Considerations for the Use of LC x LC

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The resolution improvement of a 2DLC system makes it an extremely powerful separation tool. Exploiting 2DLC is vital for separations demanding higher peak capacities than afforded by 1D approaches. Valve operation is a crucial aspect of 2DLC and achieving the best out of various configurations available to operate different modes of 2DLC (heart cutting and comprehensive) will be discussed. We will also highlight specific applications to demonstrate the power of 2DLC to separate complex samples.

1. Introduction

Two-dimensional liquid chromatography (2DLC) is a high-resolution separation tool best exploited for complex samples where peak capacity powers beyond the grasp of conventional 1D methods are required. The power of 2DLC to practically resolve chemically similar species is achieved by transferring aliquots from the first dimension separation system via a sampling/fractioning device into the second dimension separation system [1].

The peak capacity (maximum number of resolvable peaks) of a 2D separation ($n_{c,2D}$) defined by Guiochon et al. [2] and Giddings [3] is equal to the multiplication of the first dimension peak capacity (^1n_c) and the second dimension peak capacity (^2n_c); shown in Eq. (1) using the nomenclature from Stoll et al. [1]:

$$n_{c,2D} = {}^1n_c \times {}^2n_c \quad (1)$$

Comprehensive online 2DLC (LC × LC) is the most powerful 2D separation modes with respect to peak capacity per unit time 2D [1]. During LC × LC the sampling time (t_s) of the first dimension is equal to the second dimension total cycle time (^2t_c), which equates to the second dimension separation time (^2t_g) and the second dimension re-equilibration time ($^2t_{reeq}$) shown in Eq (2) [4]:

$$t_s = {}^2t_c = {}^2t_g + {}^2t_{reeq} \quad (2)$$

Eq. (1) over-estimates the LC × LC practical peak capacity. Stoll et al. defined the effective peak capacity of a LC × LC separation as follows [5]:

$$n_{c,2D}^{o'} = {}^1n_c \times {}^2n_c \times f_{coverage} \times \frac{1}{\langle \beta \rangle} \quad (3)$$

where $f_{coverage}$ takes into account the use of the entire 2D separation space and β the under-sampling of the first dimension [5]. Under-sampling often occurs, as most LC × LC studies do not abide the Murphy-Schure-Foley rule, that the loss of resolution can be avoided if the first dimension peak width is sampled at least 4 times across a $8\,{}^1\sigma$ peak width, where $^1\sigma$ = peak standard deviation [6].

In cases where under-sampling occurs, the Davis-Stoll-Carr under-sampling correction factor [7] must be applied, shown in the re-written form in Eq. (4) [4,7], and is used in various forms by other prominent 2D research groups [9-11]:

$$\beta = \sqrt{1 + 3.35\left[\frac{{}^2t_c\,{}^1n_c}{{}^1t_g}\right]^2} = \sqrt{1 + 3.35\left[\frac{{}^2t_c}{{}^1w}\right]^2} \quad (4)$$

where ^1w is the 4σ peak width of the first dimension and ^1t_g is the first dimension gradient time. Assuming that $f_{coverage}$ was 1.0 and that severe under-sampling occurred Li et al. approximates that the corrected peak capacity shown in Eq. (5) [4]:

$$n'_{c,2D} = \frac{{}^1t_g\,{}^2n_c}{1.83\,{}^2t_c} \quad (5)$$

2. The resolving power of LC × LC

The "crossover" time (τ) is the best way to illustrate the power of 2D over a 1D method by calculating the analysis time when peak capacity of a LC × LC and 1D optimised approach are equal for gradient separations (LC elution conditions for maximum peak capacity). The analysis time includes the practical aspects of a gradient separation: the system dwell time, column re-equilibration and the separation window (actual gradient time).

A study by Huang et al. experimentally investigated the overall resolving power of LC × LC (using Eq. (5)) and the effect of the sampling time of the first dimension [12]. Their findings experimentally validated that the maximum resolving power of LC × LC is achieved at an intermediary sampling rate in line with previous theoretical studies [4,13,14], of 12-21 s for all ^1t_g runs [12]. For their intermediary sampling rates (when t_s = 12 and 21 s) crossover times were calculated to be in the range of 5-7 min in line with previous experimental and theoretical findings [5, 15].

Most recently a study by Potts and Carr theoretically compared the performance of an optimised 1D vs. LC × LC method [16], the crossover time and the effect of ^1n_c, ^2t_c, ^2n_c and $f_{coverage}$ were studied. When the $f_{coverage}$ = 0.4-0.7 (based on previous results that studied the effect of the first dimension phases and mobile phase eluent strength for LC × LC [17,18]) the crossover time was calculated between 3-8 min. With the increase of $f_{coverage}$ (a more "orthogonal" separation) a direct and fast reduction of the crossover time occurred.

