Recent Advances in Two-dimensional Liquid Chromatography

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Two-Dimensional Liquid Chromatography (2D-LC) has become a relatively mature research, development and application area due to significant advances made in hardware and software in recent years. 2D-LC is typically applied for the separation of very complex samples or when target components are very hard to resolve with a one-dimensional liquid chromatography (1D) separation. Details on the 2D-LC technology have recently been reviewed by Stoll and Carr in a tutorial article [1]. This present review article, covering publications from early 2016 until mid-2017, highlights recent applications using different 2D separation modes, such as comprehensive analysis and various types of heart-cutting. It also includes the latest developments in terms of modulation, i.e. the interfaces between the 1st and 2nd dimension, and software related to 2D-LC method development.

Comprehensive 2D-LC

In comprehensive 2D-LC, often referred to as LC x LC, the entire effluent from the first dimension (^{1}D) is transferred to the second dimension (^{2}D) and the sample is subjected to separation in both dimensions. Depending on how the ^{1}D effluent is transferred to ^{2}D, LC x LC can be further categorised into 'offline' and 'online' mode. Online LC x LC is the focus in this review, because in contrast to offline LC x LC, online LC x LC is faster, easier to automate, and is less vulnerable to sample loss or degradation. Compared to offline LC x LC, online LC x LC is more difficult to employ and has much higher requirements on hardware and software. Recent advances in 2D-LC instrumentation and software, especially recent commercial offerings have significantly reduced the time and expertise required for the development of industrial applications and was critical for the proliferation of the technique.

The biggest advantage of LC x LC to one-dimensional LC (1D-LC) is the greatly increased peak capacity (n_c). In order to maximise the peak capacity, selecting stationary and mobile phases with different separation mechanisms for the two dimensions to achieve orthogonal separations is the key. Orthogonality in chromatography is a measure of how different the selectivity is between two separation dimensions. LC techniques encompass a wide variety of separation mechanisms, such as reversed phase (RPLC), normal phase (NPLC), hydrophilic interaction liquid chromatography (HILIC), ion exchange (IEC), size exclusion (SEC), etc. To achieve orthogonal separations, different combinations can be used with careful consideration of mobile phase compatibility, column dimension and flow rate match, and column regeneration if gradient elution is used. In this review, some recently reported LC x LC applications are highlighted about the combination of different separation mechanisms.

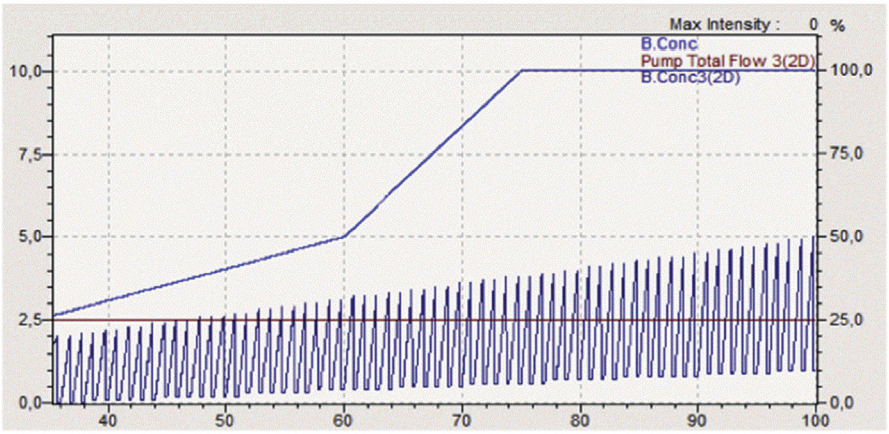

Figure 1. ^{2}D shift gradient program used in LC x LC separation of red wine sample. Adapted from reference [2].

