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Review

The Versatility of Two-Dimensional Liquid Chromatography

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This review deals with two-dimensional liquid chromatography (2D-LC) separations encompassing target heart-cut (LC-LC), multiple heart-cut (mLC-LC), non-targeted comprehensive (LC × LC), and selective comprehensive (sLC × LC) analysis. It presents an overview of basic concepts and emphasizes the versatility of the applications gained by going from one-(1D) to two-dimensional (2D) separations. This review also discusses target analysis of achiral and chiral drugs for different applications and the use of 2D-LC in zonal bioaffinity chromatography. Advances in instrumental and column technologies have widened the application of LC × LC and sLC × LC separations, and we will discuss some of them.

Keywords: bioanalysis, chiral samples, food samples, natural products, bioaffinity columns

1. Introduction

The versatility of two-dimensional liquid chromatography (2D-LC) is widely acknowledged, despite usually being used to only analyze complex mixtures with a large number of peaks.[1-3] Nevertheless, it must be borne in mind that, in some cases, a difficult separation does not mean that the mixture contains a large number of analytes, but that the difficulty stems from the physicochemical or stereochemical parameters of the analytes and the application of the separation.[4]

When we searched Web of Science™ by using the term "two-dimensional liquid chromatography" (in all fields), we retrieved 5,178 results in the period spanning from 02 Jan 2000 to 17 Apr 2023 (index date). According to Web of Science™, the retrieved papers are categorized as depicted in Figure 1.

Most of the retrieved papers are in the field of analytical chemistry or biochemical research methods, but some publications concern different application fields.

2D-LC can be conceived off-line and on-line. In this review, we only consider the on-line approaches and the categorizations target heart-cut (LC-LC) and comprehensive (LC × LC) separations; we also discuss the two-hybrid modes multiple heart-cut (mLC-LC) and selective comprehensive (sLC × LC) 2D separations.[3]

Knowing when to go from one- (1D) to two-dimension (2D) separation helps the appropriate 2D-LC approach to be selected. Some of Stoll and co-workers[1-3] papers depict the fundamentals and the actual notations of 2D-LC separation.[1-3]

Technological advances in chromatographic columns and in LC systems have broaden the interest and diminished the main drawbacks (e.g., sample dilution, modulation, data-analysis, and visualization software)[3] in using 2D-LC and, thus, have produced a wide variety of applications mainly on the basis of commercially available instruments.[5-8]

2. LC-LC and mLC-LC

LC-LC or single heart-cut analysis has been most used in bioanalysis, and examples of its applications date back to the 80s.[9] Most of the applications include on-line sample cleanup with columns for depleting protein in the first dimension (^{1}D).[10-13] LC-LC is also the mode of choice for separating achiral impurities in a mixture of chiral molecules.[4,14]

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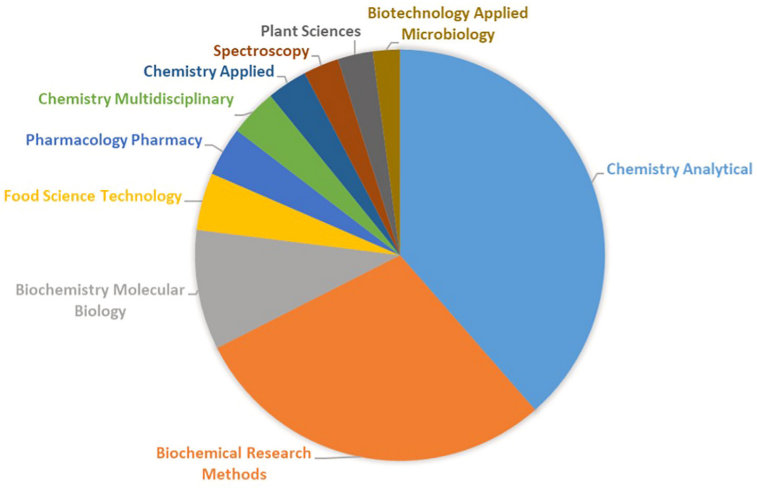

Figure 1. Ten Web of Science™ Core Collection categories that most use 2D-LC in the period spanning from 02 Jan 2000 to 17 Apr 2023. The term "two-dimensional liquid chromatography" was used in all fields.

In LC-LC, peak capacity is not important because the target analytes are the separation space. Usually, one or few peaks are targets, and the entire peak(s) or a fraction of it is transferred. For quantification, the target analyte is transferred with a fraction volume that is larger than the peak volume. Although this warrants accuracy, it can deleteriously affect separation in the second dimension (^{2}D).[15,16] Although transfer can be centered from the middle of the target peak, in a much smaller fraction, any slight shift in the retention time at the ^{1}D will affect the quantification precision.[2]

The columns are coupled through different configurations of a switching valve, and the peaks are transferred employing the back or forward-flush mode as illustrated in Figure 2. By using six-, eight-, or ten-port valves, the peaks are transferred directly from ^{1}D to ^{2}D without loops or trap columns. Under these configurations, the target analytes are usually transferred in a single heart-cut.[2,3,11,17]

The use of loops, trap columns, or parallel column arrays in the second dimension allows multiple cuts to be transferred without some of the targets being missed.[2,8,18-20] Well-designed mLC-LC applications based on multiloop deck or trap columns for collecting the peaks from the ^{1}D have been reported.[20-22] In the case of column arrays in the second dimension in either alternating, sequential, or simultaneous configuration increases the number of applications and have been exploited for achiral-chiral screenings.[23,24]

The main problems encountered when using mLC-LC, as well as LC × LC and sLC × LC, are the software for processing the multiple chromatograms in the second dimension and the plugins for hyphenating the 2D-LC system with the mass spectrometer system software.

3. LC × LC and sLC × LC

Given that LC × LC has high peak capacity, this mode is the most frequently used to analyze highly complex mixtures consisting of different classes of molecules and with broad retention time span. In LC × LC separation, the eluate is completely transferred from ^{1}D to the ^{2}D for further analysis. To avoid remixing or undersampling, four fractions *per* 8σ peak width are collected in the ^{1}D and transferred into the ^{2}D.[2,3] The modulation interface for sample transfer can vary, but they are mostly based on six-, eight-, or ten-port switching valves, with either loops or trap columns.[2,25,26] The modulation strategies should not focus only on obtaining high peak capacity because other important metrics such as separation time or detection could be compromised.[25,26] The fractions must be transferred from ^{1}D to ^{2}D without the undersampling effect being elicited,[2,11] which can be achieved by fast ^{2}D separation. Therefore, columns comprised of fully porous (FPP) or core shell (superficially porous-SPP) silica particles (with particle size varying from sub-2 to sub-3 μm) have been preferred.[27,28]

The main advantage of LC × LC is that it produces well-resolved peaks at a rate of about one peak *per* second, whereas one-dimensional LC separations produce well-resolved peaks at a much slower rate.[3]

For this, orthogonality between the two dimensions promotes separation of high heterogeneity mixture.[27] Despite moderate peak capacity in a hydrophilic interaction liquid chromatography (HILIC) × reversed-phase liquid chromatography (RPLC) separation, the orthogonality between the two dimensions allowed speciation based on the degree of ethoxylation to propoxylation for a polyether polyol synthetic formulation.[7]