The effect the fraction of time dedicated to the separation (gradient time/analysis time) denoted by λ for the first dimension λ was

also studied [16]. For the case of the first dimension increasing the λ decreases the crossover time from 3.89 min to 1.89 min (when λ is increased from 0.60 to 0.80).

The effect of the second dimension parameters that make up the ^2t_c (^2t_g and $^2t_{reeq}$) on the crossover time are as follows: a decrease in the $^2t_{reeq}$ significantly reduced the crossover time; ^2t_g experienced a minimum (between 5-15 s) for crossover times between 1-10 min; shorter ^2t_g were more sensitive to the $^2t_{reeq}$ (faster increase in crossover time with an increase of $^2t_{reeq}$) [16].

Potts and Carr's study [16], used the f_{1D} term Neue et al. and Fairchild et al. [19,20] that showed the dependence of the ^1n_c on ^1t_g. As well as a fitting equation from the work of Huang [12] and Li [4] (^2n_c versus ^2t_g) to derive the crossover time (τ), for cases of severe first dimension under-sampling, with the aid of Solver function in Microsoft Excel™ to approximate:

$$\tau \cong \frac{1.83 f_{1D}(\lambda_{1D} \times \tau) \times {}^2t_c}{{}^2n_c \times f_{coverage} \times {}^1\lambda} \quad (6)$$

Additionally Potts and Carr created a new α parameter by lumping (^2t_c, f_{1D}, ^2n_c and $f_{coverage}$) into a single parameter - α, shown in Eq. (7) [16]:

$$\alpha \equiv \frac{1.83 \times {}^2t_c}{{}^2n_c \times f_{coverage} \times {}^1\lambda} \quad (7)$$

To re-write [16]:

$$\tau = \alpha \times f_{1D}(\lambda_{1D} \times \tau) \quad (8)$$

Doing so allowed them to explore the effect of the ^1n_c on the effective 2D peak capacity which had little effect even in the case that the first dimension experienced severe under-sampling, in agreement to previous findings [4,21]. They recommend the use of Eq. (8) to calculate the crossover time and determine if any practical changes to the 1D or 2D method are worthwhile. Potts and Carr conclude that with future innovation to achieve faster first dimension sampling rates (decrease in ^2t_c) and orthogonal separations ($f_{coverage}$ approaching unity) would result in a further decrease of the τ [16]. The peak capacity power of the LC × LC approach overtakes 1D methods at short analysis times, hence should be the technique of choice for complex separations demanding high-throughput, high-resolution analyses.

3. The LC × LC valve

All of the hyphenated LC x LC methods employ switching valves to allow the sample to be switched between the 1st and the 2nd dimension columns. The switching valve can come in different configurations, comprising of 6, 8 and 10 ports, with the 2 position being the most popular for 2D LC. The valve itself has several components, with different manufacturers having slightly different designs; however, the basics of the valve design are that it comprises of three major components;

- Valve motor
- Rotor
- Stator

The rotor and stator can be made of different materials and careful choice of the material can significantly reduce the levels of carryover [23].

Chromatographers who are new to hyphenation often find the use of valves daunting as the amount of tubing seems to increase, however all LC systems already use valves to allow the sample to be introduced into the fluidic stream, and so understanding how a valve works and some of the limitations actually helps improve the understanding of a standard LC system.

There are many different configurations that chromatographers have employed for 2D and comprehensive 2D LCxLC separations, involving the use of a single valve or multiple valves. The increasing

Table 1. Method development

2DLC related Topic	Year	Title	Main Author(s)	Citations	[Ref]
Review	2007	Fast, comprehensive two-dimensional liquid chromatography	D.R. Stoll et al.	173	1
	2008	Implementations of two-dimensional liquid chromatography	G. Guiochon et al.	104	a
Method Development	2011	Peak capacity optimization in comprehensive two dimensional liquid chromatography: A practical approach	H. Gu et al.	15	15
	2011	Effect of first dimension phase selectivity in online comprehensive two dimensional liquid chromatography (LCxLC)	H. Gu et al.	8	18
	2011	Effects of first dimension eluent composition in two-dimensional liquid chromatography	X. Li, P.W. Carr	6	17
	2011	Improving Peak Capacity in Fast Online Comprehensive Two-Dimensional Liquid Chromatography with Post-First-Dimension Flow Splitting	M.R. Filgueira et al.	13	b
	2011	Perspectives on recent advances in the speed of high-performance liquid chromatography	P.W. Carr et al.	38	c
	2006	A protocol for designing comprehensive two-dimensional liquid chromatography separation systems	P.J. Schoenmakers et al.	77	d
	2005	Viscous fingering induced flow instability in multidimensional liquid chromatography	K.J. Mayfield et al.	32	e
Data processing	2013	Data Processing for 2D-LC: where are we heading?	P.G. Stevenson	-	f
	2008	Recent advancements in comprehensive two-dimensional separations with chemometrics	K.M Pierce et al.	93	g

number of valves increases the complexity of the tubing but does give the user greater flexibility or allows for parallelisation of the separation process. Figure 1 has two examples of the simpler but more common valve configurations using a single 2 position, 10 port valve, comprising of a first dimension column, 2 sample loops and two second dimension columns. This approach has been proven to be the most popular; however there are inherent issues with this. Van der Horst [22] has demonstrated that the direction of the flow in the sample loop can have an effect on the retention time of the compounds eluting from the sample loops, and hence would advise against the use of the asymmetrical valve arrangement in particular where the first dimension is operated very slowly.

