Fundamental issues in the implementation of a two-dimensional LCxLC separation.

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This article aims at presenting the fundamental steps when implementing a two-dimensional on-line separation (2D-LC) of complex samples. Firstly, an approach is proposed to make the search for orthogonal conditions easy. It is based on the treatment of retention data acquired from generic gradient separations of test mixtures in various systems (stationary phase / mobile phase / temperature). The regression coefficient of 2D retention plots and the practical peak capacity are both determined. The instrumental design and the choice of analytical conditions are also discussed. This article demonstrates that an ultra-fast second dimension combining high temperature and ultra high pressure (HT-UHPLC) is very attractive. Finally, relevant choices are illustrated by a 2D separation of a protein digest.

Key-words
Two-dimensional on-line liquid chromatography (2D-LC); HT-UHPLC ultra-fast second dimension; orthogonal systems; instrumental design

Introduction

In conventional liquid chromatography, the theoretical peak capacity is limited to about 1500. In addition to the use of very long columns, hours or even days may be required to attain such high values [1]. In two-dimensional liquid chromatography (2D-LC), the total peak capacity is theoretically given as the product of the peak capacities in each dimension [2] (Equation 1), thereby leading to impressive peak capacities.

$$n_{c,total} = n_{c,1} \times n_{c,2} \quad \text{(Equation 1)}$$

On the other hand, 2D-LC can be a powerful method for checking the peak purity. An example is given in Figure 1 for the 2D separation of aromatic compounds using two different RPLC systems.

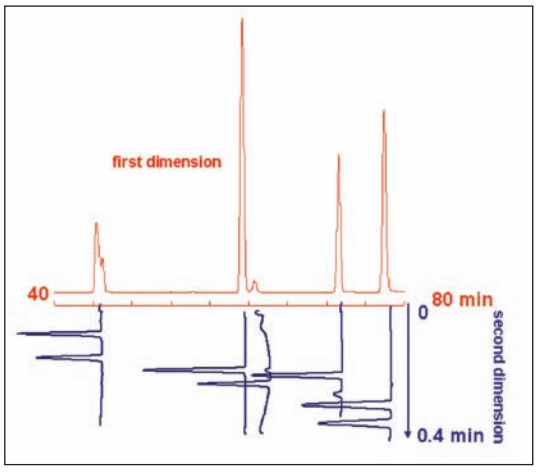

Figure 1. 2D-separation of aromatic compounds. First Dimension, 150 mm×2.1 mm i.d. BetaBasic; 0.08 mL/min; 0–70% Methanol in 87 min; 30 ○ C; Second Dimension, 50 mm × 2.1mm i.d. Acquity BEH C18; 1.45 mL/min; 12–65% Acetonitrile in 0.27 min; 90 ○ C; detection at 220 nm.

2D-LC techniques include two different modes [3]: the "heart-cutting" (LC-LC) where only a few fractions of the first separation are sent to the second separation and the "comprehensive" (LCxLC), where the whole sample is subjected to both separations. The latter is intended for the screening or the analysis of complex samples such as pharmaceutical, environmental or biological ones.

The transfer of fractions between the two columns can be operated either on-line or off-line. In on-line transfer, the two separations occur concurrently. Although the latter approach usually generates lower peak capacities, it offers many advantages: (1) it prevents sample contamination or sample loss; (2) it allows automatable analysis; (3) it leads to more reproducible and faster separations. However more complex instrumentation is required. Moreover, data handling and optimization of operating conditions become critical issues.

The multiplicative rule (Eq.1) implies two criteria to be fulfilled [4]. On the one hand, selectivities in each dimension must be different in order to reach a sufficient degree of orthogonality. Orthogonality has mainly been studied by comparing retention data of two different separations and by assessing their degree of orthogonality with the regression coefficient value r^2, which should be as small as possible [5]. Chemometric techniques have also been investigated [6]. On the other hand, the sampling rate of the first dimension peaks must be suitable in order not to lose the resolution in the second dimension. Murphy et al. [7] determined an adequate sampling rate as 3-4 cuts per peak.

