

# INTRODUCTION TO TWO-DIMENSIONAL LIQUID CHROMATOGRAPHY—THEORY AND PRACTICE

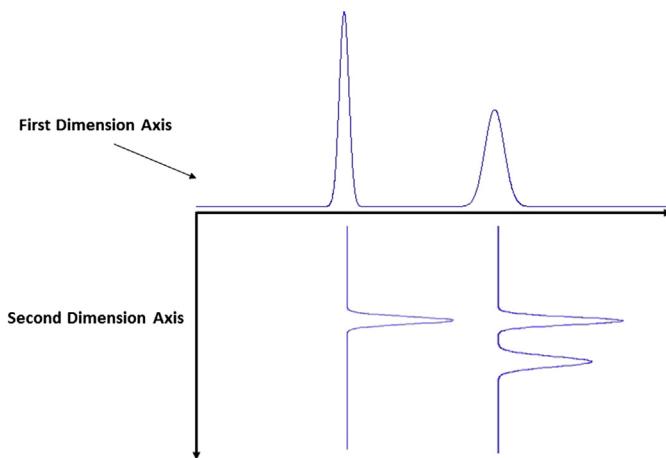
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## 1. TWO-DIMENSIONAL SEPARATIONS: CORE CONCEPTS

### 1.1 VALUE OF A SECOND DIMENSION OF SEPARATION

Our interest in liquid chromatography carried out in two dimensions is motivated by the occasions when one-dimensional (1D) separations do not allow us to achieve our separation goals, or at least not in an efficient way (Snyder et al., 1981). The reasons for failure of 1D separations fall into two broad categories: (1) cases where the sample at hand is very heterogeneous, containing hundreds, thousands, or even tens of thousands of compounds; (2) cases where the sample contains one or more pairs or groups of compounds that are chemically homogeneous and therefore difficult to resolve. The simplest view of the benefit of the added second dimension (<sup>2</sup>D) separation is shown in Fig. 7.1. The idea here is



**FIGURE 7.1**

Simple view of the benefit of a second dimension of separation. The idea is that compounds not separated by the first dimension (<sup>1</sup>D) column are transferred as mixtures to the second dimension (<sup>2</sup>D) column for further separation.

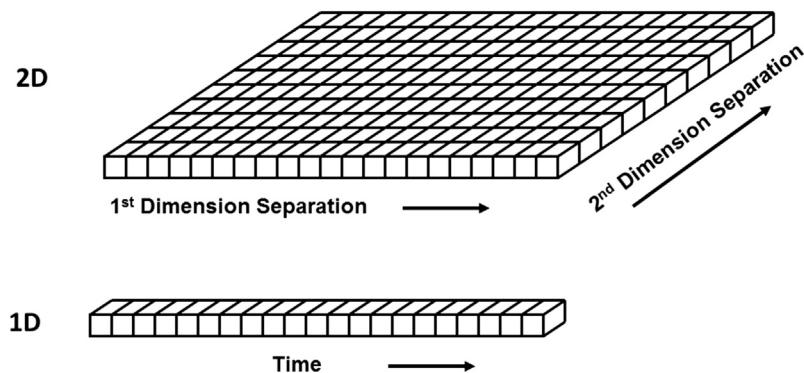
that compounds not separated by the first dimension (<sup>1</sup>D) column will be transferred as a mixture to the second dimension and separated there prior to detection at the outlet of the <sup>2</sup>D column.

## 1.2 CONCEPT OF PEAK CAPACITY

Historically, the benefit of the added <sup>2</sup>D separation has been quantified using the concept of peak capacity, especially in the case of LC × LC separations where the <sup>2</sup>D is employed during the entire <sup>1</sup>D separation. The distinctions between different types of two-dimensional liquid chromatography (2D-LC) separations and different ways of measuring 2D separation performance are discussed in detail in [Sections 5 and 6](#). [Fig. 7.2](#) illustrates one way to view the concept of peak capacity in 1D and 2D separations. The idea in the case of a 1D separation is that each bin is equivalent to one unit of peak capacity and that this bin is wide enough to accommodate exactly one chromatographic peak, where the peak width is measured at the  $4\sigma$  level ([Giddings, 1967](#)). It is important to note that the peak capacity is a theoretical maximum number of components that can be resolved. In practice the number of components that can be resolved in real samples is usually much less than the peak capacity because elution times of real components are disordered ([Davis and Giddings, 1983](#)). This issue is discussed briefly in [Section 1.2](#). In the case where gradient elution LC is used, such that the widths of peaks are nominally the same throughout the chromatogram, the 1D peak capacity ( $n_{c,1D}$ ) is simply the ratio of the size of the separation space to the average peak width ( $w_{avg}$ ), as shown in [Eq. \(7.1\)](#). In the simplest case, the size of the separation space can be taken as the gradient time ( $t_g$ ); however, for more accurate estimates factors including the column void and reequilibration times and the actual elution window of sample components should be considered ([Wang et al., 2006](#)).

$$n_{c,1D} = \frac{t_g}{w_{avg}} \quad (7.1)$$

As shown in [Fig. 7.2](#), what draws us to 2D separations is that the peak capacity is expanded from a 1D array of bins to a 2D array of bins. This means that the peak capacity of the 2D separation is not the



**FIGURE 7.2**

Illustration of the concept of peak capacity in one-dimensional and two-dimensional separations.

Adapted from Giddings, J.C., 1987. Concepts and comparisons in multidimensional separation. *HRC CC J. High Resolut. Chromatogr. Chromatogr. Commun.* 10, 319–323. <http://dx.doi.org/10.1002/jhrc.1240100517>.

sum of the peak capacities of the two 1D separations (i.e., the  $^1\text{D}$  and  $^2\text{D}$  peak capacities,  $^1n_c$  and  $^2n_c$ ) that contribute to the 2D separation, but the product of them (Giddings, 1987; Karger et al., 1973). We quantify this relationship using what we refer to here at the product rule, shown in Eq. (7.2).

$$n_{c,2\text{D}} = ^1n_c \times ^2n_c \quad (7.2)$$

It is critically important to recognize that the product rule provides an estimate of the *maximum number* of compounds that can be resolved with a 2D-LC method. In real separations, the actual numbers of peaks that are resolved are much lower than this for a few reasons. First, as is the case in 1D separations, the distributions of compound elution times are usually not highly ordered, leading to some regions of unused separation space, whereas other regions are overcrowded. Second, it is generally more difficult to effectively use all of the available separation space in 2D separations than in 1D ones. In other words, spreading peaks across the bins of a 1D array is easier than spreading them across a 2D array of bins, which effectively reduces the amount of usable 2D peak capacity. This issue is discussed in more detail in Sections 5.1.2 and 7.4. Finally, in the process of transferring fractions of  $^1\text{D}$  effluent to the  $^2\text{D}$  column for further separation, there can be remixing of sample components that had been already separated by the  $^1\text{D}$  column. This too effectively results in a loss of peak capacity and thus detracts from the potential indicated by Eq. (7.2). This issue is discussed in more detail in Section 5.2. Consideration of these practical details is important for developing high-performing 2D-LC methods and fair assessment of their performance.

Peak capacity is a very convenient metric for assessing the performance of 2D-LC separations carried out in the comprehensive mode. However, as discussed in Section 6.1, probably less than half of all 2D-LC in practice is done in the comprehensive mode. For other modes of 2D separation, other metrics of performance are more appropriate. Nevertheless, the fundamental concepts that come together in evaluating peak capacity—namely the complementarity of separation mechanisms and undersampling—are still critically important to noncomprehensive 2D separations, even if they are more difficult to quantify in those cases.

### 1.3 INTRODUCTION TO STATISTICAL OVERLAP THEORY FOR CHROMATOGRAPHY

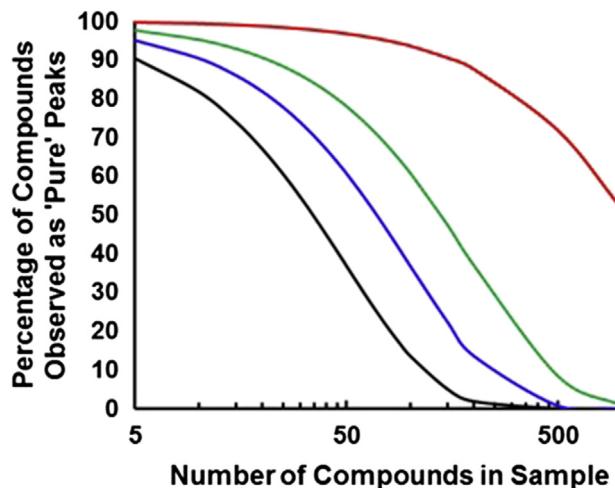
Given that our interest in 2D separations is largely predicated on the limitations of 1D separations, it is useful to have an appreciation for these limitations in quantitative terms. In the preceding section, I alluded to the fact that peaks in real chromatograms tend not to be highly ordered. A practically important consequence of this is that the number of peaks we observe is less—sometimes much less—than the estimated peak capacity of the separation. A very useful framework for thinking about these issues is the so-called *statistical overlap theory* (SOT), which was developed by Davis and Giddings (1983) for 1D separations, and subsequently extended to 2D separations (Davis, 1991). The rate at which we can produce peak capacity using one-dimensional liquid chromatography (1D-LC) is discussed in more detail in Section 5.3. Here, it is useful to know that for small nonpeptide molecules, we can achieve peak capacities of about 150, 250, and 350 in 1D analysis times of 5, 20, and 60 min. SOT enables us to answer a variety of questions about how this peak capacity can be used to work toward particular separation goals. For example, for a given peak capacity one can estimate what fraction of components in a sample would be observed in a chromatogram as pure, single-component peaks, or as peaks that are multiplets (i.e., a peak that contains multiple, coeluting compounds). For practical problems, perhaps the most instructive question is—what is the likelihood of

chromatographically resolving *all* of the components of a particular sample, given the peak capacity of a particular separation method? We can answer this question using the principles of SOT and Eq. (7.3):

$$s = m \times \exp\left(\frac{-2m}{n_c}\right) \quad (7.3)$$

where  $s$  is the number of observed peaks that are singlets (i.e., pure peaks with no coelution),  $m$  is the number of components in the sample, and  $n_c$  is the peak capacity of the method. Fig. 7.3 shows the percentage of components in a sample that are resolved as single-component peaks for several practically relevant combinations of sample complexity and available peak capacity. In the context of a discussion about 2D separations, the most important observation from this figure is just how unlikely complete resolution (i.e., 100% of peaks are singlets) is for samples of even moderate complexity with the peak capacities provided by the best available 1D chromatography in reasonable analysis times.

Resolving more than 90% of the compounds in a 20-component sample is only achievable, from a probabilistic point of view, in a reasonable analysis time using 2D-LC.



**FIGURE 7.3**

Percentage of sample constituents that are resolvable as chromatographically distinct peaks (“singlets,” with a minimum resolution of 1.0) as a function of the number of compounds in a sample and different separation peak capacities. Effective peak capacities of **100** and **200** (dark gray in print versions) are easily achievable within 15 min using modern particle and instrument technologies. A peak capacity of **400** (light gray in print versions) is more difficult to reach for small molecules, but can be reached within an hour for peptides. An *effective peak capacity* of **3000** (gray in print versions) is not accessible by one-dimensional liquid chromatography in a practically reasonable time, but can be achieved by two-dimensional liquid chromatography in 1–2 h. Singlet peak numbers were calculated assuming the same concentrations for all components and using Eq. (7.3) from Davis and Giddings.

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## 2. SCOPE OF THIS CHAPTER

In this chapter, I have aimed to provide prospective and current users of 2D-LC with a resource that is rich with practical guidance for developing effective, high-performing 2D-LC methods, and informed by guiding theoretical principles. As the field of 2D-LC develops, research in some aspects matures more quickly than others. I have touched on the more mature aspects only lightly, providing more room for discussion of those areas that are very active right now and likely will be for the foreseeable future. The chapter is not intended as a comprehensive review, thus the literature is not cited exhaustively in all areas. I have not dedicated too much space to the most basic operating principles of 2D-LC—the first diagram of a complete 2D-LC instrument is shown in [Fig. 7.26](#). Readers looking to pick up some of these more basic details are referred to the review of [Malerod et al. \(2010\)](#), which gives a very concise but effective overview. As for specific applications, this chapter only provides an overview of the types of 2D-LC methods that have been implemented in such fields as pharmaceutical and food analysis (see [Section 10](#)), with occasional examples being used to illustrate important principles from theory and/or practice.

Importantly, I have chosen to focus the chapter nearly entirely on “online” 2D-LC separations—that is, cases where the entire 2D-LC separation is executed in a single contained flow path, without collecting fractions of  $^1\text{D}$  effluent into vials or plates for storage prior to  $^2\text{D}$  separation. While there undoubtedly is a place for off-line 2D-LC separations, commercial instrumentation and current user preference are tending strongly toward online operation for most 2D-LC work. Readers interested in off-line 2D-LC separations are referred to review articles that treat this subject in detail ([Guiochon et al., 2008](#)).

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## 3. NOMENCLATURE

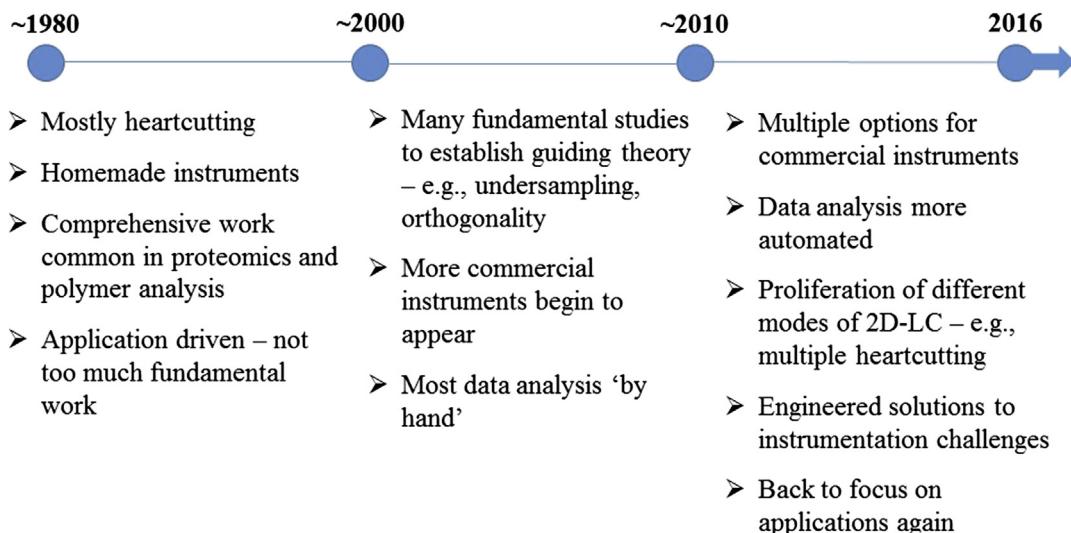
In this chapter, I have generally used the nomenclature suggested by Marriott and Schoenmakers for 2D separations ([Marriott et al., 2012](#); [Schoenmakers et al., 2003](#)). Particularly important in this scheme is the distinction between  $^1\text{D}$  and 1D and  $^2\text{D}$  and 2D. The abbreviations  $^1\text{D}$  and  $^2\text{D}$  are used to refer to components of the first and second dimensions of a 2D-LC system, respectively. So, we might refer to a  $^1\text{D}$  column or a  $^2\text{D}$  flow rate. These would be the column used in the first dimension, and the flow rate used in the second dimension. On the other hand, the use of **1D** and **2D** is reserved for situations where we want to refer to the entire system as being either one- or two-dimensional. For example, we may refer to the *peak capacity of a 1D separation*, or a *2D separation of natural products*.