Among the different separation modes, RPLC x RPLC techniques were mostly practiced since fully compatible mobile phases are employed. Separation between both dimensions is achieved by using combinations of RP columns with different phase chemistry and selectivity. Recent publications include using cyano propyl stationary phase with C18 columns for phenolic samples [2], diol and C18 columns for phenolic compounds in canes [3], pentafluoro phenyl (PFP) with C18 columns for metabolite profiling [4], amino with C18 columns for bioactive compounds in fruits [5], and C18 with phenyl-hexyl columns

for Chinese medicines [6], etc. Fast chiral separations have been employed in second dimension for separation of pharmaceuticals and synthetic intermediates for chiral and achiral drugs and metabolites as well as constitutional and stereo isomers [7]. Although RP x RP is not considered the most orthogonal ^{2}D separation, advanced instrument features such as shift gradient for second dimension separation have been applied to maximise the utilisation of the two-dimensional separation space. An example ^{2}D shift gradient program is given in Figure 1 and a comparison of LC x LC separation of red wine using full gradient and shift gradient is shown in Figure 2 [2]. The shift gradient accounts for changes in polarity of constituents eluting from ^{1}D and enables better separation in ^{2}D. Besides different combinations of stationary phase chemistry, using different mobile phase pH for the two dimensions in RPLC x RPLC mode was also demonstrated to be an effective way to gain orthogonality for peptide samples due to the zwitterionic nature of the peptide [8].

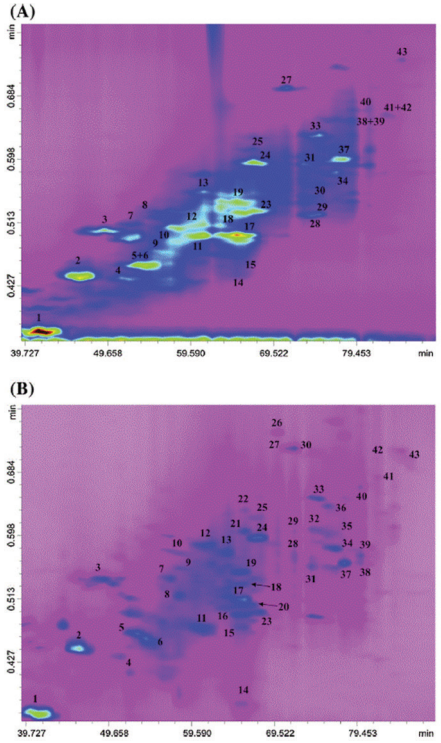

Figure 2. RPLC x RPLC analysis of red wine with a cyano column in ^{1}D and a C18 column in ^{2}D under optimised full gradient program (A) and shift gradient program (B). Adapted from reference [2].

Optimisation of LC x LC method can be laborious as many parameters can affect the overall performance of the LC x LC methods, such as column dimensions, particles