The first 2D-LC separation was reported in 1978 by Erni and Frei [8]. Since then, many improvements have been made [9, 10]. The emergence of ultra-fast LC thanks to instruments withstanding high temperatures (HTLC) and/or very high pressures (UHPLC) which allow separations in few dozens of seconds [11] was a big step forward [10].

The present study focuses on on-line comprehensive 2D-LC for the separation of complex samples of charged compounds in a reasonable time. Thus, gradient elution was preferred to isocratic elution in both dimensions in order to get faster analysis in addition to higher peak capacities and better sensitivity. Similarly, high temperature was combined with very high pressure (HT-UHPLC) in the second dimension in order to reduce the overall analysis time. The orthogonality of different reversed phase systems is discussed from the practical peak capacity approach. The choice of RP conditions in both dimensions was directed by the high efficiencies and the excellent mobile phase compatibility that can be expected with RP-separations. Moreover RPLC provides a large set of analytical conditions (mobile phase, stationary phase, temperature) which can be efficiently tuned to vary selectivity.

Experimental

Solutes

Representative mixtures of small ionisable compounds were selected according to the diversity of their physico-chemical properties (pKa and logP). Solutes were obtained from Sigma Aldrich (Steinheim, Germany) and included: acetyl salicylic acid, phenol, methylparaben, 4-nitrophenol, benzoic acide, atenolol, nadolol, pindolol, propranolol, procaine, codeine, chloroprocaine, diphenhydramine, protriptyline, imipramine, clozapine, NN-dimethylaniline, amitriptyline. Uracil was used to measure the column dead volume.

For the 2D-LC separation, the sample was a tryptic digest of bovine serum albumin (BSA). The protocol of digestion included denaturation with dithiothreitol (DTT), followed by alkylation with iodoacetamide and finally digestion with tripsyn (mass ratio protein/trypsin of 70). The sample was filtered on 0.22 µm before injection. All reagents were obtained from Sigma Aldrich (Steinheim, Germany).

Columns

Table 1 lists the different columns used in this study and their geometry. All columns are silica-based except the Hypercarb column which is based on porous graphitic carbon.

Column (manufacturer)	di (mm)	L (cm)	dp (µm)
Hypersil Gold C18 (Thermo Fisher)	1	10	1.9
Hypercarb (Thermo Fisher)	3	10	5
Gemini (Phenomenex)	2	15	3
Acquity BEH C18 (Waters)	2.1	5	1.7
Acquity BEH Shield C18 (Waters)	2.1	5	1.7

Table 1. Columns, internal diameter (di), length (L) and particle diameter (dp)

Mobile phases

The gradient runs were performed with mixtures of acetonitrile and water or methanol and water. The solvents were HPLC grade from SDS (Peypin, France). Water was obtained from an Elga water purification system (Veolia water STI, Le Plessis Robinson, France). The mobile phase pH was controlled thanks to various additives: trifluoroacetic acid 0.05% (TFA, pH 2.4); formic acid 0.1% (pH 2.7), ammonium acetate 10mM (pH 6.8), all from Sigma Aldrich (Steinheim, Germany). Eluents prepared from salts were filtered through a 0.2 µm nylon filter before use. In order to keep the ionic strength constant all along the gradient, the pH adjuster was added in both aqueous and organic phases except for ammonium salts which are not soluble in organic solvents at such concentrations.

Generic gradients were programmed from 0% to 100% organic modifier with a normalised gradient slope of 5%.

