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REVIEW

Practical considerations in comprehensive two-dimensional liquid chromatography systems (LCxLC) with reversed-phases in both dimensions

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Abstract Online comprehensive two-dimensional liquid chromatography (online LCxLC) presents high peak capacity compared with one-dimensional liquid chromatography, and reasonable operation time compared with an offline or stop-flow mode. Among various combinations, coupling of two reversed-phase (RPxRP) columns generates a high peak capacity product rate. Its wide applicability made RPxRP a promising technique in separation of complex samples. This review discusses the practical considerations in development and application of an RPxRP system, including systematic investigation of column stationary phase chemistry, column combinations, mobile phase system, interface, gradient types, and achieving fast analysis in LCxLC. In addition, many efforts are given to methods that increase the fraction coverage because this is the main obstacle caused by the correlated separation mechanism in RPxRP.

Keywords Comprehensive two-dimensional liquid chromatography · LCxLC · RPxRP · Fraction coverage · Gradient type · Column combinations

Introduction

Online comprehensive two-dimensional liquid chromatography is gaining ever-growing interest in analysis of a large number of complex samples because of its high separating power compared with one-dimensional liquid chromatography (1D-LC) [1–3]. The theoretical peak capacity of comprehensive two-dimensional separation is the product of each dimension as described by Giddings [4]. The biggest advantage of this technique is that a very high peak capacity can be generated in a relatively short time compared with noncomprehensive two-dimensional liquid chromatography (2D-LC) operated in off-line or stop-flow mode. Carr and Potts have reported [5] that on-line LCxLC analysis can lead to a better performance in comparison with one-dimensional LC at analysis time ≥2 min. In particular, LC techniques offer a wide variety of separation mechanisms, such as normal phase (NP), reversed phase (RP), size exclusion (SEC), ion exchange (IEX), or affinity chromatography (AC), characterized by different selectivities [6]. Consequently, LCxLC can be theoretically applied in various combinations, employing increased peak capacity, selectivity, and resolution [7]. However, when counting the separation modes (up to May 2014), 72.9 % of publications adopted RP in the second dimension, as shown in Fig. 1. The main cause for the use of RP mode in LCxLC systems can be attributed to the following conditions:

(1) Wide applicability: The applicability of RP conditions to a variety of samples, detailed knowledge of the RP retention mechanisms over 30 years, and the commercial availability of several hundred different stationary phases.
(2) Fast equilibration: The equilibration time can be as short as one column volume [8]. HILIC also has high performance as RPLC, however, the slow equilibration [9] is a challenge for the fast second-dimensional separation.
(3) High efficiency: RPLC can generate high column efficiency [10]. Especially with the new generation of sub-2 μm packing, the separation was sped up (e.g., by a factor of nine and often without a loss in peak capacity or change in selectivity) [11].

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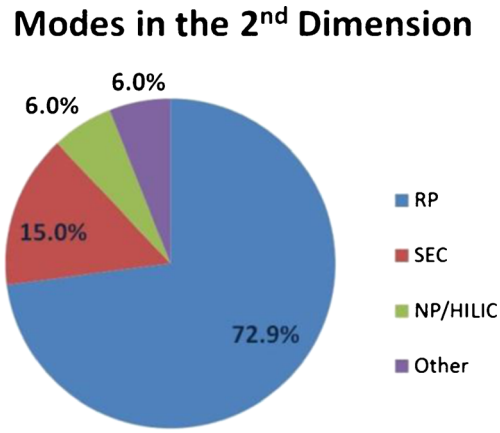

Fig. 1 Modes in second dimension of comprehensive two-dimensional liquid chromatographic system (LCxLC). Literature (up to May 2014) summarized according to valve-based online comprehensive two-dimensional liquid chromatography

(4) Difference in selectivity: A great number of various stationary phases featured significant differences in selectivity. The stationary phases show differences in the matrix (silica, polymer, inorganic oxide) and, e.g., linked functional groups (C18, Cyano, Phenyl etc.) and various chemical modifications to C18 (like endapping and polar embedded groups etc.).

(5) Compatibility with MS: As has been pointed out by Guiochon et al. [12], "However, we must keep in mind that any serious advance in chromatographic resolution should be made through approaches that permit coupling to mass spectrometry" the compatibility with MS is another advantage over other LC modes like IEX.

Therefore, RP is the primary mode used in second dimension of LCxLC systems. The aim in construction of an LCxLC system, to some extent, turns out to find a proper first-dimensional separation. The combination of certain LC modes can result in a series of difficulties, if not impossibilities, for example, mobile phase immiscibility, column efficiency, etc. [7]. Although the specificity of a separation mechanism to certain compounds is a priority concern, like SEC for polymer separation according to molecular weight [13], IEX to biological samples according to charge state [14] and NPLC/HILIC to organic samples according to functional groups [15], massive effort was put in the development of RPxRP. This is because of the high efficiency of RP mode, when calculating the total peak capacity of LCxLC system by product rule, and the good compatibility of the two dimensions [16–18]. Hence, 32.3 % of LCxLC applications used RP in the first and second dimension, as shown in Fig. 2.

Unlike SEC, IEX, and NP/HILIC combined with RP, the combination of RP with RP encounters a correlation in the separation mechanism and leads to a decrease in the practical peak capacity [19]. Therefore, the primary objective of this

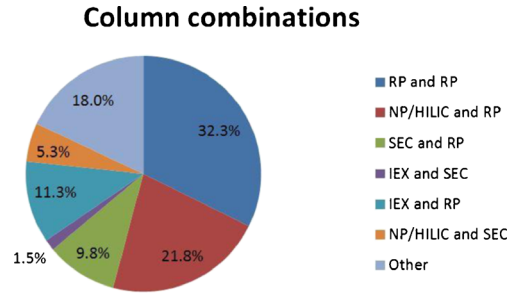

Fig. 2 Column combinations in LCxLC systems (up to May 2014)

review is to explain the theory and experimental aspect for increasing the practical peak capacity in online comprehensive RPxRP systems. More specifically, the characterization of column selectivity, calculation of orthogonality (or fraction coverage), and experimental considerations in stationary phase chemistry, mobile phase systems, programmed gradients, and achieving fast analysis in LCxLC will be discussed.

For more interest in the theory [16, 20], practical consideration [1, 21], and optimization of general 2D-LC systems [22, 23], readers are recommended to some excellent papers. For applications on polymers [24], proteomes [25], and non-proteomics applications [26], readers are recommended to recent publications. In this review, "online" means that the transfer of first-dimensional effluent fractions to a second-dimensional column is realized in real time, using an automated valve (interface), and "comprehensive" is used here as described by Schoenmakers and coworkers [27, 28].

Evaluation of orthogonality/fraction coverage

The interest in comprehensive two-dimensional separations is based on the peak capacity product rule [4], as shown in Eq. 1:

$$n_{2D} = {}^{1}n \times {}^{2}n \quad (1)$$

with n_{2D} as the theoretical peak capacity of an LCxLC analysis, ^{1}n and ^{2}n as the peak capacity of the first and second dimension, respectively. But this equation is only correct, if the separations in the two dimensions are completely independent from each other [10, 29] and if there is no loss of separation as a result of under-sampling from the first to the second dimension [30, 31]. This is rarely possible [31]. Losses of resolving power are encountered in any implementation of two-dimensional chromatography as a result of back-mixing of the fractions in the sample loops.

