Modes of operation and parameter selection in on-line comprehensive two-dimensional liquid chromatography
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Chapter 2

Theories to support method development in comprehensive two-dimensional liquid chromatography

Abstract

On-line comprehensive two-dimensional (2D) LC techniques promise to resolve samples that current one-dimensional liquid chromatography (1D-LC) methods cannot adequately deal with. To make full use of the potential of 2D-LC optimization is required. Optimization of 2D-LC aims to predict combinations of stationary and mobile phases, column formats and chromatographic conditions that maximize resolving power and minimize analysis time. In on-line 2D-LC dilution-related issues play also an important role and should be taken into account when developing optimization strategies. In this chapter, state-of-the-art strategies that support method development for on-line 2D-LC through a rigorous choice of chromatographic parameters are critically reviewed. The final aim is to provide practitioners with a clear understanding of which aspects can be optimized using current on-line 2D-LC strategies (and which ones cannot). In 2D-LC, maximizing resolving power for a given analysis time and dilution requires optimizing efficiency, selectivity and retention. While great strides forward have been made in the optimization of efficiency-related issues, considerable effort needs still to be made in terms of (1) developing models that can predict the retention factors that given stationary/mobile phase systems can provide and (2) using this information for choosing the two ones which maximize 2D-LC orthogonality. Because of this limitation, in 2D-LC this aspect is typically dealt with a posteriori through examining chromatograms. This chapter clearly shows that important progress in the optimization of on-line 2D-LC has recently been made.¹

¹ This chapter is based on the following paper: Theories to support method development in comprehensive two-dimensional liquid chromatography – A review, Bedani, F., Schoenmakers, P. J., Janssen, H.-G., J. Sep. Science, accepted for publication.
Theories to support method development in LC × LC

Contents

2.1 Introduction

2.2 Quality descriptors in LC × LC optimization: the role of peak capacity, time and dilution factor
   2.2.1 Resolving power in LC × LC
   2.2.2 Maximizing \([n_{c,tot}]_{eff}\): the role of non-ideality correction factors \(1\alpha\), \(2\gamma\) and \(f\)
      2.2.2.1 Effect of \(^1\)D undersampling on \([n_{c,tot}]_{eff}\)
      2.2.2.2 Effect of \(^2\)D injection band broadening on \([n_{c,tot}]_{eff}\)
      2.2.2.3 Orthogonality
         2.2.2.3.1 Qualitative and (semi-)quantitative strategies to predict orthogonality
         2.2.2.3.2 Calculating orthogonality from LC × LC chromatograms
   2.2.3 Analysis time
   2.2.4 Detection issues in LC × LC

2.3 Optimization aspects in LC × LC: strategies for a rigorous choice of experimental parameters
   2.3.1 LC × LC optimization strategies leading to simultaneous optimization of peak capacity, analysis time and dilution
   2.3.2 A practical optimization approach for LC × LC

2.4 Conclusions

2.5 References

List of symbols(*)

dc: column diameter

DF: dilution factor

DF_{tot}: total dilution factor

dp: particle diameter

f: degree of orthogonality

(*): The superscripts \(^1\) and \(^2\) before a symbol in the text mean that this symbol corresponds to the first- or to the second-dimension of a two-dimensional separation, respectively.
Chapter 2

$F$: flow rate

FF: focusing factor

$^{2}k_{1e}$: retention factor at the inlet of the second-dimension column with the first-dimension mobile phase

$^{2}k_{2e}$: retention factor with the second-dimension mobile phase

$L$: column length

$N$: column efficiency, number of theoretical plates of a chromatographic system

$n_{c}$: peak capacity

$n_{c,tot}$: total peak capacity

$[n_{c,tot}]_{eff}$: effective total peak capacity

$R_{s}$: chromatographic resolution

$t_{G}$: gradient time

$t_{rec}$: reconditioning time

$t_{R,first}$: retention time of the first eluting peak

$t_{R,last}$: retention time of the last eluting peak

$t_{s}$: sampling time

$t_{tot}$: total analysis time

Greek letters

$^{1}\alpha$: first-dimension $n_{c}$ correction factor due to first-dimension undersampling

$<^{1}\alpha>$: average first-dimension $n_{c}$ correction factor due to first-dimension undersampling

$^{2}\beta$: second-dimension $n_{c}$ correction factor due to second-dimension transfer band broadening

$\delta_{inj}^{2}$: parameter related to the shape of the injected band

$\sigma_{t}$: peak standard deviation in time units

$\sigma_{chrom}^{2}$: peak variance due to the chromatographic process

$\sigma_{inj}^{2}$: peak variance due to injection

$\bar{\sigma}$: average peak width
2.1 Introduction

Along its history, one-dimensional (1D) liquid chromatography (LC) has experienced a tremendous improvement in performance. This development has mostly been driven by the effort to extend the technique’s applicability to the separation of samples of ever increasing complexity. Despite all progress made, samples from many fields (e.g. food, environmental analysis, and life sciences) are far too complex to be adequately resolved by current 1D-LC methods [1]. Due to these limitations, analysts have been challenged to introduce new LC methods with higher resolving power. Promising pioneering achievements [2, 3] and theoretical considerations [4] have directed attention towards two-dimensional (2D) LC techniques. Obviously, in order to exploit the full potential of the 2D LC methods, optimization is required. Due to the many parameters and processes involved, this task is not trivial.

2D-LC techniques have recently been the subject of an excellent publication by Guiochon et al. [5]. In their extensive work, these authors have reviewed most of the existing implementations in 2D-LC and even suggested additional ones. Main focus of their attention was on-line comprehensive 2D-LC (LC × LC, following the nomenclature of Schoenmakers et al. [6]). “On-line” means that transfer of effluent fractions from the first- to the second-dimension is executed within the confinement of the LC × LC system. Then, a rapid separation is performed such that the second-dimension (2D) column is ready to receive the next fraction and perform a new separation. “Comprehensive” means that throughout the LC × LC run all the 1D effluent is transferred to the 2D column. Obviously, the transfer of the effluent of the 1D column to the 2D column cannot be continuous, but must be performed periodically in fractions of a finite volume. This requires the use of an interface between the two dimensions.

LC × LC operation can be performed in two different ways. The most-common method is the so-called continuous low-flow operation [7]; the other option is that of alternating high-flow and zero-flow operation, known as the stop-flow method (see Chapter 3). Whereas practical aspects of LC × LC have been the subject of several recent reviews [8-11], contributions focusing on approaches to method optimization are still scarce.

In conventional one-dimensional chromatography, the optimization of the separation conditions has long been a central issue. Main aim here is to predict the optimum settings
to achieve a desired resolving power in the shortest possible analysis time, or to estimate the resolving power needed to separate the target compounds in a given analysis time. As known, resolving power is the result of efficiency, chromatographic selectivity and retention. In 1D-LC, elegant theories have been derived accounting for efficiency-related [12] and selectivity/retention-related [13] issues. For systems operating under isocratic or gradient elution, efficiency-related theories allow choosing the most suitable mobile-phase flow rate and gradient program and comparing the characteristics of different particle designs and column [14, 15]. Selectivity/retention-related models focus on understanding the types and relative strengths of the chemical interactions that control retention and selectivity [13, 16]. In LC × LC developing a “general” model to optimize the separation is much more complex than in 1D-LC. A first obvious reason for this is the higher number of parameters involved. Secondly, LC × LC should be optimized as a complete system with changes in one parameter in one dimension often having consequences for several other parameters in both dimensions [11]. Thirdly, the difference in selectivity between the two chosen stationary/mobile phase systems needs to be maximized, an aspect which is not adequately supported by any current theory. Fourthly, because of sample dilution detection issues are much more serious in LC × LC than in 1D-LC [17]. In spite of the many efforts that have been devoted to these issues and of the promising results achieved, a unified theory for LC × LC optimization is still lacking.