Apparatus

An Acquity UPLC liquid chromatograph (Waters, Milford, MA, USA) was used. This instrument included a high-pressure binary solvent manager with a maximum delivery flow-rate of 2 mL/min, an autosampler with a 5 µL injection loop, a column oven with a maximum temperature of 90 °C and a UV–vis detector with a 500 nL flow-cell. Data acquisition with a 40 Hz sampling rate (time constant at 25 ms) and instrument control were performed by Empower software. The maximum backpressure was 1000 bar for flow-rates up to 1 mL/min, 800 bar up to 1.5 mL/min and 630 bar up to 2 mL/min. The Waters Acquity system included an oven with a maximum temperature of 90 °C. Mobile phase was preheated prior to entering the column thanks to a coiled stainless steel tube (50 cm × 0.127 mm) located between the injection valve and the column inlet. The measured dwell volume was 120 µL. The needle wash cycle included a strong wash using water/acetonitrile (20/80 v/v) and a weak wash (80/20 v/v).

On-line 2D-LC

The first dimension consisted in a micro pump Series 200 HPLC instrument (Perkin Elmer, Waltham, Etats-Unis), and an autosampler Series 225. The pumps could deliver flow-rates between 1 µL/min and 3 mL/min and were controlled directly on the instrument screen. The injector was equipped with a 50 µL-loop. The autosampler temperature could be regulated from 4 to 40 °C. The needle was washed with acetonitrile/water (80/20 v/v). The autosampler was controlled by 225-275-Flexar Service Manager software and the beginning of the gradient was synchronised with the time of injection via an electric signal.

The second dimension was a Waters Acquity UPLC. Fractions were transferred between the two instruments thanks to a high-pressure two-position ten-port valve which was equipped with two identical loops (Figure 2). In position A, a fraction from the first column filled the injection loop 1. After rotation (position B), the loop 1 was sent along with the second dimension mobile phase to the second column. Meanwhile, the second loop was filled with the subsequent fraction from the first column. The symmetric configuration providing the same direction of flux in both positions has been proven to be the most efficient one [12]. Acquisition data at 210 nm were exported using Waters Empower software, converted to

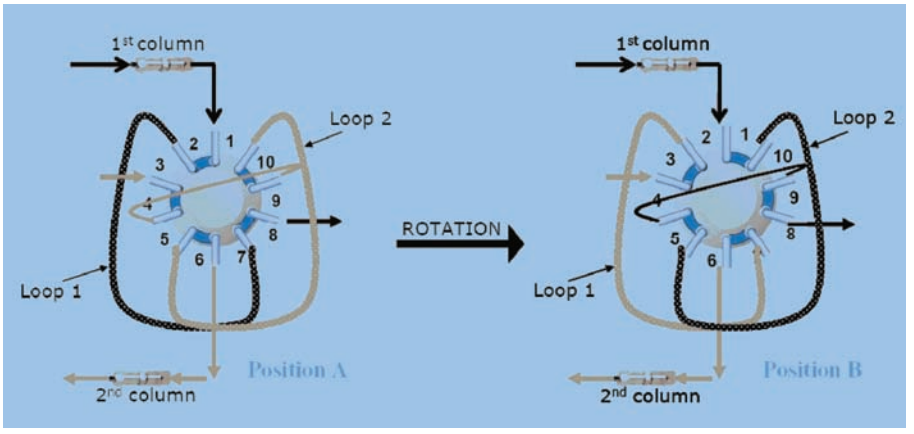

Figure 2. Configuration of the ten-port high pressure valve in the-two positions.

Excel files and further processed to obtain a 3D-plot using Matlab 6.5.

Results and Discussion

Orthogonal systems

RP-systems differing in stationary phase and/or mobile phase and/or temperature were compared using a mixture of 17 ionisable compounds, which were separated by gradient runs using a normalized gradient slope of 5%. The degree of orthogonality between two given RP-systems was assessed by the Pearson regression coefficient r^2 calculated from the 2D-retention plot of compositions at peak elution. Furthermore, as the interest of 2D-LC mainly relies on its high resolution power, the practical peak capacity was also considered. Only few studies have dealt with this critical parameter [13, 14]. In this study a novel method was proposed to determine the practical peak capacity from the 2D retention plots using the confidence envelopes of the regression straight line. The peak capacity in each dimension is derived from the Snyder's sample peak capacity (Equation 2) [15] which is related, for a given sample, to both the retention range $\Delta t_{r,i}$ and the mean peak width according to (Equation 2)