Therefore, a term of practical peak capacity (n^{*}_{2D}) was used as defined in Eq. 2 [32, 33]:

$$n^{*}_{2D} = {}^{1}n \times {}^{2}n \times \frac{1}{\beta} \times f \quad (2)$$

Undersampling ($1/\beta$) is a fairly well accepted concept [19, 33–37]. Average band width of a first dimension before sampling ($^1\sigma$) is broadened by re-mixing of the collected fraction [21]. This is characterized by the first-dimension broadening factor (β) in Eq. 3 [19]. Therefore, a shorter sampling time (t_s) decreases first-dimension broadening factor and thus increases in practical peak capacity.

$$\beta = \sqrt{1 + 0.21\left(\frac{t_s}{^1\sigma}\right)^2} \tag{3}$$

Factor f is related to spreading angle [38], weighting factor [39], fraction coverage [33, 40], and, more often discussed, orthogonality [10]. The following context discusses more details.

Orthogonality in two-dimensional chromatography is used as a value of differences between the separation properties of the two coupled dimensions [21]. Only for fully orthogonal systems, where the retention mechanism in the two dimensions can be treated as statistically independent and also without back-mixing of the fractions, the product rule for two-dimensional peak capacities, theoretically predicted by Eq. 1, can be used. However, even carefully planned experiments often cannot completely avoid selectivity correlations for real samples [21, 41].

The degree of orthogonality between both dimensions is a critical factor but is not sufficient to appreciate the potential of a given system [42]. In Giddings's intent in the definition of orthogonality [33], full orthogonality implies that the separation space must be fully accessible [33, 43]. It is reasonable to use the fraction of separation space that can be in principle covered by peaks, and term the correction factor as the fraction coverage, $f_{coverage}$. Fraction coverage appears to be a good metric to discuss how well the two-dimensional separation space is filled, regardless "orthogonality" in mathematical meaning [44, 45]. This definition simplifies the explanation of the high practical peak capacity obtained by using programmed gradient elution [46–48]. Bedani et al. used this as "column set quality" parameter to identify the best LCxLC combinations [40].

One of the earliest methods was proposed by Liu et al. [38], describing the degree of similarity of the separation systems within the boundary line. The effective area is spanned by the peak spreading angle θ, which is potentially available for separation in the two-dimensional space, as shown in Fig. 3a [38, 49, 50]. The fraction coverage was expressed in Eq. 4 [33, 38].

$$f_{\text{coverage}} = 1 - \tan\left(\frac{\pi}{4} - \frac{\theta}{2}\right) \tag{4}$$

A completely independent (or orthogonal) separation system is obtained when a 90° angle is observed. Then, in two identical separation dimensions, the angle θ equals to zero. The proposed spreading-angle methods may not successfully describe the orthogonality for the situation, where analytes are not distributed along a diagonally line in the two-dimensional separation space.

Therefore, in one of the most cited paper in LCxLC [10], Gilar et al. defined orthogonality as the normalized area covered by the eluting peaks in a separation plane [10]. The separation plane was divided into a discrete number of space elements (bins), and each bin can contain at least one sample compound. Therefore, the fraction coverage in a two-dimensional system can be estimated as the percentage of coverage of the bins as expressed in Eq. 5 and shown in Fig. 3b [10, 33].

$$f_{\text{coverage}} = \frac{\sum bins}{n_{2D}} \tag{5}$$

An ideally orthogonal separation in which bins are randomly occupied was considered to provide 63 % coverage of a square retention space [10]. Owing to several limitations [21, 51], a modification of Gilar's approach was proposed by Stoll et al. (Gilar-Stoll surface coverage) [33, 51]. It employed a user-selected bin size and includes bins that are not occupied but with the perimeter of the Gilar plot. However, the metric is strongly dependent on the bin size and is rather subjective [33]. Zeng et al. [45] divided orthogonality into two parts, the peak coverage percentage (C_{pert}) and the two-dimensional distribution correlation of compounds (C_{peaks}). The advantage of this method is the use of separation properties of comprehensive two-dimensional separation to characterize two-dimensional peak distribution and does not rely on assumption or any imposed limitations.

Gilar et al. [32] have compared metrics including correlation coefficients, mutual information, box-counting dimensionality, and surface fractional coverage with different hull methods. Among these methods, surface coverage is particularly attractive because the fractional surface coverage value is useful for calculating the achievable peak capacity from Eq. 2. The convex hull method can be used to calculate the precise fractional coverage [52]. Rutan [33] compared minimum convex hulls, convex hull peels, α-hull, three variations of local hull methods, and a kernel method. Results showed that the minimum convex hull is the most precise method, which gives similar results as the Stoll-Gilar method. However, topological methods [53] or ecological home range computations require specialized algorithms [54]. Therefore, a simple vector method

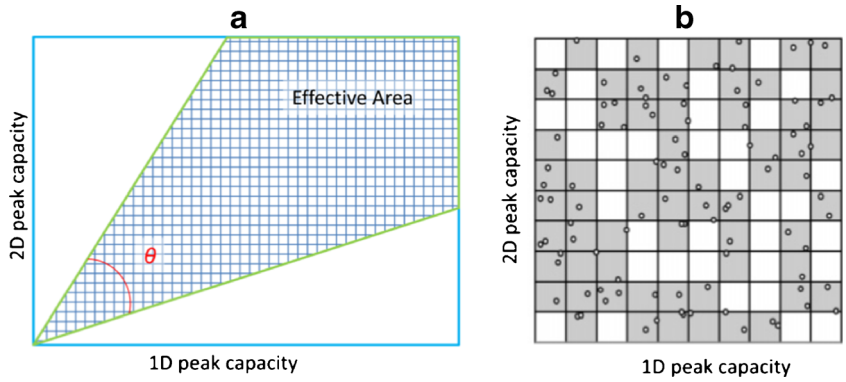

Fig. 3 Geometric approaches in calculating surface fractional coverage. Peak spreading angle (a); bin methods (b). The figures were adapted from References [38, 10]

was proposed by Dück et al. [55] to calculate practical peak capacity for various peak distribution patterns (e.g., PAR, parallelogram). For a PAR, the pattern is shown in Fig. 4, the determination was simplified to Eq. 6:

$$n^*_{2D} = \frac{1}{2}\left|\vec{a} \times \vec{c}\right| + \frac{1}{2}\left|\vec{c} \times \vec{b}\right| = \frac{1}{2}\left[\left({}^1n^a - {}^1n^b\right)\times {}^2n^c + \left({}^2n^b - {}^2n^a\right)\times {}^1n^c\right] \quad (6)$$

where $\vec{a}$, $\vec{b}$ and $\vec{c}$ correspond to different vectors, and the right superscript of n corresponds to practical peak capacity of vector $\vec{a}$, $\vec{b}$ and $\vec{c}$, respectively.

Systematic investigation of column selectivity

RPxRP often shows a significant correlation between both dimensions [56, 57]. The challenge is to find different types of columns that can uniformly spread the component peaks across the separation space to optimize the peak capacity. This leads to the requirement that the two separation dimensions should be as dissimilar as possible, or just uncorrelated [58].