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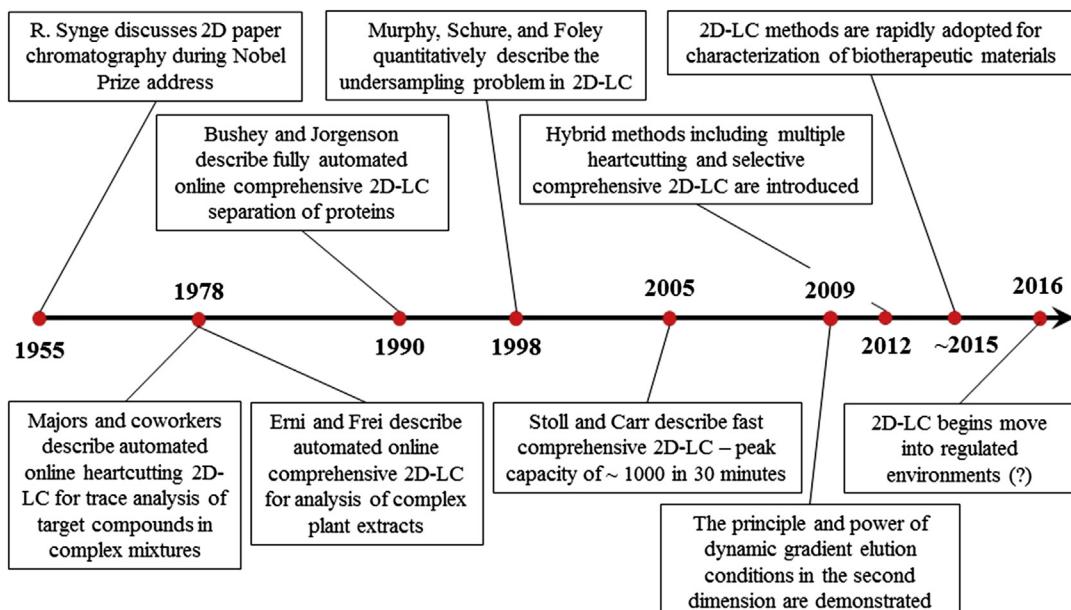
## 4. BRIEF HISTORY AND DEVELOPMENTAL MILESTONES

As we consider the state of the art of 2D-LC, and where the technology may develop in the future, it is useful to have a sense for how the technique has developed over the years up to this point. [Figs. 7.4 and 7.5](#) show some of the characteristics of the three stages of development over the past 35 years, and some of the milestones that have been especially important along the way.

The first era, from the late 1970s to about 2000, was dominated by heartcutting applications, with the exception of comprehensive work in the areas of proteomics and polymer analysis. In this period most instruments were “home-made,” or highly specialized commercial systems, and the majority of the literature was focused on applications of 2D-LC rather than fundamental studies.

**FIGURE 7.4**

Some characteristics of the different eras of development of two-dimensional liquid chromatography (2D-LC).

**FIGURE 7.5**

Some key milestones in the development of two-dimensional liquid chromatography (2D-LC). References for these events by year are: 1955 (Synge, 1952), 1978 (Appfel et al., 1981; Erni and Frei, 1978), 1990 (Bushey and Jorgenson, 1990), 1998 (Murphy et al., 1998), 2005 (Stoll and Carr, 2005), 2009 (Bedani et al., 2009; Jandera et al., 2010), 2012 (Groskreutz et al., 2012a; Zhang et al., 2013a), 2015 (Stoll et al., 2016), and 2016 (Largy et al., 2016).

The decade from about 2000 to 2010 was a very important period. In this era, several research groups engaged in fundamental studies that led to the principles (see [Section 5](#)) we now use to guide method development and assess the performance of the resulting 2D-LC methods. As it became clear that 2D-LC would be competitive with 1D-LC, even at analysis times typical of high-performance liquid chromatography (HPLC) (e.g., 15 min), instrument manufacturers began making significant investments in the development of dedicated instrumentation for 2D-LC.

Users engaging 2D-LC technology now for the first time will experience a landscape that is very different from what we observed as little as 5 years ago. In the current era, we find that several mainstream instrument vendors now offer both hardware and software that have been developed for 2D-LC applications. In some cases, they have addressed engineering challenges that are unique to 2D-LC to produce hardware that is more robust compared to the repurposed components from 1D-LC instruments that we used in past construction of home-made instruments. Now that users can simply buy a ready-made 2D-LC instrument, we are seeing the focus of research shift back to applications. Very importantly, we are seeing a proliferation of what I call “hybrid modes” of 2D-LC that are intermediate between the extreme implementations of single heartcutting and fully comprehensive 2D separation. These are discussed in detail in [Section 6.1](#) and include approaches such as *multiple heartcutting* and *selective comprehensive* ( $sLC \times LC$ ) 2D separations. Ultimately, these hybrid modes provide users tremendous operational flexibility to use the power of 2D separation to address the needs of their application in the most effective way. Indeed, this is an exciting time for 2D-LC.

[Fig. 7.5](#) is a timeline that lists some of the studies that have had the most impact on the development of 2D-LC and its adoption by users in a variety of fields. The principle of 2D separation was initially demonstrated over 70 years ago using paper chromatography for the separation of amino acids by [Consden et al. \(1944\)](#). The performance potential of 2D separation was highlighted again nearly 10 years later by [Synge \(1952\)](#) in his address on his acceptance of the Nobel Prize. However, the landmark publications demonstrating the feasibility of 2D separation in liquid chromatography came around 1980 with a pair of papers—one by [Appfel et al. \(1981\)](#) and one by [Erni and Frei \(1978\)](#). These proof-of-concept papers, along with several influential papers from this period on the theory of 2D separation ([Giddings, 1987, 1984; Guiochon et al., 1983; Snyder et al., 1981](#)) undoubtedly captured the imagination of a generation of chromatographers. The demonstration of *heartcutting* 2D-LC ( $LC-LC$ ) by Majors et al. led to the development of thousands of methods for quantitation of target compounds in complex mixtures, sometimes at the trace level. This type of method has come to be known by a number of different names, such as *column switching*, and is still in common use today. The extreme opposite of  $LC-LC$  is the so-called *comprehensive* mode of 2D separation ( $LC \times LC$ ), where all of the material eluting from the  $^1D$  column is subjected to further separation using the  $^2D$  column. In this way, we gain a more comprehensive view of what the sample contains, and this is what was demonstrated by Erni and Frei through their  $LC \times LC$  separation of a plant extract. Although some will assert that their initial work does not satisfy the modern definition of *comprehensive*, the value of this early proof-of-concept was that it showed people what was possible in the analysis of complex materials. This was followed just a decade later by the landmark paper of Bushey and Jorgenson, who demonstrated an  $LC \times LC$  separation of intact proteins in an analysis time of 6 h.

One of the most cited fundamental papers in the 2D-LC literature is from [Murphy et al. \(1998\)](#), who discussed the significance of the rate at which fractions of  $^1D$  effluent are transferred to a  $^2D$  column for further separation. In  $LC \times LC$  in particular, sampling the  $^1D$  separation at a rate that is fast enough to avoid remixing sample components that had already been separated by the  $^1D$  column is very

difficult. This results in a situation that has become known in the literature as *undersampling*, and in their landmark paper Murphy et al. quantified this effect for the first time. The influence of this work on the development of 2D-LC cannot be overstated; it has led several groups to study ways to mitigate the effects of undersampling by developing high speed  $^2\text{D}$  separations, and other approaches such as  $\text{SLC} \times \text{LC}$ . A detailed discussion of the undersampling problem is provided in Section 5.2.

In 2005 we began describing  $\text{LC} \times \text{LC}$  separations with very fast  $^2\text{D}$  gradient elution separations, on the order of 20 s, to mitigate the undersampling problem (Stoll et al., 2006; Stoll and Carr, 2005). Back then, this was difficult because of the large gradient delay volumes associated with high-pressure pumping systems. These delay volumes have dropped dramatically with the current generation of pumping systems, such that these fast  $^2\text{D}$  separations are becoming common (Gargano et al., 2016; Vanhoenacker et al., 2015). An unexpected, but very important outcome of this period of our work was the finding that the performance of  $\text{LC} \times \text{LC}$ , as measured by peak capacity, begins to exceed that of conventional 1D-LC at analysis times around 10 min. This means that 2D-LC has the potential to displace 1D-LC not only at long analysis times, but also at intermediate times that are associated with typical chromatography methods.

Before 2009, most work in 2D-LC involved separations where the  $^2\text{D}$  elution conditions were fixed throughout the 2D analysis. That is, even if gradient elution was used in the  $^2\text{D}$ , a particular set of elution conditions was used repeatedly through the analysis. Work by Bedani et al. (2009) and the Jandera group (Jandera et al., 2010) first showed that dynamically adjusting  $^2\text{D}$  gradient elution conditions during the 2D analysis is feasible. They then went on to discuss the value of this capability, as it enables more complete usage of the 2D separation space. These concepts and capabilities have fundamentally changed the way we approach the optimization of 2D-LC methods. Some of the practical details are discussed in more detail in Section 7.4.

Informed by the challenges associated with undersampling and motivated by the need to develop 2D separation strategies for samples of intermediate complexity, hybrid modes of 2D separation began to appear around 2012, and are rapidly being adopted in a variety of industries. Specifically, multiple heartcutting ( $\text{mLC-LC}$ ) (Zhang et al., 2013a) and selective comprehensive ( $\text{sLC} \times \text{LC}$ ) (Groskreutz et al., 2012a) are approaches that maximize the benefits of the more extreme implementations of  $\text{LC-LC}$  and  $\text{LC} \times \text{LC}$ , while minimizing their weaknesses. Introduction of these alternative modes of 2D separation has resulted in a shift in mindset that encourages users to be open-minded and select the mode of operation that is most suitable for their analytical problem. The details associated with different modes of implementation are discussed in Section 6.1.

Finally, one of the areas of fastest adoption of 2D-LC is currently in the biopharmaceutical industry. The complexity of the samples (e.g., peptides, large proteins, drug formulations) encountered in this space demands high-resolution separation techniques. Importantly, the properties of the molecules and samples also lend themselves nicely to 2D separation in many cases. For example, antibody-based drugs in a formulation can vary significantly both in their size (because of aggregation) and charge (because of amino-acid sequence differences), thus separation by size-exclusion (SEC) in one dimension and ion-exchange (IEX) in a second dimension could provide a very effective 2D separation of these molecules. We are currently seeing development of 2D-LC methods for these applications that span the full range of implementations ranging from  $\text{LC-LC}$  to  $\text{LC} \times \text{LC}$ ; a recent review article summarized this work as of 2015 (Stoll et al., 2016). Perhaps most important to the future of 2D-LC is that we are now seeing the movement of 2D-LC into regulated environments as

quality assurance and quality control methods ([Largy et al., 2016](#)). If this trend continues, it will cement the role of 2D-LC as an important, mainstream technique for the foreseeable future.

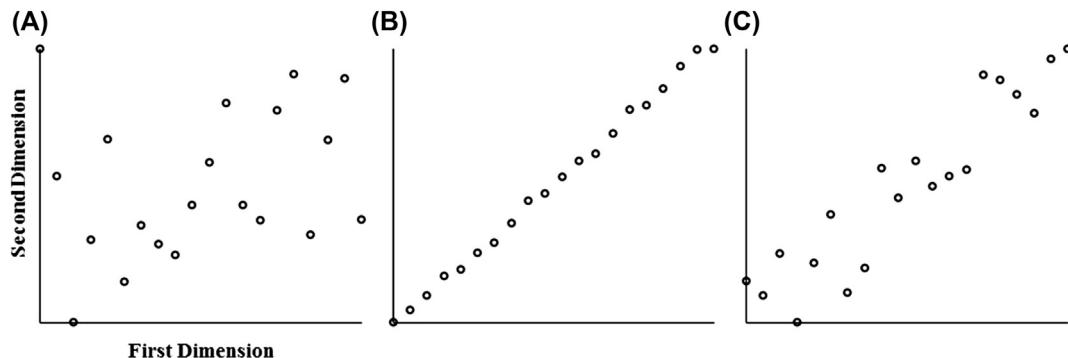
## 5. PRIMARY THEORETICAL GUIDING PRINCIPLES

Readers interested in quickly assimilating the essential guiding principles in 2D-LC are encouraged to read the excellent, succinct review of [Bedani et al. \(2012\)](#). In this section, we first quickly review the core concepts that underpin method development decisions concerning choice of separation modes and stationary phases, and the rate at which the  $^1\text{D}$  separation is sampled. We then provide updates from the literature on these topics from the last 5 years or so, and go on to discuss ideas that inform decisions about whether to even do 2D-LC separations, or just stick with conventional 1D-LC. In reading the last section in particular, it is important to not lose sight of the metrics of separation that are most important for you and your applications. Just because certain aspects of 2D chromatograms are easy to measure—such as peak capacity and coverage of the separation space—they may not be as important as other metrics, such as detection sensitivity or resolution of particular sets of compounds in a mixture.

### 5.1 COMPLEMENTARITY OF RETENTION MECHANISMS

#### 5.1.1 Estimation of Separation Space Usage

One of the essential concepts in any 2D separation is that the separation modes or mechanisms used in the two dimensions must be different enough that there is a reasonable chance that two compounds coeluting will be separated on the second, complementary column ([Giddings, 1987](#); [Karger et al., 1973](#)). This point is frequently made graphically using something like that shown in [Fig. 7.6](#). Panel A shows the best case scenario, where there is no evident correlation between retention on the first column with retention on the second column. In this case there is a very good chance that two compounds coeluting on the first column will be separated on the second column, provided the separation efficiency of the second column is high enough. Panel B shows the worst-case scenario, where



**FIGURE 7.6**

Illustration of the complementarity of retention mechanisms in 2D separations. (A) Example of peak pattern obtained with highly complementary  $^1\text{D}$  and  $^2\text{D}$  separations; (B) peak pattern obtained with separations that are not complementary; and (C) example of peak pattern obtained with separations that are somewhat complementary.

retention on the two columns is very highly correlated. In this case 2D separations are usually a waste of time, and better results can be obtained with highly optimized 1D separations. One notable exception to this exists when closely eluting peaks vary dramatically in their concentration in the sample. Readers interested in this concept are referred to the work of [Venkatramani et al. \(2014\)](#), who have demonstrated the point effectively, using samples of interest to the pharmaceutical industry. Panel C reflects the situation we face in 2D-LC analyses of real samples. In these cases, there often are some areas of the chromatogram where there is a high density of peaks, whereas other areas are sparsely occupied.

If the goal in 2D separations is to choose separation modes and stationary phases that are highly complementary, then the challenge we face is evaluating this degree of complementarity using data from chromatograms like that shown in Panel C. Early in the development of this aspect of the 2D-LC literature the term “orthogonality” was used to describe the extent to which two separation modes were complementary. Over the years many methods have been proposed and evaluated for assessing orthogonality/complementarity, ranging from very simple yet intuitive methods to much more sophisticated mathematically oriented methods. The principles of these different approaches and their advantages and disadvantages have been discussed at length elsewhere and these details are not repeated here. Readers interested in this topic are referred to a number of reviews and other articles ([Camenzuli and Schoenmakers, 2014](#); [Carr et al., 2012](#); [Gilar et al., 2012](#); [Schure and Davis, 2015](#)). [Figs. 7.7 and 7.8](#) illustrate two of the more intuitive yet effective methods. [Schure and Davis \(2015\)](#) have suggested that optimization of 2D-LC separations should be guided using information from both of these methods. [Fig. 7.7](#) shows the implementation of a so-called “box-counting” method, which was initially described by [Gilar et al. \(2005\)](#) and adapted by [Davis et al. \(2008a\)](#). In this approach the separation space is divided into a number of discrete boxes, and the fraction of the boxes containing peaks is calculated relative to the total number of boxes in the space. The results obtained from this type of approach are sensitive to the box dimensions ([Rutan et al., 2011](#), p. 267), thus this should be considered carefully.

[Fig. 7.8](#) illustrates the implementation of convex hulls as a way to estimate the fraction of 2D separation space that is used for the analysis of a particular sample. In simple terms, this amounts drawing a polygon around the peak pattern observed, where each vertex in the polygon is centered on a chromatographic peak. Because there is no decision to be made about box dimensions, this approach is even more straightforward than the box-counting methods.

Both the box-counting and convex-hull methods are prone to overestimation of the effectiveness of the separation space usage in cases where there are a few outlying peaks surrounding a small space occupied by a much larger number of peaks. In other words, these methods are insensitive to the distribution of peaks across the space that they occupy. Other methods, such as the so-called asterisk equations developed by [Camenzuli and Schoenmakers \(2014\)](#), more effectively address the issue of peak distribution, but are a bit less intuitive.