$$n_{c,i} = \frac{\Delta t_{r,i}}{0.93 \times w_{10\%,i}} = \frac{\Delta C_{e,i}}{P_i} \frac{t_{0,i}}{0.93 \times w_{10\%,i}}$$

where $w_{10\%,i}$, $\Delta Ce_{,i}$, P_i and $t_{0,i}$ are respectively the mean peak width at 10%

where $w_{10\%,i}$, $Ce_{,i}$, P_i and $t_{0,i}$ are respectively the mean peak width at 10% of the peak height, the range of composition at elution, the normalised gradient slope and the column dead time in the ith dimension. The practical peak capacity is given by the product of the sample peak capacity in each dimension as illustrated in Figure 3 which displays an example of a 2D retention plot, showing the confidence envelopes and the composition range in each dimension

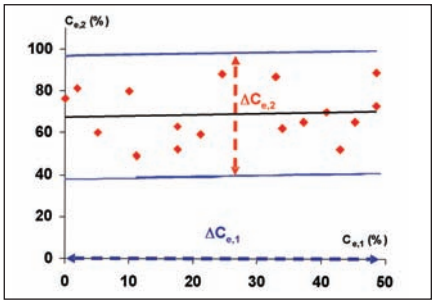

Figure 3. Representation of a 2D retention plot comparing compositions at elution in two selected systems.

Regression coefficients and practical peak capacities of different combinations of RP-systems for a sample of 17 ionisable compounds are given in Table 2.

2D systems	Dimension 1	Dimension 2	r^2	$n_{c,\ total}$
1	Gemini pH 2.7 30°C	Acquity Shield pH 2.7 30°C	0.97	850
2	Gemini pH 2.7 30°C	Acquity Shield pH 2.7 90°C	0.82	1700
3	Gemini pH 2.7 30°C	Hypercarb pH 2.7 90°C	0.02	1846
4	Gemini pH 2.7 30°C	Acquity Shield pH 6.8 90°C	0.01	5700

Table 2. Combinations of different RP-systems characterized by their degree of orthogonality (regression coefficient r^2) and their practical peak capacity ($n_{c,\ total}$)

When comparing two different silica-based stationary phases in the same conditions of both temperature and mobile phase (2D-system #1), the degree of orthogonality is very poor (r^2 = 0.97) and the resulting peak capacity is therefore disappointing (nc = 850). When the temperature is raised (2D-system #2), the orthogonality is not much better (r^2=0.82 vs. 0.97) but the peak capacity is significantly increased (n_c = 1700 vs. 850). This is mainly due to the improvement in peak shape at elevated temperature and hence the decrease in the ratio of the peak width to the column dead time (Equation 2). For combinations involving a stationary phase based on another type of material such as the Hypercarb column (2D-system #3), it can be observed that although the orthogonality is excellent (r^2 = 0.02), the practical peak capacity is not significantly higher than the one obtained with the preceding 2D system. In case of such materials, the peak efficiency is very poor for ionisable compounds and as a result the ratio of the peak width to the column dead time is dramatically high. The variation of the mobile phase pH is also a very efficient way to get orthogonal combinations (2D-system #4). As soon as the pH is different from one dimension to another, the regression coefficient becomes close to 0. In addition, due to the very good peak efficiency with silica-based columns, especially when the temperature is increased, the practical peak capacity is impressive (n_c = 5700).

Influence of the injection volume

In 2D-LC, the mobile phase of the first dimension becomes the injection solvent of the second separation. Eluent compatibility is critical for the peak shapes and resulting efficiencies. When using RPLC in both dimensions, the eluents are of the same type and hence highly compatible. However, the maximum possible injection volume has to be determined for a proper design of the instrumentation.