The degree of orthogonality assesses to what extent the selectivity between two considered systems is different. This is useful in predicting complementary combinations of chromatographic phases and for maximizing practical peak capacity. Undoubtedly, the column stationary phase chemistry plays a primary role in the optimization of the selectivity. Therefore, the packing characterization methods, which are known from LC, can be helpful for the construction of an RPxRP system. For systematic characterization of columns, for example, Neue et al. reported a method based on the retention of seven compounds in an isocratic mode [59]. The descriptors, hydrophobicity, silanophilic activity, polar selectivity, and phenolic selectivity were evaluated at pH 7 with a test mixture including uracil (void volume marker), naphthalene and acenaphthene (purely hydrophobic compounds), propranolol and amitriptyline (basic compounds), butylparaben and dipropylphthalate (polar compounds). Another method is the linear solvation energy relationship (LSER) model [60] using multiple correlations between retention and so-called solvatochromic parameters, which characterize the solubility and solvation of the solute and the stationary phase, as expressed in Eq. 7, [61, 62]:

$$\log k = \log k_0 + \frac{m_1 V_X}{100} + s_1\pi + a_1\alpha + b_1\beta \quad (7)$$

The solvatochromic parameters of Eq. 7 characterize various properties influencing the retention: the volume of solvated solute V_X, polarity π, hydrogen bonding basicity β, and hydrogen bonding acidity α. The coefficients m_1, s_1, a_1, and b_1 provide values for the response of the system stationary/mobile phase to the selective properties of analytes and can be therefore used to compare the suitability of the columns in two-dimensionanl systems [62, 63]. The LSER model was later modified to the hydrophobic subtraction model (HSM) [64], as described with Eq. 8 [18]. The five phase coefficients represent the five dominant solute–column interactions: hydrophobicity (η′H), steric resistance (σ′S*), hydrogen-bond acidity (β′A), hydrogen-bond basicity (α′B), and cation-exchange activity (κ′C). Here, k is the retention factor of an analyte, k_{EB} is the retention factor of ethylbenzene as the reference non-polar solute, measured under the same conditions.

$$log\left(\frac{k}{k_{EB}}\right) = \eta' H - \sigma' S^* + \beta' A + \alpha' B + \kappa' C \quad (8)$$

In addition, visual approaches can be applied for the classification of stationary phase selectivity, like a triangle [18, 65]

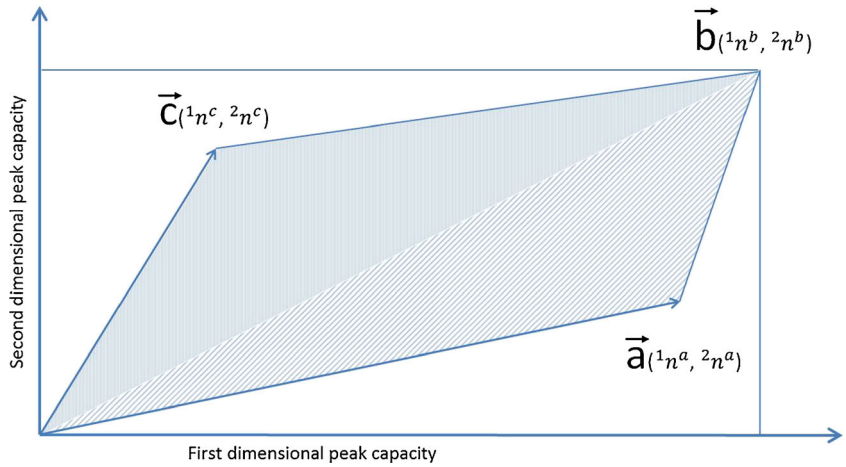

Fig. 4 Vector method for the calculation of practical peak capacity

and radar plot, and system selectivity tube [66]. To quantitatively compare the selectivity of two phases, based on HSM [67], a single parameter called "column selectivity function F_s" was defined by Zhang et al. [18], as shown below:

$$F_s = \left\{[12.5(H_2-H_1)]^2 + \left[100\left(S_2^*-S_1^*\right)\right]^2 + [30(A_2-A_1)]^2 + [143(B_2-B_1)]^2 + [83(C_2-C_1)]^2\right\}^{1/2} \quad (9)$$

where the subsript 1 or 2 correspond to column 1 or 2, respectively. Large values of F_s are realized with "complementary columns," which means two columns with very different selectivity [67–69]. However, other experimental conditions, such as changes in the concentration of organic modifier [40] or the gradient program, complicate the comparison of different column combination [68, 70]. In LCxLC, as much as in LC, the retention and selectivity depend not only on the stationary phases but also on mobile phase system [46, 57, 62, 65]. Moreover, the described methods do not take into account the practical retention space. Various types of peak distribution patterns [55] would appear and not take full coverage of the retention space.

A recent report by Le Masle et al. [71] and D'Attoma et al. [42], investigated the combination of stationary phase chemistry, organic solvent, pH additives, and temperature. Two preliminary gradient runs allow determining the degree of orthogonality, O_d [42]. However, the highest orthogonality does not lead to the best practical peak capacity, as shown in Fig. 5. Users have to make a decision between orthogonal separation and higher practical peak capacity.

Since these experiments were all performed independently, the results do not counter the practical consequences related to comprehensive operation. Their results provide only useful information on which combination of stationary phases a high orthogonality in an on-line system can be realized. It has to be noted that two separation systems might show a high orthogonality for one sample but a lower one for another sample [12, 65], and it depends strongly on "sample dimensionality' [43]. Because LCxLC samples are usually very complex mixtures and because retrieving useful selectivity information from "crowded" 1-D LC chromatograms can be arduous, analysts would like to predict fraction coverage using representative compounds of the sample [16]. However, if standards cannot be used to adequately describe the entire sample matrix, the sample itself should be used and, also, the separation should be optimized for regions of interest, not necessarily the separation as a whole [72].

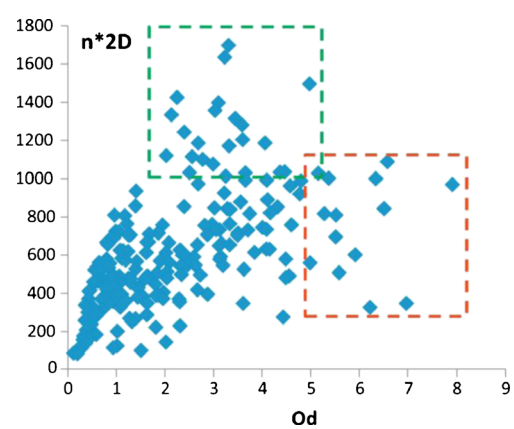

Fig. 5 Practical sample peak capacity versus degree of orthogonality for 237 considered 2D-systems [71]. Note: x-axis is the degree of orthogonality; y-axis is the practical peak capacity

Construction RPxRP system

Column combinations

The total LCxLC peak capacity is weakly dependent on the first dimension peak capacity [73]. But the selectivity of the first-dimension stationary phase does impact on the orthogonality and thus on the total LCxLC peak capacity, as discussed above. The majority of studies have focused on the effect of different stationary phase chemistry [39, 63, 71, 74].