The main consequence of the difficulty to elaborate models to optimize LC × LC separations is that the choice of the experimental conditions is still mostly based on analyst experience rather than on rigorous theory-supported strategies. Recognizing this drawback, several research groups have developed theories to help analysts in the complex task of system optimization. In the first part of this chapter, the main issues related to LC × LC optimization will be identified and discussed. In the second part, state-of-the-art strategies that support LC × LC method development through a rigorous choice of chromatographic parameters are critically reviewed. Final aim of this exercise is to provide practitioners with a clear understanding of which aspects of method development can be optimized using current LC × LC strategies (and which ones cannot).
2.2 Quality descriptors in LC × LC optimization: the role of peak capacity, time and dilution factor

In chromatography, optimization refers to the process of selecting the system settings that result in the highest value of one or more quality descriptors. In LC × LC analysts typically aim to achieve the highest possible resolving power in a given time or to reach the desired resolution in the shortest possible time. Thus, resolving power and time are quality descriptors of LC × LC separations. However, a third aspect is worth considering. During LC × LC experiments analyte concentrations often decrease due to dilution. In carefully optimized experiments this decrease is generally between 10 to 50 times [18], but dilution may get much worse in case of poorly selected transfer conditions [17, 19]. As a result, detection of low-concentration analytes is compromised. Hence, detection limits are also a quality descriptor of LC × LC separations. Good methods to describe the quality of an obtained LC × LC separation in terms of separation characteristics have been derived by Carr and coworkers [20-23]. The other part of optimization, i.e. the selection of the parameter settings, receives far less attention in the work by Carr. For this reason, when tackling LC × LC optimization aspects (see Section 2.3), more emphasis will be placed on critically reviewing other works, even when those might be less advanced in their methods for expressing the actual quality obtained. Ideally, Carr’s methods to express separation quality should be incorporated in the advanced methods for parameter selection we will largely focus on.

The main aim of LC × LC optimization strategies is to rigorously predict the chromatographic conditions that yield optimum values of the three quality descriptors (resolving power, time and detection limits). In order to do so, the quality descriptors must be unambiguously defined and reliable equations relating resolving power, time and detection limits to chromatographic parameters and settings need to be established [18, 24]. In Sections 2.2.1-2.2.4, the chromatographic objectives that analysts need to optimize when setting up LC × LC experiments are identified and discussed. In Section 2.3, strategies supporting LC × LC optimization through a rigorous choice of chromatographic parameters are critically reviewed.
2.2.1 Resolving power in LC × LC

Peak capacity is the key *quality descriptor* of any LC separation. It provides chromatographers with an objective measure of resolving power [25-29] and, hence, it is a prime factor in system optimization. 1D-LC peak capacity, $n_c$, is defined as the maximum number of peaks with a pre-specified (equal) resolution that can be located in a given retention window [30]. The peak capacity in LC × LC separations, $n_{c,tot}$, is related to the peak capacity of the two underlying 1D separations combined by the well-known “product-rule” [31-33]:

$$ n_{c,tot} \approx 1^{n_c} \times 2^{n_c} $$  \hspace{1cm} (2.1)

where $1^{n_c}$ and $2^{n_c}$ represent the peak capacity in the first- and second-dimension, respectively. The total peak capacity of Eq. 2.1 can only be utilized in case of complete orthogonality and in case the resolving power generated independently by the two columns can be maintained. Unfortunately, meeting these two requirements simultaneously is a very complex matter: the quest for fully orthogonal columns is hindered by (1) the lack of theories that can be used to maximize differences in selectivity and (2) sample complexity [34]. Maintaining the peak capacities of the two individual columns is hampered by undersampling of 1D peaks and by 2D injection band broadening [18]. As a result, $n_{c,tot}$ is lower than calculated using Eq. 2.1 [18, 20, 22, 35]. A more reliable equation for predicting $n_{c,tot}$ is obtained when including “non-ideality” factors in Eq. 2.1 to yield the *effective* peak capacity $[n_{c,tot}]_{eff}$:

$$ [n_{c,tot}]_{eff} = 1^{n_c} \times 2^{n_c} \times 1^{\alpha} \times 2^{\gamma} \times f = n_{c,tot} \times f $$  \hspace{1cm} (2.2)

where $1^{\alpha}$ and $2^{\gamma}$ account for corrections to $1^{n_c}$ and $2^{n_c}$ due to efficiency-related 1D undersampling and 2D transfer band broadening, respectively, and $f$ is the selectivity-related separation space coverage, which measures the degree of orthogonality. All three factors $1^{\alpha}$, $2^{\gamma}$, and $f$ range between 0 and 1. If $1^{\alpha} = 2^{\gamma} = f = 1$ Eq. 2.2 equals Eq. 2.1. For non-optimized experiments $1^{\alpha}$ and $2^{\gamma}$ alone can be responsible for a loss in peak capacity.
Theories to support method development in LC × LC

by up to 75% [18]. Eq. 2.2 reveals that choosing very efficient columns for the two dimensions does not suffice to maximize \([n_{c,tot}]_{\text{eff}}\) if this results in low values of \(^{1}\alpha\), \(^{2}\gamma\) and/or \(f\). Minimizing the loss in \([n_{c,tot}]_{\text{eff}}\) due to these three undesired effects is therefore a key objective of LC × LC optimization. In Section 2.2.2, strategies aimed at maximizing the product of \(^{1}n_{c},^{2}n_{c},^{1}\alpha,^{2}\gamma\) and \(f\) are discussed.

2.2.2 Maximizing \([n_{c,tot}]_{\text{eff}}\): the role of non-ideality correction factors \(^{1}\alpha,^{2}\gamma\) and \(f\)

Eq. 2.2 indicates what should be done to maximize \([n_{c,tot}]_{\text{eff}}\), but it does not provide with clear strategies on how to do so. In other words, Eq. 2.2 says nothing about how to select mobile and stationary phases, column formats and experimental conditions that maximize \([n_{c,tot}]_{\text{eff}}\). It does not reveal anything about the optimal \(^{2}\text{D}\) analysis time, the optimal transfer volume to the second-dimension, nor about how to achieve complete orthogonality. Also, Eq. 2.2 does not say how to avoid detection problems. As further discussion shows, most of these issues are fundamentally intertwined. Hence, they should be optimized together. However, for the sake of clarity, the optimization of the LC × LC objectives – i.e. the choice of optimal (1) \(^{1}\text{D}\) sampling time, (2) \(^{2}\text{D}\) transfer volume and (3) stationary/mobile phases – that can lead to maximized \([n_{c,tot}]_{\text{eff}}\) will be discussed separately (Sections 2.2.2-2.2.4). An overview of state-of-the-art LC × LC optimization schemes aimed at rigorously selecting the chromatographic conditions that maximize \([n_{c,tot}]_{\text{eff}}\) while minimizing analysis time and dilution is presented in Section 2.3.

2.2.2.1 Effect of \(^{1}\text{D}\) undersampling on \([n_{c,tot}]_{\text{eff}}\)

In LC × LC optimization it has long been recognized that transferring fractions of the \(^{1}\text{D}\) eluent to the \(^{2}\text{D}\) column leads to some recombination of already separated compounds [18, 36, 37]. This phenomenon is known as \(^{1}\text{D}\) undersampling and it results in losses in \(^{1}n_{c}\). One simple and rather intuitive way to minimize \(^{1}\text{D}\) undersampling is to shorten the period of \(^{1}\text{D}\) fraction collection. However, the sampling period determines the allowable elution time of the last \(^{2}\text{D}\) peak. The shorter this is, the lower the \(^{2}\text{D}\) peak capacity that can be achieved. Therefore, one of the most important features of LC × LC optimization consists of finding a compromise between losses in the overall peak capacity due to either (a) \(^{1}\text{D}\) undersampling or (b) short \(^{2}\text{D}\) separations. This optimization aspect is of
Chapter 2

paramount importance when operating the system in continuous low-flow mode, but less so when the stop-flow mode is employed. This arises from the fact that – in stop-flow mode – the 1D sampling is basically decoupled from the 2D analysis. However, when many fractions are collected from the 1D, the overall analysis time becomes very long. Mainly due to this reason, stop-flow mode has not become widely accepted and will not be here further discussed. A second relevant aspect concerns the optimization of the 2D transfer volume. Too large a volume can result in excessive band broadening and to losses in $^2n_c$; too small a volume may cause sensitivity problems. Clearly, analysis time and volume can be independently varied by adapting the first-dimension flow rate. We will deal with these issues in Sections 2.2.2.2 and 2.2.4.