### 5.1.2 Correction of Peak Capacities for Incomplete Usage of Separation Space

Both of the methods discussed above and illustrated in [Figs. 7.7 and 7.8](#) enable estimation of the fraction of the 2D separation space that is occupied by peaks in the analysis of real samples. Obviously, maximizing this fractional coverage ( $f_{cov}$ ) of the separation space is an important goal in method development. Equally important is the idea that this  $f_{cov}$  value can be used to make a correction to the

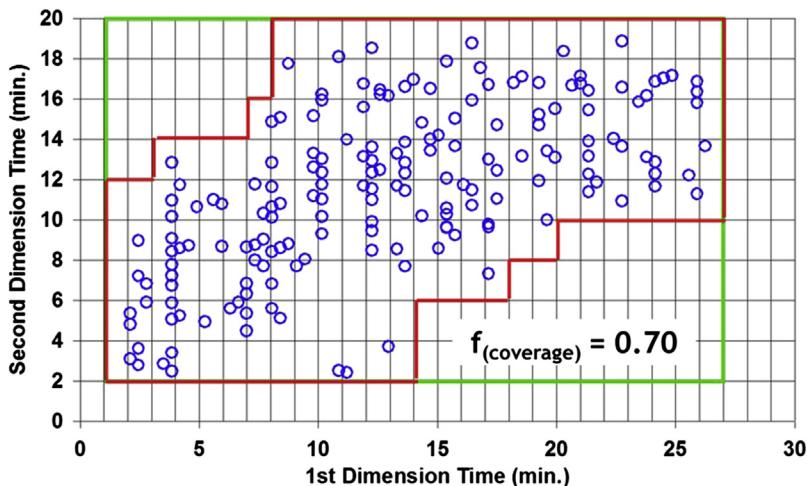
**FIGURE 7.7**

Illustration of a box-counting method, which counts the fraction of bins in a two-dimensional separation bed that is occupied by peaks. This fraction enables correction of the total peak capacity for incomplete usage of the separation space. Circles represent coordinates of observed peaks in an experimental LC  $\times$  LC separation. The red (gray line in print versions) line defines the perimeter around the bins that are considered to be occupied. LC, liquid chromatography.

*Reprinted with permission from Davis, J.M., Stoll, D.R., Carr, P.W., 2008a. Dependence of effective peak capacity in comprehensive two-dimensional separations on the distribution of peak capacity between the two dimensions. Anal. Chem. 80, 8122–8134. <http://dx.doi.org/10.1021/ac800933z>. Copyright 2008, American Chemical Society.*

2D peak capacity estimated using the product rule (see Eq. 7.2) (Liu et al., 1995; Stoll et al., 2008). This is done by simply multiplying the 1D and 2D peak capacities by the  $f_{\text{cov}}$  value as in Eq. (7.4).

$$n_{c,2D}^* = {}^1n_c \times {}^2n_c \times f_{\text{cov}} \quad (7.4)$$

Given that  $n_{c,2D}^*$  is directly proportional to  $f_{\text{cov}}$ , judicious choice of complementary <sup>1</sup>D and <sup>2</sup>D separations, along with optimization of elution conditions, can be a powerful way of increasing the practically useful peak capacity.

### 5.1.3 Combinations of Modes

There are many ways that one can imagine combining two complementary separations in a 2D separation scheme; Giddings (1984) discussed the scope of these thought experiments in the broadest terms. However, many of the conceivable combinations are not at all practical, for example, due to excessive dilution of the analyte (Schure, 1999) or very low separation efficiency in one or both of the separation steps. To help guide method development, we can score the commonly used and discussed combinations of modes using several attributes that are important in practice. Table 7.1 is an attempt at such scoring. It is important to recognize that the scores represent generalizations—indeed there are some niche applications where the combination of IEX and SEC may be very important (e.g., in

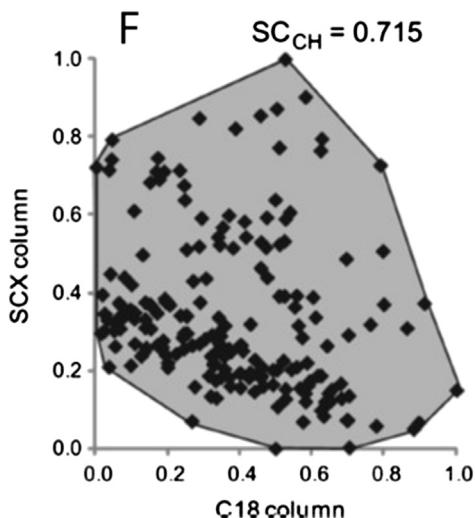
**FIGURE 7.8**

Illustration of the use of convex hulls to estimate the fraction of separation space occupied by peaks.

*Reprinted with permission from Gilar, M., Fridrich, J., Schure, M.R., Jaworski, A., 2012. Comparison of orthogonality estimation methods for the two-dimensional separations of peptides. Anal. Chem. 84, 8722–8732. <http://dx.doi.org/10.1021/ac3020214>.*

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determination of the molecular weights and charge-state distributions of a protein mixture). To commit to a combination with a low score in developing a new method, there should be a compelling reason to choose that combination that overcomes other poor attributes of the combination. One trend that emerges from this analysis is that pairing two reversed-phase (RP) separations is attractive for a variety of reasons. This may seem counterintuitive because historically RP phases have been perceived as

**Table 7.1 Comparison of Different Possible Combinations of Liquid Chromatography Separation Modes**

Modes <sup>a</sup>	IEX × RP	SEC × RP	NP × RP	RP × RP	HILIC × RP	HILIC × HILIC	AC × RP	SEC × NP	SEC × IEX
Orthogonality	++	++	++	+	+	-	++	+	+
Peak capacity	+	+	+	++	+	+	-	-	-
Peak capacity/time	-	-	+	++	+	+	-	-	-
Solvent compatibility	+	+	-	++	+	++	+	+	+
Applicability	+	+	-	++	+	-	+	-	-
Score	4	3	1	9	5	2	2	-2	-3

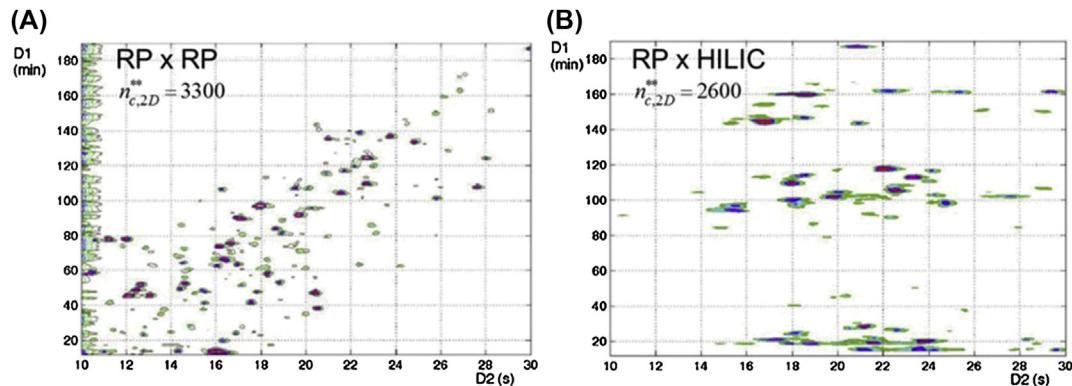
<sup>a</sup>AC, argentation; HILIC, hydrophilic interaction liquid chromatography; IEX, ion-exchange; NP, normal phase; RP, reversed-phase; SEC, size-exclusion.

having similar selectivity. Indeed, choosing two RP phases that are different enough to be useful in a 2D separation is an important consideration—this is discussed in more detail in [Section 7.2](#). Nevertheless, there are several other attributes of RP separations that make the RP × RP combination very attractive, including high efficiency, miscibility of mobile phases used in the two separations, compatibility with mass spectrometric (MS) detection, and compatibility with large biomolecules (e.g., proteins).

This has led us and others to focus on the use of RP × RP separations to address a variety of separation problems ranging from the analysis of biological extracts ([Stoll et al., 2007](#)) to peptide separations ([Sarrut et al., 2015; Vanhoenacker et al., 2015](#)) and analysis of environmental contaminants ([Simpkins et al., 2010](#)). Readers interested in practical details associated with these types of separations are referred to the review of Li and Schmitz that is focused on this topic ([Li et al., 2014b](#)).

#### **5.1.4 Separation Modes Should Be Chosen Carefully**

In this [Section 5.1](#) we have emphasized the importance of choosing  $^1\text{D}$  and  $^2\text{D}$  separation modes that complement each other in terms of selectivity. However, we must also be careful not to sacrifice other measures of 2D separation performance in the name of obtaining high fractional coverage. [Fig. 7.9](#) from the work of the Heinisch group clearly makes this point by way of example LC × LC separations of peptides ([D'Attoma and Heinisch, 2013](#)). Coupling RP–LC and hydrophilic interaction liquid chromatography (HILIC) separations together for 2D-LC is attractive from the point of view of selectivity because their separation mechanisms are known to be very different ([Separation mechanisms in hydrophilic interaction chromatography, 2013](#)). However, in the case of these peptide separations, it is evident that the  $^2\text{D}$  peaks are much wider when HILIC is used in the second dimension compared to reversed-phase liquid chromatography (RP-LC). The authors offer possible explanations



**FIGURE 7.9**

Comparison of LC × LC separations of peptides using RP–LC in the first dimension and either RP–LC (A) or HILIC (B) separation in the second dimension. In spite of the higher fractional coverage in the RP × HILIC case, the effective 2D peak capacity is lower because of the low peak capacity of the HILIC  $^2\text{D}$  separation. *HILIC*, hydrophilic interaction liquid chromatography; *LC*, liquid chromatography; *RP*, reversed-phase.

*Reprinted with permission from D'Attoma, A., Heinisch, S., 2013. On-line comprehensive two dimensional separations of charged compounds using reversed-phase high performance liquid chromatography and hydrophilic interaction chromatography. Part II: application to the separation of peptides. J. Chromatogr. A 1306, 27–36. <http://dx.doi.org/10.1016/j.chroma.2013.07.048>.*

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for the wider peaks, but the cause is irrelevant to the main point here. Specifically, the advantage of the increased orthogonality offered by the HILIC separation is outweighed by the disadvantage of its poorer peak width, resulting in a lower effective 2D peak capacity for the RP  $\times$  HILIC separation compared to the RP  $\times$  RP separation.

## 5.2 THE CONCEPT OF UNDERSAMPLING

In 1998, Murphy et al. pointed out in their seminal paper that the rate at which fractions of <sup>1</sup>D effluent are transferred to the <sup>2</sup>D column for further separation, relative to the native <sup>1</sup>D peak width, has an impact on the effective peak capacity of the <sup>1</sup>D separation. The development of our understanding of this issue between then and now has been discussed extensively elsewhere (Carr et al., 2012; Davis et al., 2008b; Horie et al., 2007; Seeley, 2002) and is not repeated here. In the extreme case where the time period over which a single fraction of <sup>1</sup>D effluent collected is large relative to the native widths of <sup>1</sup>D peaks, there is remixing of the <sup>1</sup>D peaks collected in that time interval as they are transferred to the <sup>2</sup>D column for further separation. In other words, this part of the <sup>1</sup>D separation is “undone,” which is obviously counterproductive. Fig. 7.10 illustrates this point graphically. In cases where the <sup>2</sup>D separation does not contribute significantly to the total 2D resolution, using a sampling time ( $t_s$ ) that is large relative to the native <sup>1</sup>D peak width results in serious loss of resolution.

Another perspective on this issue is shown in Fig. 7.11, which shows reconstructions of the <sup>1</sup>D separation after sampling at different rates. This makes it clear that the loss in 2D resolution originates from loss of resolution at the <sup>1</sup>D stage of the separation.

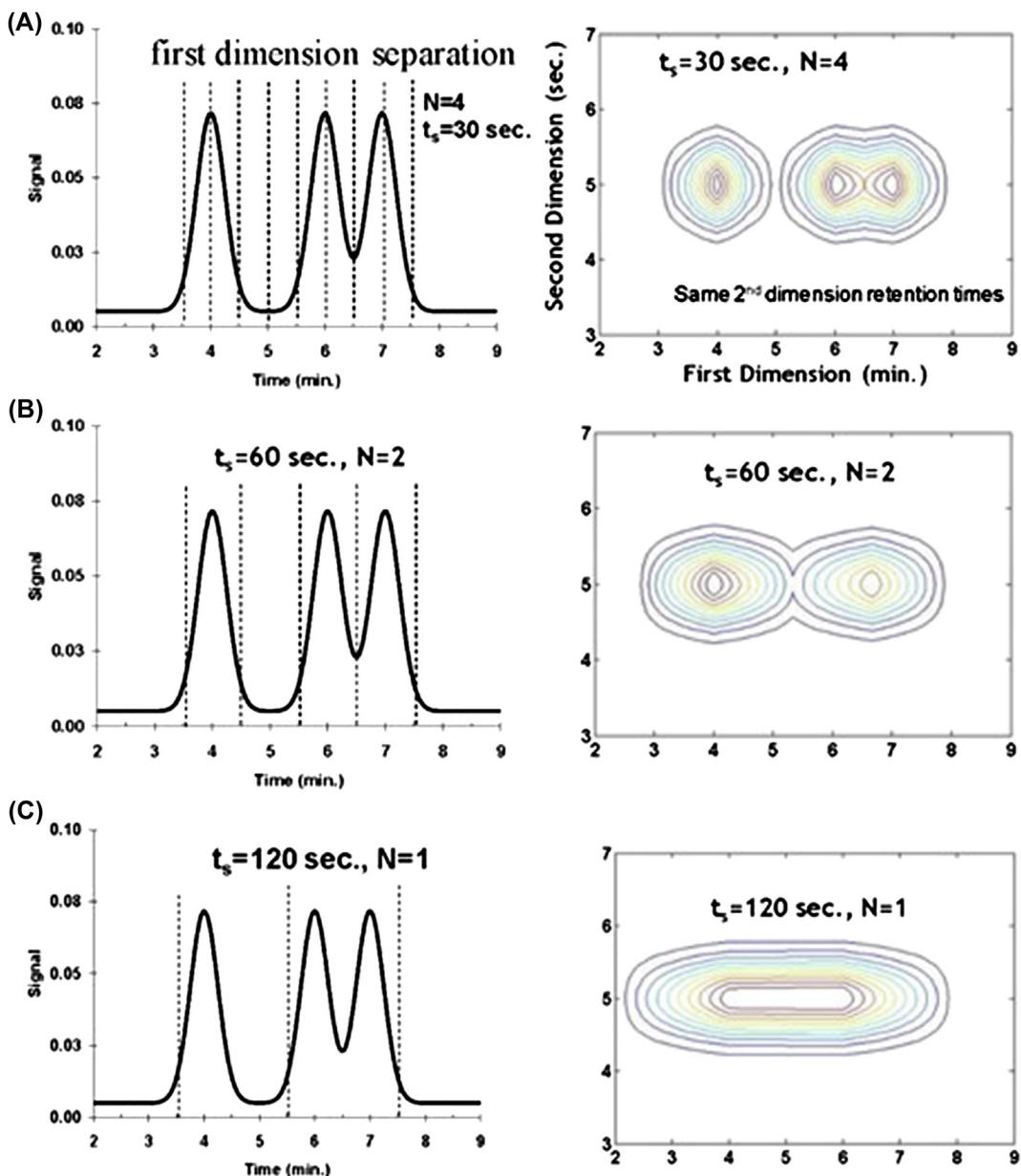
Using simulations (Davis et al., 2008b) we developed an empirical relationship that helps us understand the magnitude of the loss of <sup>1</sup>D peak capacity as the sampling time is increased. Eq. (7.5) gives an expression for  $\langle\beta\rangle$ , which is the average ratio of <sup>1</sup>D peak widths after and before sampling, as a function of the sampling time ( $t_s$ ) and the native <sup>1</sup>D peak width (<sup>1</sup> $\sigma$ ). This  $\langle\beta\rangle$  value can then be used to make a correction to the <sup>1</sup>D peak capacity that quantitatively accounts for the remixing of <sup>1</sup>D peaks, as in Eq. (7.6).

$$\langle\beta\rangle = \sqrt{1 + 0.21\left(\frac{t_s}{1\sigma}\right)^2} \quad (7.5)$$

$${}^1n_{c,corrected} = \frac{{}^1n_c}{\langle\beta\rangle} \quad (7.6)$$

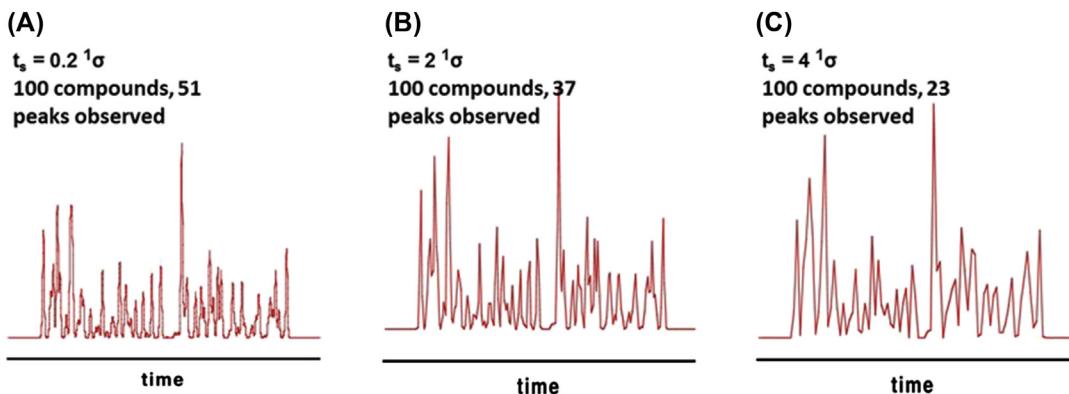
With the illustrations in Figs. 7.10 and 7.11 and Eq. (7.5) in mind, it is tempting to come to the conclusion that one should use as small a sampling time as possible to minimize the effect of undersampling on the <sup>1</sup>D and thus the 2D peak capacity. However, the problem with this in the extreme is that very short sampling times require very short <sup>2</sup>D separation times, and thus low <sup>2</sup>D peak capacities. This is most evident in LC  $\times$  LC separations where the sampling time and <sup>2</sup>D analysis time are very tightly coupled. Hybrid modes of 2D separation such as sLC  $\times$  LC relax this linkage to a large degree, as discussed in Section 6.1.

