
Antioxidants (*Malus hupehensis*)	F	-	M	HILIC	RPLC	MS, UV−vis		122
Breast milk (fluoxetine, norfluoxetine)	N	-	P	SEC	Chiral	MS		123,124
	N	-	P	SEC	RPLC			
Flavonoids	F	-	M	HILIC	RPLC	MS		125
Flavonoids	N	×	P	RPLC	RPLC	UV−vis	Microprep. to investigate recovery.	126
Flavonoids from licorice	F	-	M	NPLC	RPLC	MS, UV−vis	Preparative scale.	127
Furanocoumarins	N	×	P	RPLC	RPLC	UV−vis	Compared multivariate curve-resolution strategies in LC × LC.	87
Lipidomics (brain tissue)	N	-	S	HILIC	RPLC	MS		128
Lipidomics (human plasma)	N	-	V	NPLC	RPLC	MS		55,129
Lipidomics (mice serum)	N	-	V	NPLC	RPLC	MS		56,130
Lipidomics (rat plasma)	F	-	M	MM	RPLC	MS		131
Lipidomics (cyanobacteria)	N	-	V	NPLC	RPLC	MS		132
Lipidomics (human plasma)	N	×	P	RPLC	HILIC	MS	Comparison with LC-TIMS-MS.	13
Lipids (rice)	N	×	P	RPLC	HILIC	MS		133

DOI: 10.1021/acs.analchem.8b04841
Anal. Chem. 2019, 91, 240−263

Table 3. continued

application	mode			^{1}D	^{2}D	detection	remarks	ref
Metabolites								
Metabolites (*Escherichia coli*)	N	×	P	RPLC	RPLC	MS, UV−vis		134
Metabolites (green cocoa beans)	N	×	S	HILIC	RPLC	MS, UV−vis		40
Metabolites (licorice)	N	×	P	HILIC	RPLC	MS, UV−vis	ZIC-HILIC	135
Metabolites (microbial)	N	×	S	RPLC	RPLC	UV−vis		136
Metabolites (*Panax notoginseng* leaves)	F	×	M	HILIC	RPLC	MS, UV−vis		137
Metabolites (rice plant)	N	×	P	HILIC	RPLC	MS		138
Metabolites (Vitamin D, human serum)	N	-	S	RPLC	RPLC	MS	PFP in ^{1}D, C18 in ^{2}D	139
Metabolites and lipids (human plasma)	N	-	P	RPLC	RPLC	MS	Online prefractionation before ^{1}D	140
Metabolomics (*Glycyrrhiza glabra*)	N	×	P	RPLC	RPLC	MS, UV−vis	Multisegment shifting gradients.	141
Phenolic acids	N	×	E	RPLC	RPLC	UV−vis	Evaporative membrane modulation.	50
Phenolic acids and flavonoids	N	×	P	MM	RPLC	UV−vis	Simultaneous HILIC and RP in ^{1}D	142
Phenolic compounds (Grapevine canes)	N	×	P	HILIC	RPLC	MS, UV−vis	Compared a number of selectivities.	143
Plant extracts and coffee	N	-	P	RPLC	RPLC	MS	Special concept of LC+LC.	144
Polyphenols	N	×	S	HILIC	RPLC	MS		6
Polyphenols in red raspberry fruits shoots	N	×	P	RPLC	RPLC	MS		145
Procyanidins (cocoa)	N	×	P	HILIC	RPLC	UV−vis	Reported kinetic optimization tool.	80
Proteinogenic amino acids	N	-	P	RPLC	Chiral	UV−vis		146
Pyrrolizidine alkaloids	N	-	P	RPLC	RPLC	MS	1D at pH = 3, 2D at pH = 10	147
Steroids	N	×	P	TRLC	RPLC	UV−vis	TRLC as ^{1}D facilitates ^{2}D focusing.	77
Testosterone (human serum)	N	-	P	RPLC	RPLC	MS		148
Urine (bovine)	N	×	S	RPLC	RPLC	MS		41
Urine (Steroids, Sulphonamides)	N	×	P	RPLC	RPLC	MS	CN, BEH and Phenyl studied for ^{1}D	149
Miscellaneous								
Bioactives in plant extracts	N	×	S	RPLC	RPLC	ELSD	Fractionation system	150
Heavy-oil fractions	N	×	P	NA-RPLC	NA-RPLC	CAD, UV−vis	CN, PFP and BiPh studied for ^{1}D	151
Household dust and dryer lint	N	×	P	RPLC	RPLC	MS		152
Lignin phenols	N	×	S	RPLC	SFC	UV−vis		153
Synthetic cannabinoids	N	×	P	RPLC	RPLC	MS		154
Synthetic dyes	N	×	P	SAX	IP-RPLC	UV−vis		5
Tobacco	N	-	S	SCX	RPLC	MS		46,155
Tobacco (snus)	N	-	P	RPLC	RPLC	MS		156
Vacuum-gas oil fraction	N	×	S	RPLC	RPLC	MS	Pulsed elution in ^{1}D	42
Natural Medicines								
Phlorotannins in brown algae	N	×	P	HILIC	RPLC	MS, UV−vis	Use of Hansen solubility parameter to study extraction selectivity.	157
Brown seaweed	N	×	P	HILIC	RPLC	MS, UV−vis		158
TCM (Additives)	N	-	S	Affinity	RPLC	UV−vis		159,160
TCM (*Dracocephalum heterophyllium*)	F	-	M	RPLC	HILIC	MS, NMR		161
TCM (*U. rhynchophylla*)	F	-	M	MM	MM	UV−vis		162
TCM (*Curcuma kwangsiensis*)	N	×	P	RPLC	RPLC	MS		163
TCM (Flos Carthami, dried flowers)	N	×	S	MM	RPLC	UV−vis	^{1}D: SEC-RPLC	164
TCM (*Gardenia jasminoides* Ellis)	F	×	M	RPLC	HILIC	MS		165
TCM (Gegen-Qinlian Decoction)	N	×	P	RPLC	RPLC	MS		166
TCM (Notoginseng total saponins)	F	×	M	HILIC	RPLC	MS		167
TCM (*Oxytropis falcata*)	F	-	M	RPLC	RPLC	UV−vis	Preparative scale	168
TCM (Salvia miltiorrhiza)	F	×	M	HILIC	RPLC	MS		169
TCM (Salvia miltiorrhiza)	N	×	P	RPLC	RPLC	MS		170
TCM (sapinins and alkaloids)	F	-	M	RPLC	RPLC	MS		171
TCM (saponins in *Gleditsia sinensis*)	N	×	P	RPLC	RPLC	MS		172
TCM (*Sphaerophysa salsula*)	F	-	M	RPLC	HILIC	NMR,UV−vis	Preparative scale	173
TCM (toad skin)	F	×	M	NPLC	RPLC	MS		174
TCM (Tropane alkaloids)	N	-	P	RPLC	SCX	UV−vis		175
TCM (Xuebijing)	N	-	P	RPLC	RPLC	MS, UV−vis		176
TCM (Zhibai Dihuang Granule)	F	×	M	SCX	RPLC	MS		177
TCM (Denzhan Shenmai)	N	×	P	RPLC	RPLC	MS	Integrated data from LC × LC with that from heart-cut 2D-LC.	16
	N	-	P	RPLC	Chiral			