In selecting orthogonal combinations of RPxRP, different types of stationary phases were used [75]. Because of the high efficiency of the C18 phase, various systems adopted C18 in one of the two dimensions. For example, using a C18 column in the first dimension, cyano [76, 77], amino [77], SB-phenyl [78], polyamine [79], carbon clad zirconia column [80], or C18 monolithic [77] are used in the second dimension. With C18 in the second dimension, fluoroalkylsilyl [81], cyano [40, 48, 55], polyethylene glycol (PEG) [62, 63, 82–85], zirconia-carbon [86, 85], porous graphitic carbon (PGC) [87], human serum albumin (HSA) [88, 89], immobilized liposome chromatography [90], PBB [56], or PFP [55] were used in the first dimension. The column pair for coupling can be selected by approaches in section of Systematic investigation of column selectivity. In order to get a better band suppression effect, a higher hydrophobic column should be placed in the second dimension [40, 48]. As an example, a cyano phase in the first dimension and a C18 phase in the second dimension were often adopted in practical applications [40, 48, 91–93].

The combination of non-silica-based columns, like porous graphitic carbon (hypercarb), zirconia, and organic polymers (PLRP) with a variety of common types of reversed phases can lead to a reasonable orthogonality because of their rather different selectivity [62, 65, 94]. Therefore, RPxRP with a non-silica-based column in one of the two dimensions is a very promising technique [74, 95]. Carbon-clad zirconia (or graphitic carbon) was often chosen as a second dimension phase for its good chemical and mechanical stability at high temperature and high flow rate conditions [8, 71, 96–98].

Several columns can be coupled to increase the selectivity, like PEG-C18 in the first dimension and zirconia–carbon in the second dimension [85]. But the use of a single chromatographic mode in first dimension has been limited to the separation of components by their individual characteristics, such as hydrophobicity, ionic properties, etc.. The use of mixed-mode stationary phases has revealed opportunities to combine different retention mechanisms but leads to a decrease in orthogonality. Mixed mode stationary phase with ion-pairing reagent as an integral part of the hydrophobic chain [99], or tandem of IEX column and RP column runs in changing organic modifier and buffer concentrations [100], offers unique selectivity. In these examples, the use of mixed-mode columns will combine the selectivity of hydrophobic interaction and ion exchange.

Mobile phase system

The effect of the mobile phases' composition on the selectivity is considered to be slightly less than the effect of stationary phases. However, variation of the mobile phase chemistry and pH often leads to a change in the retention. Actually, the variation of organic solvents in RPLC is really limited. Most commonly, methanol, acetonitrile, isopropanol, and THF are taken as organic solvents [101]. Many other solvents cannot be used because of their high viscosity in mixtures with water or their high UV cut-off value. In common practice, the use of acetonitrile in first dimension and methanol in second dimension (vice versa) was adopted in presenting different selectivity by the mobile phase system [48]. As discussed by Li and Carr [101], the use of unusual organic solvent modifier has the potential to change the selectivity. The first dimension in on-line LCxLC is carried out at low velocity and, thus, pressure drop is seldom a limiting issue. Different organic modifiers have the potential to change the selectivity and, thus, the orthogonality and the practical peak capacity. However, this is strongly dependent on the sample set as well as the used stationary phase.

Mobile phase pH is an important factor and in many cases changes the separation selectivity [17]. For analytes with a wide range in acidity/basicity, a change in pH can achieve a high degree of orthogonality even if similar columns are used in both dimensions [101]. Owing to the ionic nature of peptides, the use of different pH values ensured enough selectivity between the two dimensions, although both consist of the same stationary phase [102–104]. Which type of additive should be used for a pH change is dependent on the sample (e.g., for analysis of peptides, the use of ammonia in the eluate of either the first or the second dimension leads to the highest degree of orthogonality [42]).

Elevating column temperature is one of the easiest and most straightforward parameters to vary the chromatographic selectivity. The ionization equilibria of polar and ionizable compounds are temperature-dependent. Therefore, changing the temperature is especially useful in tuning their retention [105]. At a higher temperature, the analyte transfer from the mobile phase to the stationary phase and vice versa is more efficient because of a higher diffusion coefficient. This leads to a flatter van Deemter curve (reduced C-term) and allows higher flow rates without strong reduced efficiency. Because the viscosity and the backpressure are reduced at increased temperature, separations with unusual solvents such as ethanol and isopropanol can be realized [65, 105]. In addition, the high temperature in the second dimension helps to achieve fast second-dimensional separation [71] and, therefore, high

temperature is normally applied to second-dimensional separation [106], and the first dimension is kept at lower temperature.

Interface–the valve

For the coupling of two dimensions in LCxLC, trap-column [82, 85], parallel-column [56, 82, 85, 107], and loop valve-based interfaces were adopted in the beginning of LCxLC development. However, due to difficulties in trap- and parallel-column interfaces, loop-valve-based solutions are the most frequently used interfaces today (Fig. 6). The optimization of the loop volume in LCxLC systems was well discussed in several reviews [12, 108], and the configuration of the valve plays an important role in obtaining symmetric second-dimensional retention [109].

In the early stage of LCxLC development, two position eight-port valves were adopted [109, 110]. However, the analysis direction of the two loops in this configuration is reversed [12], as shown in Fig. 7a, which results in asymmetric elution in odd and even runs [109]. Another new valve commercialized by Agilent (Waldbronn, Germany) featured a dual four-port valve. The valve allows configuring both loops, either homodromous or heterodromous, as shown in Fig. 7b and c. In homodromous configuration, the fraction undergoes a "first in-first out" process (forward flush), whereas in heterodromous configuration, the fraction undergoes a "first in-last out" process (reversed flush). Moreover, all the flow paths are equal, and no additional loops are needed as a bridge between different ports [48]. Most publications (up to 67 %) adopted 10-port valves (Fig. 6). Figure 8 shows several reported 10-port valve setups. For an easier identification of the difference between several valve configurations, the flow paths of different valve configurations are also shown in Fig. 8. However, if given a close examination on the configurations in 10-port valves, it is still not fully symmetric, because a jumper (bridging capillary) is used.

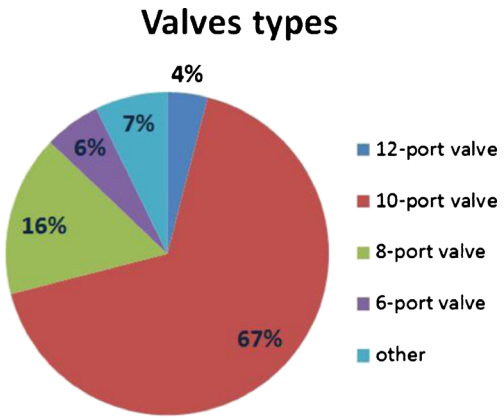

Fig. 6 Valve types adopted in LCxLC (up to May 2014)

If the jumper does not have a negligible volume in comparison with the loop volume, and the loop volume is not taken into account, the valve configuration of Fig. 8c and d will have unequal volume for adjacent two fractions. The difference in extra-column volumes between the two flow paths in the second dimension must be minimized in these configurations. Another possibility for Fig. 8c and d is to take the jumper volume into account for the loop volume. That means the sum of the volume of loop 2 and the volume of jumper is equal to the volume of loop 1. If the jumper volume can be neglected, either homodromous configuration or heterodromous configuration results in a symmetric elution.