Many strategies have dealt with 1D undersampling. A common feature is the derivation of band-broadening factors, $^1\alpha$, to account for slow 1D sampling. Most authors studied the impact of 1D undersampling on a single pair of equal-size peaks [36-38]. Others developed their theory by evaluating the effect of 1D undersampling on the overall chromatogram [39].

For gradient $\times$ isocratic separations, Murphy et al. [36] showed that a significant loss in $^1n_c$ - 25% or more - occurs only if less than three samples are analyzed on the second column during the $8^1\sigma_t$ base width of the peaks in time units and that little resolution is gained if this analysis frequency is raised to five or more. The guideline that 1D peaks should be sampled three or four times across their bandwidth has been acknowledged by many practitioners [24, 40, 41]. Most authors – including us (see Chapter 3) - have applied Murphy’s conclusions assuming that 1D peak widths are only $4^1\sigma_t$. This erroneous interpretation has therefore resulted in many authors considering $^1\sigma_t = t_s$, $t_s$ being the sampling time, as the fundamental equation of LC $\times$ LC optimization [24]. It is now widely recognized that this equation implies a too drastic constraint on 2D separation time and that it should not be generally employed. Murphy’s findings were later confirmed by Seeley across a wide range of $t_s/\sigma_t$ values [37].

For LC $\times$ LC set-ups comparable to that of Murphy et al. [36], Horie et al. studied both the influence of 1D undersampling on $^1n_c$ and of $t_s$ on $^2n_c$ [38]. Their conclusions differ from those of Murphy et al. in two main respects, i.e. (i) when $t_s \leq 1.5^1\sigma_t$, $n_c_{tot}$ drops dramatically as a result of the decrease in $^2n_c$ and (ii) the optimal value of $t_s$ for which
Theories to support method development in LC × LC

$n_{c,\text{tot}}$ is maximized is between 2 and 3.6 times $1\sigma_t$. The conclusions of Horie et al. have been confirmed by Vivó-Truyols et al. [18] and for the relevant case of gradient × gradient LC × LC by several other authors [18, 22, 35].

Davis et al. [39] have recently developed an equation to quantitatively estimate an average value of $1\alpha$, $<1\alpha>$, as a simple function of $t_s$ and $1\sigma_t$:

$$<1\alpha> = \frac{1}{1 + 0.21 \times (t_s/1\sigma_t)^2}$$

(2.3)

These authors [39] showed that, over a wide practical range, $<1\alpha>$’s values are, in the worst case scenario, 35% larger than $1\alpha$’s values determined by Murphy et al. [36]. As it provides a straightforward way to estimate $1\alpha$, Eq. 2.3 has quickly become very popular and has been employed by many authors [18, 20, 21, 23, 35]. Expanding on Davis et al.’s theory, Gu et al. have recently developed a protocol for maximizing $n_{c,\text{tot}}$ in “fast” gradient × gradient LC × LC [22]. The equation that they use to optimize $n_{c,\text{tot}}$ is:

$$n_{c,\text{tot}} = \frac{1^t_{R,\text{last}} - 1^t_{R,\text{first}}}{(1^\sigma)^2 + 3.35(2^t_G + 3)^2} \times 44.05 \times (1 - \exp(-0.04 \times 2^t_G))$$

(2.4)

where $1^\sigma$ is the average 1D peak width, $2^t_G$ is the 2D gradient time and $1^t_{R,\text{first}}$ and $1^t_{R,\text{last}}$ are the retention times of the first and the last 1D peaks, respectively. ($1^t_{R,\text{last}} - 1^t_{R,\text{first}}$) accounts for the effective retention time window. Three important conclusions can be derived from their work: (1) the optimum sampling rate is slower than previously reported. The initial rapid increase in $2^t_n_c$ with time available for the 2D separation makes it possible to tolerate some 1D resolution loss when using a relatively long sampling time; (2) the optimum sampling rate increases with longer first-dimension gradient times, $1^t_G$; (3) the optimum conditions depend strongly on the retention characteristics of the solute mixture. For instance, under certain circumstances, the 1D undersampling correction makes it possible to obtain a higher $n_{c,\text{tot}}$ by deliberately using a less than optimum first-dimension peak capacity. The importance of establishing a trade-off between $t_s$ and $2^t_n_c$ is conveniently shown in Figure 2.1, where, for a typical $t_{\text{tot}}$, the “corrected” $1^t_n_c$, $1^t_n_c(\text{corr.}) =$
$n_c \times \alpha \times \sigma_t$ and $n_{c,tot}$ are plotted as a function of $t_s/\sigma_t$. In order to get to this result, equations derived by Gu et al. [22] and by Horváth et al. [42] were combined. Fig. 2.1 confirms that for $t_s/\sigma_t \sim 4$, $n_{c,tot}$ goes through a maximum. These results are thus in line with what is predicted by state-of-the-art theories [18, 38, 39]. In order to meet the sampling requirement, current technology requires that (1) the 1D separation is slowed down and often be run at sub-optimum efficiencies [43] and, yet, that (2) the second-dimension is both fast and efficient [20, 21]. Short, but efficient 2D columns should be used. Successful strategies here employ monoliths [44, 45] or columns packed with small (porous-shell fused-core) particles [46-48] in combination with high temperatures [20, 21, 41, 49, 50] or high pressures [51]. In most current LC × LC applications, 50 to 100 fractions are transferred from the first- to the second-dimension. If – as is most common - the goal is not to exceed $t_{tot} = 2$ h, a sufficiently high $n_c$ should thus be reached in $t_s$ less than 2.4 min. Consequently, 1D separations should be slowed down to provide a $\sigma_t$ in the order of about 0.5-1 min. As a consequence, in many cases it is not useful to pursue a value of $n_c$ higher than 50.

**Figure 2.1:** Total peak capacity, $n_{c,tot}$ (solid line), “corrected” $n_c(corr.)$ (dotted line) and $n_c$ (dashed line) plotted as a function of $t_s/\sigma_t$; $t_{tot} = 2$ hr; $n_c$ is calculated according to: $n_c = 175 \times 1G/(7.8 + 1G) [35]$; $n_c(corr.) = 1G/\alpha$, where the correcting factor $\alpha$ is calculated according to: $\alpha = (1+(0.21 \times t_s/\sigma_t)^2)^{1/2} [39]$; $n_c$ is calculated according to: $n_c = 174.2 \times 2G/(1.39 + 2G)$, where $2G = t_s - 2t_{rec}$, $2t_{rec}$ being the 2D reconditioning time [42]. $[n_{c,tot}]_{eff} = n_c(corr.) \times n_c \times f \times \gamma$, with $f = 2G/\gamma = 1$ (see Eq. 2.2).
In conclusion, recent results suggest that sampling times between 2 and 3.6\(\sigma_t\) provide the best trade-off between the decrease in \(n_c\) due to 1D undersampling and the decrease in \(n_c\) due to “short” 2D separations. Ignoring this aspect is likely to lead to drastically decreased values of \(n_{c,tot}\) (see Fig. 2.1).