DOI: 10.1021/acs.analchem.8b04841
Anal. Chem. 2019, 91, 240−263

Table 3. continued

application	mode			^{1}D	^{2}D	detection	remarks	ref
Natural Medicines								
TCM (*Ginseng* powder)	N	-	P	HILIC	RPLC	UV−vis		52
TCM (*Ginkgo biloba*)	F	×	P	HILIC	RPLC	MS		178
Peptides								
Peptide biomarkers (rat urine)	F	-	M	RPLC	RPLC	MS		179
Peptic digests	N	-	S	RPLC	MM	MS		180
Peptides	N	×	P	RPLC	RPLC	MS, UV−vis		14
Protein digests (plasma)	F	-	M	RPLC	RPLC	MS		181
Tryptic peptides	N	-	S	SCX	RPLC	MS	DCM instead of Gradient	182
Tryptic peptides (Cortical neurons)	N	×	S	SCX	RPLC	MS		183
Tryptic peptides (*Elaeis Guineensis* Jacq)	N	-	S	RPLC	RPLC	MS		184
Tryptic peptides (*Flammulina velutipes*)	F	-	M	RPLC	RPLC	MS		185
Tryptic peptides (human tissue, cancer)	N	-	P	RPLC	RPLC	MS		186
Tryptic peptides (mitochondrial proteins)	F	-	M	SCX	RPLC	MS		187
Tryptic peptides (lung adenocarcinoma)	F	-	M	SCX	RPLC	MS		188
Tryptic peptides (*Proteus mirabilis*)	F	-	M	RPLC	RPLC	MS		189
Tryptic peptides (*Salmonella* bacteria)	F	-	M	SCX	RPLC	MS		190
Tryptic peptides (serum)	F	-	M	SCX	RPLC	MS		191
Tryptic peptides (serum)	F	-	M	SCX	RPLC	MS		192
Peptides	N	×	P	RPLC	SEC	UV−vis	Studied stop-flow effects in ^{1}D	193
Peptides	N	-	P	Chiral	RPLC	FLD, MS		194
	N	-	P	Chiral	Affinity			
Peptides	N	×	P	SEC	RPLC	UV−vis	^{1}D: Stop-flow; ^{1}D dispersion studied.	195
Peptides	N	-	P	RPLC	RPLC	MS, UV−vis	2D-LC used as desalting tool.	196
Pharmaceuticals								
Antibiotic drug (cefonicid sodium)	N	-	P	RPLC	RPLC	MS		197
Antibiotic residues in dairy products	N	-	P	HILIC	RPLC	MS		198
Antibiotics	N	-	P	RPLC	RPLC	MS		199
Beta-blockers in human plasma	N	-	P	SEC	RPLC	FLD	RAM as ^{1}D for prefractionation.	200
Desonide cream	N	-	P	RPLC	RPLC	MS	Strong salt buffer in ^{1}D	201
Parental drug microdosing vehicle	N	-	P	RPLC	RPLC	MS, UV−vis		202
Pharmaceutical materials	N	-	P	RPLC	RPLC	MS, UV−vis		203
Pharmaceuticals	N	-	P	RPLC	SFC	UV−vis	sLC × SFC	204
Pharmaceuticals	N	×	P	Chiral	Chiral	UV−vis		205
	N	-	P	RPLC	Chiral			
Pharmaceuticals	N	-	S	RPLC	SFC	MS, UV−vis		18
Pharmaceuticals	N	×	P	RPLC	RPLC	MS, UV−vis	Applied theoretical optimization.	206
Pharmaceuticals, Metabolites	N	-	S	RPLC	Chiral	MS	Chiral at SFC conditions.	45
Therapeutic drug (in human plasma)	N	-	S	RPLC	RPLC	UV−vis	SCX as trapping column.	207
Vidarabine monophosphate	N	-	S	RPLC	MM	MS		208
Vitamins	N	-	A	HILIC	RPLC	UV−vis	sLC × LC	37
Polymers								
Oligomers (Oxidzed waxes)	N	×	P	NPLC	SEC	ELSD	^{1}D and ^{2}D at high temperature.	209,210
Polyether polyols	N	×	P	HILIC	RPLC	MS		9
Polymeric nanoparticles	N	×	S	HDC	SEC	UV−vis		43
Synthetic polymers (Polystyrene/Polybutadiene Block Copolymers)	N	×	S	NA-TGIC	SEC	UV−vis		211
	N	×	S	RP-TGIC	LCCC			
Synthetic polymers (novolac)	N	×	A	SEC	RPLC	UV−vis		36
Synthetic polymers (branched poly (bisphenol A-carbonate))	N	×	P	LCCC	SEC	LS, RID, UV−vis, VI	MALDI used off-line. LC × LC Correlated with Monte Carlo simulations.	212,213
Synthetic polymers (HEUR)	N	×	P	SEC	RPLC	ELSD		214
Synthetic polymers (nonlinear)	N	×	P	NPLC	SEC	LS, RID, VI		215
Synthetic polymers (poloxamers)	N	×	P	LCCC	LCCC	ELSD	Use two ^{2}D columns.	216
Synthetic polymers (polystyrene)	N	×	P	NA-RPLC	SEC	UV−vis	^{2}D-col with longitudinal porosity gradient.	217

DOI: 10.1021/acs.analchem.8b04841
Anal. Chem. 2019, 91, 240−263

Table 3. continued

application	mode			^{1}D	^{2}D	detection	remarks	ref
(Intact) Proteins								
Intact histone proteoforms	N	×	S	MM	RPLC	MS	Nanoflow LC × LC; ^{1}D WCX-HILIC	44
Intact proteins	N	×	P	SCX	RPLC	UV−vis	Application of multichannel detector[218]	218−220
Intact proteins and protein digests	N	×	P	SCX	IP-RPLC	MS, UV−vis	Photografted monolith for SCX. Optimized various parameters.	221
Metaproteomics (soil)	N	-	S	SCX	RPLC	MS		48
Proteins in human plasma	N	×	S	SAX	RPLC	None	Array (8) of ^{2}D columns, Fractionation	222
Surfactants								
Ethoxylate phosphate surfactants	N	×	S	HILIC	RPLC	UV−vis		4
Ionic surfactants	N	×	P	MM	RPLC	CAD	^{1}D: WCX-RPLC	2
Nonionic surfactants in pharmaceuticals	N	×	P	HILIC	RPLC	ELSD, MS		223
Polymeric dispersants in detergents	N	-	P	SEC	RPLC	ELSD		224

[a]N = Online, F = Offline, × = Comprehensive, - = Heart-cut, P = Passive modulation (empty loops), A = Active-Solvent Modulation (ASM), E = Evaporation membrane, S = Active stationary-phase assisted modulation (SPAM), V = Vacuum-evaporation modulation (VEM), M = Manual/No modulation. CAD = charged-aerosol detection, ELSD = evaporative light-scattering detection, FLD = fluorescence detection, LS = light scattering, MS = mass spectrometry, RID = refractive-index detection, VI = viscometry. LS, RID, and VI are typically combined to obtain "triple detection". See Modulation section for a detailed discussion of the different modulation techniques.

effluent without compromising the performance of the ^{2}D separation.

In addition to avoiding undersampling, as already discussed above, one of the attractive aspects of the sLC × LC mode of separation is that it enables a kind of piecewise transfer of the entire volume of a ^{1}D peak that would otherwise be too large to transfer in a single fraction. For example, suppose one wants to study an unknown peak that suddenly appears in an existing 1D separation that is carried out using a 4.6 mm i.d. column at 1 mL/min. If the peak of interest is 10-s wide at the base (i.e., 6σ level), then transferring "all" of the peak would require a sample loop of about 200 μL. However, in the sLC × LC mode the peak can be split up into a number of fractions with volumes that are more convenient. For example, the peak may be sampled five times, each time using a 40-μL loop. This kind of flexibility is valuable from a method-development point of view and enables precise quantitation, even for ^{1}D peaks with large volumes.[64] However, this line of thinking quickly leads to the question, what is the "right" number of fractions to transfer per ^{1}D peak to use as a starting point during method development? In a series of two recent papers, Davis and Stoll[3,65] addressed this question using simulations to study the effect of the number of transfers of each ^{1}D peak on the probability of fully resolving sample mixtures of increasing complexity by mLC-LC/sLC × LC. Interestingly, they found that, from a probabilistic point of view, making a single transfer of each ^{1}D peak maximizes the probability that the resulting 2D separation will fully resolve a random mixture of analytes. This outcome is observed even after undersampling is accounted for. Nevertheless, here are two important caveats to keep in mind here. First, the simulations used in this work did not take into account the volume of ^{1}D peaks, and therefore splitting the ^{1}D peak into multiple fractions may still be warranted for practical reasons, even if this is not optimal from the point of view of resolving power. Second, the conclusions of these studies are based on the outcomes of tens of thousands of simulations of 2D separations of random mixtures. Even if the simulations suggest the use of a single transfer of each ^{1}D peak as a starting point, there undoubtedly will be specific separation scenarios where transferring multiple fractions of a ^{1}D peak is needed to avoid undersampling and the concomitant loss of ^{1}D resolution.