The opposing effects of sampling time (and its connection to <sup>2</sup>D analysis time) on effective <sup>1</sup>D peak capacity and <sup>2</sup>D peak capacity lead to an optimum sampling time as shown in Fig. 7.12. While these results are based on theory, the optimum <sup>2</sup>D analysis time of about 10–20 s predicted from this type of calculation has been confirmed by experimental LC  $\times$  LC separations (Huang et al., 2011). It is important to keep these trends in mind in the process of method development and selection of instrument hardware for 2D-LC.

**FIGURE 7.10**

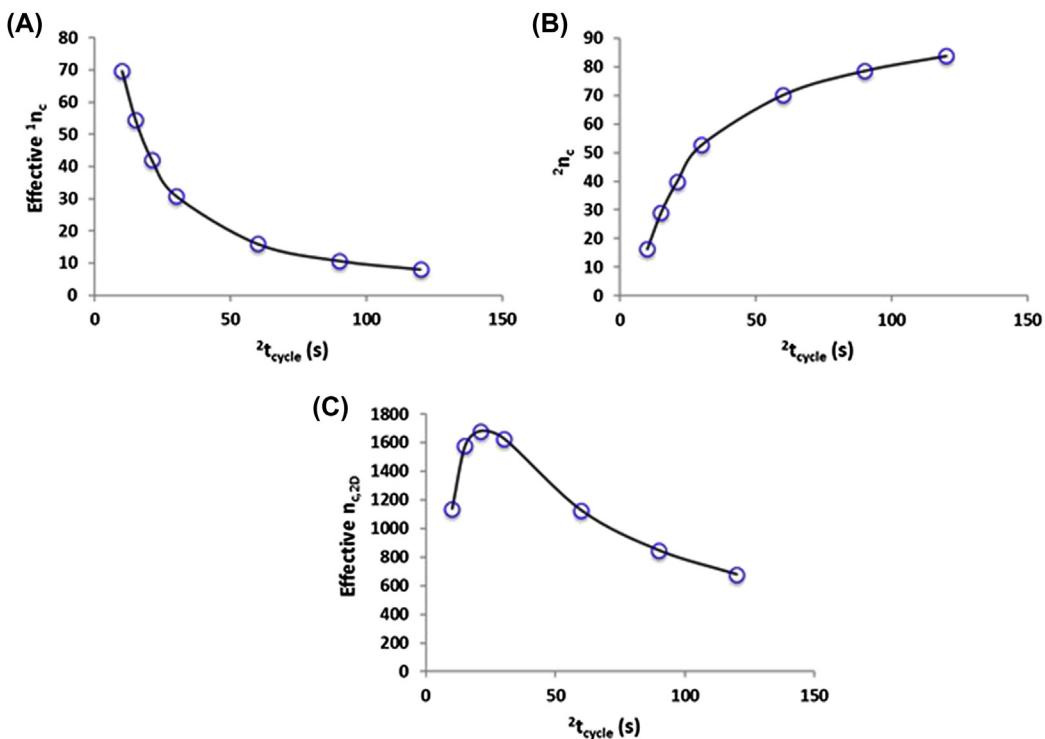
Effect of sampling time (rate) on resulting LC  $\times$  LC contour plot. Panel (A) sampling at the Murphy–Schure–Foley recommendation of  $t_s = 2^{-1}\sigma$ ;  $N = 4$  samples/ $8^{-1}\sigma$ . As the sampling time is increased there is more remixing of  $^1\text{D}$  peaks, and resolution in the two-dimensional chromatogram is lost; panel (B) sampling at  $N = 2$ ; and panel (C) sampling at  $N = 1$ .

*Reprinted with permission from Carr, P.W., Davis, J.M., Rutan, S.C., Stoll, D.R., 2012. Principles of online comprehensive multidimensional liquid chromatography. In: Gruska, E., Grinberg, N. (Eds.), Advances in Chromatography. CRC Press, Boca Raton, FL.*



**FIGURE 7.11**

Effect of undersampling on effective one-dimensional (1D) peak capacity. Reconstructed 1D chromatograms for a hypothetical sample containing 100 randomly spaced compounds show that sampling at a rate of 5 samples per  $\sigma$  ( $t_s = 0.2 \sigma$ ) yields a separation with 51 peaks. Increasing the sampling time to  $2 \sigma$  (Panel B) or  $4 \sigma$  (Panel C) yields 37 or 23 peaks, respectively.



**FIGURE 7.12**

Illustration of the dependence of effective two-dimensional (2D) peak capacity ( $n_{c,2D}$ ) on second dimension ( ${}^2D$ ) analysis time ( ${}^2t_{\text{cycle}}$ ). Calculations were made as follows: the native first dimension ( ${}^1D$ ) analysis time and peak capacity (i.e., without undersampling) were assumed to be 30 and 100 min, respectively, and the effective  ${}^1D$  peak capacity (A) was calculated using Eq. (7.5); the dependence of  ${}^2D$  peak capacity (B) on  ${}^2D$  cycle time is the same as that reported previously (Davis and Stoll, 2014); finally, the effective 2D peak capacity (C) is calculated as the product of the effective  ${}^1D$  peak capacity and the  ${}^2D$  peak capacity at each value of  ${}^2t_{\text{cycle}}$ .

Adapted from Bedani, F., Schoenmakers, P.J., Janssen, H-G., 2012. Theories to support method development in comprehensive two-dimensional liquid chromatography – a review. *J. Sep. Sci.* 35, 1697–1711. <http://dx.doi.org/10.1002/jssc.201200070>.

### 5.3 DECIDING WHETHER TO DO ONE- OR TWO-DIMENSIONAL LIQUID CHROMATOGRAPHY

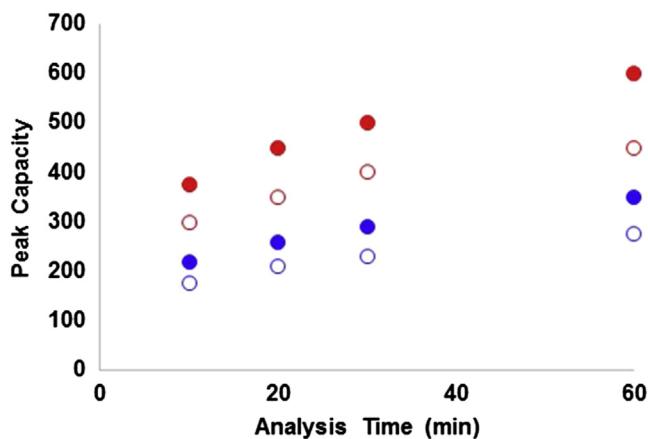
Although it is certainly true that the barriers new users of 2D-LC face are much lower than they were a decade ago, the decision to develop and implement a 2D-LC method is still not a trivial one. Moving from 1D- to 2D-LC requires a significant financial commitment to acquire and maintain a 2D-LC instrument, and users of the instrument must be trained and acquire practical experience with the methodology. Given these commitments, one should be convinced that the performance of 2D-LC will very likely be superior to 1D-LC for the application at hand. And, “performance” and “success” can be measured in very different ways depending on where the method will be deployed, for what purpose, under certain financial and time constraints, and so on. There is no question that in cases where the sample of interest is very complex, the goal is to learn as much about the sample as possible, and long analysis times are not prohibitive, 2D-LC will yield results superior to those from 1D-LC. But, on the continuum of all LC applications, this type of scenario is not very common. Rather, there are many more situations in the “middle ground” where sample complexity challenges the capabilities of 1D-LC, but analysis time is still a concern, and minimizing it is a primary method development goal. On the other end of the continuum, where the sample is simple (e.g., one to five compounds) and very fast analysis is particularly important, 1D-LC is king—with current technology it is difficult to imagine this changing in the foreseeable future. This then sets up the key question—how does one decide whether to do 1D- or 2D-LC?

The literature on this topic contains a blend of experimental and theoretical studies. The two performance metrics that have been used to guide discussion are peak capacity and time to resolve  $n$  compounds. The concept of peak capacity is most useful for comprehensive separations and situations where a primary goal of the method is to simply separate as many compounds as possible without regard for the resolution of specific pairs of compounds (e.g., in proteomics). On the other hand, for less complex samples a common method development goal is to arrive at a method that resolves all of the compounds in a sample (or a specific subset of compounds) in the shortest possible time. This is what we refer to here as “time to resolve  $n$  compounds.”

#### 5.3.1 Comparison of One- and Two-Dimensional Liquid Chromatography in Terms of Peak Capacity

High-performance liquid chromatography (HPLC) theory predicts that the chromatographic efficiency (i.e., plate number) of columns used for 1D-LC increases with the  $\frac{1}{2}$  power of analysis time (Carr et al., 2009). Taken together with the fact that peak capacity in 1D-LC in gradient elution mode also depends on the  $\frac{1}{2}$  power of the plate number (Giddings, 1967), this means that peak capacity in 1D-LC increases with the  $\frac{1}{4}$  power of analysis time. This is a very weak dependence. It means that to double the peak capacity in 1D-LC we must increase the analysis time 16-fold. Using typical chromatographic parameters yields plots like those shown in Fig. 7.13 where we have peak capacity as a function of analysis time. We see that even when allowing for high-pressure operation up to 1000 bar, the peak capacities we can expect in 1 h of analysis time are only on the order of 350 and 600 for small nonpeptide molecules (e.g., benzene) and peptides, respectively.

The product rule (Eq. 7.2) teaches us that if we take an existing 1D-LC separation with a peak capacity of 200 and add a  $^2\text{D}$  of separation, even if the peak capacity of that  $^2\text{D}$  is only 15, we will increase the peak capacity to 3000. This, of course, assumes that we properly optimize the system to

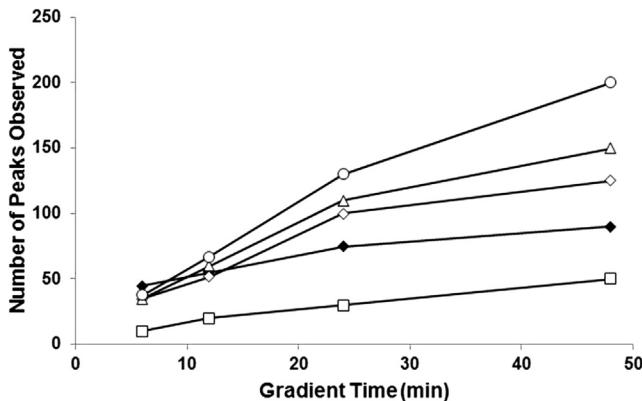
**FIGURE 7.13**

Peak capacity as a function of analysis time for one-dimensional liquid chromatography with different pressure limitations and for different analyte types. Blue (dark gray in print versions) circles are for small nonpeptide molecules with  $d(\ln k)/d(\phi) = 8$ , and red (light gray in print versions) circles are for peptide molecules with  $d(\ln k)/d(\phi) = 25$ . Open circles are for a pressure limit of 400 bar, and filled circles are for 1000 bar. Plate numbers are first estimated for dead times that are 10% of the analysis time assuming three-parameter optimization at 40°C (Carr et al., 2009), and then gradient peak capacity is calculated from these plate numbers using the approach prescribed by Neue (2005).

avoid serious degradation of the <sup>1</sup>D performance due to undersampling. But, this is stunning potential—increasing the peak capacity of 1D-LC by a factor of 15 would require an increase in analysis time by a factor of 15 to the fourth power.

If 1D-LC is clearly superior at very short analysis times, and 2D-LC is superior beyond an analysis time of 1 h, then there must be a region of analysis time in between where the performances of the two techniques cross over. The determination of this crossover time has been studied from both theoretical (Potts and Carr, 2013) and experimental points of view (Huang et al., 2011; Stoll et al., 2008). Fig. 7.14 shows results from the experimental work of Huang et al. who studied the effect of the <sup>2</sup>D cycle time on the effective peak capacities of LC × LC separations of plant extracts, and compared those to peak capacities for 1D-LC analyses of the same samples at comparable analysis times. More important than peak capacities are the numbers of peaks observed in real separations—these are plotted in Fig. 7.14. We see that at the shortest total analysis time of 6 min, the 1D-LC method outperforms the 2D one, no matter the <sup>2</sup>D cycle time. However, starting at an analysis time of 12 min, the 2D method outperforms the 1D one, provided optimal <sup>2</sup>D cycle times in the range of 12–20 s are used. LC × LC methods with shorter or longer <sup>2</sup>D cycle times of 6 or 40 s yield results that are inferior to those from the 1D method at that time. At the longest analysis time in the study of about 50 min, the LC × LC method yields threefold more peaks compared to the 1D method.

The essential message from this and related studies is that the performance crossover happens at relatively short times—10 min, rather than something longer like 100 min. This means that LC × LC should be considered as a viable option to either achieve increased resolving power compared to 1D-LC

**FIGURE 7.14**

Numbers of peaks observed in separations of plant extracts. For LC  $\times$  LC separations, the  $^2\text{D}$  cycle time was varied to demonstrate the optimum cycle time— $^2\text{D}$  cycle times are: 40 (open diamonds), 21, (open circles), 12 (open triangles), and 6 (open squares) sec. At analysis times of about 10 min, the two-dimensional liquid chromatography method outperforms the one-dimensional liquid chromatography method (filled diamonds) and remains superior at all longer times.

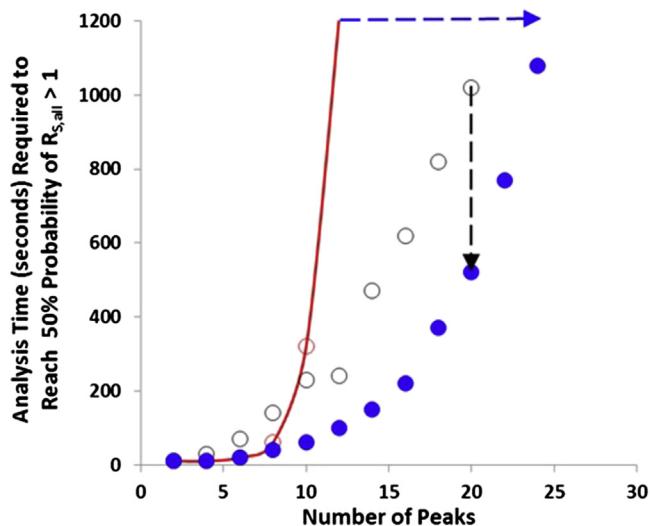
*Reprinted with permission from Huang, Y., Gu, H., Filgueira, M., Carr, P.W., 2011. An experimental study of sampling time effects on the resolving power of on-line two-dimensional high performance liquid chromatography. J. Chromatogr. A 1218, 2984–2994.*

*<http://dx.doi.org/10.1016/j.chroma.2011.03.032>. Copyright 2011, Elsevier.*

at a given analysis time or perhaps reduce the analysis time of an existing method, even for analyses in the range of 10–60 min. The theoretical study of Potts and Carr (2013) suggests that highly optimized LC  $\times$  LC methods may even be competitive with 1D-LC at analysis times as short as 3 min.