In Fig. 8a and d, the valve's loop collects fraction in the "first in-first out" manner, whereas in Fig. 8b, c, and e, the valve's loop collects in the "first in-last out" manner. A little extra broadening and a second dimension retention time shift between neighboring second dimension cycles was observed using the "first in-first out" valve configuration [96]. No extra broadening was reported on "first in-last out" configuration, probably because the back flush remixes the collected fraction. It is possible to connect 10-port valves in an asymmetric way, just like the eight-port valve [111, 109], which also results in an asymmetric elution.

Note: Figure 7a is a typical configuration of a conventional eight-port two-position valve. Loop 1 runs in homodromous (the eluent was in first in-last out mode), while loop 2 runs in heterodromous (the eluent was in first in-last out mode). Two loops are not in a symmetric pattern. In Fig. 7b and c, a newly designed dual four-port valve is presented. Either in homodromous or heterodromous, both loops run with the same pattern.

Note: Figure 8a [112] and d, first in-first out [113–115]; b [82], Fig. 8c and e [96], first in-last out. For a and b, the difference in the configuration is the position exchange of second dimension and pump; the difference between c and d in the configuration is the same between a and b. For setup of a and b, the jumper was involved only in filling process, but not in the analysis process; for setup c and d, jumper was involved in loop 2 but not in loop 1. For setup of e, the jumper was involved in filling process but not in analysis process. In the analysis process, loop was connected with second-dimensional column and pump. Regarding higher pressure in second dimension, the absence of jumper in the analysis process would facilitate a stable injection plug.

Fully symmetrical configuration was reported by Carr and co-workers [8] using two six-port valves, as shown in Fig. 9. Although the configuration is more complicated compared with one valve, it accomplished a full symmetrical path for both loops.

Other configurations, including two position 12-port valves [77, 78], two four-port switching valves, and two second dimension columns connected in parallel [80], and other [117] will not be discussed in more detail in this review.

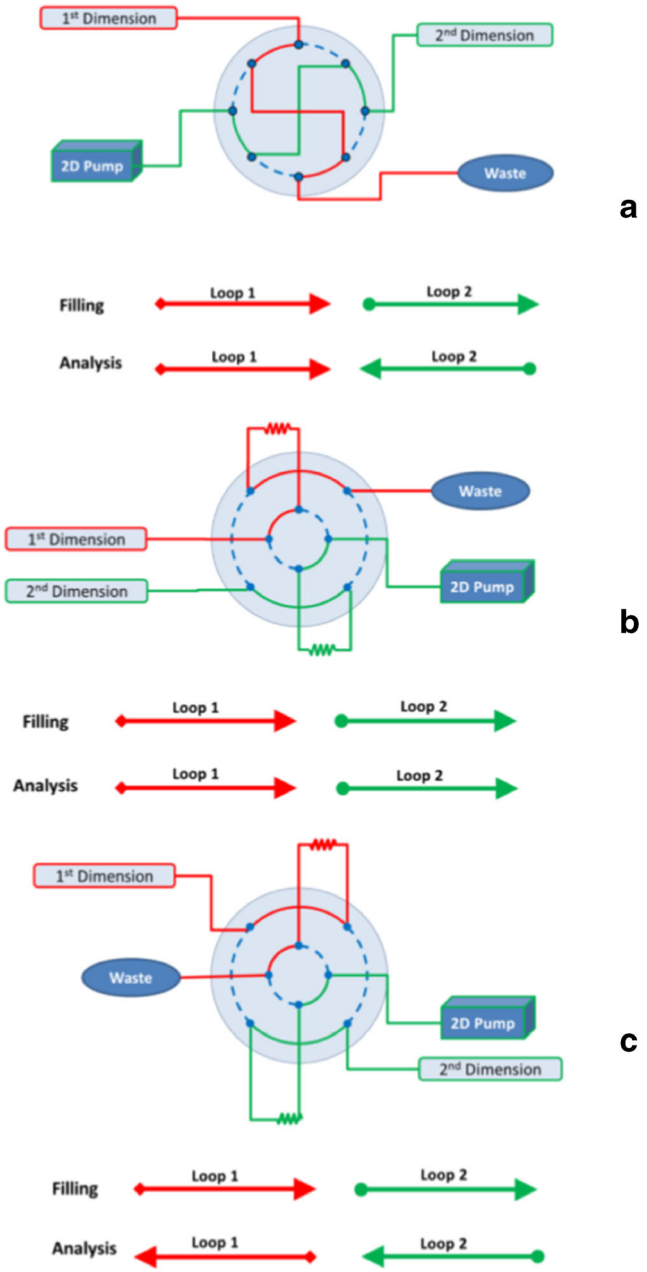

Fig. 7 Setups of different eight-port valves in LCxLC

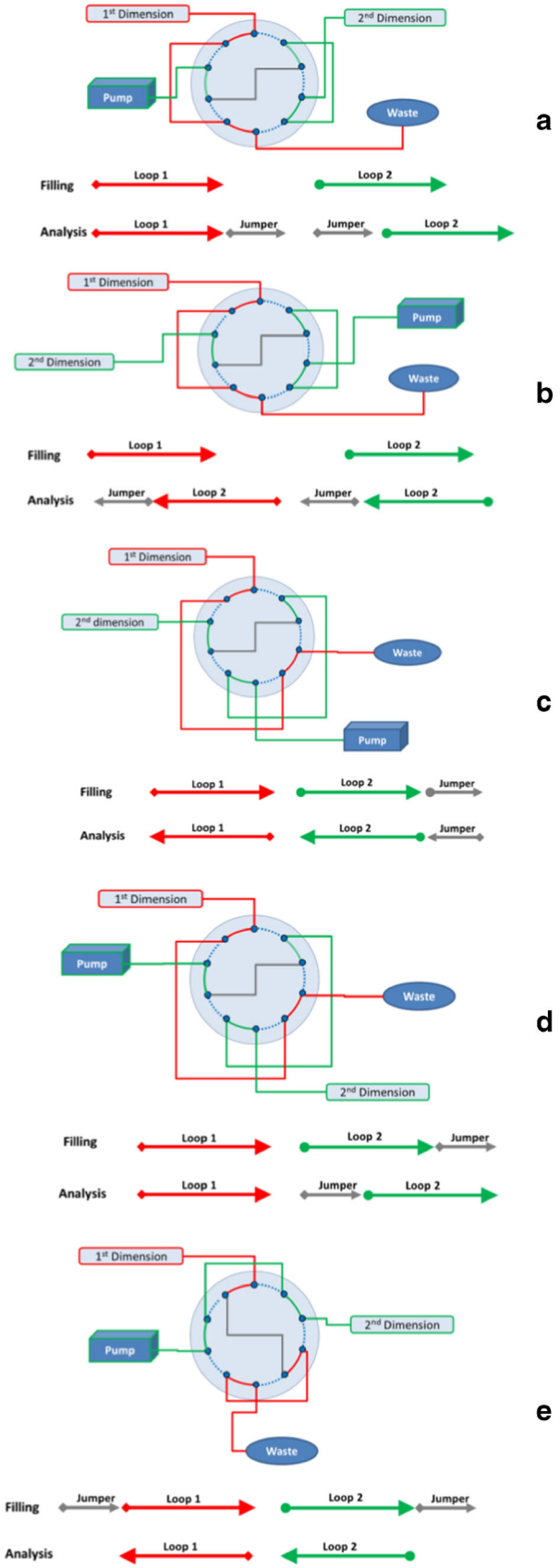

Fig. 8 Ten-port valve setups in LCxLC

Gradient type

In developing an LCxLC system, the elution in both dimensions can be optimized separately, either in an isocratic or gradient mode. Gradient elution is mainly adopted in reversed phase LC for higher peak capacity, elimination of the carry-over effect, and a better band compression effect [114]. In a traditional LCxLC operation, the second-dimensional gradient covers a wide range of mobile phase composition in a short time, and repeats the same gradient program in the next modulation time. This type of gradient program is called full