We investigated which volume can actually be injected relative to the column volume when the compositions at elution for a given solute are the same in both dimensions. The study was conducted on a typical second dimension column (Acquity BEH C18 50 x 2.1mm; 1.7 µm). As can be seen in Figure 4, the peaks superimpose up to 15% of the column dead volume (V_0) injected. Beyond this limit, the peak shape is significantly affected and the loss of resolution becomes unacceptable. Consequently, in these conditions, an injection volume up to 0.15 x V_0 is appropriate. In isocratic elution, it was observed that due to the lack of focusing effect, much smaller volumes have to be injected otherwise a strong deformation of peaks occurred (results not shown). It should be underlined that when the eluent strength of the injection solvent is higher than the composition at elution in the second dimension, smaller injection volumes are required. This issue which is

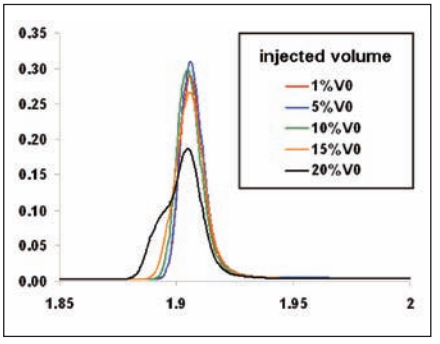

Figure 4. Peak shape of butylparaben depending on the injection volume (expressed as a percentage of the column dead volume). Acquity BEH C18 50 x 2.1mm; 1.7 µm, 30 °C, 0.5 mL/min, 5-85% methanol in 1.65 min, The injection solvent is the same as the composition at elution in the second dimension (78% methanol). The injected quantity is constant irrespective of the injection volume

critical in HILIC x RPLC, is under investigation in our laboratory.

Instrumental design

The optimisation of the instrumental design was performed assuming orthogonal combinations of two RP-systems. The Van Deemter coefficients were determined with neutral compounds. The objective was to maximize the practical peak capacity, taking into account numerous constraints. Some of them are inherent to successful 2D-LC separations and include (1) a suitable sampling rate (around 3) as well as (2) an injection volume compatible with the second column dimensions (V_{inj} <0.15 x V_0). In addition, many constraints result from the instrument: (1) the minimal flow-rate for reproducible gradients in the first dimension (20 µL/min); (2) the maximal allowable

	a		b		c		d	
	dim 1	dim 2	dim 1	dim 2	dim 1	dim 2	dim 1	dim 2
T (°C)	30	30	30	30	30	30	30	*80*
L (cm)	10	5	10	5	10	5	10	5
di (mm)	2,1	2,1	*1*	2,1	1	2,1	1	2,1
dp (µm)	5	5	5	5	5	*1,7*	5	1,7
P_{max}	400	400	400	400	400	1000	400	1000
sampling rate	3		3,1		3		3,2	
V_{inj}/V_0 (%)	0,14		0,09		0,13		0,14	
peak capacity	598		1779		4130		6511	

Table 3. Optimisation of the instrumental design. The steps presented consist of a) conventional conditions in both dimensions, b) reduction of the first column diameter, c) UHPLC conditions in the second dimension, d) HT-UHPLC conditions in the second dimension.

	dimension 1	dimension 2
stationary phase	Hypersil Gold	Acquity BEH Shield
column geometry	100 x 1mm; 1.9µm	50 x 2.1mm; 1.7µm
mobile phase	ammonium acetate 10mM / acetonitrile	TFA 0.05% / acetontitrile
flow-rate (µL/min)	20	1300
composition range (%)	1 to 50%	1 to 30%
normalized gradient slope	0.2	5.5
temperature (°C)	30	80

Table 4. Selected conditions for on-line 2D-LC separation of the BSA digest.