Comprehensive Mode. The step toward comprehensive 2D-LC (LC × LC) is accompanied by a number of additional constraints on the method. For the method to be truly comprehensive, it is required that all of the analytes are transferred completely from the ^{1}D effluent to the ^{2}D column. In the case of passive modulation, this requires that the ^{1}D flow rate is adjusted to match the size of the loop.

One focus within the chromatographic community has been the development of faster ^{2}D separations. Shorter modulation times imply that more fractions can be collected and transferred, thus decreasing the likelihood of undersampling of the ^{1}D separation and/or reducing the total analysis time. In this context it is not surprising that RPLC proves to be the most popular mechanism in LC × LC (see Table 3 below). Indeed, RPLC offers many advantages when used as ^{2}D separation mechanism[2], including very fast gradient-elution separations, thanks to the swift column equilibration.[66] The use of an ion-pairing reagent does not necessarily lead to impractically long gradient times. For the separation of synthetic dyes by SAX × IP-RPLC,[5] Pirok and co-workers combined a programmed increase of the fraction of organic modifier in the ^{2}D mobile phase with a proportional decrease in the ion-pair concentration.

The acceleration of size-exclusion chromatography (SEC) for ^{2}D separations has recently also received attention. Separation in SEC is thought to be promoted by the largest possible pore volume,[67] and as a result the polymer-separation community has largely ignored the introduction of core−shell (or superficially porous) particles, which are now routinely applied in LC due to their superior efficiency.[68,69] Recent studies suggest that, despite the decrease in pore volume, the increase in efficiency yields similar or better resolution in a shorter time.[70,71] This type of stationary phase was applied in a second-dimension separation of acrylate polymers. The authors exploited the possibility of overlapping injections, as no separation is expected to occur outside the SEC range of the column.[43]

In this context, the demonstration of subsecond (isocratic) HILIC, chiral, and achiral separations by Wahab et al. represent an attractive concept.[72] The authors packed superficially porous particles in 5 mm-long columns. While the results were promising, it was noted that modified UHPLC

DOI: 10.1021/acs.analchem.8b04841
Anal. Chem. 2019, 91, 240−263

hardware with minimized extra-column dispersion was required to successfully realize subsecond separations. In addition, sufficiently high detection sampling frequencies are required to accurately describe the shape of elution bands.

A completely different approach to LC × LC is based on spatial separations. In a spatial separation, analytes are not eluted from a column but separated "spatially" because they reach different locations. In a second step they may be eluted from a flat bed or a series of channels in an orthogonal direction. In the latter case there is the potential for a third dimension. The fundamental advantage of using a spatial separation in the first dimension is that all ^{2}D separations can be performed simultaneously, rather than sequentially. As a result, spatial 2D (and 3D) separations are fundamentally very attractive, up to a point that a peak capacity approaching one million has been predicted for a spatial 3D-LC.[73] However, spatial separations at elevated pressures have hardly been developed yet. Early studies illustrate that some serious obstacles need to be overcome.[74,75] Most importantly, to realize successful LC × LC with a spatial ^{1}D separation we need to find ways to confine the flow to the ^{1}D separation channel.

Very recently Adamopoulou et al. described a potentially revolutionary two-dimensional insertable separation tool (TWIST).[76] The ^{1}D spatial separation can take place in the TWIST channel. After turning the TWIST, a series of fractions can be sent simultaneously from different locations along the ^{1}D channel to a series of ^{2}D channels. This approach has potential advantages, due to its simplicity and the potential for highly selective (and ultimately high-resolution) separations in a relatively short time. However, the concept is quite new and still highly immature.

Other technological advancements include the introduction of new modulation strategies to help overcome incompatibility and undersampling issues, as discussed in the previous section. Baert et al. recently advocated the use of temperature-responsive LC (TRLC) in purely aqueous environments as ^{1}D separation, in combination with RPLC in the second dimension.[77] Their concept relied on the use of temperature-responsive stationary phases, which exhibit strong changes in retention behavior upon small changes in temperature. The purely aqueous ^{1}D mobile phases were ideally compatible with a ^{2}D RPLC separation. Using a sample containing neutral organic analytes the authors demonstrated that undersampling constraints could be relaxed and complete analyte refocusing could be achieved.

Optimization. There are a variety of useful studies that provide general guidelines for developing LC × LC methods.[2,23,78] However, genuine optimization of methods is much more complex.[2,79] This is reflected in different interpretations of the word "optimization". Chromatographers may have specific objectives, such as improving peak shapes or enhancing resolution in targeted sections of the separation space. Specific adjustments based on a trial-and-error approach may suffice to answer the analytical question at hand. In untargeted optimization, the most-efficient route toward optimal conditions is less-clearly defined. In the absence of a specific question, this type of optimization encompasses maximizing or minimizing quality descriptors (e.g., peak capacity and analysis time, respectively).

Quality descriptors are objective parameters that quantify specific performance properties, such as orthogonality, peak capacity, dilution factors, resolution, etc. Readers seeking to learn more about these and other quality descriptors are referred elsewhere.[2] Often a known (theoretical) relationship exists between a method parameter and a quality descriptor. For example, increasing the particle diameter of the packing material of either dimension (while keeping all other parameters constant) results in a reduced pressure drop but also in a decreased efficiency and peak capacity. Changing one parameter typically also affects other parameters. In the case of passive modulation, changing the ^{1}D flow rate affects the required loop volume, which in turn affects the suitable ^{2}D column dimensions and, consequently, the ^{2}D flow rate. Sarrut et al. tried to create a network diagram to visualize the relations between a large number of parameters.[7] They certainly succeeded in illustrating the complexity of LC × LC method development. The number of possible method parameters is much larger in 2D-LC than in 1D-LC, where method development is already thought to be challenging. This sheer complexity greatly encumbers the method development, increasing the time, effort, and knowledge required to arrive at an acceptable solution. When developing a method "manually", using a trial-and-error process as a solution is unlikely to be truly optimal. Therefore, several groups work on computer-aided method-development tools, which aim to significantly accelerate method development using multi-objective algorithms.

Vivó-Truyols et al. proposed the use of Pareto optimization for sample-independent optimization of two-dimensional chromatographic separations.[8] In this approach, multiple method parameters (e.g., gradient slope, column dimensions, flow rates) are varied and, using theoretical relations, their impact on objective parameters is assessed. The Pareto-optimization strategy involves defining two or more of such objective parameters. A point is considered Pareto optimal if no other point exists that has superior values for all objective parameters. All other points are suboptimal. The collection of Pareto-optimal points forms the Pareto-optimal front. Vivó-Truyols and co-workers argued that this approach was eminently suited to deal with the trade-offs between different objectives, such as peak capacity, analysis time, and dilution factors.[8] They took the loss of peak capacity due to undersampling the ^{1}D separation and due to large injection volumes in the ^{2}D into account. The method yielded optimal values for a large number of parameters including the ^{1}D and ^{2}D column particle sizes and diameters and the modulation time. Sarrut et al. applied a systematic optimization approach to a real RPLC × RPLC separation of peptides.[7] More recently, Muller et al. reported a predictive kinetic optimization program to derive the optimum column combinations and chromatographic conditions for a HILIC × RPLC separation of procyanidins.[80] The method was also applied in a different study where a phenolic extract of red wine was separated using the Pareto-optimized chromatographic method but now hyphenated with IMS-MS (Figure 9).[81]

The optimization of physical properties, as described above, is pivotal for the design of efficient 2D-LC separation systems. Sample-independent optimization procedures typically aim to maximize quality descriptors that are largely independent of the sample, such as the peak capacity. Resolution in chromatography is known to depend on three factors, viz., selectivity, retention, and efficiency. In sample-independent optimization, the focus is on efficiency, maximizing the plate counts in the two dimensions and ultimately the overall peak capacity. As we discussed in the introduction, the peak capacity