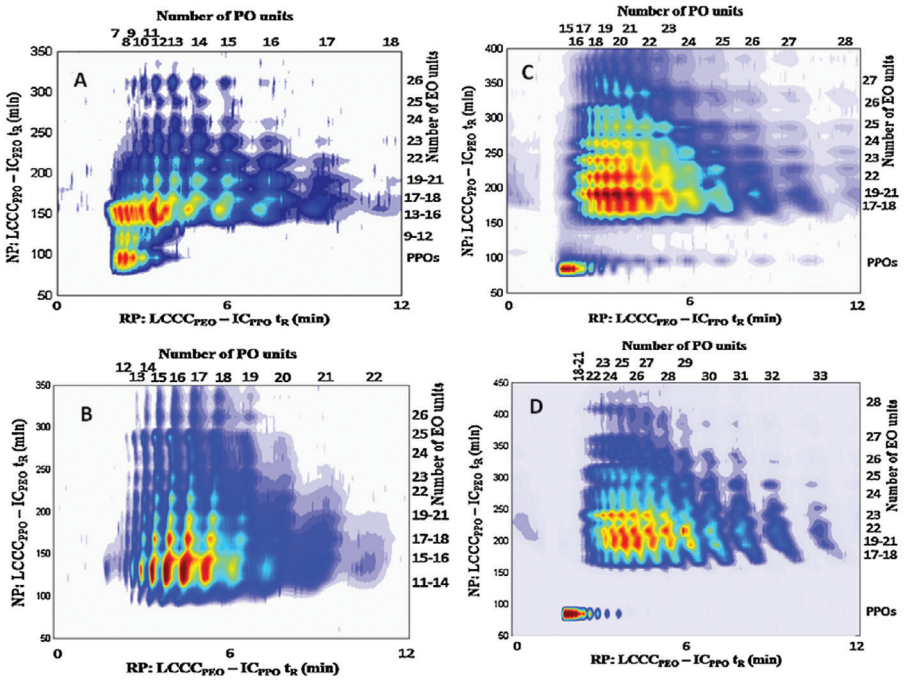

Figure 3. ^{2}D NPLC x RPLC separation of Poloxamer samples (A) Pluronic-10R5, (B) Pluronic-135, (C) Pluronic-1740, (D) Pluronic-17R4. Adapted from reference [19].

sizes, flow rates, modulation time, ^{2}D injection volume, gradient-elution program, temperature, etc. Modulation describes the event of sample collection from ^{1}D and subsequent injection onto ^{2}D. For LC x LC modulation time typically takes 30s – 1.5 min, which means the ^{2}D separation needs to be very fast. To make the LC x LC method development more practical and less time-consuming, simulation and computer programs have been developed for the prediction of 2D-LC method performance [9, 10]. These models have been successfully applied to RP x RP type systems.

Another very promising combination of separation mechanisms for LC x LC involves the use of HILIC for ^{1}D and RP conditions for ^{2}D. Although the hyphenation of HILIC and RP in LC x LC is more complicated due to the mismatch of solvent strength used in each dimension and the relatively long column equilibration time for HILIC separation, HILIC x RP is considered more orthogonal compared to RP x RP and have been reported several times for metabolite profiling, bio-active compounds and phenolics and flavonoid compounds [11-14]. Strategies such as using trapping column to replace the loop or using micro-flow rate to reduce ^{2}D injection volume have been exploited to minimise the strong solvent going from ^{1}D to ^{2}D column [12, 13, 15]. Alternating ^{1}D separation mode between HILIC and RP using zwitterionic monolithic micro columns combined with a shift gradient for ^{2}D has been reported as a novel system to obtain three-dimensional data in a relatively short time for the separation of flavones and related polyphenolic compounds [12].

Normal phase and reversed phase combinations (NP x RP) are also considered highly orthogonal, but the coupling of these two separation modes is more challenging due to the incompatibility of ^{1}D and ^{2}D mobile phases. Most of the reported NP x RP applications were not carried out in full comprehensive mode [16, 17]. Online NPLC x RPLC using vacuum evaporation assisted adsorption interface was recently reported for the isolation of toad venom. On-line solvent exchange within the two dimensions was achieved to eliminate the problem with incompatible mobile phases [18]. Separation of triblock copolymers composed of poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO) blocks with regard to block composition has been reported using NP x RP with LC at critical conditions (LCCC) of PEO block and interactions of PPO block for ^{1}D, and LCCC of PPO block and interactions of PEO block for ^{2}D [19]. Two short columns of identical stationary phase are used for trapping the ^{1}D analyte a few times before injecting onto the ^{2}D column in order to conduct the separation in a comprehensive manner with a relatively slow second dimension separation. Using this technique, full resolution of all oligomers was accomplished (Figure 3).