### 5.3.2 Time to Resolve $n$ Compounds

As discussed in the preceding section, typically the goal of LC  $\times$  LC is to maximize the effective peak capacity of the separation. For samples of moderate to low complexity, peak capacity may not be the most relevant performance metric, especially if we are concerned with the resolution of two or several neighboring peaks. In these cases, defining and measuring success can be highly variable, and is often dependent on the sample at hand. In our work simulating such scenarios, an important metric of performance that we have settled on is the *time to reach a specified probability of resolving all of the compounds in a particular mixture* (Davis and Stoll, 2014). Using this metric enables us to fairly compare different separation methodologies including 1D-LC and LC  $\times$  LC, but also others such as multiple heartcutting, selective comprehensive, and stop-and-go 2D-LC. Fig. 7.15 shows the analysis time required to reach a 50% probability of resolving all of the components in a mixture as a function of the number of sample components, for different LC techniques. We see that even with a peak capacity of about 200 for 1D-LC at an analysis time of 20 min, we cannot expect more than 50% probability of resolving all components in the mixture if there are more than 10 components in the sample. In the same analysis time, moving (see horizontal blue arrow) to LC  $\times$  LC provides 50%

**FIGURE 7.15**

Time required to reach 50% probability of resolving all of the components in a mixture for different liquid chromatography (LC) techniques: one-dimensional liquid chromatography [○ (light gray in print versions)],  $LC \times LC$  (○),  $sLC \times LC$  [● (dark gray in print versions)]. The red (gray in print versions) line is an interpolation between the times for 10 and 12 sample components.  $sLC \times LC$ , selective comprehensive two-dimensional liquid chromatography.

Adapted from Davis, J.M., Stoll, D.R., 2014. Likelihood of total resolution in liquid chromatography: evaluation of one-dimensional, comprehensive two-dimensional, and selective comprehensive two-dimensional liquid chromatography. *J. Chromatogr. A* 1360, 128–142. <http://dx.doi.org/10.1016/j.chroma.2014.07.066>.

probability of success for about 20 compounds, and with  $sLC \times LC$  about 25 compounds. The other way to interpret these results is to ask the question—how much can analysis time be reduced for a given separation goal by changing techniques? One answer to this question is illustrated by the vertical black arrow, which shows that for a 20-component mixture, moving from  $LC \times LC$  to  $sLC \times LC$  enables a 50% reduction in analysis time.

## 6. IMPORTANT PRACTICAL DETAILS ASSOCIATED WITH IMPLEMENTATION

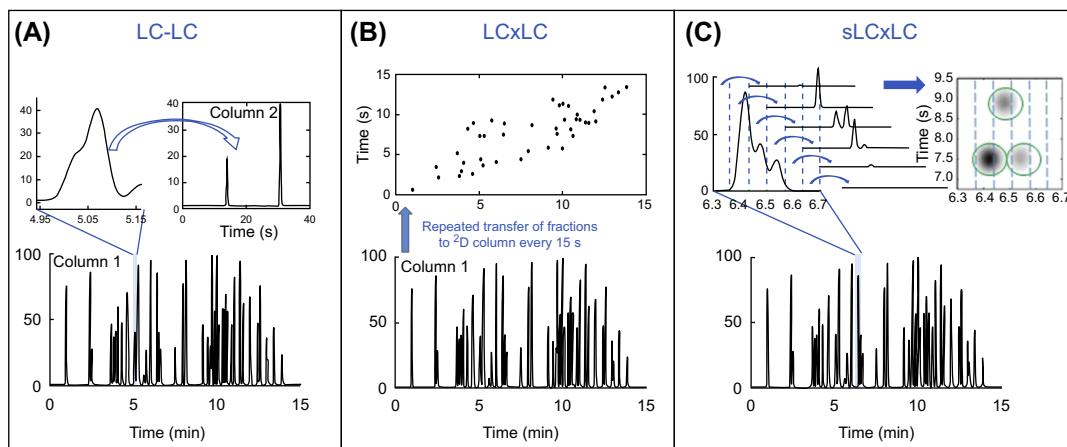
In this section we will discuss some of the most practically important details associated with setting up a 2D-LC system and implementing methods for the analysis of real samples. At the writing of this chapter 2D-LC is still a rapidly evolving technique and our community still has a lot to learn and discover about how to best implement 2D-LC methods; however, we have learned a lot in the last decade or so, especially with commercially available instrumentation. What follows is a major fraction of the distillate of those experiences.

## 6.1 CONCEPTS FOR DIFFERENT MODES OF TWO-DIMENSIONAL LIQUID CHROMATOGRAPHY SEPARATION

The first online 2D-LC separations were demonstrated in the 1970s and were focused on what we now refer to as “heartcutting” methods (Apffel et al., 1981), for which we use the symbol LC–LC. In this mode of 2D-LC, most of the components of a sample are neglected as they elute from the  $^1\text{D}$  column, and a select portion of  $^1\text{D}$  effluent containing analytes of interest are transferred to a  $^2\text{D}$  column for further separation. As pointed out by Snyder et al. in their early description of “boxcar chromatography,” the main reason for doing analysis by LC–LC over conventional 1D-LC is the potential for significant savings in analysis time (e.g., tenfold or more). Indeed, LC–LC has been incredibly effective for analysis of one or a few compounds in complex mixtures ranging from milk to plasma and environmental samples (Cassiano et al., 2012). Recently, we demonstrated that the concept can be extended to three dimensions, still with automated equipment, albeit at the cost of increases in the complexity of the instrumentation and method development (Simpkins et al., 2010). However, the fact that LC–LC methods ignore most of the content of the sample being analyzed limits them to a relatively small portion of the application space addressable by LC. Given this, and the fact that LC–LC concepts have been worked out in detail for decades, in this chapter we focus on modes of 2D-LC that are capable of addressing a larger fraction of the sample being analyzed. From the early 1980s until about 2010 the complement to LC–LC was comprehensive 2D-LC ( $\text{LC} \times \text{LC}$ ), which as the name suggests is capable of addressing the components of a sample in a more comprehensive way. In the past 5 years or so there has been significant development of, and interest in, modes of 2D-LC that are intermediate between LC–LC and  $\text{LC} \times \text{LC}$ . These include multiple heartcutting (mLC-LC) (Zhang et al., 2013a), stop-flow (Bedani et al., 2006), and selective comprehensive (sLC  $\times$  LC) (Groskreutz et al., 2012a) 2D-LC. We now have at our disposal a variety of modes of 2D-LC that span a continuum running from LC–LC at one extreme to  $\text{LC} \times \text{LC}$  at the other. The trade-off for increased information resulting from analysis by 2D-LC is complexity of the instrumentation, method development, and data analysis associated with a particular mode of 2D-LC.

Fig. 7.16 shows conceptually how the LC–LC,  $\text{LC} \times \text{LC}$ , and sLC  $\times$  LC, modes of 2D-LC separation compare to each other. These have also been discussed extensively in a recent review article (Stoll and Carr, 2016). Fig. 7.17 shows examples of the valve interfaces that can be used for these approaches. Either of the configurations shown in Fig. 7.17 can be used for simple single heartcutting (LC–LC). A variety of other valve interfaces have been described in the literature, particularly for  $\text{LC} \times \text{LC}$ . The biggest single collection of descriptions of interfaces that have been used appears in a book chapter by Francois et al. (2011).

The primary advantage of the mLC–LC approach is that it is tremendously flexible. Provided the interface is equipped with enough loops and there is adequate spacing between target peaks in the  $^1\text{D}$  separation, one can easily target 10  $^1\text{D}$  peaks, and one can imagine targeting many more. The primary disadvantage, however, is that a single fraction of each targeted  $^1\text{D}$  peak is transferred to the  $^2\text{D}$  for further separation. This disadvantage manifests itself in two very different ways. First, in cases where the fraction volume is small relative to the peak volume, the quantity of analyte transferred from the target  $^1\text{D}$  peak to the  $^2\text{D}$  column will be very sensitive to exactly where on the peak a cut is made, and therefore very sensitive to  $^1\text{D}$  retention time stability in successive analyses, which can severely

**FIGURE 7.16**

Conceptual comparison of LC–LC, LC × LC, and sLC × LC modes of two-dimensional liquid chromatography (2D-LC) separation. Panel A shows the case of LC–LC (heartcutting 2D-LC) where, in this case, only a single fraction is injected into the 2D column. Note that in multiple heartcutting (mLC–LC, not shown) more than one region of the first dimension (<sup>1</sup>D) effluent would be injected onto the second dimension (<sup>2</sup>D) column. Panel B shows the case of LC × LC (comprehensive 2D-LC) where the entire <sup>1</sup>D effluent is sequentially injected. Panel C shows the case of sLC × LC (selective comprehensive 2D-LC) where selected segments of the <sup>1</sup>D effluent are injected comprehensively on to the <sup>2</sup>D column.

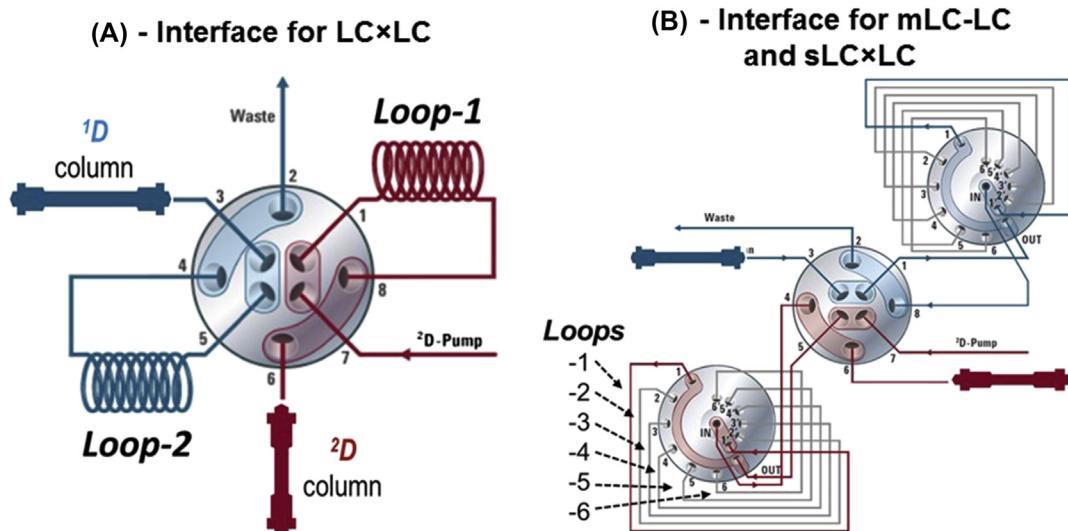
Reprinted with permission from Stoll, D., Carr, P., 2016. Two-dimensional liquid chromatography: a state-of-the-art tutorial.

Anal. Chem. (submitted for publication). <http://dx.doi.org/10.1021/acs.analchem.6b03506>. Copyright 2016, American Chemical Society.

degrade quantitative precision (Pursch and Buckenmaier, 2015). Second, mitigating the sensitivity to retention time stability is possible by making the fraction volume large relative to the peak volume; however, this inevitably results in mixing analytes from the target peak with neighboring <sup>1</sup>D peaks prior to transfer to the <sup>2</sup>D column. In other words, the target <sup>1</sup>D peak is severely undersampled in this case, defeating the progress made toward resolution by the <sup>1</sup>D column. These consequences are shown graphically in Fig. 7.18.

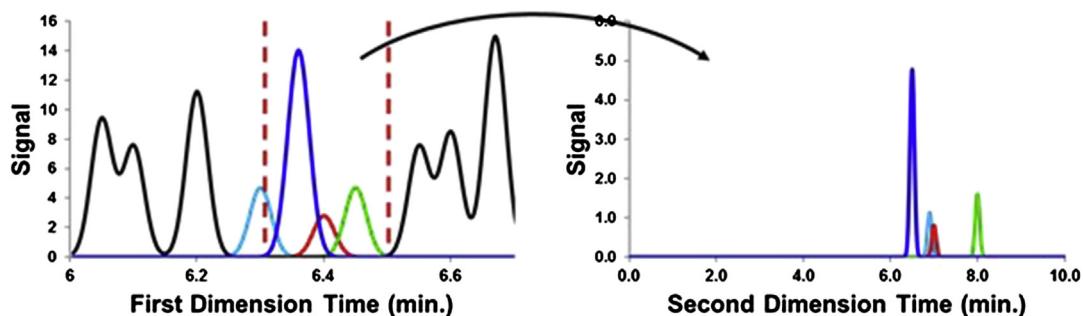
Recognizing the limitations of mLC–LC motivated us to develop sLC × LC. The principal difference between mLC–LC and sLC × LC is that in the case of sLC × LC multiple fractions of each target <sup>1</sup>D peak or region are transferred to the <sup>2</sup>D column in a comprehensive way—this is shown graphically in Fig. 7.16. This effectively solves the problems associated with shifting <sup>1</sup>D retention times making quantitation more robust (Pursch and Buckenmaier, 2015). This also avoids undersampling <sup>1</sup>D peaks, which is valuable in cases where the contribution of the <sup>1</sup>D separation to the overall resolution of the compounds of interest is important.

Stop-flow 2D-LC is an interesting concept that has received relatively little attention; interested readers are referred to a few papers in the literature on this topic for more detail (Bedani et al., 2006; Guiochon et al., 2008; Hou et al., 2015; Kalili and de Villiers, 2013a,b).

**FIGURE 7.17**

Example valve interfaces used for mLC–LC, sLC  $\times$  LC, and LC  $\times$  LC separations. Either configuration can also be used for simple single heartcut operation (LC–LC).  $^1D$ , first dimension;  $^2D$ , second dimension; LC, liquid chromatography.

Graphics courtesy of Agilent Technologies.

**FIGURE 7.18**

Consequence of using a large sampling window [indicated by the red (dark gray in print versions) dashed vertical lines] in the case of single heartcut operation of two-dimensional liquid chromatography. Sample components that had been separated from the target compound (red peak (dark gray in print versions)) by the  $^1D$  column are remixed and some are not reseparated (the teal peak (gray in print versions) here) from the target analyte by the  $^2D$  column.

## 6.2 IMPORTANT CHARACTERISTICS OF INSTRUMENT COMPONENTS

The operation of instruments assembled for 2D-LC places demands on the individual instrument components that are unique to 2D-LC and not normally encountered in the operation of a chromatograph for conventional 1D-LC. Thus, it is important to examine some of these challenges so that the user can make informed decisions when choosing which instrument components to use in their 2D-LC work. In the early days of the development of 2D-LC most instruments were “home-built” using components repurposed from existing 1D-LC instruments. Now, with the involvement of several major instrument manufacturers designing instrument components with 2D-LC in mind, we have a better understanding of some of the critical components and more options when assembling an instrument for 2D-LC.

### 6.2.1 The Sampling Interface

We refer to the valve and associated components that are used to transfer fractions of <sup>1</sup>D effluent to the <sup>2</sup>D column for further separation as the *sampling interface*, or simply *the interface*. This is without a doubt the most critical component of a 2D-LC system, as it ties the two separation dimensions together. The characteristics of the interface influence most aspects of the performance of a 2D-LC method including quantitation, retention repeatability, column stability, and detection sensitivity. Quantitation is discussed in [Section 9.3](#), and detection sensitivity is discussed in [Sections 6.2.3 and 8.4](#). Here, we discuss the topics of retention repeatability and column stability as they relate to the characteristics of the interface. As discussed in [Section 6.1](#), many different valve types and configurations have been used as the interface for 2D-LC. In the following two sections we compare the characteristics of two particular valve types, as examples of the kinds of differences users can expect to see that depend on their choice of instrument components.

#### 6.2.1.1 Effect of Interface Characteristics on Retention Repeatability

[Fig. 7.19](#) shows the flow paths through two different valve designs in common use today. The difference between them that we highlight here is that the flow paths through the valves are asymmetric in

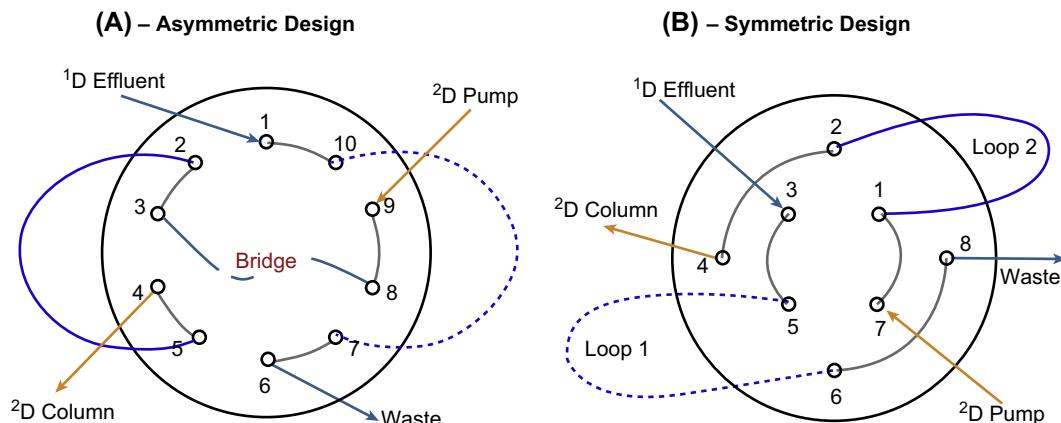


FIGURE 7.19

Flow paths through two different valve designs in common use. The valve shown in Panel A has an asymmetric design, whereas the valve shown in Panel B has a symmetric design. <sup>1</sup>D, first dimension; <sup>2</sup>D, second dimension.