DOI: 10.1021/acs.analchem.8b04841
Anal. Chem. 2019, 91, 240−263

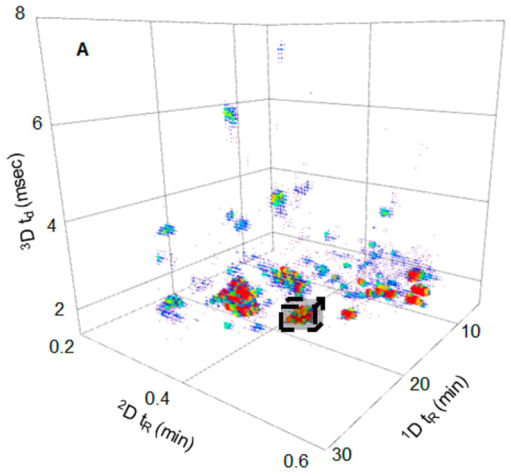

Figure 9. HILIC × RP-LC × IMS-MS TIC separation of a phenolic extract of red wine. The chromatographic method was optimized using predictive Pareto optimization.[80] Reproduced from Venter, P.; Muller, M.; Vestner, J.; Stander, M.A.; Tredoux, A.G.J.; Pasch, H.; de Villiers, A. *Anal. Chem.* **2018**, *90*, 11643−11650 (ref 81). Copyright 2018 American Chemical Society.

must be very high relative to the number of analytes in the mixture if the analytes follow Poison statistics. However, very large gains are possible if the retention and selectivity are optimized. If we understand the properties of the sample, conditions can be selected such that all components are eluted with optimal k values. In LC this is often achieved by establishing suitable gradient parameters. If the number of analytes is manageable or if (group-type) selectivity is sought for a limited number of component classes, the selectivity of the LC × LC separation may be optimized.

Such sample dependent optimization is being pursued by Pirok and co-workers.[82] Their PIOTR program is based on the realization that a linear relationship between a retention parameter (such as k or log k) and a composition parameter (such as the volume fraction of modifier in RPLC) can be accurately described based on two (gradient-elution or isocratic) measurements. Furthermore, Pirok et al. realized that a single LC × LC experiment in principle provides retention data in two dimensions for all analytes. They demonstrated the principle for a separation of a complex mixtures of dyes with gradient-elution IEC (where log k is expected to vary linearly with the log of the counterion concentration) and gradient-elution ion-pair LC. An approach like this, based on modeling the retention of large numbers of analytes, in principle allows a great acceleration and improvement of LC × LC method development. To achieve this, accurate retention models are needed for all popular mechanisms. While a (log-) linear model for RPLC usually suffices, models for, for example, HILIC are still under study

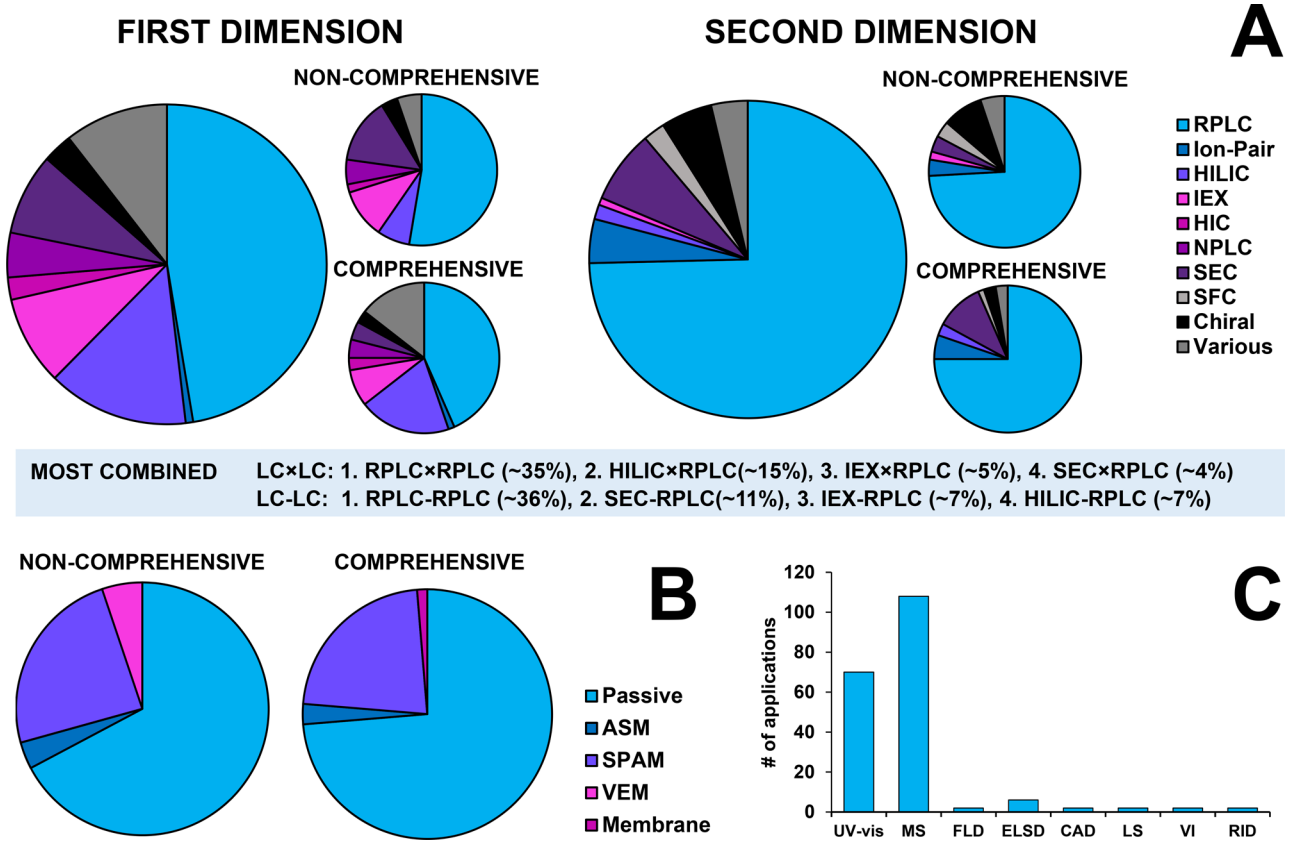

Figure 10. (A) Overview of applied retention mechanisms in the first (left) and second (right) dimensions. (B) Use of modulation strategies in noncomprehensive (left) and comprehensive (right) applications (see Modulation section for a technical clarification). (C) Overview of applied detection techniques. Note that one application may use more than one detection technique so that a pie chart is less appropriate in this case. Total number of applications: 161 (online noncomprehensive, 58; online comprehensive, 76; offline, 27. CAD = charged-aerosol detection, ELSD = evaporative light-scattering detection, FLD = fluorescence detection, LS = light scattering, MS = mass spectrometry, RID = refractive-index detection, VI = viscometry. Data covers all online applications from Table 3.

DOI: 10.1021/acs.analchem.8b04841
Anal. Chem. 2019, 91, 240−263

and not necessarily linear.[83,84] The principle of a program like PIOTR is hardly affected, provided that it allows establishing nonlinear retention models based on three or four experiments. To obtain correct input data for retention modeling, "peak matching" is a critical requirement.[85] While it is not necessary to identify all peaks in the input (LC × LC) chromatograms, it is vital to know which peaks refer to the same analytes. Given the large numbers of analytes typically encountered in the complex samples subjected to LC × LC, another requirement is a high degree of automation of the entire optimization process.

Work has also been invested in studying some of the quality descriptors themselves. Leonhardt and co-workers investigated the performance of different orthogonality metrics.[86] They noted that the convex hull and bin-counting methods do not provide information on the peak distribution, whereas the asterisk method does not work optimally when only a limited number of components are used for method development. The authors therefore introduced a new concept for peak distribution assessment across the separation space of two-dimensional separation systems in combination with clustering detection.

While, LC × LC separations typically deal less with coelution relative to 1D-LC, overlapping peaks often still occur in LC × LC data sets. Indeed, for quantification it is imperative to be able to deconvolute overlapping peaks. One frequently applied tool is multivariate curve-resolution alternating least-squares (MCR-ALS). Cook et al. recently compared several MCR-ALS strategies.[87]

Automated optimization programs typically need to revert to data analysis strategies. One interesting example is the work of Navarro-Reig et al., where the authors proposed an untargeted protocol that used MCR-ALS on region-of-interest (ROI) compressed data to find relevant information from the increasingly highly complex LC × LC-MS data sets. The authors applied this protocol on HILIC × RPLC-MS/MS data from separations of arsenic-exposed rice samples and found that arsenic exposure had significant effects on the rice lipidome.