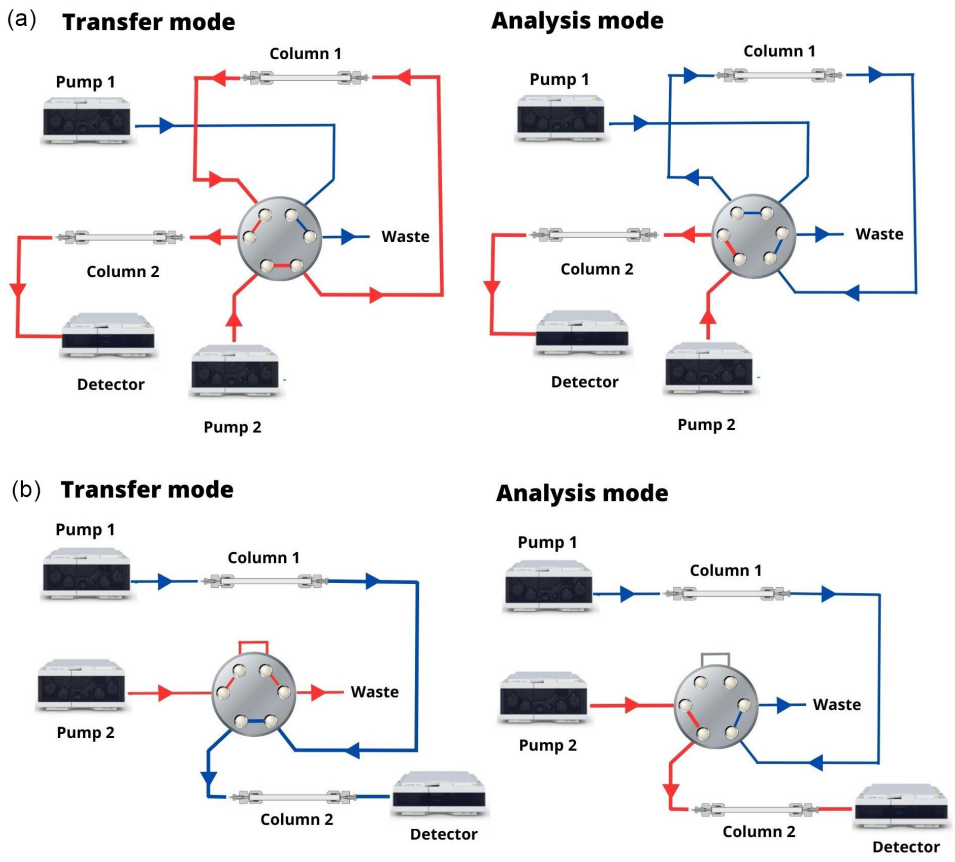

Figure 2. Schematic switching valve system in the transfer and analysis mode for the (a) back- or (b) forward-flush mode transfer.

The use of different elution modes between the two dimensions can promote much higher orthogonality. Despite of this, several examples can be found for RPLC in both dimensions.[2,29,30] For that, different column selectivity and chromatographic elution conditions are needed. In this regard, quality descriptors such as peak capacity, analysis time, dilution factor, number of runs in the second dimension, and injection volume have been determined for small molecules and peptides.[29,30] The preference for RPLC is justified by solvent compatibility, which facilitates the interface between the two dimensions.[3,27]

In sLC × LC, targets region of the ^{1}D chromatogram is comprehensively sampled to the ^{2}D without the resolution of the target analytes being diminished.[2]

When the fraction is collected and transferred, the fractions are not remixed regardless of peak width. Although the mLC-LC instrumentation hardware can be used for sLC × LC, they are fundamentally different. Moreover, sLC × LC holds great advantage over mLC-LC in quantification analysis. In heart-cut analysis, a fraction volume that is much larger than the peak volume of the target analyte must be transferred, which can crowd separation in the ^{2}D.[2]

Bearing in mind that only multiple peaks are transferred from the ^{1}D to the ^{2}D, during method development it is important to see how practical constraints of current sLC × LC system affect the performance of the separations. In other words, the number of fractions that can be transferred, the size of the sampling window, and whether the transfer is serial or parallel must be considered.[31,32] As example of application, sLC × LC has been used to profile impurities in synthetic and therapeutic peptides.[33]

The workflow for modelling chromatographic conditions for analysis of therapeutic peptides has been recently updated.[34,35] The other sections of this review deal with separation examples covering all 2D-LC categories.

4. LC-LC and mLC-LC Applications

Bioanalysts have taken advantage of the advances in LC-LC methods and employed them to analyze plasma and other biological matrixes directly, that is, without adopting laborious pretreatment steps.[36-38] Yamaguchi *et al.*[39] developed an LC-LC method to determine total phenylephrine (conjugated and free PL) in human serum. The method did not require extensive sample pretreatment procedures and was based on fluorescence detection. Initially, the authors deproteinized with acetonitrile and hydrolyzed conjugated with diluted hydrochloric acid. After

the solution was dried, the authors reconstituted the residue and analyzed it by LC. Analysis of the residue in a single C18 column lead to large interfering peaks to coelute with PL and prevented the required sensitivity and selectivity from being achieved. Therefore, the authors transferred the eluate fraction containing the analyte initially separated on a ^{1}D C18 column to the ^{2}D C18 column by valve operation. This LC-LC method provided the required selectivity and sensitivity for determining the total PL concentration after oral administration of PL hydrochloride, and the limit of quantification (LOQ) was 5 ng mL^{-1}.

To gain throughput and reproducibility in bioanalysis while decreasing solid or chemical waste during analysis, restricted-access materials (RAM) columns have been used in the ^{1}D of a 2D-LC separation to retain small molecules selectively while macromolecules are excluded in the void volume of the column.[11] In this configuration, single heart-cut transfers the targets analytes to the analytical column in the ^{2}D by forward or back flush.[13,17]

The stereoselective metabolism of lansoprazole, omeprazole, and pantoprazole in healthy subjects has been investigated.[40-42] To this end, the plasma samples were analyzed by directly injecting them into a bovine serum albumin RAM (RAM-BSA) column in ^{1}D, followed by single forward-flush transfer of the analytes to polysaccharide columns in the ^{2}D. Despite the structural similarity of the proton pump inhibitors, the chiral columns used for lansoprazole[40] and pantoprazole[42] differed from the column used for omeprazole.[41]

RAM-BSA columns can be employed during environmental analysis by direct sample injection. Barreiro *et al.*[43] coupled a RAM-BSA column with a polysaccharide-based chiral column to quantify simultaneously pantoprazole and lansoprazole enantiomers fraction in native aqueous matrixes. The RAM-BSA column allowed humic substances to be excluded, while the polysaccharide-based chiral column enabled the enantiomers of both pharmaceuticals to be separated. The LC-LC method provided an analysis time of 40 min, did not require any sample pretreatment, and proved a useful tool to assess biotic and abiotic enantioselective degradation and temporal changes of the enantiomeric fractions.