gradient (full in fraction [46, 83]) as shown in Fig. 10a. Another type of gradient is called segment gradient (Segment in fraction [46, 83]). For the first segment (the early fractions) the segment gradient uses a lower gradient coverage and a higher coverage in the second segment (Fig. 10b). Parallel gradient mode uses a gradient program, which is independent on second-dimensional run [47, 63, 83] and the parallel gradient mode shows more or less isocratic condition for each second-dimensional run. Finally, the second dimension can run with a narrower gradient program, but changing concentration over the whole analysis time. This is termed as shift gradient in the Reference [48]. The advantages and disadvantages of each mode are discussed below:

Figure 10a, full gradient: A very steep gradient runs in a very short time, which offers a high bandwidth suppression effect. Very narrow peak width can be achieved and thereby very high second-dimensional peak capacity. Because the gradient is always the same, the probability of wrap-around behavior of some strongly retained compounds is increased. Furthermore, the analytes, which eluted earlier from first-dimensional RP column have also weak retention on second-dimensional RP column; the analytes eluted later have also strong retention on the second-dimensional RP column. Therefore, the compounds do not fill the available two-dimensional retention area and tend to cluster more or less around a diagonal line, which connects the lower left with the upper right corner of the two-dimensional contour-plot.

Figure 10b, segment gradient: The segment gradient, although somewhat less steep than the full gradient, provides significant bandwidth suppression effect. Moreover, the probability of wrap-around behavior is reduced because organic solvent concentration ranges can be adjusted to suit the retention of the sample. Therefore, an increase in the fraction coverage is often observed.

Figure 10c, parallel gradient [62]: Quasi-isocratic elution shows larger bandwidths, due to a reduced bandwidth suppression effect as compared with a gradient run. And, thus, it results in somewhat lower total two-dimensional peak capacity. The advantage of quasi-isocratic elution is to provide longer second dimension elution time as post-gradient equilibration is not necessary within the individual fraction cycles. The gradient can be programmed according to the retention characteristics of the first-dimensional elution pattern and, thus, to improve the effective separation space.

Figure 10d, Shift gradient: The use of a second-dimensional gradient facilitates bandwidth suppression effects and the continuous change of the gradient program reduces the probability of wrap-around behavior and the increasing organic solvent concentration can be adjusted to suit the retention of the sample. Therefore, the second-dimensional peak capacity is often higher in comparison with all other gradients and the fraction coverage increases.

Furthermore, LCxLC could be optimized with more complex gradient programs. An almost orthogonal LCxLC run was realized for the separation of phenolic and flavone natural antioxidants [84].

HPLC can run either with a solvent gradient or a temperature gradient [118]; the latter is called temperature programmed HPLC [105]. Although the advantage of high temperature has been widely applied to LCxLC [8, 71, 84, 86, 112, 119–121], normally the second-dimension was kept at constant higher temperature and in LCxLC no temperature gradient is used. The advantage of temperature programmed HPLC is not completely investigated [105, 122, 123]. The use of a systematic temperature program (changing the second-dimension temperature over the whole run, not in one second-dimensional run), should lead to an increase in fractional coverage and could be a promising technique for further increase in separation power.

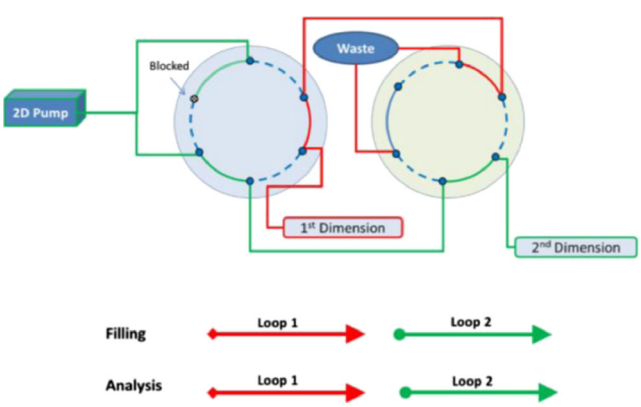

Fig. 9 Setup of the two-six-port valves system [8, 116]

Achieving fast LCxLC analysis

The advantage of LCxLC compared with heart-cutting and stop-flow is the speed. More specifically, the peak capacity production rate is very high [124]. The speed of first-dimensional separation determines the whole analysis time, whereas the speed of second-dimensional separation affects the sample rate to first-dimensional eluent. The newly commercialized ultra-high performance liquid chromatography (UHPLC) facilitated fast analysis by providing state-of-art hardware. The UHPLC instruments have a very higher pressure limit (up to 1000 bar), higher sampling rate (up to 80Hz), etc., and in order to perform fast gradient, reduce system volume, and reduce system extra column, broadening is especially important. More details can be found in a recent excellent paper [125].

Fast first-dimensional separation

In online LCxLC, the first-dimension separation is often conducted at conditions where flow rates are below the optimum velocity of the van Deemter curve [126]. The loop size and

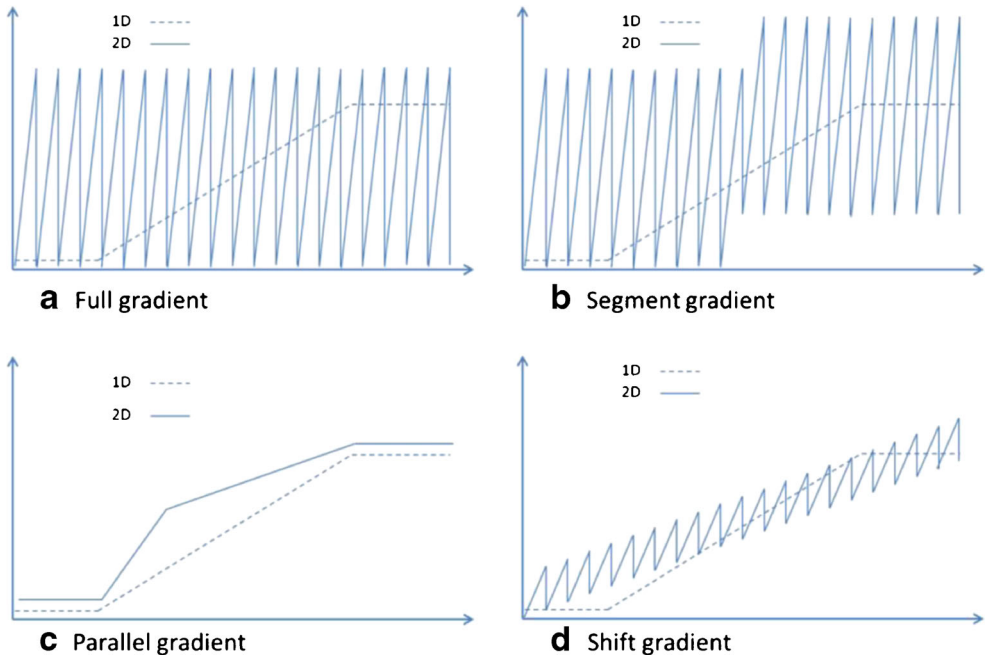

Fig. 10 Second-dimensional gradient types in LCxLC. Note: In both axes, the label is time