2.2.2.2 Effect of 2D injection band broadening on \([n_{c,tot}]_{eff}\)

The search for the best compromise between the decrease in 1D peak capacity due to 1D undersampling and the decrease in \(n_c\) due to the short available 2D analysis time (see Section 2.2.2.1) can result in relatively large fractions of the 1D effluent being injected in the 2D column. As a result, the 2D peak width in time units, \(2\sigma_t\), is likely to suffer from additional band broadening due to injection. Thus, when optimizing LC \(\times\) LC experiments, this aspect should also be considered. Assuming extra-column contributions to be negligible, \(2\sigma_t\) can be calculated from:

\[
2\sigma_t = \sqrt{2\sigma_{chrom}^2 + 2\sigma_{inj}^2}
\]  

(2.5)

where \(2\sigma_{chrom}^2\) is the 2D peak variance due to the chromatographic process and \(2\sigma_{inj}^2\) is the 2D variance due to injection. \(2\sigma_{inj}^2\) can be calculated via Eq. 2.6 [18, 52, 53]:

\[
2\sigma_{inj}^2 = \frac{1}{2} \frac{F^2}{F^2} \left( \frac{2k_{1e} + 1}{2k_{1e} + 1} \right) \delta_{inj}^2 = \frac{1}{2} \frac{F^2}{FF} \frac{1}{FF} \frac{t_s^2}{t_s^2}
\]  

(2.6)

where \(\frac{1}{F}\) and \(\frac{1}{F}\) are the flow rates in the first- and in the second-dimension, \(2k_{1e}\) is the retention factor at the inlet of the 2D column with the 1D mobile phase, \(2k_{2e}\) is the retention factor with the 2D mobile phase, \(FF = (\frac{2k_{1e} + 1}{2k_{2e} + 1})\) is the focusing factor and \(\delta_{inj}^2\) is a parameter related to the shape of the injected band. \(\delta_{inj}^2\) equals 12 for the ideal rectangular shape (see Sternberg, [54]), but is in practice closer to 4 (see Blumberg, [55]). When \(2\sigma_{inj}^2\) is not negligible, \(\gamma\) in Eq. 2.2 is lower than 1 and will lead to a decrease in \(n_{c,tot}\) [18].
Chapter 2

Figure 2.2: (A) Total $^2\sigma_t$, plotted as a function of the $^1F$, for the two cases of absence ($^2k_{1e} = ^2k_{2e}$) (solid line) and presence ($^2k_{1e} = 10; ^2k_{2e} = 1$) (dotted line) of on-column focusing. Parameters used for the calculations: $^2F = 2 \text{ mL min}^{-1}; ^2\sigma_{\text{chrom}} = 0.025 \text{ min}; ^2\sigma_{\text{inj}} = 4; t_s = 2 \text{ min}; ^2t_{\text{rec}} = 3 \text{ sec}.$

Eq. 2.6 provides us with clear strategies on how to minimize $^2\sigma_{\text{inj}}$ and with valuable indications on how to choose mobile phases to do so. For constant $t_s$, a first way to minimize $^2\sigma_{\text{inj}}$ consists of maximizing the flow rate ratio $^2F/^1F$. This can be achieved
through maximizing the ratio of column diameters, $d_c/\sqrt{d_c}$ [24]. However, since larger $d_c$ values also imply greater sample dilution [18], detection limitations are aggravated. For the sake of clarity, this second aspect is examined in Section 2.2.4. On the other hand, LC × LC systems where $d_c$ is smaller than $d_c$ are appealing from the point of view of detection, but less so from the point of view of 2D band broadening. In this case, the injection of too large a volume is likely to result in increased $\sigma_{inj}^2 (= \text{lower } \gamma)$ and in decreased $n_{c,tot}$ [18]. A second way to minimize $\sigma_{inj}^2$ is to maximize FF [56]. When analytes dissolved in a solvent with lower eluting strength than that of the mobile phase are transferred to the 2D, they are “focused” as a narrow band on the top of the analytical column. Subsequently, the solute band is eluted by the mobile phase with higher eluting strength travelling behind it. This phenomenon is generally referred to as “on-column focusing” [18, 52, 56]. Clearly, Eq. 2.6 also suggests that, if $k_{1e} < k_{2e}$, $\sigma_{inj}^2$ will increase.

For typical LC × LC experimental conditions, the impact on $\sigma_t$ of changing the flow rate $F$, for $F = \text{const.}$, is plotted in Figure 2.2 for the two cases of (a) absence ($k_{1e} = k_{2e}$) and (b) presence ($k_{1e} = 10, k_{2e} = 1$) of on-column focusing. In case on-column focusing cannot be exploited, Fig. 2.2 shows that, for $F = 0.1 \text{ mL/min}$ and $t_s = 2 \text{ min}$ – and thus a transferred volume $V_{tr} = 0.2 \text{ mL}$ –, the relative contribution to $\sigma_t$ due to the injection volume already amounts to ~30% (see Figure 2.2(B)). Under the particular 2D column format and experimental conditions used, $n_c$ decreases from 19 to about 14, $\gamma$ in Eq. 2.2 amounts to $14/19 = 0.74$ and $[n_{c,tot}]_{\text{eff}}$ is 26% lower than $n_{c,tot}$ as predicted by Eq. 2.2. In this first case, splitting of the 1D eluate before the system interface can reduce the decrease in $[n_{c,tot}]_{\text{eff}}$ due to high $\sigma_{inj}^2$ values at the expense of aggravated detection problems. Therefore, between-column splitting can only be applied if (a) the concentration of analytes is sufficiently high and/or (b) high-sensitivity detectors are used. In case on-column focusing can be exploited, Fig. 2.2 also shows that, for the same experimental conditions as before (and a focusing factor FF of 5.5), the increase in $\sigma_t$ due to the injection volume only amounts to ~2% ($\gamma \approx 0.98$). In LC × LC optimization, the use of on-column focusing is particularly useful when, due to low analyte concentrations (trace analysis) and/or detection issues, large-volume transfer is required. Although the chosen values for this exercise are particularly favourable (FF=5.5) and
cannot be expected to hold for all transferred analytes, the conclusion to be drawn here is that on-column focusing is an important tool for the chromatographer to reduce the potentially excessive increase in peak width due to fraction transfer. LC × LC optimization is simplified if focusing can be exploited.

2D-transfer band broadening can be responsible for a decrease in $n_{c,tot}$ of 20% or more (see Fig. 2.2). When optimizing LC × LC separations, mobile phases in the two dimensions should - whenever possible - be chosen in such a way to favour focusing. In case on-column focusing cannot be exploited, splitting of the 1D eluate before the system interface combined with a high sensitivity detector may be the only viable strategy to prevent a drastic decrease in $n_{c,tot}$ due to high $2\sigma_{inj}$ values.

2.2.2.3 Orthogonality

From the discussion so far it is clear that optimizing LC × LC systems is a complex exercise, which involves accounting for efficiency-, retention-, and selectivity-related issues. In Sections 2.2.2.1 and 2.2.2.2, strategies to minimize losses in $n_{c,tot}$ due to efficiency-related factors ($1\alpha$ and $2\gamma$; see Eq. 2.2) have been discussed. In Section 2.2.2.3.1, we will focus on approaches to maximize the selectivity-related degree of orthogonality $f$ so as to achieve the highest possible effective peak capacity $[n_{c,tot}]_{eff}$ (see Eq. 2.2). Since it is related to selectivity, the reliable prediction of $f$ requires information on how the sample interacts with the two stationary/mobile-phase systems [34]. In other words, in LC × LC as much as in LC, what really determines retention and selectivity is not just columns but the phase systems (stationary and mobile phase) used. Because LC × LC samples are usually very complex mixtures and because retrieving useful selectivity information from “crowded” 1D-LC chromatograms can be arduous, analysts would like to predict $f$ using model compounds representative of the sample. However, while such a selection may be feasible if the sample composition is accurately known, the problem gets intangible when little is known about the sample [34]. In such cases, the sample itself should be used to optimize $f$. In any case, due to the intrinsic complexity of this issue, general models which for a given sample allow predicting $f$ values for any combination of two columns and mobile phases have not yet been developed. As a result, columns have so far mostly been selected experimentally and $f$ values estimated a
posteriori via the examination of LC × LC chromatograms. This second aspect is the subject of Section 2.2.2.3.2.