The enantiomeric shifts of propranolol and its hydroxy metabolites, namely 4-, 5-, and 7-hydroxy propanolol (HOPL) have been quantified by mLC-LC.[44] Achiral separation of racemic propranolol and its metabolites 4-HOPL, 5-HOPL, and 7-HOPL was achieved with gradient elution on a phenyl-hexyl column in ^{1}D. The analyte fractions were cut out and parked separately in several 40 μL loops. Then, each fraction was transferred to the ^{2}D containing a glycopeptide teicoplanin-based chiral column, which separated the matrix residues from the analyte and discriminated between enantiomers. The enantiomers of propranolol and its hydroxy metabolites were successfully separated and quantified in urine samples. The separation and quantification evidenced that (*R*)-5-HOPL and (*R*)-7-HOPL were excreted in higher excess than their respective enantiomers, while (*R*)-propranolol and (*R*)-4-HOPL and their (*S*)-enantiomers had similar excretion rate.

Food analysis involves very complex samples by high-throughput analytical procedures. Therefore, LC-LC methods offer the required resolving power to assess food safety, food quality, and the relationship between health and food and to characterize particular groups of food components.[45] For instance, the use of antibiotics for treating inflammatory diseases or in cattle breeding can contaminate milk, which is a problem for the milk processor and the consumer. An LC-LC method has been developed to monitor 20 antibiotics of seven (7) categories in milk and powder milk.[46] The analytes, which had a wide polarity range, were separated by coupling an HILIC column with an RP C18 column. For the first time, this LC-LC method allowed the 20 selected antibiotics, including β-lactams, tetracyclines, macrolides, aminoglycosides, amphenicols, quinolones, and sulfonamides, to be separated and detected simultaneously.

An LC-LC method involving HILIC in ^{1}D combined with an RPLC column in ^{2}D by LC-LC has been applied to determine the level of *N,N*-dimethyltryptamine (DMT) in plasma and brain samples; α-methyltryptamine (AMT) was used as internal standard.[47] DMT is an endogenous hallucinogen that is present in various mammals tissues such as the brain, pineal gland, and lung and in body fluids like urine, cerebrospinal fluid, and blood plasma. Selective and sensitivity assays are required for determining DMT in experimental models of cerebral ischemia/reperfusion by DMT administration. DMT and AMT eluted from the HILIC column were trapped in a C18 trap column and, after the valves were switched, the trap column was connected to the RP analytical column. This LC-LC method was significantly faster (10 min) and exhibited better sensitivity than many published 1D-LC methods.[47]

2D-LC has found widespread use in several industries, especially for producing biotherapeutics (e.g., peptides, proteins, and drug formulations). Recombinant monoclonal antibodies (mAbs) are highly heterogeneous proteins, whose characterization requires a battery of analytical techniques. Antibody-based drugs, for example, can have varied size (due to aggregation) and charge (because of amino-acid sequence differences), which makes their separation by size-exclusion

chromatography (SEC) in ^{1}D and ion-exchange (IEC) in ^{2}D a very effective approach for analyzing them.[48]

Although IEC and SEC usually provide optimal separation of mAb variants, the mobile phase employed in these methods contains salt and other non-volatile buffers that are not compatible with on-line mass spectrometry (MS) detection, a technique that plays an essential role in the mAb structural elucidation. Therefore, mAb charge and size variants can be characterized by 2D-LC, by coupling an SEC or IEC column with an RPLC and interfacing the system with a high-resolution mass spectrometer (HRMS). SEC and IEC are highly orthogonal to RP, i.e., they are complementary separation modes, so they can spread sample constituents out over the entire ^{2}D separation space.[49] Thus, fractions from ^{1}D can be directly transferred to the RP gradient, which employs MS-compatible solvents. Then, the fractions can be further analyzed, providing highly resolved peaks and structural information through top-down analyses.[50]

By using a similar approach, an SEC-RPLC-HRMS has been employed to separate and to characterize polymerized impurities in cephalosporins.[51] The mobile phase of ^{1}D (SEC) consisted of phosphate buffer solution and acetonitrile in gradient elution. Individual analysis of cefodizime, cefonicid, and cefmenoxime exhibited two polymerized peaks detected before each cephalosporin peak, which were loaded into six loops by using a seven-port switching valves (sample collection configuration, Figure 3). After that, the effluent of each impurity peak was transferred to the C18 column with an MS-compatible mobile phase to enable mass spectrometry detection (LCMS analysis, Figure 3). Eleven allergic impurities were separated, and their structures were annotated by MS^{n} data. Nine of these impurities were polymerized impurities.

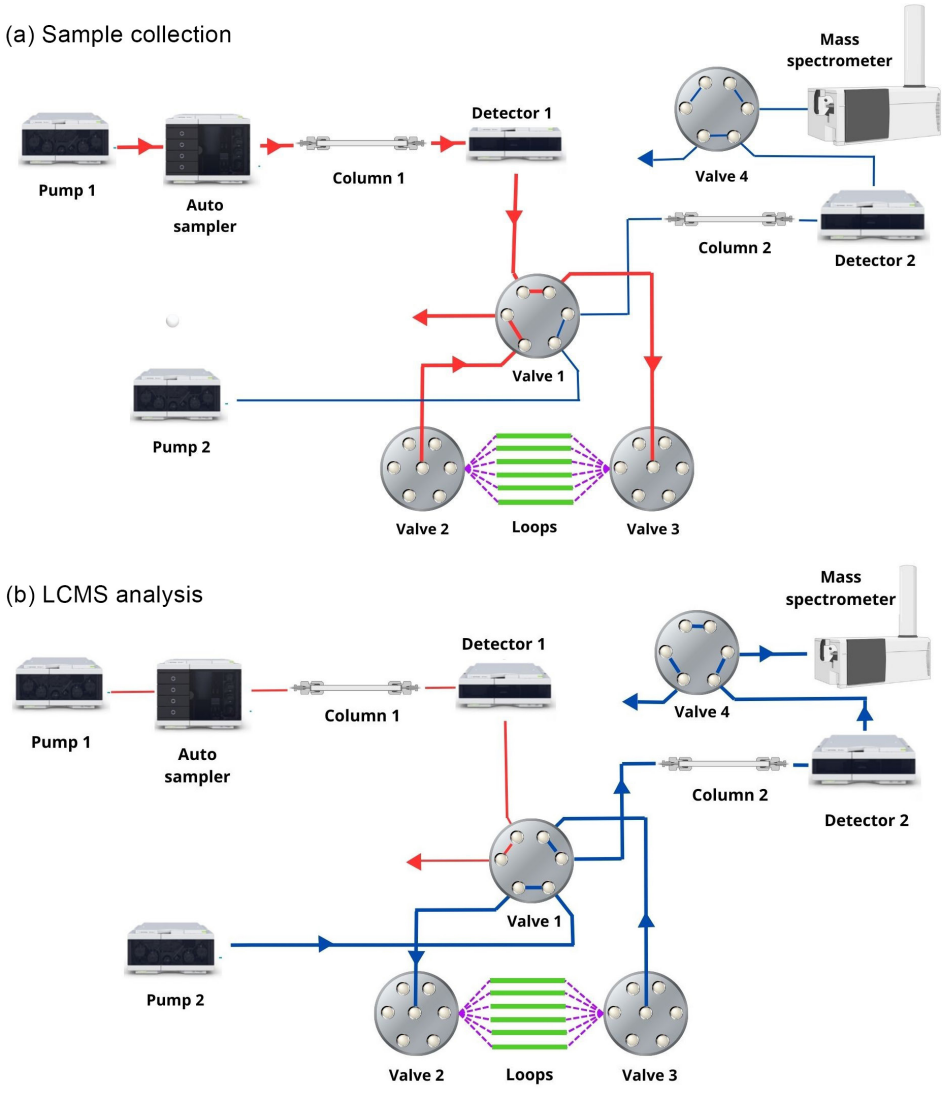

Figure 3. LC-LC instrumental set-up employed to separate and to characterize polymerized impurities in cephalosporins (adapted from Xu *et al.*[51]).