and thus ensuring that the sampling rate of the second dimension is commensurate with the elution rate of the first dimension column. The sampling rate also dictates the use of two second dimension columns, as this will allow all of the eluant from the first column to be analysed. The development valve configurations which utilise two second dimension columns in combination with sample loops or a trapping column of some description offers some advantages. In particular the use of two second dimension columns allows for the separation time to be double that of the fractionation time which effectively doubles the peak capacity of the second dimension chromatographic system [24-27], dramatically increasing the overall separation capability of the chromatographic system.

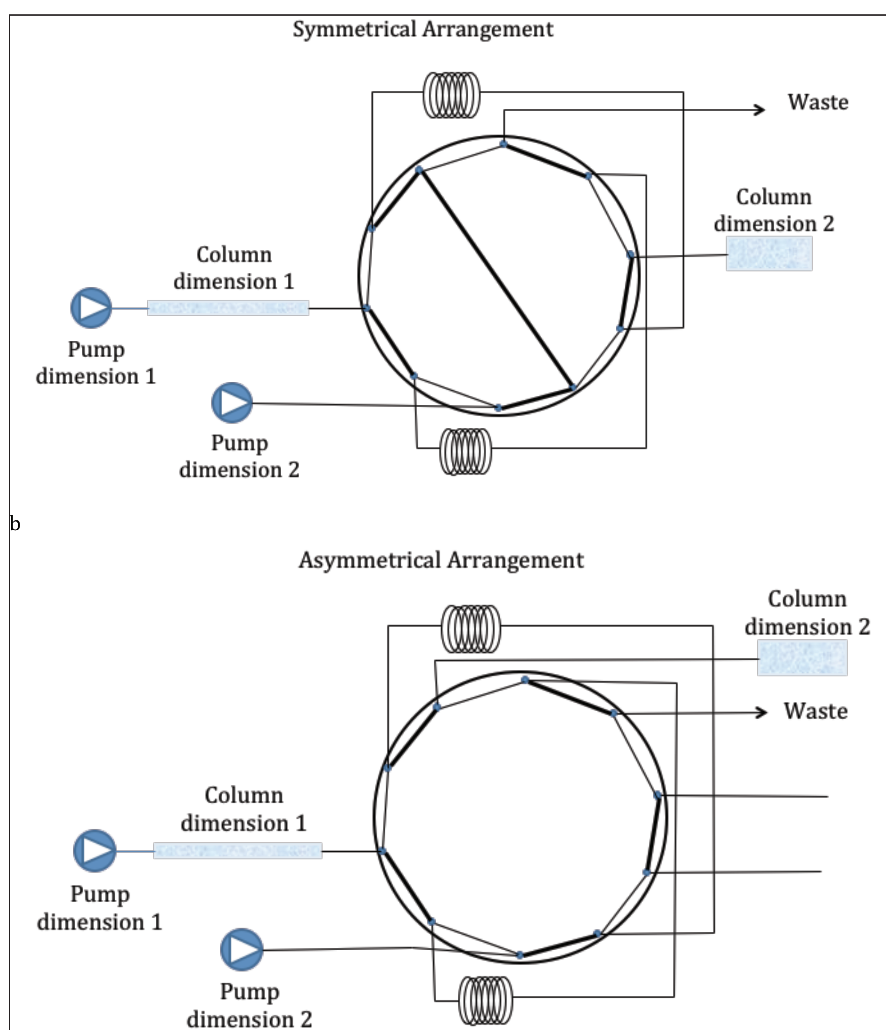

Figure 1 Common valve arrangements for 2D LC, showing a – Symmetrical, and Asymmetrical configurations

As has been previously stated it is important to consider not only the stationary phases being employed but also the dimensions of the columns that are employed. In general the second dimension column will be short and also have a wider diameter. This allows the flow rates to be optimised

The use of a focussing device between the first dimension column and the second dimension column is very popular and this can come in a variety of formats but typically will be a trapping column, or a sample loop packed with a suitable stationary phase. The use of a trapping device between the first and second dimension columns presents many challenges since any retention that is observed in the trapping media has to be negated in a manner that will not result in the elution of the analytes from the second dimension column. There are different approaches that can be employed to ensure that the compounds are retained on the second dimension column, but can still be eluted from the trapping cartridge. One approach is to add a diluent stream to the eluant from the trapping column. This approach has several disadvantages, in that;

- the peak is diluted,
- the transfer time from the trapping column to the second dimension column is typically increased in accordance with the flow rate ratio of the eluant from the trapping column to that of the diluents stream,
- the flow rate of the second dimension column will mean that a larger id column will have to be used or a UHPLC system will have to be employed which may not be compatible with the valve configurations being used.
- Requires more complex plumbing

There are other approaches that could be employed but as of yet there is limited academic literature to support these ideas.