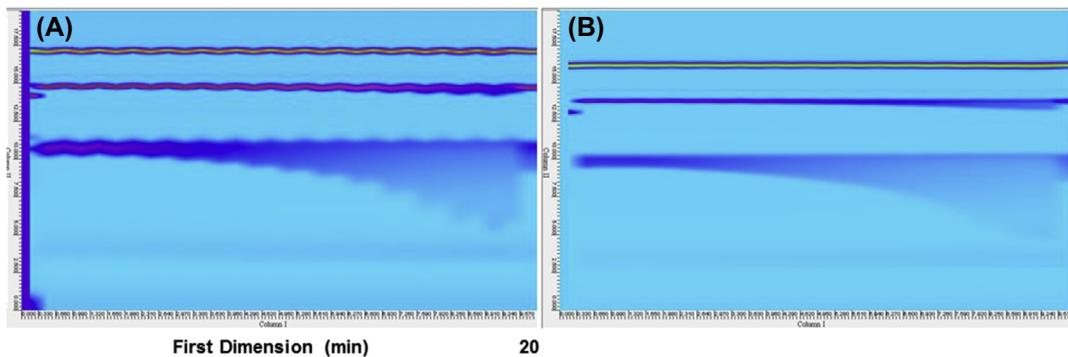
**Table 7.2 Delay of First Dimension Effluent Fraction Arrival Times as a Function of Crossover Connector Dimensions and Second Dimension ( $^2\text{D}$ ) Flow Rate**

$^2\text{D}$ Flow Rate (mL/min)	Bridge Connector Volume ( $\mu\text{L}$ )	Retention Shift (min/s)
3.0	1.3 <sup>a</sup>	0.00043/0.026
2.0	1.3	0.00065/0.039
1.0	1.3	0.0013/0.078

<sup>a</sup>Volume of a 100 mm  $\times$  120  $\mu\text{m}$  i.d. piece of connecting tubing.

one case (Panel A) and symmetric in the other (Panel B). When the valve in Panel A is in Position 1 the path traveled by a fraction of  $^1\text{D}$  effluent toward the  $^2\text{D}$  column is slightly longer than when it is in Position 2 because the bridge connector between ports 3 and 8 is positioned between the sample loop and the  $^2\text{D}$  column rather than between the sample loop and the  $^2\text{D}$  pump. This slight difference in the arrival of the  $^1\text{D}$  effluent fraction at the  $^2\text{D}$  column inlet directly translates to a difference in the perceived retention times of analytes eluting from the  $^2\text{D}$  column. Whether or not this difference is practically significant depends on a number of factors, including the dimensions of the bridge connector, the flow rate, and the dimension of the  $^2\text{D}$  column. Table 7.2 shows the delay times for different combinations of bridge connector dimensions and flow rates. Whether this delay time will be observable as a retention time shift depends on the magnitude of the shift relative to the  $^2\text{D}$  peak width and retention time precision when the valve asymmetry is not a factor.

Fig. 7.20 shows experimental results obtained using two 2D-LC systems differing only in the interface valve used. The goal of this experiment was to show the impact of the bridge connector on the



**FIGURE 7.20**

Pseudo two-dimensional liquid chromatography (2D-LC) chromatograms obtained from two nominally identical 2D-LC systems differing only in the type of valve used in the interface. A three-component analyte mixture was infused directly into the valve, resulting in repeated injection of the sample every 20 s throughout the analysis. The result in Panel A involved an asymmetric interface valve, whereas the result in Panel B involved a symmetric valve.

$^2\text{D}$  retention time repeatability. In this case a simple mixture of three neutral analytes was infused directly into the valve interface with a syringe pump, and  $^2\text{D}$  separations of the sample were completed by gradient elution in 20-s cycles. In this way the analytes appear in these images as streaks rather than spots because they are repeatedly injected into the  $^2\text{D}$  column every 20 s. In comparing Panels A and B we see that the retention time repeatability is much better in the case of Panel B, which involves the symmetric valve design. We attribute this difference to the difference in valve designs.

In 2D-LC separations of real samples, often the composition of the sample is not fully known. So, the problem with  $^2\text{D}$  retention times that vary depending on which loop they are injected from is as follows. For two peaks that appear in consecutive  $^2\text{D}$  separations and have slightly different  $^2\text{D}$  retention times, it is difficult to know with certainty if the difference in retention is due to the valve or a difference in chemical identity. In other words, are we actually seeing the same compound twice with the difference in retention time because of the valve design, or are we actually seeing two different compounds? The ability to use a symmetric valve design simply and effectively removes this source of uncertainty from the analysis of the chromatographic data.

#### 6.2.1.2 Effect of Interface Characteristics on Second Dimension Column Stability

The design and operation of the interface valve can also have a significant impact on the behavior of instrument components connected to the valve. In this section we discuss one way that the valve design can impact the stability of  $^2\text{D}$  columns. In a recent study in my laboratory (Talus et al., 2015) we evaluated the stability of  $^2\text{D}$  columns operated in two 2D-LC systems varying only in the type of interface valve used. The two valve designs were the same as those shown in Fig. 7.19. Fig. 7.21 shows

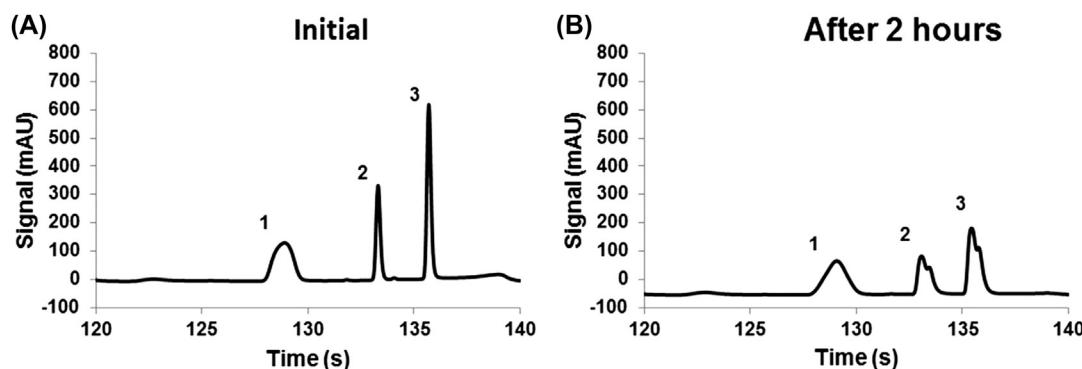


FIGURE 7.21

Second dimension ( $^2\text{D}$ ) chromatograms showing the change in peak shape observed for the  $^2\text{D}$  column after 2 h of continuous LC  $\times$  LC operation (360 cycles) using an electrically actuated 10-port/2-position valve in the interface. The sample injection occurred at 120 s relative to the start of the LC  $\times$  LC separation (2.0–2.33 min). Panel A shows the initial performance of the column during the very first LC  $\times$  LC separation, and Panel B shows the performance of the same column after 2 h of use. The probe compounds are (1) nitropropane, (2) nitropentane, and (3) dipropyl phthalate. All of the  $^2\text{D}$  columns used in this work were 30 mm  $\times$  2.1 mm i.d., packed with sub-three micron particles with a C18 stationary phase.

Reprinted with permission from Talus, E.S., Witt, K.E., Stoll, D.R., 2015. Effect of pressure pulses at the interface valve on the stability of second dimension columns in online comprehensive two-dimensional liquid chromatography. *J. Chromatogr. A* 1378, 50–57. <http://dx.doi.org/10.1016/j.chroma.2014.12.019>. Copyright 2015, Elsevier.

representative chromatograms for the three-component mixture used to evaluate the performance of the <sup>2</sup>D columns during many hours of continuous operation of the 2D-LC systems. Each <sup>2</sup>D separation cycle was 20 s, thus for every hour of operation of system 180 injections were made into the <sup>2</sup>D column. In this case the chromatogram in Panel A shows the initial performance of the column, and the chromatogram in Panel B shows the performance of the same column after 2 h of continuous operation of the system where the valve in use was the one shown in Fig. 7.19A.

It is clear from this result that after just 2 h of operation the performance of the <sup>2</sup>D column was severely compromised. To summarize the results of many stability tests of this type, we plotted the percent increase in peak width and the change in peak symmetry factor, as shown in Fig. 7.22. Significant changes in peak width and shape begin after just an hour of operation and within about 2 h the performance is severely compromised.

To understand the cause of these column failures we measured pressures in the system at several points in and around the interface valves, as shown in Panels A and B of Fig. 7.23. Panels C and D of the same figure show the pressure traces recorded as a function of time during a switch from Position 1 to

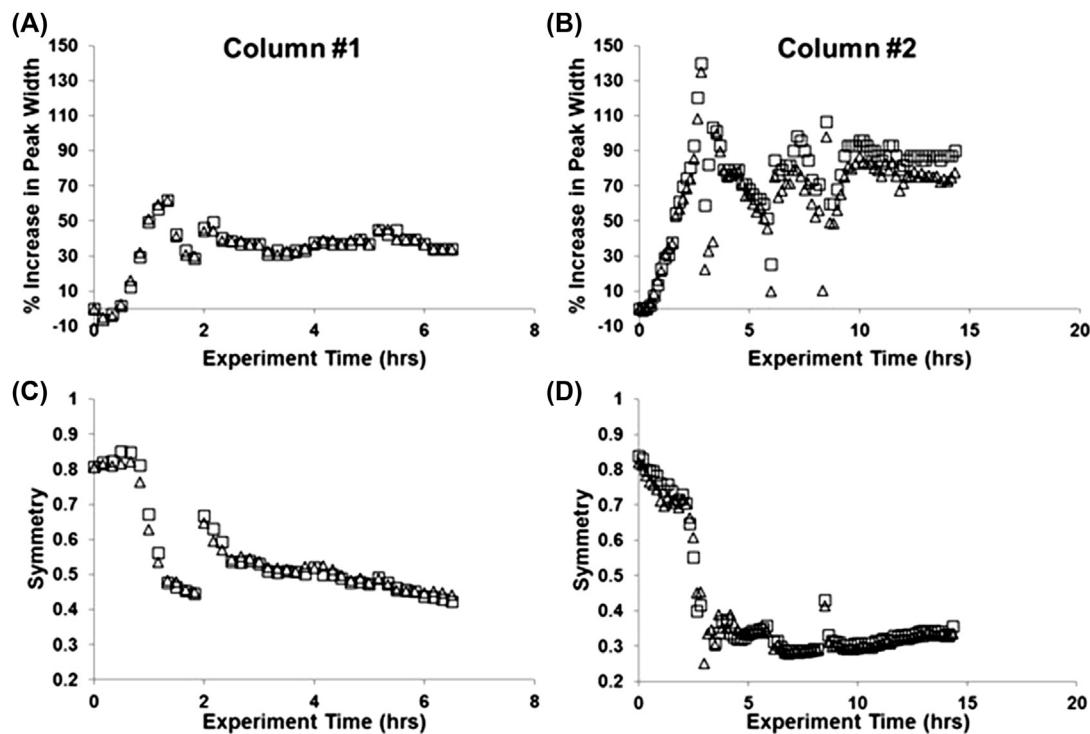
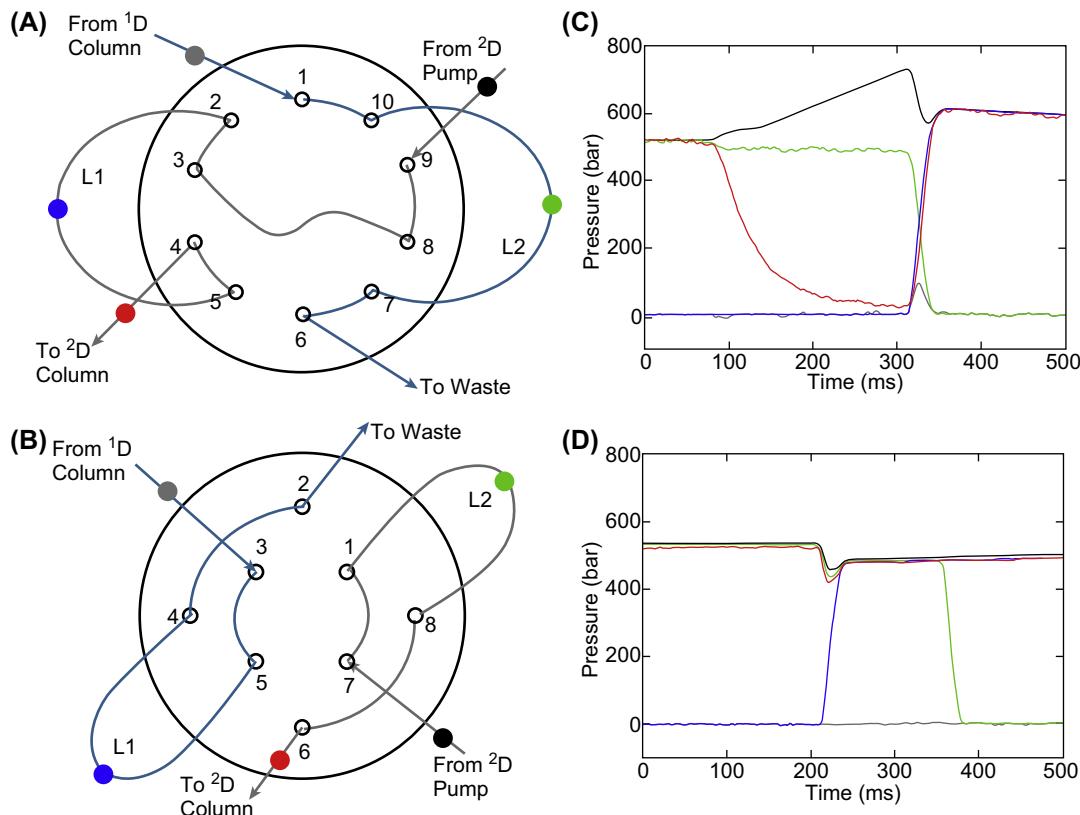


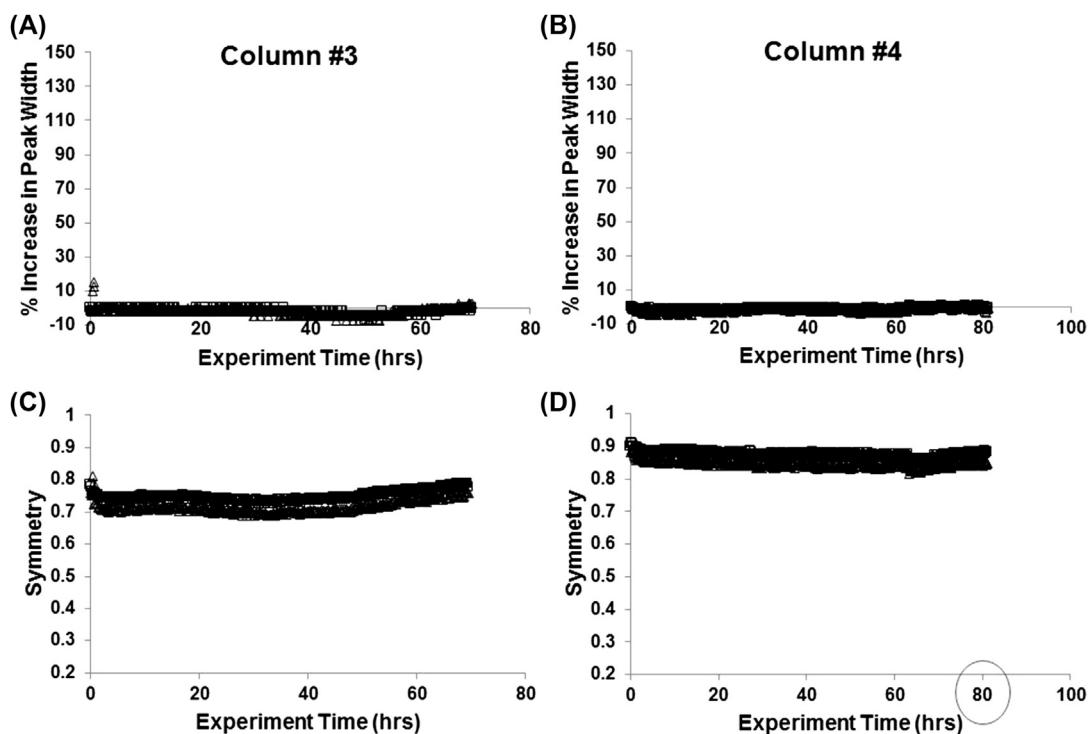
FIGURE 7.22

Changes in peak width (A, B) and symmetry (C, D) for the probe compound nitropentane using two nominally identical columns prepared with sub-three micron superficially porous particles and C18 bonded phase, and the 10-port/2-position valve design shown in Fig. 7.19A. Evidence of column failure appears after just an hour of two-dimensional liquid chromatography operation.