Synthetic oligonucleotides for pharmaceutical applications in humans and clinical trials must exhibit high purity. Ion-par reversed-phase is the predominant separation mode for characterizing synthetic oligonucleotides, but this mode can suppress ionization during MS detection. Thus, an mLC-LC method that uses an RP/weak anion exchange in ^{1}D has been developed to separate closely structurally related oligonucleotide sequences and deletions selectively.[8] Heart cuts of the oligonucleotide peaks were transferred to ^{2}D, via a five-position-10-port valve connected to two six-position-14-port valve carrying six 40 µL loops each; and the phosphate buffer was removed by using an RP column. This allowed oligonucleotides to be sensitively detected by electrospray ionization mass spectometry (ESI-MS).

Considering that many of the chiral drugs are currently used as racemates, and that enantiomers display different biological activities, suitable analytical methods for monitoring stereoisomeric impurities are still needed. In this context, an achiral-chiral LC-LC method has been described for enhanced pharmaceutical impurity analysis.[23] RP separation, employed in ^{1}D, is the most used to analyze impurities in active pharmaceutical ingredients (APIs), and it can remove interfering compounds before chiral separation. A multi-column selection approach was designed for the ^{2}D-the multi-column arrangement comprises a set of six polysaccharide-based chiral columns operating in reversed-phase mode, which allowed five racemates with a wide range of polarity to be separated. This automated ^{2}D chiral screening provided highly efficient selectivity tuning and Rs values of up to 17.21 for some of the racemates.

2D-LC has also been employed to isolate the active constituents of natural products. Wong and Shalliker[52] reported an LC-LC method to isolate the major bioactive compound (xanthine oxidase inhibitor) in a crude extract of *Clerodendrum floribundum*. Chromatographic separation using 1D gradient elution helped to identify the target bioactive compound. However, because this compound eluted in a region with a vast array of minor components, it could not be isolated with high purity. When the heart-cut approach with a nitrile column in ^{1}D and a C18 column in ^{2}D was used, the purity of the isolated sample was greater than 99%, and the recovery was 95%. Under the same loading factor, the 1D gradient elution method gave a final product with purity lower than 95% and recovery lower than 70%. Moreover, because the ^{1}D separation took less time than the ^{2}D separation, the sample could be re-injected into the system before separation was achieved in the ^{2}D, to yield a brief cycle time with full use of the separation space.

5. LC × LC and sLC × LC Applications

LC × LC is a versatile technique that has been applied in many analytical fields, to analyze various types of sample and analytes. It provides higher separation and identification capabilities than traditional 1D-LC.[53] Food analysis also takes advantage of the high-resolution power of LC × LC to overcome the low peak capacity of 1D-LC methods, which cannot often separate complex matrixes.[54] From the omics/foodomics perspective, Montero *et al.*[45] overviewed some of the most notable 2D-LC applications developed from 2009 to 2019.

Cacciola *et al.*[55] discussed method optimization and modulation approaches by using an sLC × LC[31,56] longitudinal on-column thermal modulation device to analyze red wine samples.[57] Applications of sLc × LC in food analysis include analysis of polyphenols, lipids, and carotenoids. The two most commonly used approaches for polyphenol analysis in food and natural products, RPLC × RP-LC and HILIC × RPLC, have recently been reviewed[58] and investigated.[59] The mobile phases compatibility between HILIC and RPLC makes this combination valuable for application of different purposes. The mismatch in mobile phase strength is, however, something not to be overlooked.

HILIC × RPLC approaches based on dilution of the ^{1}D effluent and large injection volume provide powerful and relatively fast analysis for detailed screening of the phenolic content in several natural products. As the sample eluting from the ^{1}D should be in a weaker mobile phase than the one entering the ^{2}D, these aqueous-rich fractions are not suitable when an HILIC column is used in the ^{2}D position. In contrast, RPLC is equally used as ^{1}D or ^{2}D, allowing numerous HILIC × RPLC or RPLC × RPLC combinations.[58]

Incompatible solvent strength limits HILIC × RPLC and requires modulation strategies.[3] Reduced orthogonality is an issue with RPLC × RPLC methods. To improve orthogonality, tailored ^{2}D gradient programs can be employed.[3,55] Montero *et al.*[60] used a LC × LC-DAD system consisting of two columns with complementary separation behavior in ^{2}D. The columns were automatically alternated according to the chemical characteristics of the compounds eluted from the ^{1}D. Direct injection of crude vermouth samples (a beverage containing a complex mixtures of phenolic compounds) without sample preparation have been analyzed. The setup uses an RP column pentafluorophenyl (PFP) in the ^{1}D and a ZIC-HILIC column and C18 column in ^{2}D. A two positions/four-ports dual valve with 60 µL sampling loops was employed to couple the ^{1}D to the ^{2}D. To select the column in ^{2}D automatically, an additional two-

position/six-port valve was connected to the modulation valve exit. Gradient elution in both ^{2}D columns was conducted by using water and acetonitrile in different strength order, modulation time of 2 min, and elution rate of 1.8 mL min^{-1}. The switching time for the selector valve was set at 30 min, which resulted in high HILIC column efficiency for analysis of polar compounds in ^{2}D. From 30 min to the end of the analysis time (120 min), the C18 column separated the medium and less polar compounds well. The peak capacity ($^{2D}n_c$) and orthogonality increased compared to individual LC × LC methods providing maximum sample separation in a single analysis.

To address the growing demand for eco-friendly separation techniques, researchers have developed a new LC × LC approach that replaces acetonitrile with propylene carbonate (PC), to efficiently separate a mixture of 39 drugs of various pharmaceutical classes. Two LC × LC methods were devised using PC: ethanol, at a PC/ethanol ratio of 60:40 in the ^{1}D, and ethanol alone in the ^{2}D. The compounds were separated by using a C18 column in the ^{1}D and either a C18 column (method A) or Pinnacle DB PFPP (method B) column in the ^{2}D. Compared to traditional conditions that use acetonitrile, using PC reduced the analysis time (32 min *versus* 53 min with acetonitrile) while the peak capacity and orthogonality were maintained. This study demonstrates the potential use of PC as an organic modifier in RPLC × RPLC separations to make LC × LC a greener method.[61]

Anti-doping analysis requires exceptional accuracy and precision. Whereas the world anti-doping agency (WADA) currently relies on chromatography (LC or GC) hyphenated with mass spectrometry and immunological methods,[62] LC × LC methods have also been reported for anti-doping analysis, such as the quantification of the beta blockers alprenolol and propranolol in human plasma,[63] and the multiclass screening method for quantifying prednisolone, methylprednisolone, dexamethasone, and betamethasone in urine.[64]

Protein biopharmaceuticals, such as monoclonal antibodies (mAbs) and therapeutic proteins, are widely applied to treat various life-threatening diseases,[65] so robust analytical approaches are required to characterize them. 2D-LC applications have potential use for this purpose.[66,67] LC × LC analysis for therapeutic mAbs determination based on a tryptic digest of trastuzumab and different LC × LC combinations, including strong cation-exchange (SCX) × RPLC, RPLC × RPLC, and HILIC × RPLC, has been reported.[65,68] These approaches have demonstrated the potential use of 2D-LC for characterizing mAbs and related products, such as host cell protein, antibody-drug conjugates, and small molecular drugs.[48]

sLC × LC-MS has been applied to identify the main isoforms and subunits of rituximab through a middle-up approach that incorporates cation exchange chromatography (CEX) and RPLC in ^{1}D and ^{2}D, respectively. According to Stoll *et al*.,[69] this approach offers several advantages over single heart-cut or fully comprehensive 2D separations and allows maximum information to be obtained from both separation dimensions in each analysis time. The use of CEX, a well-known strategy for separating charge variants in biopharmaceutical analysis, coupled to RPLC in the ^{2}D, as desalting step, allows MS information to be directly acquired from a CEX experiment, while improving peak capacity resolution.