APPLICATIONS

Table 3 provides a comprehensive overview of recent applications of 2D-LC. We made an effort to include every relevant paper from early 2016 until now. Two-dimensional combinations of electrophoresis with liquid chromatography are not included but have been reviewed recently.[88] Applications where a solid-phase extraction cartridge was used as "first-dimension separation" were considered to fall in the category online sample preparation and were not considered here. Yang and Pursch[89] recently provided an overview of a number of interesting applications of 2D-LC.

The applications in Table 3 are grouped in a number of categories. Some application fields (chiral separation, biopharmaceuticals, and synthetic polymers) are discussed in more detail in later sections of this review. For each application a distinction is made between online (N) or offline (F) 2D-LC. Comprehensive two-dimensional separations are denoted with "×"; all other modes (heart-cut LC-LC, mLC-LC, multiple heart-cut and selective comprehensive, sLC × LC) are grouped under "noncomprehensive" methods and are denoted with "-". Finally, the separation modes are classified through the type of modulation used, being either passive (P), active solvent modulation (A), evaporation-membrane modulation (E), stationary-phase assisted modulation (S), vacuum-evaporation modulation (V), and manual (M). The latter mode is typically associated with the offline mode. The retention mechanisms used, and the detection method are listed in the table for each application. In some cases, two applications are described in one publication or vice versa.

The popularity of different separation mechanisms is illustrated in the pie charts shown as Figure 10. RPLC is popular as first dimension technique, but it is used in less than 50% of all applications. Due to the pursuit of orthogonal mechanisms, other mechanisms are of considerable interest in 2D-LC and most of these are more suitable as ^{1}D separations than as ^{2}D separations.[2] In a significant number of cases (some 35% of all online applications) RPLC is used in both dimensions, with very different mobile phases or different stationary phases. Other popular ^{1}D techniques include HILIC, ion-exchange methods (grouped under IEX) and SEC. IEX and SEC using aqueous solvents are highly compatible with a ^{2}D RPLC separation, thanks to inherent analyte focusing at the inlet of the ^{2}D column. HILIC is popular as ^{1}D technique, especially in comprehensive 2D-LC. When combining ^{1}D HILIC with ^{2}D RPLC some form of active modulation is highly desirable.

RPLC is the dominant technique used in the second dimension. This is easily explained by the advantageous behavior of RPLC systems under gradient conditions. Column equilibration is much faster than in other modes of LC and gradients from high to low percentages of water allow analytes with a very broad range of polarities to be eluted under optimal conditions. One the other hand, NPLC, HIC, IEX, and HILIC are almost exclusively used as ^{1}D techniques, because of slow equilibration and more-limited applicability. Other popular ^{2}D techniques include SEC, which is an isocratic method, allowing simpler instrumentation and a wider selection of detectors to be used. Ion-pair (RP)LC is used as ^{2}D separation in a substantial number of cases, probably because of the greatly different selectivity it offers for ionic analytes in comparison with RPLC. Chiral separations are used as ^{1}D and, especially, ^{2}D separations, mainly in heart-cut 2D-LC.

Figure 10B summarizes the use of different types of modulation systems. Passive modulation is still dominant, which may at least in part be explained by the recent emergence of some active-modulation strategies. Stationary-phase assisted modulation (SPAM) is by far the most popular active-modulation technique. Vacuum evaporation modulation has been used in some noncomprehensive applications. It may at present still be too slow to be used in LC × LC applications, while analyte recovery may also be an issue. Membrane evaporation modulation has been used in one LC × LC application. Active solvent modulation may be used more often in forthcoming years.

The dominant detection techniques in 2D-LC applications are UV spectrometry and, especially, mass spectrometry (Figure 10C). Various types of MS analyzers (quadrupole, time-of-flight, Orbitrap, and ion-trap) and hybrid systems (such as Q-TOF) are all frequently used.

In the following sections, we will discuss a number of application fields from Table 3 in more detail. Readers interested in other application areas are referred to the recent reviews on herbal medicines,[90,91] food,[92,93] and pharmaceuticals.[94]

Chiral Separations. The use of 2D-LC for pharmaceutical applications was reviewed recently by Iguiniz and co-

DOI: 10.1021/acs.analchem.8b04841
Anal. Chem. 2019, 91, 240−263

workers,[225] including the development of 2D-LC separations involving chiral separations in one or both dimensions. Here, we are focusing our discussion on developments in this area to the most recent years. Recent developments have been focused on two primary areas of research, separations of chiral amino acids, and separations of pharmaceutical materials having at least some components of the sample that are chiral.

Although the use of 2D-LC for separations of amino acids actually has a considerable history,[226] the recent developments in this area are particularly exciting, because they reflect and leverage recent developments in 2D-LC technology to produce some impressive separations. Chen and Liao have demonstrated the utility of LC-LC separations, with chiral columns in one or both dimensions, for bottom-up sequencing of short peptides that yields not only the sequence but also knowledge of the enantiomeric composition of each amino acid in the peptide.[194] In a different study, Wang and co-workers used offline LC × LC separations to characterize the enantiomeric composition of free amino acids in tea.[121] Amino acids were derivatized with 9-fluorenylmethoxycarbonyl (FMOC) chloride prior to 2D-LC using the RP mode in the first dimension followed by the chiral mode in the second dimension. Woiwode et al. describe what they refer to as "two-dimensional correlation liquid chromatography" for characterizing the chirality of peptides and proteins by chiral × chiral separations of their constituent amino acids.[120] In this work they deployed stationary phases for the ^{1}D and ^{2}D separations that were very similar except for the configuration of the chiral selector bonded to the stationary phase. In this way they refer to "R columns and S columns". This is quite clever in that this particular configuration makes interpretation of the resulting 2D chromatograms quite straightforward. Molecules that are achiral all elute along a diagonal line in the 2D plot because the selectivities of the R and S columns are nominally identical for these molecules. On the other hand, all D-amino acids elute one side of this diagonal line, while the L-amino acids elute on the other side of the line. According to the authors, this clear separation of the enantiomers makes it possible to quantify the enantiomeric composition of each amino acid present in the peptide or protein that it came from. Finally, in a different paper from the same group, the authors demonstrate the ability to resolve and quantify both the D- and L-forms of all 20 proteinogenic amino acids (plus five others) using a fully automated,[146] online 2D-LC separation operated in multiple heartcutting and selective comprehensive modes. In this case, the amino acids were derivatized prior to analysis using 2,4-dinitrobenzene, and the ^{1}D and ^{2}D columns were RP and chiral, respectively. Figure 11 is a representative chromatogram from this work that shows where each cut is made over the course of the 2D separation, and the corresponding ^{2}D separations of the D- and L-amino acids.

In the pharmaceutical domain, Barhate and co-workers have demonstrated a variety of 2D separations of chiral small pharmaceutical molecules that leverage very fast ^{2}D chiral separations (e.g., 30−60 s per 2D cycle).[205] These fast ^{2}D chiral separations enable a number of possibilities for online 2D separations, including the use of chiral separations in one or both dimensions, and different 2D separation modes, ranging from simple single heart-cut all the way up to fully comprehensive separations. Furthermore, the fast ^{2}D separations render the overall analysis time for these 2D separations much shorter than what has been possible historically.