2.2.2.3.1 Qualitative and (semi-)quantitative strategies to predict orthogonality

For a resolution $R_s = 1$ and assuming peaks are equally dispersed, the total LC × LC separation space can be visualized as a space of $1n_c × 2n_c$ tiled boxes of area $4^1\sigma × 4^2\sigma$. When the whole separation space is “accessible”, $f$ assumes its maximum value of unity and the two separation systems are “orthogonal” (Figure 2.3(C)). “Full” orthogonality can only be achieved if the retention factors in the two dimensions are completely independent. Conversely, if the retention factors in the two dimensions are completely correlated, peaks will gather along a line in the separation space (see Figure 2.3(A)). In the case of Fig. 2.3(A), the 2D separation hardly adds any further information to the 1D one and $f = 0.1$ [31]. As a result, successful LC × LC operation requires a high degree of orthogonality as much as high efficiencies in both dimensions. Yet, how can orthogonality be achieved? Qualitative and (semi-)quantitative approaches to answer this question are discussed next.

![Figure 2.3](image)

Figure 2.3: The geometric orthogonality concept. Hypothetical separation of 100 analytes in a 10 × 10 normalized separation space. (A) Non-orthogonal system, $f = 0.1$. (B) Hypothetical ordered system, $f = 1$. (C) Random, ideally orthogonal system, $f = 0.63$. Reprinted, with permission, from ref. [79]. Copyright 2005 American Chemical Society.

When sample properties are accurately known, the “sample dimensionality” concept can provide some qualitative guidance in column selection [57]. The main idea here is that the retention of analytes arises based on so called “sample dimensionality”, which Giddings defined as “the number of independent variables that must be specified to identify the components of the sample” [57]. For example, the retention times of a sample consisting of saturated straight-chain fatty acids are dominated by molar mass (or carbon
Chapter 2

number), and for this reason such a sample is “one-dimensional”. On the other hand, a mixture of peptides has at least two dominant dimensions, molar mass, which correlates with molecular size, and net charge. Increasing the number of dimensions of a separation system (i.e., moving from 1D-size exclusion chromatography (SEC) to, for example, ion-exchange chromatography (IEC) × SEC) will only result in increased chromatographic information if the sample dimensionality is greater than or equal to the dimensionality of the separation system and if the separation system is sensitive to the relevant sample “independent variables” [57]. Sample dimensionality has been used successfully to maximize $f$ values in the separation of copolymers of ethylene and propylene oxide [58] and of butyl-terminated oligostyrene mixtures [59]. However, mechanisms regulating retention can rarely be neatly reduced to single physical properties (mass, charge, hydrophobicity, etc.). For example, SEC and RPLC display some level of correlation, because retention in RPLC is also dependent on molecular weight. Therefore, the quantitative use of sample dimensionality to predict $f$ values does not seem straightforward.

Some “more–quantitative” approaches have recently been reported which aim to maximize $f$ values for gradient × gradient RPLC × RPLC systems [16, 53, 60]. RPLC × RPLC systems show several advantages: (1) a wide variety of samples can be separated in the RP mode; (2) RP stationary phases are not only efficient, but also provide marked differences in selectivity [16]; (3) theoretical models seem to suggest that these selectivity differences can be further enhanced [16]; (4) RP mobile phases play a big role in varying both retentivity and selectivity [61]. Zhang et al. developed a simple graphical method based on the hydrophobic subtraction model (HSM) [62] for visually comparing a large number of RP stationary phases simultaneously [16]. Their approach shows that, due to their high retentivity and hydrophobicity, carbon clad columns provide rather different selectivities than the vast majority of alkyl bonded RP columns. The conclusion of Zhang et al. that these columns should be used as the 2D column in RPLC × RPLC systems has recently been adopted by Gu et al. [63]. Jandera and coworkers used linear-free-energy relationships (LFER) [60, 64] as a tool for comparing the selectivity of several LC columns [53, 65, 66]. Based on this approach, these authors showed that the combination of polyethylene glycol (PEG) columns in the 1D and porous-shell fused-core
C18 columns in the 2D provides a high degree of orthogonality when separating complex mixtures of phenolic and flavonoid natural antioxidants [65]. Jandera et al. have also developed the method of “parallel gradients” in the two dimensions [60], which has led to improved coverage of the LC × LC retention space [53, 65-67].

In conclusion, maximizing $f$ is an important aspect of LC × LC optimization. In order to do so, sample information is a necessary, yet not a sufficient condition. Our view is that, in order to reliably predict - and thus maximize - $f$, theories should first account for the choice of model compounds that accurately “mimic” sample behaviour. Based on this choice, theory should predict differences in retention that different mobile/stationary phase systems generate. The less the retention obtained on two systems correlate, the more orthogonal they are (and the closer $f$ to 1). Unfortunately, at present no current theory is adequate for the purpose. Sample dimensionality [57] is largely a qualitative tool, which is only applicable to samples whose retention is dominated by “clear” properties (carbon number, hydrophobicity, charge, molar mass, etc.) [58, 59]. More-rigorous strategies aiming to maximize $f$ by predicting retention and selectivity have recently been proposed. These can lead to optimal column selection in case chromatographers have deliberately decided to run experiments on gradient × gradient RPLC × RPLC systems [16, 66]. Clearly, in case little is known about the sample, designing an orthogonal system becomes even harder. Still, $f$ is an important factor to express the suitability of a set of phase systems. Due to the lack of models to predict $f$, its value is usually obtained a posteriori from LC × LC chromatograms, but even this is not trivial.

2.2.2.3.2 Calculating orthogonality from LC × LC chromatograms

Due to the lack of general predictive models, the “optimal” choice of phase systems (stationary and mobile phases) has so far mostly been based on analyst experience. In these cases, $f$ values can be quantified based on the a posteriori examination of LC × LC chromatograms. Several approaches measure $f$ by means of statistical and chemometrical tools [68-78]. Among these is the mathematical correlation coefficient, which was shown to fail in many circumstances (see Figure 2.4, [10]). In addition, some “more-practical” approaches have been proposed. Gilar et al. [79] divided the normalized 2D separation
space into rectangular bins and superimposed the normalized data set on this grid. The number of rectangular bins equals the number of sample components. The fraction of bins that contain peaks, $f$, can then be determined as the ratio between the number of bins containing data points, $\Sigma \text{bins}$, and the total peak capacity, $n_{c,tot}$, here calculated as the sum of all bins. Based on this definition, these authors concluded that in real LC × LC applications, $f$ is unlikely to exceed 0.63 [79]. Some theoretical shortcomings in the work of Gilar et al. have later been corrected by Watson et al. [80]. Our view is that $f$ should be calculated as the fraction of the LC × LC separation space that can be occupied by chromatographic peaks. When this is done, the reliable estimate of $f$ can be used to define the values for the initial and final organic-modifier content needed to design optimal 2D gradient conditions. Both the way to calculate $f$ and its relationship with the 2D gradient operation will be diffusely discussed in Chapter 4.

**Figure 2.4**: Demonstration of the weakness of the correlation coefficient as a metric of separation space utilization in 2D-LC systems. Reprinted, with permission, from ref. [10]. Copyright 2007 Elsevier.

In this section, practical approaches to quantify $f$ have been discussed. As no general model allows its *a priori* prediction, estimates of $f$ are often based on examining LC × LC chromatograms. In spite of the fact that a general agreement on how to do so has not yet been reached, practical applications show that full orthogonality ($f = 1$) can rarely be achieved [79]. Thus, it is optimistic to use $n_{c,tot}$ as an indication of resolving power.
Theories to support method development in LC × LC

$[n_{c,\text{tot}}]_{\text{eff}}$, a property that also includes $f$ (see Eq. 2.2), provides a more-realistic assessment of the performance of an LC × LC system.

In Section 2.2.2, we have focused on how to reliably predict and maximize $n_{c,\text{tot}}$ and $[n_{c,\text{tot}}]_{\text{eff}}$. The next step is to relate this discussion to the second important quality descriptor, the analysis time.