**FIGURE 7.23**

Locations of pressure sensors and resulting pressure traces recorded during the switch from Position 1 to Position 2 of these valves. The traces in (C) are for the valve shown in Panel A, and the traces in (D) are for the valve shown in Panel B. The colors of the traces correspond to the locations of the sensors indicated with the same colors. <sup>1</sup>D, first dimension; <sup>2</sup>D, second dimension.

Position 2 for these valves. Although we can rationalize the shapes of all of these pressure traces, we believe the red traces are the most important to <sup>2</sup>D column stability. In Panel C we see that with the 10-port/2-position valve the red trace drops from about 500 bar to nearly zero during the valve switch, and then quickly rebounds to 500 bar at the end of the switch. This red trace shows the pressure measured between the interface valve and the <sup>2</sup>D column inlet, meaning that in this case the column itself experiences these dramatic pressure fluctuations every time a fraction of <sup>1</sup>D effluent is injected into the <sup>2</sup>D column. Panel D shows the same pressure traces collected using the 8-port/2-position valve. We see that the red trace dips by about 15% during the switch, but most of the large change in pressure during the switch is mitigated by the difference in valve design. It is important to note that during normal operation of commercial 2D-LC systems, the pressure changes between the interface valve and column are not apparent to the user because there is no pressure sensor installed at that point. It is typical to have a single

**FIGURE 7.24**

Changes in peak width (A, B) and symmetry (C, D) for the probe compound nitropentane using two nominally identical columns prepared with sub-three micron superficially porous particles and C18 bonded phase, and the 8-port/2-position valve design shown in Fig. 7.19B. The columns are stable under continuous operation for at least several days.

pressure sensor in the system, which is typically mounted onboard the pumping system. In this case, the pressure profile observed in everyday use would be the same as the black traces in Fig. 7.23.

Most importantly, we observe that  $^2\text{D}$  columns are much more stable when the pressure changes between the interface valve and  $^2\text{D}$  column are reduced. Fig. 7.24 shows the changes in peak width and symmetry observed for  $^2\text{D}$  columns when the 8-port/2-position valve was used. We see that columns are very stable for at least several days.

In our view, the results of this study are very important to advancing both our knowledge of how the characteristics of the interface affect the performance and robustness of 2D-LC instruments, and our ability to use 2D-LC methods more effectively in situations where the robustness and reliability of the method is very important.

### 6.2.2 Pumping Systems

Next to the valve interface connecting the two dimensions in a 2D-LC system, the characteristics of the pumping system used in the second dimension have the biggest influence on the overall performance

and flexibility of 2D-LC separations. As discussed in [Section 5](#), separation speed is a particularly desirable asset in the second dimension of 2D-LC systems. Setting aside decisions about column dimensions and particle size (discussed in [Section 7](#)), and particularly when gradient elution conditions are used in the second dimension, the gradient delay volume of the <sup>2</sup>D pumping system is of paramount importance. The reason for this is straightforward, but users who are unfamiliar with the mechanics of gradient elution and the way different pumping systems work are strongly encouraged to study these concepts carefully (e.g., see [Snyder et al. \(2010a\)](#)). All pumping systems that are capable of gradient elution are characterized by a finite gradient delay volume ( $V_d$ ), which is the volume of solvent that must be displaced from the pump outlet before a change in solvent composition is actually delivered to the analytical column it is connected to. The delay time ( $t_d$ ) associated with the arrival of a change in solvent composition at the column inlet is related to  $V_d$  through the flow rate ( $F$ ):

$$t_d = \frac{V_d}{F} \quad (7.7)$$

In the context of the 2D separations, what is even more important than the delay time itself is what we call the flush-out time ( $t_{\text{flush}}$ ). This is the time required for “strong solvent” (e.g., acetonitrile (ACN) or MeOH in the case of RP-LC) to be flushed from the pumping system and connections at the end of a gradient elution to begin delivering the initial solvent used in the gradient for the subsequent analysis. In our work we have taken  $2 \times t_d$  as a reasonable estimate of  $t_{\text{flush}}$  ([Schellinger et al., 2005](#)). [Table 7.3](#) shows the gradient delay times for different combinations of delay volume and flow rate. Also shown is the fraction of <sup>1</sup>D analysis or <sup>2</sup>D analysis times that remains after accounting for the loss of productivity during the flush-out time.

One of the principal conclusions that emerge from these numbers is that it is effectively impossible to do fast <sup>2</sup>D separations using older pumping systems with gradient delay volumes on the order of 1 mL. Choosing a modern pump with a low gradient delay volume on the order of 100  $\mu$ L or less should be one of the highest priorities in assembling an instrument for 2D-LC. If this cannot be done, and very long <sup>2</sup>D separations are used in the case of LC  $\times$  LC, <sup>1</sup>D peaks will be severely undersampled leading to much lower 2D separation performance than we expect. The noncomprehensive modes of 2D-LC are much more forgiving in this regard because there is generally more time available for each <sup>2</sup>D separation. Other possible solutions exist for increasing the throughput of the <sup>2</sup>D, such as using multiple <sup>2</sup>D pumps/columns in parallel ([Fairchild et al., 2009a](#); [Francois et al., 2008](#);

**Table 7.3 Gradient Delay Times for Different Combinations of Delay Volume and Flow Rate**

Gradient Delay Volume ( $V_d$ , mL)	Flow Rate (mL/min)	Gradient Delay Time ( $t_d$ )	Analysis Time	Fraction of Analysis Available for Separation (%)
1.0	0.25	4 min	30 min	74
1.0	3.0	20 s	40 s	0
0.1	0.25	24 s	30 min	97
0.1	3.0	2 s	30 s	88

The first two rows are typical of legacy high-performance liquid chromatography pumps. The first and third rows correspond to typical <sup>1</sup>D conditions, whereas the second and fourth rows correspond to typical <sup>2</sup>D conditions.

Venkatramani and Patel, 2006), but from a practical point of view these are much less desirable than simply choosing a high-performing pump from the start.

### 6.3 OPTIMIZING FOR DETECTION SENSITIVITY

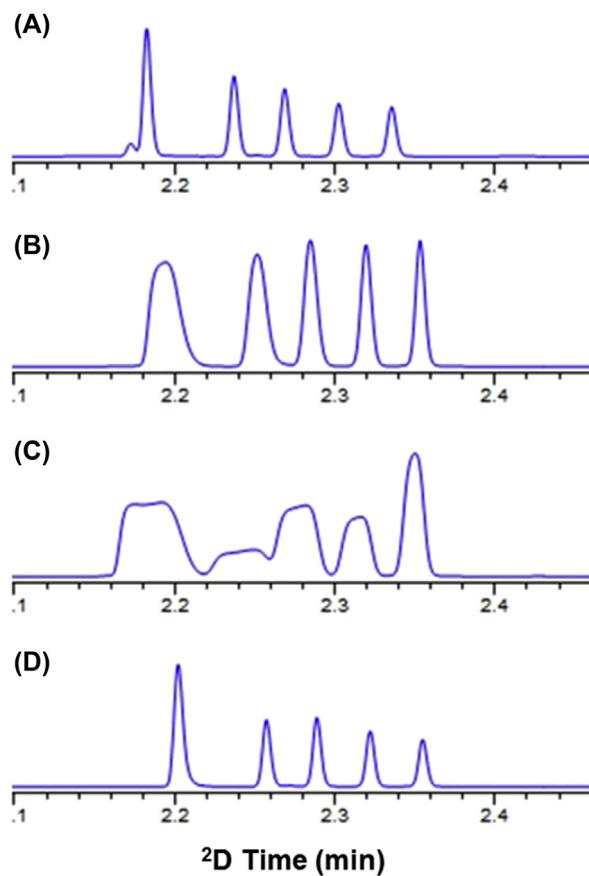
Historically, the performance of 2D-LC methods has been most commonly assessed using the metrics of peak capacity and the degree of usage of the separation space. With a few exceptions (Gargano et al., 2016; Sarrut et al., 2015; Schure, 1999; Stoll et al., 2014; Vivó-Truyols et al., 2010), detection sensitivity (i.e., detection limits) has typically been overlooked as an important performance attribute. However, with the adoption of 2D-LC by more application-driven users, detection sensitivity is being identified as an increasingly important issue.

The fundamental problem is that under most circumstances the samples we inject into columns used in 2D-LC, whether they are used in the first or second dimension, become dispersed inside the column as they migrate from the inlet to the outlet of the column. So, whatever detection sensitivity we have at the outlet of the <sup>1</sup>D column [whether in terms of mass(*t*) or concentration(*t*)], we will have less sensitivity at the outlet of the <sup>2</sup>D column because the analytes of interest are spread out over a larger eluent volume. Fortunately, we have some ability to mitigate this effect by choosing conditions that promote “compression” or “focusing” of the analyte zone between elution from the <sup>1</sup>D column and the inlet of the <sup>2</sup>D column. At the writing of this chapter there is no single approach that completely solves this problem in 2D-LC for all applications. In the past 10 years or so, a number of approaches have been proposed, and some developed and commercialized, each with its unique advantages and disadvantages. This is a broad and rapidly evolving area of research. Here, we briefly touch on the different approaches that have been described in the literature and provide some detail on an approach we have used extensively in my laboratory.

Fig. 7.25 shows the impact of the volume and composition of fractions of <sup>1</sup>D effluent on the performance of <sup>2</sup>D separations, in the context of a 2D-LC separation where RP-LC separations are used in both dimensions. Note that a short, narrow column is used in the second dimension, which is necessary for very fast <sup>2</sup>D separations. We see that when the injection volume (2 µL) is small relative to the column volume (60 µL), and the sample solvent is matched to the initial solvent used in the gradient elution program (50/50 ACN/water), we get peaks that are symmetrical, narrow, and tall (Panel A). When we simply inject more of the same sample, as we would do in the context of a 2D-LC separation, the early eluting peaks in particular begin to broaden significantly (Panel B). If we then mimic the worst-case scenario where the <sup>1</sup>D effluent contains a higher fraction of “strong solvent” (in this case ACN) than the initial solvent used in the <sup>2</sup>D separation, the peaks are severely broadened and distorted (Panel C). However, the ability to change the properties of the <sup>1</sup>D effluent fraction between the outlet of the <sup>1</sup>D column and the inlet of the <sup>2</sup>D column can be very powerful. Panel D shows that even if we use the same injection volume of 40 µL, if we are able to drop the ACN content from 50% to 30%, this produces very nice-looking peaks that are not very different from those in Panel A. In other words, adjustment of the sample composition allows us to inject a large fraction of <sup>1</sup>D effluent to improve sensitivity without compromising chromatographic resolution in the <sup>2</sup>D.

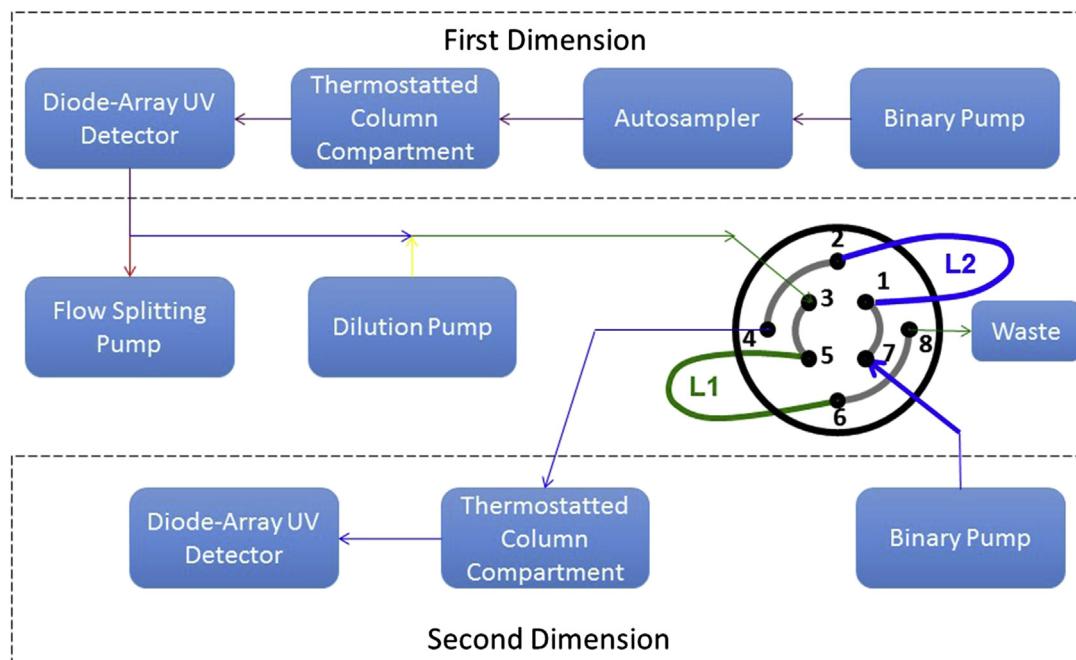
The idea of diluting the <sup>1</sup>D effluent with weak solvent prior to transfer of the mixture to the <sup>2</sup>D column has a long history (Oda et al., 1991). Readers interested in its development, and other approaches over time, are also referred to a recent review article of ours (Stoll and Carr, 2016). Here, I describe representative results from our own work that demonstrate the potential benefits of the

approach. To understand how these effects play out in 2D-LC separations of real samples, we have quantified the benefit of the ability to change the composition of the <sup>1</sup>D effluent fraction in the case of detecting trace-level components of a mixture produced by forcibly degrading an active pharmaceutical ingredient (Stoll et al., 2014). Fig. 7.25 shows that the effect of the sample solvent composition on the performance of the <sup>2</sup>D column can be minimized by injecting smaller volumes of <sup>1</sup>D effluent (Panel A), but of course this reduces detection sensitivity simply because fewer moles of the analytes are injected into the <sup>2</sup>D column. To study the interplay of these variables, we used an instrument set up like that shown in Fig. 7.26. The virtue of this particular configuration was that it allowed us to



**FIGURE 7.25**

Effect of the volume and composition of fractions collected from the one-dimensional column and injected into the second dimension (<sup>2</sup>D) column on performance of the <sup>2</sup>D separation. Conditions: 30 mm × 2.1 mm i.d. Zorbax SB-C18 column; 40°C; 2.5 mL/min; gradient elution from 50% to 90% ACN over 15 s; solutes are alkylphenone homologs. Chromatograms were obtained with: (A) 2 μL injection of sample in 50/50 ACN/water; (B) 40 μL injection of sample in 50/50 ACN/water; (C) 40 μL injection of sample in 70/30 ACN/water; and (D) 40 μL injection of sample in 30/70 ACN/water. <sup>2</sup>D, second dimension.