Biosimilars are a rapidly growing segment of the pharmaceutical market. This segment requires highly efficient analytical methods to distinguish biossimilars from reference products to ensure that they are clinically efficacious. Sorensen *et al*.[70] combined a CEX and RP platform in a middle-up approach to compare three pairs of reference/biosimilar mABs: cetuximab, trastuzumab, and infliximab.

Back in 1991, Oda *et al.*[71] demonstrated the benefits of reducing the volume of the ^{1}D effluent injected into the ^{2}D column. More recently, researchers have employed the RPLC × RPLC configuration and active solvent modulation (ASM) to separate peptides.[1] The advantages of using ASM and an HILIC × RPLC configuration to analyze the mAbs cetuximab, obinutuzumab, and atezolizumab have also been reported. These mAbs differ significantly in terms of the of *N*-glycosylation level, but the HILIC × RPLC analyses swiftly provided the degree of glycosylation on the Fc/2 and Fd subunits of each mAb. Moreover, this setup was considerably more discriminatory when it came to separating the numerous glycoforms of heavily glycosylated mAbs, such as cetuximab, as compared to other LC × LC configurations like CEX × RPLC.[72]

Most commercially available therapeutic peptides are synthetic and often contain impurities that co-elute with the main peak during purification. This demands complementary analytical methods to ensure that peptide quality is controlled beyond the typically used 1D RPLC. Karongo *et al.*[33] developed a novel generic sLC × LC (RPLC × RPLC) method with various orthogonal detection modalities including UV (diode array detector, DAD), charged aerosol detection (CAD) and HRMS, they kept the available generic 1D RPLC peptide impurity profiling method and set it as the ^{1}D separation (UV detection). Oxytocin octreotide, cyclosporin A, and proprietary peptides 1-3 were used as a proof of concept. Experiments based on ASM showed that the generic sRP × RP 2D-LC method did not require ASM for standard peptides.

However, for peptides with multiple ionizable groups (e.g., peptides that have chelating moieties and which are used for imaging), the use of ASM is highly recommended to avoid peak splitting. 2D contour plots presented impurity profiles (UV detection) that could not be identified by the generic 1D RP, except for exenatide. In the experimental multi-detector configuration, the effluent from the ^{2}D column was divided by a flow splitter at a 5:1 ratio. The smaller flow was directed to the HRMS, while the larger volumetric flow was directed to the DAD detector and CAD, which were coupled in-line.

LC × LC analysis has been used to analyze biomarkers. The normal phase (NPLC) × RPLC-HRMS method has been developed for comprehensive lipid profiling of human plasma samples collected from atherosclerosis patients to compare the differences in lipid metabolism between the control and the patient samples. This method allowed 540 endogenous lipid species from 17 different classes to be identified. Furthermore, the isomers, galactosylceramides (GalC) and glucosylceramides (GluC), were successfully separated, revealing that only the levels of GalC were significantly increased in atherosclerosis patients compared to controls (the ratio in controls *vs.* patients was 1.5-2.8 fold).[1]

Isobaric tags for relative and absolute quantification (iTRAQ) have been widely adopted as a screening assay for detecting cancer protein biomarkers. In a study by Bouchal *et al.*,[73] an off-line iTRAQ-2D-LC-MS/MS approach was proposed to identify potential biomarkers related to metastatic processes in breast cancer. Recently, Yu *et al.*[74] improved upon this approach by combining RPLC × RPLC-HRMS, creating an on-line iTRAQ-2D-LC-MS/MS platform to investigate potential serum biomarkers of pediatric Non-Hodgkin's lymphoma (NHL) in patients and controls (B-NHL *vs.* control and T-NHL *vs.* control). Samples obtained from healthy controls and children with B-NHL and T-NHL were pooled, and the 14 high-abundance proteins were eliminated using the human 14 multiple affinity removal system (MARS) before tryptic digestion and iTRAQ labelling. The peptide mixture was then analyzed by 2D-LC-MS/MS, and the bioinformatics software IPA was used to analyze the differentially expressed proteins. The candidate biomarkers S100A8 and LRG1 were selected for further validation with enzyme-linked immunosorbent assays (ELISAs), and their efficacy was evaluated by using receiver operating characteristic (ROC) curves.

An HILIC × RPLC and RPLC × RPLC-MS configuration that uses C18 trapping column modulation has been examined for separating and identifying two gastrointestinal digests of commercial microalgae formulations (*Spirulina platensis* and Klamath). The study evaluated how both configurations performed in terms of peak capacity, maximum number of identified phycocyanin peptides, and their properties. Results showed that the HILIC × RPLC approach provided higher peak capacity values (n_c HILIC × RPLC: 932) compared to RPLC × RPLC (n_c RPLC × RPLC: 701), while RPLC × RPLC identified a slightly larger number of phycocyanin-derived peptides (HILIC × RPLC: 88 *vs.* RPLC × RPLC: 103) within the same analysis time of 60 min.[75]

LC × LC methods have been used to analyze degraded APIs. Naproxen and its photodegradation products have been separated and detected by using RPLC × RPLC-UV.[76] Wicht *et al.*[77] reported an innovative LC × LC approach for analyzing API impurities. They used a temperature responsive (TRLC) column in the ^{1}D with a RPLC column in the ^{2}D. The TRLC column was made of poly (*N*-isopropylacrylamide) (PNIPAAm) silica-based material, which can change hydrophobicity retention at temperatures above ca. 32 °C.[78] This feature allows aqueous mobile phase without organic modifier, to be used and chromatographic retention can be adjusted by changing the temperature. For non-targeted screening of impurities, a mixture of 17 APIs was used,[77] and three different selective SPP columns were investigated in the ^{2}D with acetonitrile in water (0.1% formic acid) in gradient elution at 2.5 mL min^{-1}. The developed generic method effectively separated all the impurities from the overloaded APIs. The authors[77] suggested that TRLC × RPLC can be implemented on current state-of-the-art LC × LC instrumentation.