2.2.3 Analysis time

Eq. 2.2 calculates the key LC × LC quality descriptor peak capacity, taking into account correction factors for non-ideality. Strategies aimed at maximizing these factors - and thus the effective overall peak capacity - have been discussed in Section 2.2.2. In this and the next section, we focus on the other previously identified LC × LC quality descriptors, the total analysis time $t_{\text{tot}}$ and the limits of detection.

In LC × LC, the total analysis time $t_{\text{tot}}$ is given by the product of $t_s$ and the number of transferred fractions, $N'(fr)$. Thus, for a fixed $N'(fr)$, reducing $t_{\text{tot}}$ goes together with increasing the speed of the 2D separation. Because the number of transferred fractions typically amounts from 50 to 100, the 2D separation has to be fast. In Section 2.2.2.1, strategies to provide the best trade-off between $t_s$ and $2n_c$ have been discussed. Here, we want to emphasize the fact that, because of the complexity of the samples subjected to LC × LC, 2D separations are predominantly carried out in gradient mode. In gradient elution, $t_s$ should equal the sum of the gradient and the reconditioning times. If it is now assumed that the resolving power generated during the sampling time $t_s$ is sufficient, the 2D gradient time $2t_G$ can be considered optimized. In this case, the only way to minimize $t_s$ - and so $t_{\text{tot}}$ - is to minimize the reconditioning time, $2t_{\text{rec}}$. Our personal experience is that, when using conventional LC × LC set-ups [7], three 2D column volumes of initial gradient eluent suffice to provide run-to-run repeatability. By using modified 1D-LC instrumentation, Schellinger et al. have showed that a good run-to-run repeatability can be obtained after re-equilibration with just one column volume of eluent [81]. This approach has been exploited in LC × LC by Li et al., who were able to reduce $2t_{\text{rec}}$ to only 3 sec [20]. Recent theoretical considerations further suggest that beneficial effects on both $2n_c$ and $2t_{\text{rec}}$ can be obtained by employing multiple 2D columns operated in parallel [40].
2.2.4 Detection issues in LC × LC

In LC × LC dilution of the analytes occurs during the two successive elutions and possibly during the on-line collection, storage and transfer of the sample fractions. Strong dilution can lead to detection problems. Therefore, the limits of detection are also an important descriptor of LC × LC performance. In absence of on-column focusing (see Section 2.2.2.2) the total dilution factor, \( DF_{\text{tot}} \) in LC × LC is the product of the dilution factors in the two single dimensions [19], which are given by Eqs. 2.7 and 2.8.

\[
1DF = \sqrt{2\pi} \frac{1\sigma_i}{1V_{\text{inj}}} \tag{2.7}
\]

where \( 1V_{\text{inj}} \) is the 1D injection volume and

\[
2DF = \sqrt{2\pi} \frac{2\sigma_i}{2V_{\text{inj}}} = \sqrt{2\pi} \frac{2F \cdot 2\sigma_i}{1F \cdot ts} \tag{2.8}
\]

where \( 2\sigma_i \) includes the injection band broadening as calculated from Eq. 2.6, \( 2V_{\text{inj}} \) is the volume transferred to the 2D column and \( r \) accounts for the number of times \( 1F \) is split. Thus, \( 2V_{\text{inj}} = (1F/r) \times ts \) accounts here both for the case when the entire volume of effluent collected at the outlet of the 1D column is re-injected in the 2D column \((r = 1)\), as well as for the case in which the effluent of the 1D column is split and just a fraction \((1F/r; r > 1)\) is transferred to the 2D column. This second option is only attractive when highly sensitive detectors can be used.

In Section 2.2.2.2, we have seen that the band broadening induced by the transfer to the 2D column can be decreased by increasing the ratio \( 2F/1F \). This can be done through increasing the ratio \( 2d_c/1d_c \). However, as \( 2F/1F \) is also present in Eq. 2.8, increasing its value also results in higher values of \( 2DF \). In other words, the ratio \( 2F/1F \) should be chosen such as to provide the best compromise between transfer band broadening and dilution.
In optimized gradient \times gradient LC \times LC separations, \( DF_{\text{tot}} \) is not higher than about 50, but it can be considerably reduced if on-column focusing can be exploited [18]. In this case, the transfer band broadening is also reduced, which in turn decreases the need for a high value of \( 2F \). Narrower second-dimension columns (lower values of \( 2d_{c} \)) can then be used, resulting in a decrease in \( 2DF \). In conclusion, on-column focusing not only contributes to higher values of \( n_{c,\text{tot}} \) but also reduces the dilution factor, thus enhancing detectability [18].

2.3 Optimization aspects in LC \times LC: strategies for a rigorous choice of experimental parameters

In Section 2.2 the discussion about important LC \times LC optimization aspects, which followed the identification of total peak capacity, total analysis time and detection limits as the key LC \times LC separation-quality metrics, has led to some important conclusions, namely: (1) fraction widths between 2 and 3.6\( \sigma_{t} \) should be transferred to the \( 2D \) column to find a good balance between \( 1D \) undersampling and \( 2D \) resolving power (see Section 2.2.2.1); (2) the \( 2D \) separation has to be fast and efficient, which can be achieved by using, for example, short columns packed with small particles (see Section 2.2.2.1); (3) in case of gradient elution in the second-dimension, \( 2t_{\text{rec}} \) must be minimized (see Section 2.2.3); (4) the flow rate ratio \( \frac{2F}{1F} \) should be chosen in such a way as to avoid excessive \( 2D \) band broadening and as not to incur into detection issues (see Sections 2.2.2.2 and 2.2.4); (5) If not properly optimized, low values of the orthogonality factor (\( f \)) can lead to a strong decrease in \([n_{c,\text{tot}}]_{\text{eff}} \) (see Eq. 2.2); (6) as it simplifies the optimization process while providing a gain with respect to all three quality descriptors (peak capacity, analysis time, and detection limits), on-column focusing should be employed whenever possible (see Section 2.2.2.2);

As previously discussed, these conclusions certainly contribute towards the LC \times LC optimization goal of maximizing the overall peak capacity, minimizing the total analysis time, and minimizing dilution factors. However, they do not yet provide practitioners with clear indications on how to select column formats and chromatographic conditions, which lead to the best trade-off between these separation metrics. The rigorous selection of chromatographic conditions so as to reach the above mentioned optimization goal is a
recent yet important area of LC × LC research. As explained before, an obstacle to reaching this goal is that establishing optimum trade-offs between the three quality descriptors ultimately means finding the best compromise between several conflicting chromatographic objectives. A rigorous solution can only be achieved by means of simultaneous optimization. The complexity of addressing this problem and/or the lack of adequate theories (see Section 2.2.2.3) has so far prevented researchers from finding a general solution that accounts for all previously identified optimization “challenges”. However, significant steps towards reaching a general optimization theory have recently been set. In Section 2.3.1, state-of-the-art LC × LC theories are presented. In Section 2.3.2, “practical” guidelines for optimizing LC × LC experiments are proposed.

2.3.1 LC × LC optimization strategies leading to simultaneous optimization of peak capacity, analysis time, and dilution

The previous discussion has shown that maximizing \( n_{c,\text{tot}}^{\text{eff}} \) not only implies maximizing the efficiency of the two individual separation stages, but also requires maximizing their difference in selectivity and retention. In other words, when optimizing LC × LC systems, analysts have to simultaneously take the next two questions into account.

1) How can the first- and second-dimension particle sizes, column diameters and lengths, flow rates, and the modulation time be chosen in such a way as to find the best compromise between (effective) total peak capacity, total analysis time, and overall sample dilution?

2) How can stationary and mobile phases for the problem at hand be chosen in such a way that the orthogonality (\( f \)) is maximized?