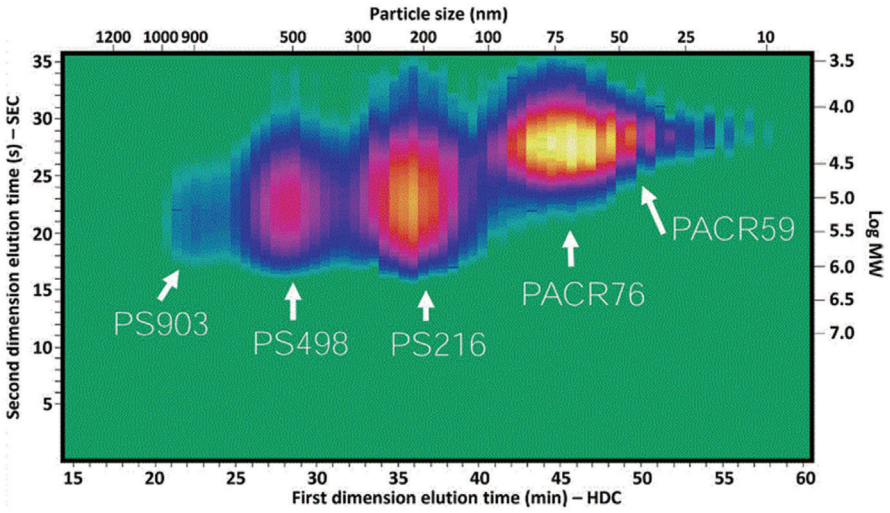

Figure 4. HDC x SEC-DAD chromatogram of a mixture of nanoparticles containing PS 903 nm, PS 498 nm, PS 216 nm, polyacrylate 76 nm and polyacrylate 59 nm. Adapted from reference [23].

Another combination of orthogonal separation mechanisms that have proven to be highly effective, especially for compounds containing both ionic and hydrophobic moieties, is ion exchange chromatography (IEC) with RPLC. IEC x RPLC has been reported for applications ranging from synthetic dyes to proteins and monoclonal antibodies [20-22]. Due to the long equilibration time of IEC stationary phase and the time constraint of ^{2}D separation, IEC is only deemed possible as first dimension in LC x LC. [21]

A unique combination of separation mechanisms worth mentioning is the recently reported LC x LC analysis of nanoparticles by using hydrodynamic chromatography (HDC) in ^{1}D and SEC in ^{2}D to gain information on particle size and hydrodynamic radius simultaneously (Figure 4) [23]. Online dissolution of the particles eluting from the HDC column was exploited using the Agilent Jet Weaver V35 or V100 mixer and tetrahydrofuran (THF) as solvent before injecting onto the ^{2}D SEC column.

As the needs for discovering new compounds (e.g. biomarkers, bioactives) continue to increase, using advanced separation techniques such as 2D-LC with high-end mass spectrometers for the identification, fractionation and isolation of complex samples such as crude natural products and extracts, biological membrane and fluids has become a promising and attractive approach [24-26]. Separation of microbial metabolites by online analytical and preparative LC x LC and isolation of milligram quantities of metabolites has been reported recently [27] and a review article on preparative ^{2}D separations was also recently published [25].

Heart-cutting 2D-LC

Heart-cutting (HC) techniques are used for target analysis of one or multiple components, mostly in complex sample matrices [1]. With the respective hardware, 2D-LC can be executed in single or multiple heart-cutting modes. In many cases a single HC experiment can be sufficient to resolve co-elution of two critical components. Recent examples include metabolite profiling of vitamin D [28] and quantitative determination N-nitrosoamines in tobacco smoke [29]. Both applications have used tandem MS as the detection mode. A combined approach using HC and column switching has been reported for simultaneous metabolomics and lipidomics analysis [30].

Heart-cutting 2D-LC is already being considered as a tool for impurity analysis in pharmaceutical QC laboratories (see Figure 5) [31]. An in-depth method validation has shown good accuracy, sensitivity and robustness. Additional applications include a validated HC 2D-LC method for determination of vancomycin in human plasma [32] and clone selection for monoclonal antibodies (mAb) [33].

Multiple heart-cutting (MHC) 2D-LC is very powerful when several components are co-eluting in the ^{1}D separation. Hereby fractions from ^{1}D are stored in loops and are analysed subsequently in ^{2}D. An advantage of multiple heart-cutting versus LC x LC operation is the decoupling of ^{2}D and ^{1}D. ^{2}D analysis times are no longer constrained to match with the modulation frequency, allowing for somewhat slower and more optimised ^{2}D runs. Method development effort is therefore reduced for MHC compared with that of LC x LC.