Another approach that has seen some success is to effectively thermally desorb the analytes form the trapping column, in a manner similar to that employed when utilising GCxGC [28]. This implies that the compounds are thermally labile, how for the field of comprehensive GCxGC. The concept here is that the eluant from the first dimension is passed through a thermal modulator, which cycles between two temperatures, to initially trap components and then release these components into the second dimension column. This approach works well with the field of GC but is harder to apply to LC for several reasons;

- LC columns have a greater thermal mass
- Utilising an appropriate stationary phase that is thermally stable
- Some compounds are thermally labile

Developments in the field of high temperature LC are already addressing many of these issues [29,30], which will result in new approaches to the 2D LC.

Another approach that could be employed is to use an electric field to retain charged compounds. This technique would be comparable to capillary electrophoresis, where analytes are retained based on their charge. This approach presents many

Table 2. Applications

Application	Year	Title	Main Author(s)	Citations	[Ref]
Food analysis	2013	Potential of comprehensive chromatography in food analysis	P.Q. Tranchida et al.	-	h
	2009	Multidimensional chromatography in food analysis	Miguel Herreroa et al.	31	i
Polymer analysis	2014	Polymer separations by liquid interaction chromatography: Principles – prospects – limitations	W. Radke	-	j
	2014	Multi-dimensional separations of polymers	P.J Schoenmakers, P.J. Aarnoutse	-	k
Environmental	2014	Development of on-line comprehensive two-dimensional liquid chromatography method for the separation of biomass compounds	A. LeMasle et al.	-	l
	2012	Selective comprehensive multidimensional separation for resolution enhancement in high performance liquid chromatography. Part II: Applications	S.R. Groskreutz et al.	7	m
Pharmaceutical	2010	Combined use of immunoassay and two-dimensional liquid chromatography mass spectrometry for the detection and identification of metabolites from biotherapeutic pharmacokinetic samples	R.E. Murphy et al.	6	n
	2013	Two-dimensional liquid chromatography for direct chiral separations: a review	M.E. León-González et al.	-	o
Metabolomics	2008	Comparison of the Practical Resolving Power of One- and Two-Dimensional High-Performance Liquid Chromatography Analysis of Metabolomic Samples	D.R. Stoll et al.	31	p
	2010	[a]Two-dimensional liquid chromatography/mass spectrometry/mass spectrometry separation of water-soluble metabolites	J.N. Fairchild et al.	12	q
Proteomics	2008	Development of a comprehensive multidimensional liquid chromatography system with tandem mass spectrometry detection for detailed characterization of recombinant proteins	T.Kajdan et al.	18	r
	2010	Multi-dimensional liquid chromatography in proteomics—A review	X. Zhang et al.	45	s

[a]Offline approach

challenges;

- The hydrodynamic force is typically very large compared to the electrophoretic effect
- Resistive heating of the mobile phase may result in eluent/sample solution out gassing

There are researchers that are looking to address these significant challenges, but this is currently not a popular approach [31].

4. Summary of key 2DLC applications.

The wealth of information available on 2DLC is vast. A literature search on multi/two-dimensional liquid chromatography via Scifinder Scholar returns over 6,000 hits. The optimum summary is to tabulate the most recent or highly cited journal articles in their respective topic. Selected articles related to method development and applications are included in Tables 1 and 2 respectively. The reader must not limit themselves to the tables but use it as an initial reference to build confidence in developing a LC × LC method. Once the technique is adopted in the lab, the peak capacity power can be applied to various complex separations demanding high resolution.

5. Conclusion

This article has looked at the hyphenation of LC and LC. There are still many challenges that need to be overcome before the technology is accepted by the majority of chromatographers as being routine, however substantial strides have been made by the many authors listed in this publication. It is envisaged that with the development of more robust stationary phases and the development of new approaches to focussing elution bands, will result in this technology becoming more widely adopted, particularly with the development of the ‘omic science which require substantial resolution of components not traditional viable using a one dimensional approach. The hyphenation of LC and LC is not at all trivial and the journey to a universally accepted solution will take some time, but the benefits of obtaining such high resolution will prove invaluable for fingerprinting where complex samples are being analysed.

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