While LC × LC theories have recently been developed that allow maximization of efficiency-related issues, the same cannot be said for selectivity/retention-related aspects. As explained before, the \emph{a priori} prediction of selectivity differences - and thus the development of a general theory – is hindered by a number of factors, including the fact that in real applications often little is known about the sample. For this reason, selectivity-related issues will not be further explored. A third important optimization aspect concerns retentivity. Clearly, compounds have to be retained on both columns.
Further, in case the two columns are correlated, analysts also have to make sure that the most retentive column is used as the 2D column. If this is not done, compounds eluting early from the 1D column are likely to be unretained on the 2D column (and thus “lost” in the solvent front). Also, using the most retentive column in the second-dimension will reduce the injection band broadening in this column (see Section 2.2.2).

As stated before, in order to maximize the efficiency-related factors, equations have to be provided that relate experimental chromatographic parameters to the three quality descriptors. In this section, theories to optimize efficiency-related LC × LC aspects are discussed. In Section 2.3.2, some general guidelines to LC × LC optimization are presented.

Optimization of LC × SEC experiments based on Poppe plots. Schoenmakers et al. [24] introduced a protocol aimed at maximizing $n_{c,tot}$, minimizing $t_{tot}$ and minimizing dilution for LC×LC systems that combine (reversed-phase or normal-phase) LC with SEC for the analysis of complex polymers. In this protocol, the analyst only has to define the maximum acceptable analysis time ($t_{tot}$), the maximum allowable pressure drop in both dimensions, and the minimum diameter of the first-dimension column ($d_1$). Then, the protocol provides “realistic” values for the column dimensions (length and diameter), particle sizes, and flow rates for the two dimensions, and the resulting 2D injection volume. In order to achieve these results, these authors [24] proposed to use so-called Poppe plots, i.e. graphs that, for a given particle size, maximum pressure drop, mobile-phase viscosity, solute molecular diffusivity, and estimates of the coefficients of the plate height equation, allow assessing the best compromise between column efficiency and speed of isocratic [14] and gradient [82] 1D-LC separations. First, these authors use Poppe plots to determine $d_p$, $N$ and $L$. Then they proceed to calculate $n_c$, $\sigma_t$ and $t_s$ [24]. Unfortunately, in doing so they considered that $\sigma_t = t_s$, which posed a far too drastic constraint on the 2D separation time (see Section 2.2.2.1). As previously discussed, a better compromise between the decrease in $n_c$ due to $\alpha$ and the decrease in $n_c$ due to “short” 2D separations would occur when $2\sigma_t \leq t_s < 3.6\sigma_t$. The longer value for $t_s$ can easily be incorporated in the protocol. Using Poppe plots again for the 2D separation, the optimum values for $L$, $d_p$ and $N$ can be calculated. The last step of the protocol consists of determining suitable $d_c$ values, flow rates and injection volumes for the two columns.
Because the maximum retention times and column lengths are known, flow rates only depend on \( d_c \) and column porosity. Once the analyst decides on a value for \( 1^{st} d_c \), the \( 2^{nd} \) injection volume is known and this value is used to select \( 2^{nd} d_c \). As previously discussed, this volume must be small enough to cause an acceptable (injection) band broadening for the second separation, but high enough not to incur into detection issues. Schoenmakers et al. suggested that the \( 1^{st} \) column be very efficient and that the \( 2^{nd} \) column be as efficient as possible within the short analysis time available [24]. In case of limited inlet pressures, highly efficient, long \( 1^{st} \) columns can be achieved by packing them with medium to coarse particles (5 to 10 \( \mu \)m). \( 2^{nd} \) columns should be short and packed with small particles, preferably between 1 and 2 \( \mu \)m, and they should be operated at high flow rates. By assuming complete orthogonality and by neglecting corrections for \( 2^{nd} \) transfer band broadening (see Eq. 2.2), Schoenmakers et al. predict that for \( t_{tot} = 220 \) min and a maximum pressure of 40 MPa, \( n_{c,tot} \) between 2500 and 5000 can be achieved [24]. However, if fewer “cuts” are made per \( 1^{st} \) peak (see above), a better compromise between \( 1^{st} \) undersampling and \( t_s \) may be achieved, which is likely to lead to higher \( n_{c,tot} \) values. Clearly, if this is done using the same \( 1^{st} \) column and chromatographic conditions as before, care must be taken that the increase in \( 2^{nd} \gamma \) does not lead to a too drastic decrease in \( 2^{nd} n_c \). As discussed in Section 2.2.2.2, the use of narrower first-dimension columns – and thus of lower values of \( 1^{st} F \) - will reduce this risk. Again, exploitation of on-column focusing can greatly simplify the choice of \( 1^{st} d_c \).

Optimization of \( LC \times LC \) experiments based on the Pareto-optimality method. The Pareto-optimality method [83] offers a convenient way to find (collections of) optimal conditions when the goal is to establish a trade-off between different conflicting objectives. Recently, Vivó-Truyols et al. were the first to apply the Pareto-optimality method to \( LC \times LC \) optimization as a strategy to achieve “the highest peak capacity within the shortest possible time and with the lowest possible sample dilution” [18]. The optimization problem can be summarized as follows. The chromatographer controls some of the parameters involved in the equations relating peak capacity, analysis time and dilution, i.e. maximum pressure drop, column diameter, gradient parameters, injection volume, column particle diameter, and modulation time. Other parameters are given by the system, i.e. coefficients for the plate-height equation, diffusion coefficients, viscosity,
retention factors for the first and last eluted compound, column porosity, column flow-
resistance factors. When substituting these parameter values, equations return values of
the column lengths, flow rates, peak capacity, analysis times and dilution factors for both
dimensions. In practice, the Pareto-optimality method allows studying the effects of the
most important chromatographic variables (column diameter, particle size, and
modulation time), on the three previously identified - total peak capacity, total analysis
time and detection limits - quality descriptors and on the recommended column lengths
and flow rates. In a first step, thousands of possible combinations of the factors that are
being optimized are generated. In a second step, the software calculates the output
parameters (dilution factors, analysis times, peak capacities, column length, etc) for each
combination of parameters. In a third step, the Pareto-optimization algorithm is applied to
discard all the cases that are not Pareto-optimal. The Pareto “front” is then constructed,
and the optimal values along this line (or surface) can be inspected. In developing their
optimization strategy, Vivó-Truyols et al. take into consideration (1) state-of-the-art 1D
undersampling theories, (2) the mode of operation – isocratic or gradient – used in each
dimension, and (3) the possibility to employ ultra-high-pressure liquid chromatography
(UHPLC) [18]. For gradient × gradient LC × LC systems, while assuming complete
orthogonality and a maximum pressure of 40 MPa in each dimension, FF = 1 (i.e., no on-
column focusing), and a transfer of about 2 cuts per 1D peak, they predicted that values of
$n_{c,tot}$ around 4000 could be obtained in 2 h and with $DF_{tot} \approx 40$. The corresponding
experimental conditions were as follows: $^1L = 500 \text{ mm}$, $^2L = 12 \text{ mm}$; $^1d_c = 1 \text{ mm}$, $^2d_c = 8
\text{ mm}$; $^1d_p = 3.3 \mu\text{m}$, $^2d_p = 1.5 \mu\text{m}$ [18]. The corresponding predicted $^1F$ and $^2F$ values were
0.029 and 1.70 mLmin$^{-1}$, respectively. For FF = 1 and $DF_{tot} \approx 10$, the attainable value of
$n_{c,tot}$ was found to decrease to about 3300. However, if FF is increased from 1 to 5, $n_{c,tot} \approx
4000$ can be obtained for $DF_{tot} \approx 10$. The chance of experiencing sensitivity problems is
then greatly reduced (see Figure 2.5). The use of UHPLC with a maximum working
pressure of 100 MPa in one of the dimensions can increase $n_{c,tot}$ by 15-20% in
comparison with an HPLC system with maximum working pressure of 40 MPa. If
UHPLC systems are used in both dimensions the potential increase in $n_{c,tot}$ will be about
25-30%. If $n_{c,tot}$ is kept constant $t_{tot}$ will decrease by 25% if UHPLC is used in one
dimension and by about 35% if UHPLC is used in both dimensions (see Figure 2.6).
Figure 2.5: (a) Pareto-optimal surface resulting from optimizing total peak capacity, total analysis time, and total dilution for a dual-gradient LC × LC system using typical chromatographic conditions. Overlaid dots represent actual Pareto experiments, whereas the surface represents the linear interpolation of the dots. Part (b) depicts the iso-dilution map corresponding to part (a) (iso-dilution lines at dilution factors of 7.5, 10 or 20 are interpolated, whereas iso-dilution lines at 30 and 40 correspond to actual Pareto experiments). Parts (c) and (d) represent the iso-dilution maps as in part (b) but considering different values of the focusing factor FF (see Eq. 2.5). Plot (c) corresponds to FF = 2 and plot (d) corresponds to FF = 5. The iso-dilution lines in parts (c) and (d) are not interpolated but represent actual computations. Adapted, with permission, from ref. [18]. Copyright 2010 American Chemical Society.