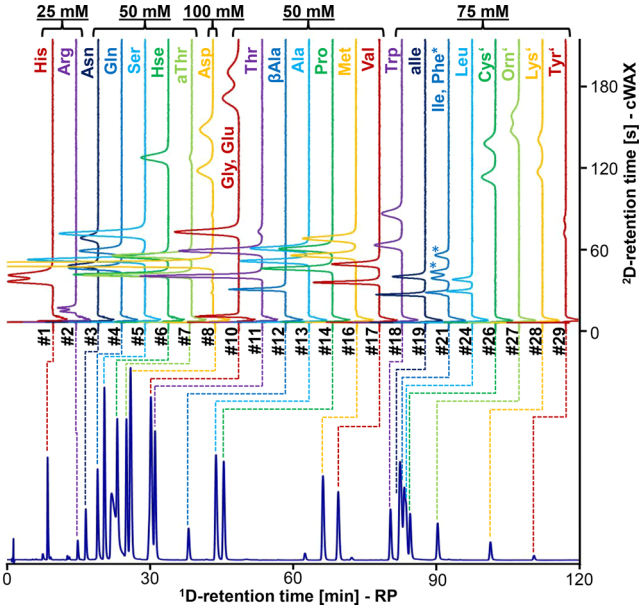

Figure 11. Online 2D-LC separation of 2,4-dinitrobenzene (DNP) derivatives of all 20 proteinogenic amino acids (plus allo-threonine, allo-isoleucine, homoserine, ornithine, and β-alanine). The D-enantiomer always elutes after L-, except for glutamine and ornithine. The ^{1}D separation is carried out in the RP mode and the ^{2}D separation in the chiral mode. Figure based on ref 146 and kindly provided by M. Lämmerhofer.

The last three papers we will discuss here involve 2D separations with achiral RP-LC separations in the first dimension, followed by chiral SFC separations in the second dimension. Although the feasibility of LC-SFC separation was demonstrated in 1992 by Cortes co-workers,[227] there has been little development in this area until recently. Venkatramani and co-workers made a significant step forward through the development of a simpler interface for the two separations that involves the use of trapping devices between the two dimensions to address the solvent incompatibility inherent to this coupling of separation modes.[228] Using a multiple heart-cutting approach, they demonstrated a fast (18 min) and efficient separation of a mixture of eight stereoisomers of a single drug substance. Whereas most work involving trapping columns in the interface has used just two traps, in this work they also demonstrated the use of an array of five traps for more flexibility in the multiple heart-cutting mode.

In a follow-up study,[45] the same group discussed the results of experiments aimed at systematically studying the variables that affect detection sensitivity in LC-SFC systems. Parameters studied included column diameters in both dimensions (2.1, 3.0, and 4.6 mm i.d.), volume of ^{1}D RP effluent transferred to the ^{2}D SFC separation, chemistry of the trapping stationary phase, dimensions of connecting tubing in the system, and detection techniques. By choosing optimal values of these variables, they established a detection limit of 10 ng/mL for the drug metabolites studied and applied the resulting system to the study of *in vivo* interconversion of a chiral drug.

Finally, Iguiniz and co-workers[229] have described the development of 2D separations involving ^{1}D achiral RP-LC separation and ^{2}D chiral SFC separation that is significantly different from the work of Venkatramani et al. First, these separations were carried out in the selective comprehensive mode (sLC × SFC), which makes the simultaneous

DOI: 10.1021/acs.analchem.8b04841
Anal. Chem. 2019, 91, 240−263

optimization of method variables more challenging. Second, they explored conditions that do not involve trapping columns in the interface. Although relatively small transfer volumes of 10 μL were used along with large ^{2}D columns, the authors report a detection limit of 0.5% of a minor enantiomer of a drug substance relative to the major enantiomer. These are interesting developments that should promote more studies comparing the advantages and disadvantages of 2D separations coupling LC to either LC or SFC for achiral−chiral analyses.

Separations of Biopharmaceuticals. Biopharmaceuticals include therapeutic peptides, oligonucleotides, monoclonal antibodies (mAbs), antibody drug conjugates (ADCs), and other therapeutic proteins. Protein biopharmaceuticals present significant challenges to analytical techniques because of their large size and the presence of many variants that result from small changes in structure (isomers, glycosylation, etc.). Increasingly, analysts are turning to 2D separation methods, often coupled with MS detection, to help characterize these complex materials. Work in this area has been reviewed many times in recent years. In some cases the focus of the review is on biopharmaceuticals, and 2D-LC is discussed among an array of other techniques.[230,231] In other reviews, the articles are organized around different 2D-LC techniques with a focus on application to biopharmaceutical problems.[61,232−234] Since these reviews are pretty recent and thorough, we will not reiterate a detailed discussion of recent work in this area here. Rather, we provide an overview of recent trends in this area, with an emphasis on the most recent publications.

Over the past few years most work in this area has been focused on mAbs and ADCs. Arguably, the single most compelling demonstration of the value of 2D-LC separations for the analysis of mAbs was published recently by Sandra and co-workers.[235] In this work they demonstrated multiple modes of 2D separation, ranging from single heart-cut to fully comprehensive separations. They showed examples of separations involving multiple combinations of different separation modes including Protein-A affinity chromatography (ProA) combined with SEC, IEX, or RP separation and RP combined with RP for LC × LC separations of peptide mixtures (in this case very different pH buffers are used in the two dimensions). Other groups have been exploring the potential of these new applications as well. Williams and co-workers discussed the development of an online ProA-SEC system for automated determination of mAb titer and degree of aggregation in a single 2D-LC analysis.[105] Gstöttner and co-workers have described the development of a complex but powerful mLC-LC approach for characterization of mAb charge variants.[108] In this work fractions from a ^{1}D cation-exchange (CEX) separation were first reduced online with dithiothreitol (DTT) and then digested online with trypsin prior to separation and identification of the resulting peptides in a ^{2}D RP-LC-MS analysis. The entire workflow is automated, which eliminates many of the manual sample manipulations associated with the characterization of mAb charge variants. In a very different study, Yan and co-workers used ion-exchange coupled with SEC separations in a mLC-LC system to study the degradants produced in a forced degradation of a mAb.[106] Whereas all prior 2D-LC separations of mAbs at the intact or subunit level have been noncomprehensive, Stoll and co-workers demonstrated both WCX × RP-MS[107] and HILIC × RP-MS[15] separations of mAbs after partial digestion with the *IdeS* enzyme. The HILIC × RP separation is particularly interesting for heavily glycosylated mAbs, because of the exquisite selectivity of the HILIC separation mode for separating forms of the proteins with subtly different glycans.

In recent years, the most active area of development for 2D-LC separations of biopharmaceuticals has involved ADCs, perhaps because an additional level of complexity is added to the already complex mAbs when small-molecule cytotoxic drugs are conjugated to the protein. This conjugation step gives rise to a number of important questions that must be answered in the course of developing an ADC as a therapeutic agent. These include questions about how much free small-molecule drug is present in the drug formulation and the extent and localization of the small molecule conjugation to cysteine or lysine residues in the protein. To quantify the level of unconjugated (free) drug present in the protein sample, several groups have used LC-LC or mLC-LC separations.[95,97,103] In the first dimension, free drug molecules are separated from protein by SEC. The fraction containing the free drug is transferred to a ^{2}D RP separation for desalting, further separation, and eventual detection by UV or MS. In the work of Goyon and co-workers, the authors report excellent quantitative accuracy, precision, and recovery for the SEC-RP method, consistent with our expectations of conventional 1D-LC around these performance metrics.[97] Since the conjugation of the small-molecule drug to the protein involves a "linker" molecule, this is an additional potential source of impurities and variation in the drug product. Venkatramani and co-workers have demonstrated the use of sLC × LC with RP separations in both dimensions to efficiently study the effect of process conditions on the impurity profile of these linker-drug intermediate molecules.[96]

Understanding the extent and localization of conjugated small-molecule drugs on an ADC is a challenging task. Although hydrophobic-interaction chromatography (HIC) is a powerful tool for separating ADCs according to the number of small molecule drugs that are attached (i.e., drug-to-antibody ratio, DAR), these separations typically require very high eluent concentrations of nonvolatile salts (often exceeding 1 M) and thus are not directly compatible with MS detection.[236] Adding a second dimension of separation by RPLC both substantially increases the resolving power of the HIC method and provides an easy and effective desalting step to enable direct MS detection. Sarrut and co-workers have demonstrated the utility of such a HIC-RP-MS system for online, automated, and thorough mapping of the extent and localization of small-molecule drug conjugation in cysteine-linked ADCs.[101,237] Ehkirch and co-workers[102] have coupled HIC and SEC separations with the SEC separation carried out under nondenaturing conditions to enable determine DAR and molecular mass of ADCs in their native state. In this work they further coupled the HIC × SEC separation to IM-MS detection, thus enabling confirmation of conformational homogeneity of each detected protein. Gilroy and Eakin studied a family of related heart-cutting approaches to assemble a detailed picture of conjugation in a cysteine-linked ADC.[98] This included HIC-SEC-MS, where the SEC conditions were nondenaturing, thus allowing the determination of the intact mass of each species observed in the HIC separation. They also used HIC-RP-MS separations under nonreducing or reducing conditions. In case of reducing conditions, an online reduction step (using DTT) was implemented after the HIC separation but before injection into the ^{2}D RP column. Whereas most conjugation-mapping work has been demonstrated using cysteine-linked ADCs,