LC × LC to analyze polymers has become increasingly popular and has been extensively documented in the literature. Various combinations of chromatographic conditions, such as SEC, RPLC, and NPLC, have been employed.[79-81] Thermal modulation, also known as cold trapping, has been suggested to separate polymers by 2D-LC fast and efficiently which can be applied to analytes that exhibit sufficiently increased retention with decreasing temperature. Pump-frequency synchronized modulation helps to minimize the observed pressure pulses resulting from the switching valve have been minimized. A 2D-LC cold-trap set-up has been used to separate a polystyrene/polybutadiene star block copolymer. Qualitative evaluation of the trapping efficiency was accomplished by monitoring the trap effluent with an evaporative light-scattering detector, while quantitative evaluation was conducted by determining the recovery of polystyrene standards from RPLC × SEC experiments.[82]

LC × LC has been used to analyze pesticides. Firstly, to samples containing both chiral and achiral compounds and to improve the resolution capacity of multiple pesticides in a

single analysis, Díaz Merino *et al.*[83] devised a chiral × achiral approach that successfully separated 24 pesticides (17 chiral and 7 achiral compounds). An active flow splitter pump was utilized to facilitate adjustments in sample volume transferred to the second dimension, as well as to independently optimize flow rates in the first dimension.

More recently, an LC × LC-MS/MS method has been developed for the simultaneous analysis of 112 pesticides in corn-based products.[84] The sample treatment was conducted using a scaled-down QuEChERS (quick, easy, cheap, effective, rugged and safe) method, which reduces the amount of sample, solvent, and sorbents required. The LC × LC method employed two RP columns: an F5 column in the ^{1}D and a short partially porous C18 column in the ^{2}D. To enhance sensitivity and minimize broadening of bands in the ^{2}D, two identical trapping columns were incorporated in the modulator. The introduction of a focusing effect resulted in reduced band broadening, as the analytes from the ^{1}D were captured in the trapping column during the collection step. The method achieved LOQ values ranging from 0.5 μg kg^{-1} (for fenoxycarb) to 43.6 μg kg^{-1} (for carbaryl). When analyzing two different samples of corn-based foods, only three pesticides out of 112 tested were detected below the LOQ and the maximum residue limit (MRL) values.[84]

Herbal medicine often contains numerous small molecules with different polarities, structures, and contents, which makes separation a challenging task. In recent years, 2D-LC has become a popular separation technique whose efficacy has been assessed in various studies on natural extracts,[85] including the screening of bioactive components from natural products[86] and preparative separations.[87] For example, Shang *et al.*[88] proposed a chemical profiling strategy for Xiaoer-Feire-Kechuan (XFK), an 11-herb Chinese medicine formula. To this end, they used LC × LC (CHS C18 × phenyl-hexyl) coupled to an Orbitrap- and triple quadrupole (QqQ-MS) platform. This system had peak capacity of 990.5 and orthogonality of 90.3%. The untargeted mass spectra data, which was collected by using data-dependent MS2 (dd-MS2) scan on the Orbitrap-MS, corresponded to 542 peaks, or four times the number detected by 2D-LC-UV (131 peaks) and annotated 108 compounds.

A low-cost and gradual gradient on-line 2D preparative LC system has been developed for the preparative separation of compounds of interest from natural products. The system consisted of medium-pressure liquid chromatography (MPLC) in the ^{1}D (RP column) and preparative LC in the ^{2}D (RP column). Two trapping columns (C18) and a makeup pump were also employed. This MPLC × preparative LC system was evaluated via Gram-scale isolation of a crude methanol extract venom from the toad *Bufo gargarizans*. By means of a single 2D separation run (345 min), 18 bufadienolides were isolated from 0.5 g of crude extract, and the purity of each compound was higher than 90%.[89]

The workflow applications of 2D-LC have been recent reviewed.[3] Table 1 provides a complementary overview of LC × LC and sLC × LC applications reported of from 2019 to 2023.

Xu *et al.*[100] created a 2D-LC column selection manual for analyzing natural alkaloids. The guide is a result of evaluating multiple columns with distinct separation mechanisms and offers a straightforward starting point for column selection. The use of this guide allowed for successful isolation of alkaloids from a *Uncaria rhynchophylla* medicinal plant sample.

6. Bioaffinity Chromatography in 2D-LC

Bioaffinity chromatography have been used for a wide variety of applications[101-103] such as producing metabolites and enantioselective synthesis[101,102] but, mainly for profiling binding events between target proteins and ligands.[104-106]

Back in 1998, Wainer and co-workers[103] used β-glucuronidase immobilized reactors (BG-IMER) to hydrolyze glucuronides on-line. For that, they used BG-IMER in ^{1}D, and the hydrolysis products (glucuronide metabolites) were directly transferred to an RP analytical column in the ^{2}D and analyzed with gradient elution. The enzymatic activity of the BG-IMER was evaluated by

Table 1. Examples of applications of LC × LC and sLC × LC

Sample	Modulation	^{1}D × ^{2}D	^{1}D separation	^{2}D separation	Detection	Remark	Reference
Campomanesia xanthocarpa Berg. (gabiroba)	P 1.0 min	Ascentis RP-Amide (250 × 1 mm, 5 μm) × Ascentis Express C18 (50 × 4.6 mm; 2.7 μm)	mobile phases: A 0.1% formic acid in water (pH 3) and B 0.1% formic acid in ACN; multi (four-step) segmented gradient: 0-5 min, 2% B; 40 min, 40% B; 50 min, 60% B; 60 min, 100% B; 90 min, 100% B; flow rate 10 μL min^{-1}	mobile phase: A 0.1% formic acid in water (pH 3), solvent B 0.1% formic acid in acetonitrile; multi (four-step) segmented gradient 0-40 min 10-16% B; 40-60 min 16-26% B; 60-70 min 30-50%B; 70-105 min 50-90% B; flow rate 2.5 mL min^{-1}	DAD; MS	identification of active polyphenols	90

Table 1. Examples of applications of LC × LC and sLC × LC (cont.)