second-dimensional modulation time are dependent on the flow rate in the first dimension. To minimize the loop size and modulation time, which increased the separation power of the LCxLC system, low flow rates in the first dimension are needed. The use of trap columns or parallel second dimension columns allows a higher flow rate in first dimension or an extended second-dimensional separation time. In both modes, the first-dimensional separation can be realized under more or less optimal one-dimensional analysis conditions. However, the finding of two identical trap-columns or parallel columns represents a challenge. Another possibility is the use of a narrow-bore column in first dimension. However, the column inner diameter influences the practical sample size, the limits of detection (sensitivity), and a possible column overload [127].

Splitting the flow after the first dimension column and performing online LCxLC on constant fraction of the first dimension effluent allows the two dimensions to be optimized almost independently [113]. Without changing the sample loop size or the modulation time, it is possible to double the flow rate in the first dimension column with a 1:1 flow split. Using such a post-first dimension flow splitter a 2-fold increase in the corrected two-dimensional peak capacity and the number of observed peaks for a 15-min analysis time was observed [113].

As the fast analysis of the LCxLC was mostly applied to the second dimension, the benefit of very high pressure was well studied and applied to the second dimension. Truyols et al. predicted the use of UHPLC in one of the dimensions increases the peak capacity by 15 %–20 %, whereas the use of UHPLC in both dimensions improves the peak capacity by close to 25 %–30 % in comparison with the original HPLCxHPLC system [22]. A UHPLCxUHPSEC was reported for the separation of polymers [126] and results in higher efficiency.

Fast second-dimensional separation

The second-dimensional separation plays a more crucial role in the total performance of LCxLC systems [37]. The speed as well as the efficiency of the second-dimensional analysis is of major importance. The speed of the second-dimension analysis indicates the flow rate of the first dimension. Moreover, the shorter the second dimension run the higher the sample rate of the first dimension effluent. Therefore, much effort was made to achieve fast second-dimensional analysis.

Monolithic columns are formed by a porous continuous gel with porosity typically 15 % higher than a conventional packed column [128]. The resulting low backpressure and good mass transfer enable the use of elevated flow rates (3 to 10 times larger as in particle packed columns). When working with a conventional column length, it allows ultrafast separations of only a few seconds [129, 130]. However, monolithic columns are not widely used because of limited column chemistry and efficiency [131].

Core-shell columns present one of the most promising technologies in this field [132, 133]. It gains ever-growing interest because conventional HPLC equipment can be used [129, 133] with more or less the same separation power of UHPLC systems with sub-2 μm particle columns. Owing to

lower particle size distribution of core-shell columns, a reduced Eddy-diffusion coefficient compared with conventional fully porous particles is observed [134, 135]. Core-shell columns have higher mass transfer rates. Therefore, a higher flow rate can be used without significant loss of column efficiency, without the generation of a high back pressure [128], and with very brief reconditioning times [136]. Also, a more homogenous packing of core-shell columns compared with conventional columns is discussed [137]. Various authors have determined plate height *h* values down to 1.5 for such columns in contrast to values of 2–2.5 for columns packed with porous particles [129]. This type of column can provide speed and efficiency similar to columns packed with sub-2 μm particles and, therefore, allows fast second-dimensional analysis [136, 138–140].

High temperature liquid chromatography (HTLC) offers significant advantages in the speed of second-dimensional separation. The solvent viscosity decreased and diffusion of analytes increased when high temperature was applied, thus increasing the optimum linear velocity. Because of these properties, it is possible to maintain resolution and increase the speed of separations by a factor of three to five (90 °C), and up to a factor of 20 (200 °C), with methanol as the organic solvent [129]. Carr and coworkers [8, 121] applied HTLC with a core-shell column for the analysis of maize extract. The second-dimensional modulation time can run as short as 12 s, with a temperature at 110 °C [96, 113]. Thus the total LCxLC run time was only 15 min. The change in selectivity correlation is another advantage of high temperature [71]. In practice, it is preferable to use lower temperature in the first dimension and higher temperature in the second dimension.

As well discussed in several papers [92, 129, 130, 132, 141], UHPLC in second dimension shows significant advantages in performing fast analysis while maintaining high efficiency, compared with HPLC [130]. When an elevated temperature is applied to UHPLC separation (HT-UHPLC), the analysis time could be further reduced [92]. As shown in Fig. 11, compared with above-mentioned methods, HT-UHPLC demonstrated maximum peak capacity and throughput for both small and large molecules [42, 129].

There is another technique to increase the speed of second-dimensional run. For example, the use of a constant pressure instead of constant flow gradient results in decreased analysis times by about 20 % [142]. Moreover, good reproducibility of response factors, peak widths, and retention times were demonstrated [143]. Columns packed with core-shell particles seem to be more suitable for constant pressure methods than those packed with fully porous particles.

The optimization of the second-dimensional operation depends not only on the speed, but also on the separation efficiency. A descriptor for the separation efficiency is the peak capacity product rate [8]. Li et al. [73] reported that the optimum value of the second dimension productivity occurs at a second dimension gradient cycle time in the range of 20 s. The interaction between high resolution and fast analysis is not easily qualified as it depends essentially on the analytical purpose.

Recent applications

Most RPxRP separations have been developed and applied for the determination of antioxidants, but also for the analysis of biological compounds [65, 114, 115], environmental compounds [71, 144], and natural products, such as Chinese herbal medicine (CHM) [48, 55].

The presence of acidic and basic functional groups in tryptic digested peptides, such as primary amines and carboxylic acids, leads to a change in the hydrophobic properties of the peptides as a function of pH, which results in an additional selectivity when applying both dimensions at acidic and basic pH conditions. While acidic and basic peptides are more retained at a low or high pH, respectively, the affinity of the relatively "neutral" peptides is altered as well at differing pH [103, 104]. Low selectivity correlation and, hence, very high peak capacities were achieved through employing first dimension under acidic (pH 1.8) and second dimension under basic (pH 10) conditions. D'Attoma et al. [124] compared the separation of tryptic digested proteins with RPxHILIC and RPxRP. In RPxHILIC, a lower peak capacity was obtained, whereas the peak coverage was better compared with RPxRP.