The previously described approaches based on (1) Poppe plots and (2) the Pareto-optimality method are useful tools for studying the interrelationships between peak capacity, analysis time, and dilution in LC × LC. Due to its extreme versatility in dealing with the simultaneous optimization of several (conflicting) objectives, the Pareto-optimality method appears to be a more suitable tool for optimization of LC × LC than
the approach based on Poppe plots. Further, as it is not specifically designed for a specific application, the Pareto-optimality method appears more general.

Figure 2.6: Pareto fronts in the optimization of the total peak capacity and total analysis time for an LC × LC system using typical chromatographic conditions. Gradient (“Grd”) or isocratic (“Iso”) elution modes (at 400 bar) have been considered in the first- and the second-dimension, as indicated. UHPLC (maximum pressure drop of 100 MPa) and HPLC (maximum pressure drop of 40 MPa) have also been considered in the first- and the second-dimension for the gradient × gradient. Adapted, with permission, from ref. [18]. Copyright 2010 American Chemical Society.

2.3.2 A practical optimization approach for LC × LC

We have previously discussed how the Pareto-optimality method can provide realistic estimates of $n_{c,tot}$ values for practical $t_{tot}$ and $D_{F,tot}$ values. In this section we want to expand on previously drawn conclusions about LC × LC optimization with the final aim to propose practical guidelines for the set-up of LC × LC experiments. In doing so, the
assumption is made that basic information about the sample (classes and approximate number of compounds, range of molecular weights, etc.) is known. As previously emphasized, no general optimization schemes presently allows choosing stationary and mobile phases in such a way as to achieve full orthogonality (see Section 2.2.2.3). When optimizing LC × LC systems, this aspect thus often needs to be addressed in a semi-empirical way.

Choice of stationary phases. For a limited number of cases the sample-dimensionality criterion constitutes a good qualitative strategy to maximize $f$ (see Section 2.2.2.3.1). However, if the sample is too complex, the choice of stationary phases has to be made empirically. For those classes of analytes for which the choice of stationary phases is limited, the same separation mechanism may be used in the two dimensions. Conditions here are that (a) the two columns still exhibit sufficient differences in retention and that (b) the more retentive column be used in the $2^\text{D}$. Given the fact that RP columns offer high efficiencies, a wide range of selectivities, and a great versatility and that, when combined smartly, they may allow exploitation of on-column focusing, the implementation of RPLC × RPLC systems has become increasingly popular. Yet, one must realize that the more orthogonal the two phase systems are, the less likely it is that focusing can be achieved for all analytes.

Choice of mobile phases. As emphasized in Section 2.2.2.2, the choice of the mobile phases for the two dimensions in LC × LC is a very important matter. We have previously stressed that, whenever possible, the sample eluting from the $1^\text{D}$ column should be injected dissolved in a weaker mobile phase than the one entering the $2^\text{D}$ column during the sample transfer. When this can be realized, the sample is focused in a narrow band on top of the $2^\text{D}$ column (see Section 2.2.2.2). As a result, the total dilution factor, $DF_{\text{tot}}$, is decreased for comparable analysis times and peak capacities. However, in some cases, such as NP (HILIC) × LC systems, the sample eluting from the $1^\text{D}$ is dissolved in a stronger solvent than the (initial) $2^\text{D}$ mobile phase. In that case, injection on the $2^\text{D}$ column may result in undesirable phenomena, such as band broadening, peak distortion, peak splitting or breakthrough [67, 84]. The result would be dramatic decreases in $2^\text{D}n_c$ – and thus in $n_{c,\text{tot}}$. These effects may be particularly detrimental when using short, highly efficient $2^\text{D}$ columns, which generate very small peak volumes [85].
In conclusion, we further observe that on-column focusing can also be exploited in LC × LC transfer interfaces by replacing sampling loops with “trapping” columns. This “application” of on-column focusing is particularly useful in the analysis of those analytes (e.g., peptides and proteins) which are susceptible of adsorption in interfaces and storage loops [86-88].

Sample concentration. In order not to incur into sensitivity problems, high sample concentrations should preferably be used. Given the high mass loadability of state-of-the-art chromatographic columns, the chance of 1D mass overloading is very low (see Chapter 5).

Clearly, the choice of stationary and mobile phases together with that of the sample analyzed largely determines whether isocratic or gradient elution will be used. Keeping the analysis time \( t_{\text{tot}} \) constant in the previously described Pareto-optimality approach to LC × LC optimization, Vivó-Truyols et al. predicted that selecting gradient elution in both dimensions would improve \( n_{c,\text{tot}} \) by around 50% in comparison with the isocratic × isocratic case [18]. If gradient elution is used in just one (typically the first) dimension, a 30% increase in \( n_{c,\text{tot}} \) relative to the isocratic × isocratic case can still be expected [18].

In the final step of the optimization process, the analyst should derive the efficiency-related chromatographic parameters which allow reaching the desired \( n_{c,\text{tot}} \) in a reasonable time and with acceptable dilution. As extensively discussed above, this requires setting up strategies which can deal with the simultaneous optimization of several conflicting objectives. The recently developed Pareto-optimality method approach described in Section 3.1 promises high flexibility in achieving such a goal. Hence, it seems a suitable candidate method to deal with the simultaneous optimization of peak capacity, time and dilution [18].

2.4 Conclusions

The optimization of LC × LC experiments is a relatively new, yet important research topic the aim of which is to rigorously select stationary and mobile phases, column formats, and chromatographic conditions that maximize peak capacity and minimize both analysis time and dilution factors. In LC × LC, maximizing the total peak capacity for a given analysis time and dilution requires optimizing efficiency, selectivity and retention.
Maximizing efficiency requires *simultaneously* optimizing several conflicting objectives, including first- and second-dimension peak capacity, 1D undersampling, and 2D transfer volume. As discussed in detail in this chapter, this result can only be achieved through multi-objective optimization strategies. From this point of view, a recent important step forward in method development was the introduction of the Pareto-optimality method in LC × LC optimization. This method allows determining (a collection of) optimal conditions that represent a trade-off between different conflicting objectives. For instance, for gradient × gradient LC × LC, the Pareto-optimality method predicts that peak capacities of about 4000 can be obtained in 2 h and with a dilution factor of 40. However, if on-column focusing is exploited, comparable peak capacities can be obtained in the same time with considerably lower dilution factors. These predictions are based on the rigorous optimization of chromatographic conditions. However, in case efficiency-related aspects are not carefully optimized, a loss in the total peak capacity of more than 50% may easily result. As far as optimizing selectivity and retention are concerned, no general theory currently allows predicting the degree of orthogonality that two given stationary/mobile phase systems can provide. One first issue is that sample properties need to be accurately known. Secondly, most of the research in this field has so far focused on predicting selectivity differences for the limited (but relevant) case of RPLC × RPLC experiments. Because of these limitations, the degree of orthogonality is typically calculated based on the *a posteriori* examination of LC × LC chromatograms. In spite of the theoretical shortcomings, this chapter clearly shows that important progress in LC × LC optimization has recently been made. Yet, in order to reach the ambitious goal of developing a general LC × LC optimization strategy much more effort is still required.
2.5 References


Theories to support method development in LC × LC