Sample	Modulation	^{1}D × ^{2}D	^{1}D separation	^{2}D separation	Detection	Remark	Reference
Two industrial hemps, both were *indica* dominant hybrid (60% *Cannabis indica*, 40% *Cannabis sativa*) strains	A 0.5 min additional pump: solvent: 0.1% (v/v) formic acid and water, flow rate of 0.020 mL min^{-1}	Kinetex PFP (150 × 2.1 mm, 1.7 µm) × Kinetex C18 (50 × 4.6 mm; 2.6 µm)	mobile phase: A 0.1% formic acid in water; B 0.1% formic acid in methanol; gradient, 0-5 min; 5-8% B, 5-7 min 25% B, 7-18 min 35% B, 18-19 min 40% B, 19-35 min; 55% B, 35-36 min 65% B, 36-52 min 85% B, 52-54 min 100% B, 54-65 min 100% B; flow rate of 0.050 mL min^{-1}	mobile phase: A 0.1% formic acid in water; B 0.1% formic acid in acetonitrile; gradient: 0-0.42 min 5-10% B; 10-12 min, 5% B; 12-12.42 min, 10% B; 12.42-33 min, 25% B; 30-33.42 min, 30% B; 33.42-40 min, 25% B; 40-40.42 min, 45% B; 40,42-51 min, 35% B; 51-51.42 min, 70% B; 51.42-60 min, 80% B; and 60-60.42 min, 100% B; flow rate: 2.5 mL min^{-1}	DAD; DAD	µLC × LC separation; a home-made program to do a "demodulation", which allows discrimination of industrial hemp strains	91
Pistacia vera L. kernel extracts	P 1.20 min	Ascentis Express Cyano (150 × 1.0 mm, 2.7 µm) × Ascentis Express C18 (50 × 2.1 mm, 2.7 µm)	mobile phases: A 0.1% formic acid in water (pH 3), B 0.1% formic acid in ACN; gradient: 0-5 min, 2% B; 5-20 min, 10% B; 20-60 min, 30% B; 60-80 min, 100% B (held for 20 min); flow rate, 15 µL min^{-1}	mobile phases: A 0.1% formic acid in water (pH 3) and (B) 0.1% formic acid in ACN; shift gradient: 0-8 min, 1% B; 8-80 min, 1-26 % B; flow rate: 0.8 mL min^{-1}	DAD, MS	polyphenolic fraction of *P. vera* extracts from diverse geographic origins detected using a shift gradient approach had greater separation space to overcome co-elution issues, resulting in identifying more compounds than conventional one-dimensional LC analysis	92
Olea europeaea L. (olive trees)	P 0.25 min	PFP Kinetex F5 column (50 mm × 2.1 mm, 2.6 µm) × Zorbax Eclipse Plus C18 (50 mm × 3 mm, 1.8 µm)	mobile phases: A 0.05% TFA in water (v/v). B 0.05% TFA in MeOH; gradient: 0-10 min 30% B; 10-25 min 60% B, 25-40 95%, it was kept during 2 min; A re-equilibration step was carried out during 14 min; flow rate: 0.1 mL min^{-1}	mobile phases A 0.05% TFA in water (v/v); B 0.05% TFA and ACN; shifted gradient 0-60 min; flow rate: 2.5 mL min^{-1}	DAD, DAD	polyphenolic fingerprints for classification of olive leaves and pulp extracts	93
Bovine serum albumin digest (one protein), yeast-proteome digest (about 6700 proteins) and human kidney-tissue (about 20,000 proteins)	P	ZIC-HILIC (200 mm × 200 mm, 5 mm) × C18 (50 mm × 4.6 mm, 3 mm; both columns packed in house	mobile phases: A 10-mM ammonium formate in water (pH 3). B, 97% ACN: 3% 10 mM ammonium formate, (pH 3); A multi-segment gradient: 0-1 min 95% B 1-2 min, 95-85% B 2-59 min 85-75% B 59-89 75-65% B 89-90 min 65-50% B 90-91 min 95% B equilibration column with 95% B for 30 min; flow rate 1 µL min^{-1}	mobile phases: A, 0.1% formic acid in water with 2% ACN B, 0.1% formic acid in 20% water and 80% ACN; gradient: 5% B to 60% B, gradient time was set equal to the modulation time minus 1 min; modulation times (5, 10, 15 and 30 min); flow rate 1.2 µL min^{-1}	DAD, MS	use of a C18 trap column to overcome dilution and solvent incompatibility; a 60% increase in peak capacity and a 17-34% increase in the number of identified proteins were achieved for the samples analyzed (2D-yeast-8280 peptides and 2D-kidney tissue-8843 peptides), without increasing the analysis time (2 h)	94
Rat plasma related to depression		SeQuant ZIC-cHILIC column (150 × 2.1 mm, 3 µm) × Kinetex C8 column (150 × 2.1 mm, 2.6 µm)	mobile phases: A 0.1% formic acid and 5 mM ammonium acetate in water: ACN (95:5) B ACN: water (95:5) with 0.1% formic acid and 5 mM ammonium acetate; gradient: 0-1.5 min 90% B, 1.5-20 min 90-60% B, 20-22 min 60% B, 22.1-40 min 90% B; flow rate 0.4 mL min^{-1}	mobile phase: A water; B ACN: isopropanol (60:40) with 5 mM ammonium acetate; gradient; 0-2 min 0-20% B, 2-18 min 20% B, 18-35 min 20-100% B, 35-39.5 100% B; flow rate 0.4 mL min^{-1}	DAD, MS	simultaneous analysis of the metabolome and lipidome; a total of 319 metabolites were determined within 40 min, including organic acids, nucleosides, carbohydrate derivatives, amino acids, lipids, and other organic compounds; 44 depression-related differential metabolites were screened; compared with conventional LC-MS-based methods, the 2D-LC method covered over 99% of features obtained by two conventional methods	95

Table 1. Examples of applications of LC × LC and sLC × LC (cont.)

Sample	Modulation	^{1}D × ^{2}D	^{1}D separation	^{2}D separation	Detection	Remark	Reference
AQC-derivatized amino acids	P	ACQUITY BEH C18 (150 × 1.0 mm, 1.7 µm) × QNAX-ZWIX(+) tandem column setup consisting of a QN-AX (50 × 3.0 mm, 2.7 µm,) column coupled to a ZWIX(+) (50 × 3.0 mm, 2.7 µm)	mobile phases: A: 0.05% formic acid + 1% MeOH in water; B: 0.05% formic acid in ACN; gradient: 0- 9.33 min 2.5% B 9-33-17.5 min 9% B 17.5-23.33 min 9% B 23.33-29.17 min 13% B 29.17-40.83 min 13% B 40.38 -44.33 min 25% B 44.33-45.50 min 50% B 45.50-46.67 min 50% B 46.67-47.83 min 0% B 47.83-58.33 min 0% B at 150 µL min^{-1}) 58.33-59.50 min 13% B 59.50-60 min 13% B; flow rate: 0.06 mL min^{-1}	mobile phases: A: 10 mM ammonium formate + 10 mM formic acid + 0.5% H_2O in MeOH; B: 50 mM ammonium formate + 50 mM formic acid + 0.5% H_2O in MeOH, gradient: 0-0.2 min 0% B, 0.2-0.5 min 100% B 0.5-1.5 min 100% B 1.5-1.6 min 0% B 1.6-5 min 0% B, flow rate: 2.5 mL min^{-1}; the final LC × LC 2D gradient without initial hold was: 0-0.35 min 0-100% B 0.35-0.83 min 100% B 0.83-0.85 min 0% B 0.85-1 min 0% B, flow rate: 2.5 mL min^{-1}	HRMS	an LC × LC HRMS method with data-independent SWATH detection for untargeted analysis of peptide derived AQC derivatized AA established for simultaneous enantioseparation of all proteinogenic amino acids, including the side chain isomeric analogues of Leu (Ile, aIle, Nle, Tle) and Thr (aThr, Hse) (a total of 25 components), within a total runtime of 60 min (including re-equilibration)	96
Honey samples	A 0.3 min	CSP-QN-EC (quinine) (100 × 2.1 mm, 5 mm), × C18 (3.3 2.1 mm, 3 µm)	mobile phases: A: 50 mM ammonium formate (pH 6.30) and B: 50:50 ACN: MeOH; 0-40 min 100% B 40-50 min 100% B; flow rate: 0.3 mL min^{-1}	mobile phases: A 50 mM formic acid; B: ACN gradient: 0 0.25 min 0-70% B; flow rate: 3 mL min^{-1}	DAD, DAD	enantiomeric separation of amino acids	97
Plastic-bonded explosive (PBX) 9501 Composition: 94.9 wt.% HMX, 2.5 wt.% Estane® 5703 (poly (ester urethane)), 2.5 wt.% BDNPA/F nitroplasticizer), 0.1 wt.% Irganox 1010 and PBNA (*N*-phenyl-naphthylamine)	P 1.6 min	Zorbax C_{18}-Extended (2.1 mm × 150 mm; 3.5 µm; × PLGel Mixed C (300 mm × 7.5 mm)	mobile phase: A water and B tetrahydrofuran; gradient: 0-150 min 10-70 % B; 150-240 min 70% B, 240-300 min 70-100% B, 300-360 min 100% B; flow rate, 0.05 mL min^{-1}	mobile phases: tetrahydrofuran; flow rate 4.0 mL min^{-1} for 1.5 min	DAD; DAD	a combination of HPLC and SEC techniques can facilitate the analysis while also yielding additional chemical insights. LC × LC analysis can be simplified with the proposed sample preparation approach	98
Acrylate-modified hyaluronic acid (HAM)	P	Zorbax RX-C8 100-5 (150 mm × 2.1 mm, 3.5 mm) × PSS SUPREMA linear M column, (50 mm × 20 mm, 10 µm)	mobile phase: A water; B ACN; gradient: 0-7 min 0% B, 7-8 min 12% B, 8-15 min 12% B, 15-25 min 40% B, 25-28 min 40% B, 28-30 min 0% B 30-45 min 0% B; flow rate 0.5 mL min^{-1}	mobile phase: ACN:H_2O (40:60 vol%) with 0.02 M ammonium acetate; flow rate: 4.0 mL min^{-1} for 3.7 min	ELSD, ELCD	HAM separation according to chemical composition followed by separation based on molar mass	99