Natural products have been a major resource for the investigation of naturally-occurring biologically active substances. In most cases, however, medicinal plants contain hundreds or even thousands of constituents, and vary greatly in their contents and their physical and chemical properties. A traditional, offline 2D-LC approach is time-consuming and labor-intensive. Online operation of LCxLC has the advantages of high-throughput and automation. Thus, characterization of natural antioxidants [94], steroid glycosides in *Anemarrhena asphodeloides* extract [79], *Stevia rebaudiana* [141], *Radix Angelicae sinensis* [55], and analysis of TCM formula [48] were done in online mode. Fractionation of a comprehensive two-dimensional liquid chromatographic analysis provides more pure compounds, which facilitate the ensuing identification process. When preparative HPLC was adopted in both dimensions, the sample could be isolated in a gram-scale. The identification by MS, ^{1}H-NMR, and ^{13}C-NMR could be achieved in a short period of time [145].

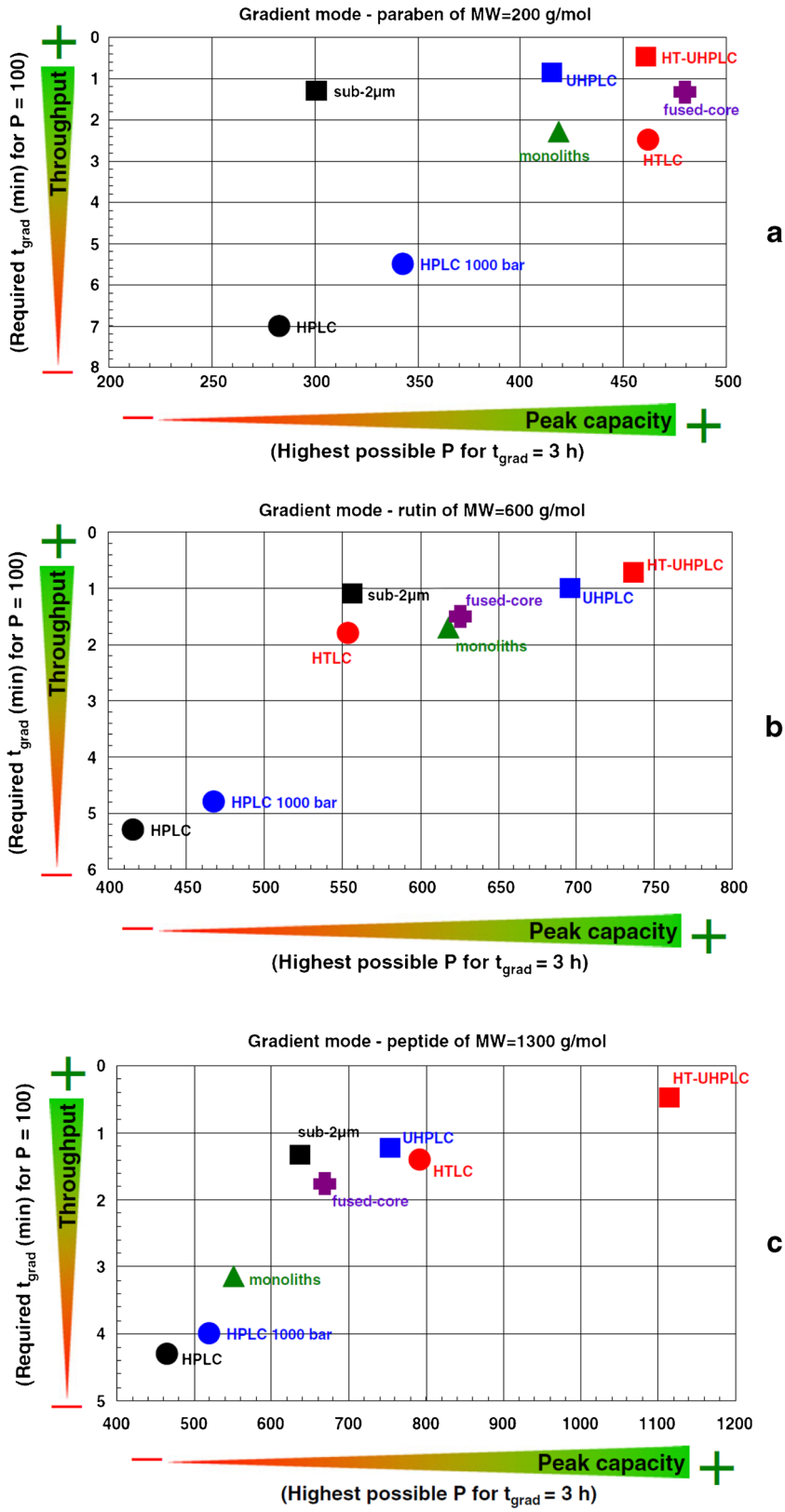

Fig. 11 Comparison on performance of LC strategies in terms of throughput and maximum resolution for model compounds. Adapted from Reference [129]

Many applications were done with comprehensive two-dimensional liquid chromatography in food analysis [146], such as beer samples [62], citrus juices [147], red wine [87], corn seed samples (or/metabolites) [65, 96–98, 101], whole-grain bread extracts [65], juice extract samples [65], yeast supernatant samples [65], phenolic acids and flavonoids [138], and polyphenols in red wines [136, 140]. More details can be found in a recent review [146].

As discussed in several applications, LCxLC systems provide very high resolving power. When coupled with a diode array detector or mass analyzer, a four-dimensional data containing two-dimensional retention times, peak intensity, and *m*/*z* ratio (or spectral) was generated [148]. These data become especially useful for non-target analysis and identification of patterns [3]. Nevertheless, due to the large amount of data from LCxLC separation and from the detector, the extraction of applicable information for the identification of patterns is still one of the major drawbacks for a wider application of this technique. Matos et al. [3] have developed a simple and fast way to build a three-dimensional fingerprint of a given sample. A data set consists of retention time at the peak maximum in the first and second dimension, and of the wavelength of the maximum UV absorption. The data set was then used to build a three-dimensional fingerprint of the given sample. It was used for identifying different patterns associated with the specific properties of four Portuguese red wine samples.

Reichenbach et al. [114] described a sample classification method to extract comprehensive non-target chromatographic features from a set of two-dimensional chromatograms. The method defines a set of chromatographic regions relative to a pattern of peaks and, hence, is robust with respect to compositional differences among samples, chromatographic variations, and co-eluted peaks. Based on the extracted features, a support vector machine successfully classified urine samples by individual, before/after procedure, and concentration with leave-one-out and replicate *K*-fold cross-validation.

For pattern matching, a smart template method [115] was reported. A template was generated through the prototypical pattern of peaks with retention times and associated metadata (such as chemical identities and classes). The template pattern is matched to the detected peaks in subsequent data. Then the metadata is copied from the template to identify and classify the matched peaks.

Final remarks

The combination of RPxRP presents a promising system providing a high practical peak capacity and wide applicability, after solving the crucial correlation problem. Many solutions were developed to increase the fraction coverage such as different columns, mobile phases, and temperature. The shift-gradient is a more direct and effective way to increase the fractional coverage. Even though already adopted as HTLC, the use of temperature-programmed elution for the construction of a more orthogonal LCxLC should be a good alternative to change stationary phases and solvents. For example, a temperature-programmed elution in first dimension with pure water as the mobile phase will facilitate the focusing effect on second dimensional column. New technologies, which are developed for LC, show also a high potential in LCxLC, such as the core-shell columns. A more attractive part is the combination of several new techniques (e.g., HT-UHPLC) to provide high efficiency separation in a short time.

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