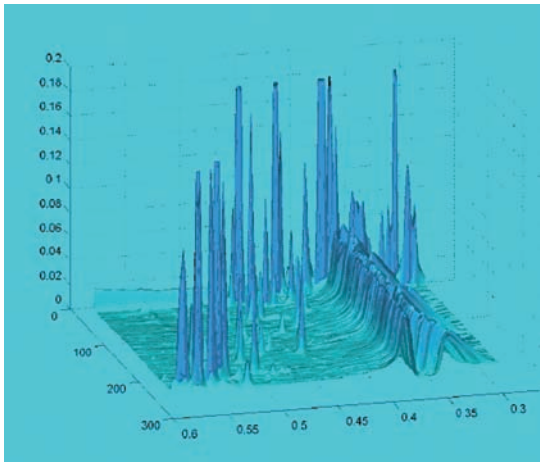

Figure 5. Zoom shot on a fraction of the 3D reconstructed chromatogram of a protein digest.

pressure in the second dimension (e.g. 1000 bar for flow-rates up to 1mL/min); (3) the maximum temperature in the second dimension (depending on the oven and the column stability); (4) the dwell volume in the second dimension. A calculation tool based on an Excel sheet was developed in our laboratory to help with dimensioning 2D separations.

Four examples of calculation are displayed in Table 3. The calculations were carried out so that the sampling rate was suitable (>3) and the injection volume was below 15% of the column dead volume. In conditions (a), the same internal diameter (2.1 mm i.d.) and the same temperature (30 °C) is used in both dimensions. This configuration leads to a very low practical peak capacity (600). In contrast, when the first column internal diameter is decreased to 1 mm, the practical peak capacity is significantly higher (conditions (b)). Changing to a sub-2 µm column and increasing the pressure up to 1000 bar in the second dimension leads to another large improvement of the practical peak capacity (conditions (c)). Finally, when the temperature is raised in the second dimension, the peak capacity is further increased (conditions (d)). To sum up, a small internal-diameter column in the first dimension associated with an ultra-fast second dimension (HT-UHPLC) is a good combination to maximise the practical peak capacity.

It must be highlighted that the columns in each dimension should be operated at their maximum pressure in order to maximise the peak capacities. Consequently, the maximal allowable pressure and/or the maximuml delivered flow-rate are limiting factors in the pursuit of very high peak capacities. Recent instruments that can withstand pressures up to 1200 bar and/or over a range of flow-rates up to 5 mL/min are hence very attractive with a view to reaching larger peak capacities. High temperatures increase the peak capacity since higher flow-rates can then be reached due to the decrease in mobile phase viscosity. In addition, increasing temperature is very attractive in case of charges compounds as it improves the peak shape as highlighted above.

Example of a 2D-LC separation

The separation of a BSA digest was performed according to the conditions given in Table 4.

The resulting 3D chromatogram, obtained after processing data with Matlab, is given in Figure 5. The optimisation of gradient conditions in both dimensions was achieved thanks to thermodynamic (retention models), kinetic (Van Deemter plots) and column permeability data. The use of HT-UHPLC in the second dimension made it possible to obtain an ultra fast separation (<0.6 min).

Conclusions

The different steps necessary for the development of an online RPLC x RPLC separation have been discussed. The orthogonality between the two dimensions is needed. It was shown that combinations involving two silica-based columns operated at different pH provide the highest degree of orthogonality and the largest practical peak capacity for a sample of 17 ionisable compounds. When dimensioning the instrumentation, many parameters have to be taken into account. In particular, the injection volume in the second dimension is a critical parameter. It was shown that a volume as high as 15% of the column dead volume can be injected in the second dimension without causing a detrimental effect on the peak shape and hence on the peak capacity.

Specific instrumental constraints are also limiting parameters. A home-made calculation tool was very helpful to deal with all these constraints and hence to optimize 2D-separations. Using HT-UHPLC in the second dimension allowed accessing impressive peak capacities as illustrated by a separation of a complex mixture (protein digest).

Acknowledgements

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