P: passive; A: active; ELSD: evaporative light scattering detector; ELCD: electrolytic conductivity detector; DAD: diode array detector; MS: mass spectrometer; HRMS: high resolution mass spectrometer; TFA: trifluoroacetic acid; IEX: ion-exchange column (columns with distinct ion-exchange strengths); SCX: strong cation exchange; WCX: weak cation exchange; SWATH: sequential window acquisition of all theoretical fragment-ion spectra; t-mAbs: human therapeutic monoclonal antibodies; AEX: anion-exchange; ACN: acetonitrile. Mobile phase composition is always in v/v.

using seven glucuronides. These configurations have been referred only as coupling columns, mainly because the IMERs at the ^{1}D have low chromatographic resolution, while the chromatographic separation is obtained exclusively at the ^{2}D.

Later on, a more complex system configuration with two LC pumps and three six-port valves was used on-line to hydrolyze of chloramphenicol-β-D-glucuronide in urine samples.[107] For that, a RAM column was used to sample clean-up for 5 min in the off-line mode (pump 1). Glucuronide was then transferred by means of a six-port valve to the BG-IMER and hydrolyzed at a flowrate of 0.25 mL min^{-1}; 0.01 M ammonium acetate (pH 6.7) was the mobile phase. Through a second six-port valve, chloramphenicol was concentrated in-line on the bed of a C8 analytical column. For the analysis, a third six-port valve was switched to pump 2, and chloramphenicol was quantified by gradient elution (acetonitrile in 0.01 M ammonium acetate (pH 5.0)) at flowrate of 1 mL min^{-1} at 280 nm.

A 2D-LC configuration was envisaged for sorting out ligands and non-ligands for a nicotinic acetylcholine receptor (α3b4-nAChR). To this end, an α3b4-nAChR bioaffinity column was coupled to a C18 column via a switching valve. Then, for detection purposes, the analytical column was hyphenated with a single quadrupole mass spectrometer. By using nicotine as probe, this system allowed 18 compounds to be ranked and identified in 32 min.[108]

In 2005, Girelli and Mattei[109] summarized publications about bioaffinity chromatography covering the period from 1994 to 2003. In this review, the authors discussed different configurations in which the bioaffinity columns could be assembled in the LC system. To try to solve inconsistences of the mobile phase between the two dimensions, trap columns have been inserted after the bioaffinity column. The mobile phases of a bioaffinity columns are usually buffers compatible with the immobilized target and containing no or very small percentage of an organic modifier; the flow rate is very low. These characteristics of the mobile phase can alter the chromatographic selectivity of the analytical column.[110]

To avoid coupled enzymatic reactions and false positive results during inhibitors screening, 2D-LC configuration combined with UV detection has been explored for monitoring enzymatic activitity.[111,112] This has been well explored for purine nucleoside phosphorylases (PNP). In this case, most assays are based on the Kalckar method wherein hypoxanthine generated by inosine phosphorolysis is oxidized by xanthine oxidase (XO), to generate uric acid, which is spectrophotometrically monitored at 293 nm. In searching for inhibitors, the use of coupled assays demands that selectivity toward both enzymes (PNP and XO) be evaluated, which is not always done.[113]

The production of uric acid by an XO capillary column in the ^{1}D using xanthine (as substrate) has been monitored in the ^{2}D allowing an allopurinol ruthenium derivative to be characterized as a selective and competitive tight binder with a true inhibition constant (K_i) value of 0.29 μM.[114] By using this same system, we have been able to disclose a 3-nitrobenzoyl 9-deazaguanine (LSPN451) from a series of 10 synthetic derivatives as a novel potent XO inhibitor, with inhibition constant of 55.1 ± 9.80 nM. The 2D-LC system with XO in the ^{1}D allowed XO inhibitors to be screened, their inhibition constants to be determined, and their inhibition modes to be characterized.[115]

The advance in 2D-LC system hardware has allowed eight compounds with acetylcholinesterase (AChE) binding affinities to be identified in a *Corydalis yanhusuo* extract. For that, a system with two parallel AChE columns (active and inactive) in the ^{1}D were used to sort out ligands from non-ligands. The ligands retained in the active AChE column were transferred to a C18 analytical column in the ^{2}D for separation and diode array-MS detection.[116]

An innovative comprehensive AChE inhibitors screening assay has recently been published.[117] The 2D-LC-MS system had a C18 analytical column in the ^{1}D while the capillary bioaffinity column (AChE-cIMER) was assembled in the ^{2}D. The system interface consisted of an eight-port/two-position high-pressure switching valve equipped with two identical sample loops. This interface allowed time-controlled fraction transfer of the ^{1}D effluent to the AChE-cIMER and acetylcholine insertion, facilitating correlation of the active fractions with the natural product library.

7. Final Remarks

The advances in 2D-LC system configurations in the last decade have allowed a wider range of applications and taken the instrumentations out of the universe of lab-assembled systems, which has its pros and cons. A commercial instrument has a settled number of switching valves, loops or trap columns and a certain modulation strategy that may not fit all purposes.

2D-LC offers high peak capacity and resolution but demands for adequate modulation strategies, otherwise it will deleteriously affect retention, separation and bandwidth in the ^{2}D. The dilution effect is other important factor to be considered, it negatively impacts sensitivity and increases the complexity of method development and affects the prevalent use of this chromatographic technique. Moreover, detection in 2D-LC is still the Achilles' heel due to plugins and software to control the LC and the mass spectrometer.

In the meantime, we expect that some current challenges will be dealt soon, and that 2D-LC will be routinely used, especially in the pharmaceutical industry.

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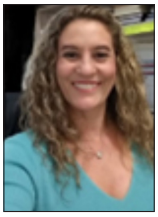

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