DOI: 10.1021/acs.analchem.8b04841
Anal. Chem. 2019, 91, 240−263

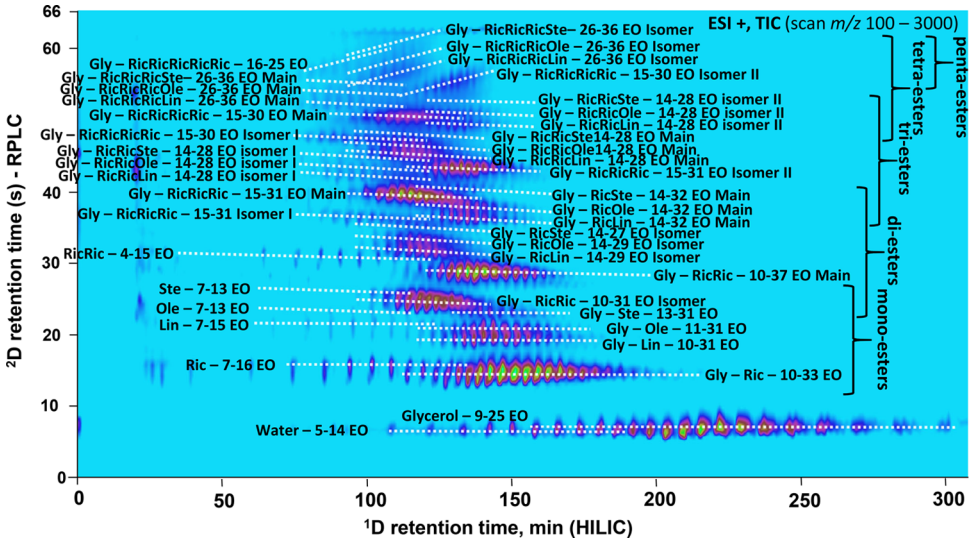

Figure 12. HILIC × RPLC-(+)HRMS separation of the castor oil ethoxylates. The [1]D HILIC dimension (horizontal) indicates the degree of ethoxylation, while the [2]D RPLC column (vertical) separates the ethoxylated species according to hydrophobicity. Various ethoxylated fatty acids as well as glycerol ethoxylated mono-, di-, tri-, tetra-, and penta-esters were identified using the obtained accurate mass and isotope distributions. Reprinted from *J. Chromatogr. A, 1569*, Groeneveld, G.; Dunkle, M.N.; Rinken, M.; Gargano, A.F.G.; de Niet, A.; Pursch, M.; Mes, E.P.C.; Schoenmakers, P.J., Characterization of complex polyether polyols using comprehensive two-dimensional liquid chromatography hyphenated to high-resolution mass spectrometry, pp. 128−138 (ref 9). Copyright (2018), with permission from Elsevier.

Sandra and co-workers have used mLC-LC and LC × LC separations to study the conjugation of the lysine-linked ADC ado-trastuzumab emtansine.[99] This is an even more challenging problem, because there are many more potential lysine conjugation sites in a typical mAb than there are cysteine conjugation sites. CEX and RP separations were used in the first and second dimensions of the mLC-LC system, respectively, to study intact and partially digested forms of the molecule, again with MS detection. Also, RP × RP-MS was used to localize sites of conjugation, following digestion of the molecule with trypsin.

To date there have not been many published reports on 2D-LC applied to the characterization of therapeutic peptides or oligonucleotides, although we expect this to change going forward. Petersson et al. have demonstrated how mLC-LC can be used to rapidly identify unknown impurities observed in 1D-RPLC separations of peptides that rely on high eluent-salt concentrations to obtain good peak shapes.[104] Adding a second dimension of RP separation with a MS-friendly eluent not only enables direct MS detection, but also provides additional selectivity that is useful in cases of coelution in the [1]D separation that would otherwise complicate interpretation of MS spectra. Finally, Roussis et al. have demonstrated the use of mLC-LC and LC × LC separations involving different combinations of AEX, RP, and IP-RP separation modes to identify impurities present in synthetic oligonucleotides as well as degradants observed in stability studies.[47] This work provides a compelling example of the ability of 2D-LC to achieve separations "...not currently possible by mass spectrometry alone or 1D-LC".

Separations of Synthetic Polymers. LC × LC is particularly relevant for the characterization of polymers for several reasons. The dimensionality of the sample[238] is relatively low. In case of homopolymers there may just be one parameter, molecular weight, that describes the nature of each molecule. In case the same homopolymer molecules have variable end groups, two parameters suffice and the dimensionality of the sample is two. Two-dimensional samples, or samples with two dominant dimensions, may yield structured and readily interpretable chromatograms in LC × LC. The dimensions of the sample may reflect key properties. For example, if a polymer is to be used to form a network in a reacting formulation, both the functional (reactive) groups and the molecular weight (length of the cross-link) reflect essential properties of the polymer. A second reason why LC × LC is attractive for characterizing polymers is that LC-MS is usually not a good alternative. In most cases online LC-(ESI-)MS is not a viable alternative. Only for relatively small and relatively polar polymers can MS be used successfully, at least for qualitative analysis. Quantitative LC-MS is always difficult, because the response depends on the exact structure (e.g., end groups) and on the molecular weight of the molecules. For the above reasons, the characterization of polymers was one of the first domains in which LC × LC started to be regularly applied and as a result a number of applications are relatively mature and routinely performed in industry. Routine applications of LC × LC for polymer characterization often involve RPLC × SEC or NPLC × SEC separations, and these are barely published anymore.[239,240] Recent publications often focus on unique (combinations of) selectivities to solve difficult problems in polymer science and industry.

Groeneveld et al. describe the separation of complex polyether polyols (polymers of ethylene oxide, EO, and propylene oxide, PO) by HILIC × RPLC.[9] One of their chromatograms is shown in Figure 12. This is a good example of highly structured 2D chromatograms of samples that are complex but have a low sample dimensionality. The relatively small and relatively polar polymers allowed the use of online high-resolution MS to characterize samples in great detail. Vanhoenacker et al. demonstrated HILIC × RPLC-separations of nonionic surfactant used in pharmaceutical formulations, using mass-spectrometric detection.[223] Malik et al. achieved impressive separations of triblock copolymers of EO and PO using an isocratic first-dimension with an acetonitrile/water

DOI: 10.1021/acs.analchem.8b04841
Anal. Chem. 2019, 91, 240−263

mixture on a silica column,[216] corresponding to the critical conditions (no retention) for the PO block and a RPLC separation with a methanol/water mixture that corresponded to critical conditions for the EO block.

Lee et al. described the separation of a series of polystyrene-polybutadiene block copolymers with temperature-gradient interaction chromatography (TGIC) in the first dimension.[211] Normal-phase TGIC was combined with either SEC or NPLC at the critical conditions in the second dimension. Likewise, RP-TGIC was combined with either SEC or RPLC at the critical conditions.

One example of newly charted territory are high-temperature LC × LC separations. High temperatures are typically required to resolve poly olefins (including ubiquitous polymers, such as poly ethene, PE, and polypropene, PP). Ndiripo and Pasch used high-temperature LC × SEC to characterize oxidized waxes at 140 °C.[209] A porous-graphitic-carbon column and a gradient from *n*-decane to *o*-dichlorobenzene were used in the first dimension. The latter solvent was also used for the second-dimension SEC separation. In a separate study, interaction LC on a silica column with a gradient from *n*-decane to *c*-hexanone was used in the first dimension,[210] either with a composition gradient at a constant temperature (110 °C) or in TGIC mode, increasing the temperature at a constant mobile-phase composition.