Recent examples include analysis of impurities in peptides and proteins by 2D-LC/MS [34]. The ^{1}D separation has a high salt content which presents a huge challenge for MS detection. The use of MHC eliminates the salt from ^{1}D and led to superior MS performance, as suppression effects are significantly reduced in ^{2}D. Additional applications of MHC are presented for the separation of target constituents in natural products-derived pesticides [35] and the determination of pyrrolizidine alkaloid isomers and their N-oximes with Q-TOF/MS detection [36]. Also, quantification of drugs in human plasma [37], determination of secondary metabolites in flowers [38] and advanced structural profiling of Chinese medicine formulas are described [6].

A related heart-cutting mode is called high-resolution sampling (or selective comprehensive 2D-LC [1]) – it is typically applied when only very narrow cuts can be made because of reduced solvent compatibility or when peak purity is studied. In such cases three or more consecutive cuts are made across one single peak [35]. On the other hand, MHC was applied in a low resolution mode (continuous MHC with modulation time of four min) for a 2D-LC ion mobility TOF MS investigation of a Ginkgo plant extract [39]. The low resolution in ^{1}D creates single peaks for MS, which facilitates structure analysis.

Modulation

The modulator is the interface between the two chromatographic dimensions. It transfers fractions of ^{1}D effluent onto ^{2}D. In most cases a 10-port valve or an 8-port duo valve is used. Some research activities are on-going in search for alternative or improved modulation strategies.

Compatibility of 1st and 2nd dimension mobile phases is sometimes an issue, as strong ^{1}D eluents (methanol, acetonitrile, THF) can cause significant peak broadening and distortion or even sample breakthrough in ^{2}D. Active modulation (AM) uses dilution of ^{1}D eluent with weaker mobile phase [40] and subsequent trapping on solid-phase extraction (SPE) cartridges [41]. Higher loop filling factors can be used which leads to shorter run times. For a separation of aromatic ethoxylates analysis time could be reduced by a factor of five from 200 min to 40 min when AM was applied [41].

Stoll and colleagues presented an improved valve-based modulation strategy recently,

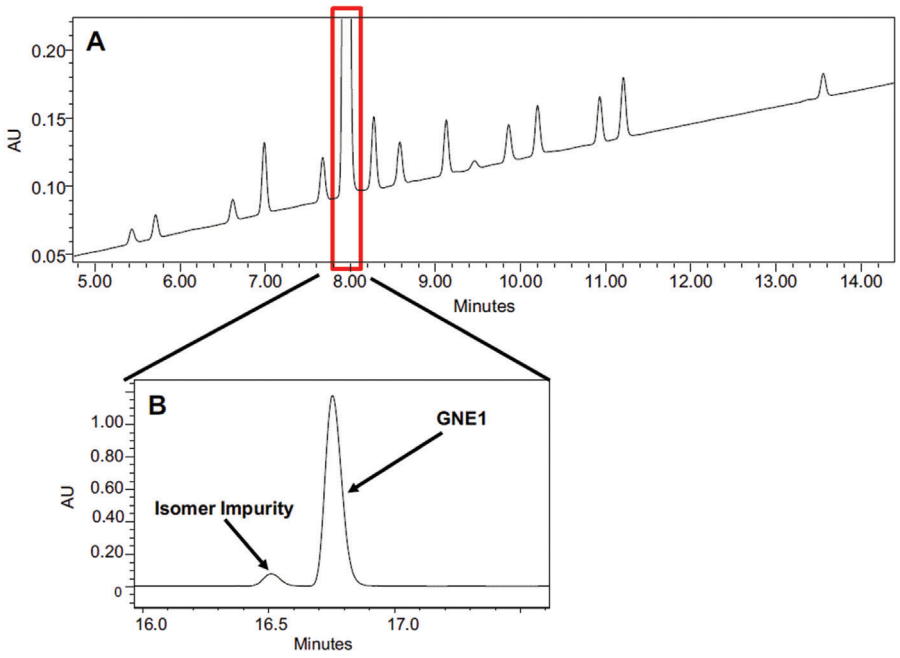

Figure 5. Impurity separation in a pharmaceutical sample by heart-cutting 2D-LC. Adapted from reference [31].

called active solvent modulation (ASM) [42]. The 8-port duo-valve contains an additional loop which is used for temporary on-line dilution of ^{1}D effluent with ^{2}D eluent. Solvent mismatch problems are reduced and much higher sensitivity can be obtained in ^{2}D. This is achieved by peak focusing with the low eluent strength mobile phase for the time when ASM is activated (see Figure 6).

An alternative concept for modulation was presented by Fornells and coworkers. Modulation can be executed via evaporation of ^{1}D mobile phase in microfluidic channels using a vacuum pump [43]. At this point, semi-volatile components were partially evaporated which led to reduced recovery.

Longitudinal thermal modulation for LC x LC was recently demonstrated [44]. A dual stage set-up for trapping and remobilisation on micro columns similar to that of GC x GC has been used. The modulator was applied to separate constituents in red wine and a peak width reduction of 30-60% was achieved due to peak focusing.

Fractionised sampling and stacking (FSS) was introduced as an interface for heart-cutting 2D-LC [45]. Here a combination of valves was used to dilute strong effluent from a ^{1}D HILIC separation with weak eluent used for RP in ^{2}D.

Pulsed elution 2D-LC was introduced by the group of Nielsen, Denmark [46]. A sequence of pulses of increasing elution strength is generated in ^{1}D. Between the pulses ^{1}D is kept at non-elution conditions using low elution strength mobile phase.

The effluent from the first dimension is actively modulated using trap columns and subsequently analysed in the second dimension. With this approach enhanced compatibility of ^{1}D and ^{2}D is obtained. Long run times were required for this proof-of-concept study – 500-900 min when no-elution times of 5-10 min are applied.

In summary, many new applications and technical developments were seen during the last 18 months. In our opinion 2D-LC will continue to be a very active research area. We expect further advances to be made in hardware and software to facilitate 2D-LC method development. These developments will result in improved qualitative and quantitative measurements for a large variety of chemical and biochemical applications.

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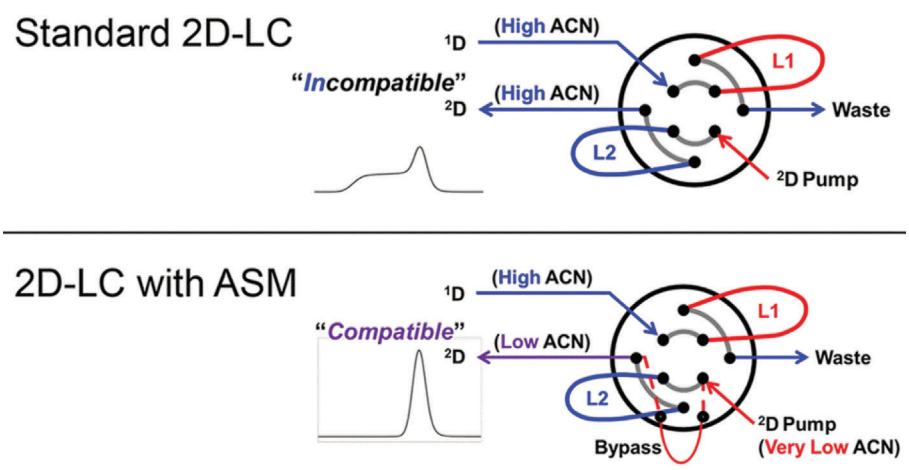

Figure 6. Valve configuration and resulting peak shape for standard 2D-LC at incompatible solvent conditions (top) and 2D-LC with ASM and compatible solvent conditions (bottom). Adapted from reference [42].

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