Apel et al. performed RPLC in the first dimension with a very shallow gradient close to the critical conditions,[212] followed by SEC in the second dimension to characterize branched poly (bisphenol A carbonate) structures. They combined this RPLC × SEC method with a triple-detector system (concentration detector, light scattering, and viscometry) and they tried to correlate the results with the outcome of Monte Carlo simulations.[213] Yang et al. used SEC × RPLC to determine the number of hydrophobic end groups in modified ethylene-oxide-urethane polymers.[214] They used an ultrahigh-performance column packed with sub-2-μm particles and a mobile phase containing 100 mM ammonium acetate in methanol in the first dimension and a gradient RPLC method in the second dimension. The authors developed a multistep second-dimension water−acetonitrile gradient to avoid breakthrough. The same group used a similar system for the separation of polymeric dispersants in detergents.[224] A very wide (8 mm i.d.) ^{1}D SEC column was used with an aqueous mobile phase and up to 60% of the effluent was diverted to waste.

Lee et al. exploited the unique selectivity of LC × LC to separate polystyrenes with interesting configurations ("figure-eight-shaped" and "cage-shaped" cyclic molecules) from branched polystyrenes using NPLC × SEC with a bare silica column (NPLC) and a *n*-hexane/THF gradient in the first dimension and SEC with THF as eluent in the second dimension.[215] Separation is based on the fact that molecules with different conformations but the same molecular weight have a different size in solution (hydrodynamic radius) and thus different elution times in SEC, whereas the extent of interaction with the silica column is not strictly related to the hydrodynamic radius.

Pursch et al. used the full capabilities of contemporary LC × LC instrumentation, including the use of ultrahigh pressures to realize SEC × RPLC separations of Novolac-type polymers within 20 min.[36] Active solvent modulation was used to successfully avoid breakthrough in the second dimension.

A unique application of LC × LC for the analysis of polymeric nanoparticles was described by Pirok et al.[43] They first separated the nanoparticles by packed column hydrodynamic chromatography (HDC) using an aqueous mobile phase. At the modulation stage, the particles were dissolved in THF to yield a solution of the constituting polymer molecules. After concentrating these on a trap column, the molecular-weight distribution was determined by organic SEC in THF.

SUMMARY AND OUTLOOK

The popularity of two-dimensional liquid chromatography (2D-LC) is on the rise, due to a range of successful applications. When two very different selectivities (retention mechanisms) are selected, heart-cut LC-LC allows target peaks to be rigorously purified and the purity of target analytes to be assessed. This is, for example, of significant interest in the emerging field of biopharmaceuticals. LC-LC also provides additional certainty of peak identification, which is relevant, for example, in forensics. Comprehensive two-dimensional liquid chromatography (LC × LC) is of general interest for analyzing complex samples that contain nonvolatile analytes or matrix compounds. Resolution for such samples can be much improved, thanks to the much higher peak capacities in comparison with conventional 1D-LC and thanks to the additional selectivity. As a result, LC × LC is potentially a much better "sample-preparation" method prior to analysis by MS or MS/MS. LC × LC-MS may thus be advantageous for the analysis of, for example, complex mixtures of peptides. When the two separation dimensions are selected judiciously, LC × LC may provide reliable fingerprints that allow rapid (often visual) and efficient comparison of samples, for example, in the field of natural medicines. For complex samples with a low dimensionality, such as synthetic polymers, LC × LC may provide highly structured, readily interpretable two-dimensional chromatograms.

The applications discussed in this review provide plenty of motivation to accelerate the development of LC-LC and LC × LC techniques. Research may be focused on a number of aspects. First and foremost, 2D-LC will thrive if the techniques (instruments, columns, modulators) are robust and reliable. Arguably, 2D-LC is a complex technique. This creates high demands on user interfaces for applications and, especially, for method development. Third-party method development (by instrument manufacturers or research groups) may accelerate the proliferation of 2D-LC techniques, provided that methods can be transferred reliably and efficiently. Technical issues that must be addressed include generic approaches to overcome compatibility issues that arise from the selection of two very different ("orthogonal") retention mechanisms. If the first-dimension effluent is not suitable as second-dimension injection solvent active-modulation techniques must be applied. These may have additional advantages. If the analyte bands are effectively focused by the modulator detection sensitivity may be enhanced, and the second-dimension separation may be performed more efficiently and on narrower columns, reducing the amounts of solvent required. Generally, 2D-LC can benefit from any progress in LC. In this century better columns (sub-2-μm particles, core−shell particles) and ultrahigh-performance instruments have emerged. Two developments are especially relevant for 2D-LC. Very fast analysis are desirable, especially in the second dimension of LC × LC methods. All 2D-LC methods can be improved by minimizing extra-column dispersion.

DOI: 10.1021/acs.analchem.8b04841
Anal. Chem. 2019, 91, 240−263

In the short period covered by this review (2016–2018) we have seen great activity in the development and application of 2D-LC techniques. Both heart-cut (LC-LC) and comprehensive (LC × LC) techniques appear to have a bright future.

AUTHOR INFORMATION

Corresponding Author

*E-mail: B.W.J.Pirok@uva.nl.

ORCID

Bob W. J. Pirok: 0000-0002-4558-3778

Dwight R. Stoll: 0000-0002-4070-9132

Notes

The authors declare no competing financial interest.

Biographies

Bob W. J. Pirok worked at Shell after obtaining his M.Sc. degree in 2014. He now is a Ph.D. student in the MANIAC (Making ANalytically Incompatible Approaches Compatible) project at the University of Amsterdam since 2015. He developed the (LC ×)LC optimization tool Program for Interpretive Optimization (PIOTR) and has published on fast size-exclusion chromatography using core–shell particles, comprehensive integrated analysis of nanoparticles and the constituting macromolecules by HDC × SEC, and the application and optimization of LC × LC separations. Bob W. J. Pirok was decorated with a Shimadzu Young-Scientist Award at HPLC2015 Beijing, the Young-Scientist-Award Lecture during the SCM-8 meeting in Amsterdam in 2017, and the Csaba Horváth Young-Scientist Award at HPLC2017 Prague.

Dwight R. Stoll is Professor and Co-Chair of Chemistry at Gustavus Adolphus College in St. Peter, MN. He has authored or coauthored 57 peer-reviewed publications, four book chapters in separation science, and over 100 conference presentations. His primary research focus is on the development of two-dimensional liquid chromatography (2D-LC) for both targeted and untargeted analyses. Within this area he has made contributions on many aspects of the technique including stationary phase characterization, biopharmaceutical analysis, new 2D-LC methodologies and instrumentation, and fundamental aspects including re-equilibration in gradient elution reversed-phase liquid chromatography and analyte focusing. He is the 2011 recipient of LCGC's Emerging Leader in Chromatography Award, the 2015 recipient of the American Chemical Society Division of Analytical Chemistry Award for Young Investigators in Separation Science, and was named to The Analytical Scientist's list of Top Ten Separation Scientists in 2017.

Peter J. Schoenmakers is Professor of Analytical Chemistry at the University of Amsterdam. He investigates analytical separations, focussing on multidimensional liquid chromatography. He studied in Delft, The Netherlands (with Professor Leo de Galan) and in Boston, MA (with Professor Barry Karger). Thereafter, he worked for Philips in Eindhoven (The Netherlands) and for Shell in Amsterdam and in Houston, TX. He was awarded the AJP Martin Medal in 2011, the John Knox Medal in 2014, and the Csaba Horváth Award and the CASSS Award in 2015. In 2016 he obtained an Advanced Grant for Excellent Research from the European Research Council.

ACKNOWLEDGMENTS

Bob W. J. Pirok acknowledges the MANIAC project, which is funded by The Netherlands Organisation for Scientific Research (NWO) in the framework of the Programmatic Technology Area PTA-COAST3 of the Fund New Chemical Innovations (Project 053.21.113). Dwight R. Stoll acknowledges financial support from the National Science Foundation (Grant CHE-1508159). Prof. Dr. Michael Lämmerhofer (University of Tübingen, Germany) is kindly acknowledged for providing Figure 11.

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DOI: 10.1021/acs.analchem.8b04841
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