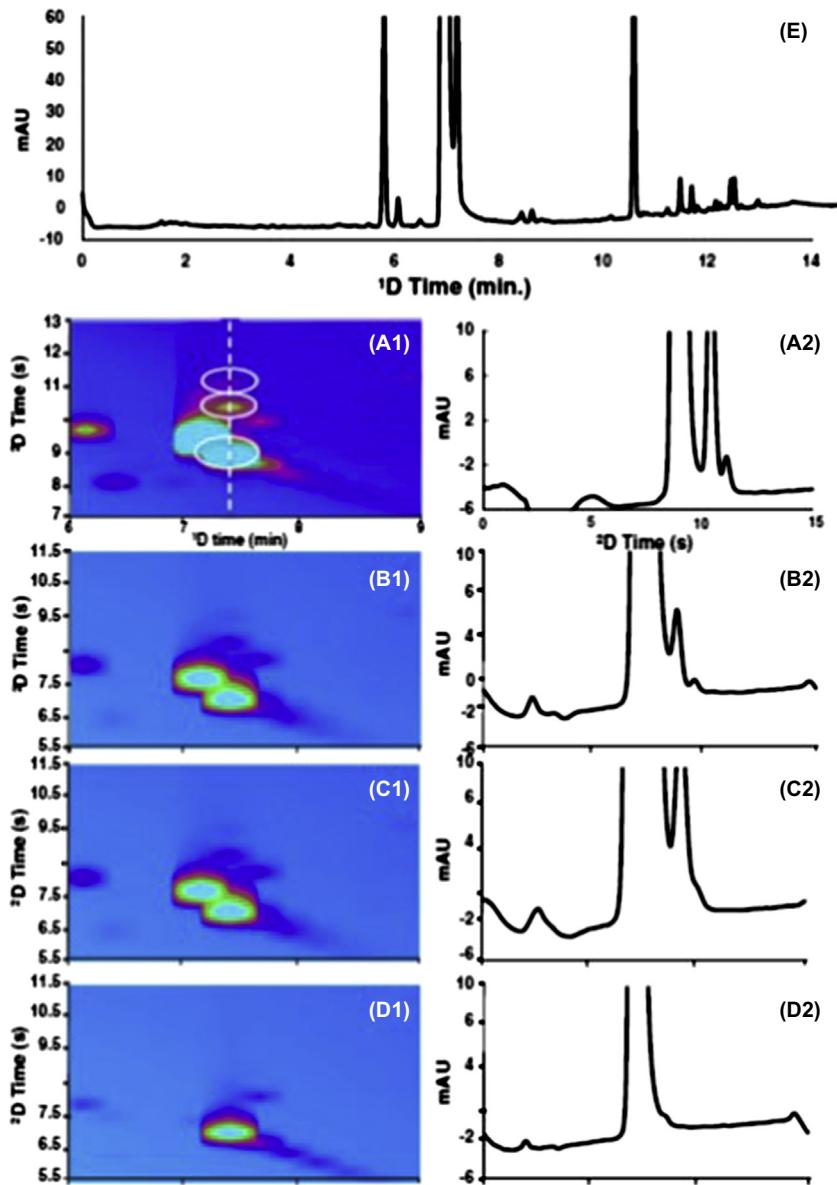
**FIGURE 7.26**

Instrument configuration used to study the effect of one-dimensional effluent fraction composition and volume on two-dimensional resolution and detection sensitivity.

*Reprinted with permission of Springer from Stoll, D.R., Talus, E.S., Harmes, D.C., Zhang, K., 2014. Evaluation of detection sensitivity in comprehensive two-dimensional liquid chromatography separations of an active pharmaceutical ingredient and its degradants. Anal. Bioanal. Chem. 407, 265–277. <http://dx.doi.org/10.1007/s00216-014-8036-9>.*

independently vary the volume and composition of the fraction of <sup>1</sup>D effluent injected into the <sup>2</sup>D column without changing the <sup>1</sup>D separation parameters.

Fig. 7.27 shows the impact of the interface conditions on the ability to both detect and resolve low-concentration degradants that coeluted with naproxen in the <sup>1</sup>D separation. Panel E shows the <sup>1</sup>D separation of naproxen and degradants produced on exposure to light, and Panels A–D show subsections of the resulting LC × LC chromatograms obtained using different interface conditions. In this case, the second dimension reveals the presence of a low-concentration degradant that coeluted with naproxen and was not visible in the <sup>1</sup>D chromatogram. Panels B–D all involve direct injection of <sup>1</sup>D effluent without any dilution but in decreasing volume. As we move from Panel B to Panel D we see that the widths of the <sup>2</sup>D peaks improve, but at the cost of detection sensitivity. In Panel A the <sup>1</sup>D effluent was diluted 1:1 with water before injection of the fraction into the <sup>2</sup>D column, and the injection volume was increased to 80 µL from 40 µL to accommodate the volume of the diluent. Even though the same volume of actual <sup>1</sup>D effluent was injected in both cases, we see that in Panel A the <sup>2</sup>D peaks are slightly taller and better resolved compared to Panel B. This is because of the focusing effect of the added water, resulting in narrower, taller, and better resolved peaks.



**FIGURE 7.27**

Effect of interface conditions on the ability to both resolve and detect trace-level degradants coeluting with the main peak (naproxen) in the first dimension of a high quality LC  $\times$  LC separation of photodegraded naproxen. The one-dimensional (<sup>1</sup>D) separation is shown in Panel E. Panels A1–D1 are subsections of LC  $\times$  LC separations focused on the region where naproxen and neighboring peaks elute. Panels A2–D2 show the two-dimensional chromatograms observed at the position indicated by the *white dashed vertical line* in Panel A1. Interface conditions were: (A1/A2) 80  $\mu$ L injection of <sup>1</sup>D effluent after 1:1 dilution with water; (B1/B2) 40  $\mu$ L injection of undiluted <sup>1</sup>D effluent; (C1/C2) 20  $\mu$ L injection of undiluted <sup>1</sup>D effluent; and (D1/D2) 7  $\mu$ L injection of undiluted <sup>1</sup>D effluent.

Reprinted with permission of Springer from Stoll, D.R., Talus, E.S., Harmes, D.C., Zhang, K., 2014. Evaluation of detection sensitivity in comprehensive two-dimensional liquid chromatography separations of an active pharmaceutical ingredient and its degradants. *Anal. Bioanal. Chem.* 407, 265–277. <http://dx.doi.org/10.1007/s00216-014-8036-9>.

The examples and data discussed so far in this section are relevant to cases where RP-LC separations are used in both dimensions. Of course, not all 2D-LC applications rely on RP-LC in both dimensions; some combinations of separation modes suffer from the effects of the injected <sup>1</sup>D effluent on <sup>2</sup>D performance more than others. Fig. 7.28 makes the point that the strength of the <sup>1</sup>D effluent relative to the eluent used in the <sup>2</sup>D separation has a direct effect on detection sensitivity at the outlet of the <sup>2</sup>D column. In some cases, such as the combination of IEX separation, where eluents are primarily composed of aqueous solvents, and RP-LC, these solvent effects are minimal, and in fact fraction volumes that are several-fold larger than the <sup>2</sup>D column volume can be used, which translates to great sensitivity. One could argue that this is a major reason why 2D-LC separations of peptides involving IEX and RP-LC have been so successful. On the other hand, when coupling either HILIC or NP-LC separations in the first dimension to RP-LC in the second dimension, these solvent effects can be even more detrimental than they are in the case of coupling two RP-LC separations. This has led the 2D separations community to describe such combinations of separation modes as “incompatible” (Kivilompolo et al., 2011). In fact, 2D-LC separations involving such combinations of modes have been demonstrated (Dugo et al., 2004; François et al., 2006), but at great cost in terms of detection sensitivity. In these cases, the injected volume of <sup>1</sup>D effluent must be a small fraction of the <sup>2</sup>D column volume to avoid serious deterioration of the <sup>2</sup>D column performance.

In addition to the effect of the organic solvent/water composition of the <sup>1</sup>D effluent, the pH and concentration of buffering components of the <sup>1</sup>D effluent can also significantly impact the performance

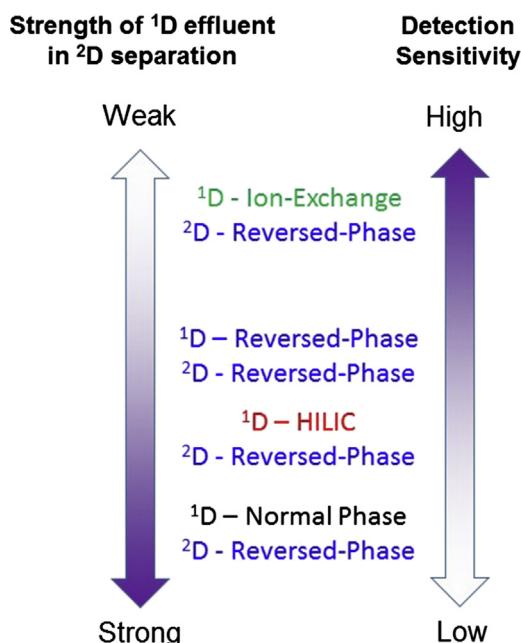


FIGURE 7.28

Effect of the solvent strength of the <sup>1</sup>D effluent in the <sup>2</sup>D separation mode on detection sensitivity at the outlet of the <sup>2</sup>D column. *HILIC*, hydrophilic interaction liquid chromatography.

of  $^2\text{D}$  separations. In our study of buffer effects on 2D-LC separations of carboxylic acids using RP-LC in both dimensions, we found that these effects can actually be more serious than the effects of the organic solvent/water ratio, causing peak splitting in  $^2\text{D}$  columns with injection volumes as low as 5  $\mu\text{L}$ . On the other hand, the problem is more easily addressed in this case, because the  $^1\text{D}$  effluent properties can be adjusted easily through the addition of a small amount of highly concentrated buffer solution. Readers interested in more details on this topic are referred to papers in the literature (Li et al., 2014a; Stoll et al., 2015).

In addition to the online dilution scheme described here, a variety of other approaches have been demonstrated. Ding et al. (2010) and Tian et al. (2006) have used interfaces that allow partial evaporation of the solvent in a fraction of  $^1\text{D}$  effluent prior to injection into the  $^2\text{D}$  column. Temperature modulation has been used to first focus analytes in a trapping cartridge mounted on the valve interface and maintained at low temperature, and then release those analytes into the  $^2\text{D}$  by raising the temperature of the trapping cartridge (Sweeney and Shalliker, 2002; Verstraeten et al., 2011).

The concept of using a solid-phase adsorbent to focus and trap analytes in the interface between dimensions was demonstrated at least 25 years ago in a heartcutting application by Oda et al. (1991). This was also demonstrated in LC  $\times$  LC applications with relatively slow  $^2\text{D}$  separations (Cacciola et al., 2007, 2006; Holm et al., 2005; Li et al., 2011; Mihailova et al., 2008; Pepaj et al., 2006; Vonk et al., 2015; Wilson et al., 2007). More recently, however, such trapping functionality has been incorporated into LC  $\times$  LC systems with faster  $^2\text{D}$  cycle times, and in a system coupling LC and SFC in the first and second dimensions, respectively (Venkatramani et al., 2016). Figs. 7.29 and 7.30, from the work of Gargano et al. (2016), show an illustration of such a system involving traps in the interface, and comparison of LC  $\times$  LC separations obtained for a surfactant mixture with either open loops or traps used in the interface. They refer to this approach as “active modulation.” The use of the traps provides additional flexibility in method development (e.g., through use of higher flow rates in the  $^1\text{D}$ ), and can reduce the volume of sample injected in each  $^2\text{D}$  cycle after the analytes have been focused in the trap.

This aspect of the development of 2D-LC is a very active area of research right now, and we can expect significant gains in the way of improvements these developments bring to the technique, including improvements in detection sensitivity, ease of use, and system robustness.

## 7. METHOD DEVELOPMENT

The topic of method development in 2D-LC could easily be expanded to fill an entire large chapter by itself, thus a detailed discussion of the topic is beyond the scope of this contribution. From the point of view of an analytical chemist, 2D-LC is a very powerful tool because of its flexibility—there are many variables that contribute to the outcome of a separation, and these can be adapted to a wide variety of analytical problems. However, from a user’s point of view, the number of considerations can be overwhelming. The challenge, then, is to identify the most important decisions and focus on those first, leaving the other variables for further optimization later in the development process. In the last 10 years there have been several journal articles (Bedani et al., 2012, 2006; Fairchild et al., 2009b; Schoenmakers et al., 2006; Vivó-Truyols et al., 2010) and a book chapter (Murphy and Schure, 2008) describing method development strategies and protocols that can serve as guides to the method development process. Interested readers are encouraged to engage the details of these prior publications. However, some of them are becoming obsolete quickly because of advances in instrumentation

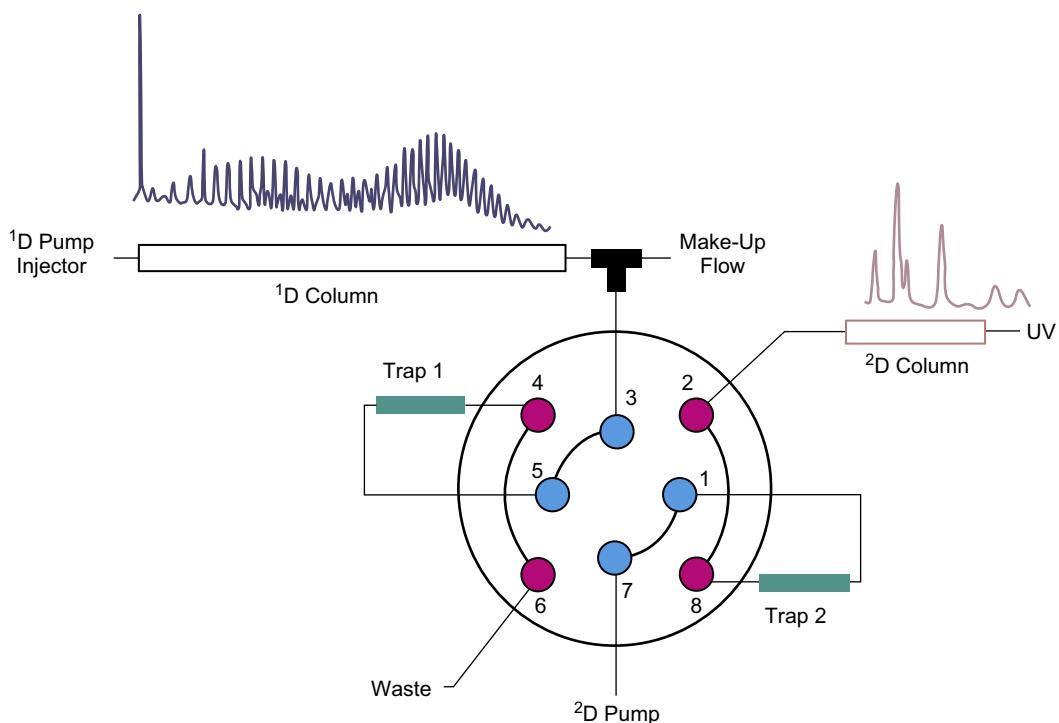
**FIGURE 7.29**

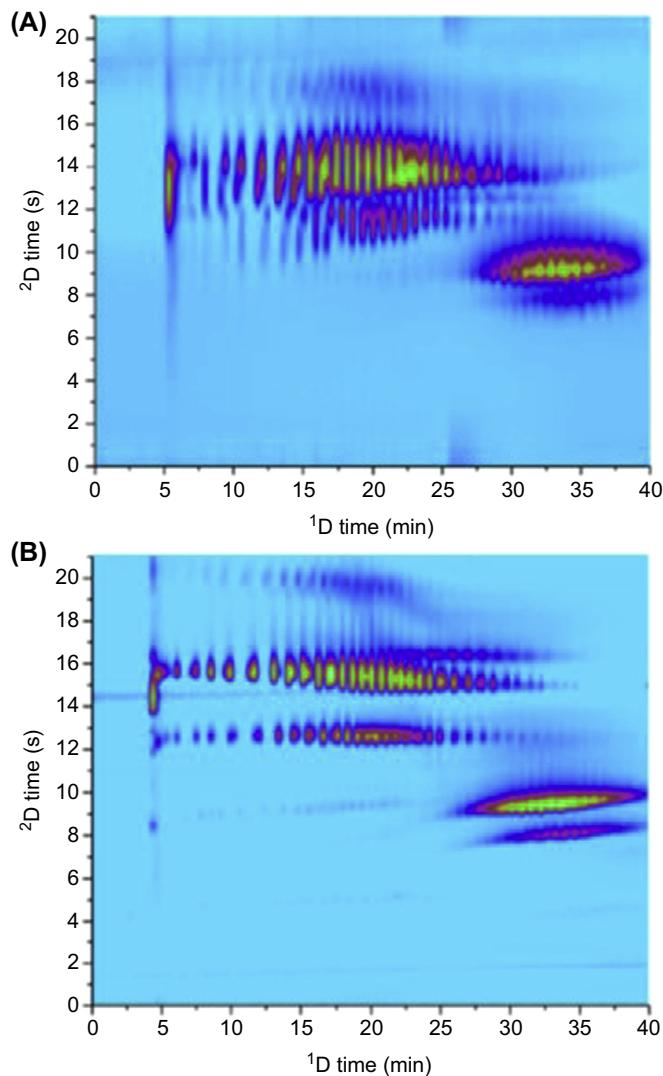
Illustration of a system for two-dimensional liquid chromatography capable of both online dilution between dimensions and the use of packed traps instead of open sample loops in the valve interface.  $^1D$ , first dimension;  $^2D$ , second dimension.

*Reprinted with permission from Gargano, A.F.G., Duffin, M., Navarro, P., Schoenmakers, P.J., 2016. Reducing dilution and analysis time in online comprehensive two-dimensional liquid chromatography by active modulation. Anal. Chem. 88, 1785–1793. <http://dx.doi.org/10.1021/acs.analchem.5b04051>.*

and development of new strategies for dealing with some of the long-standing challenges in method development (e.g., solvent “incompatibility”). In the following sections, I briefly describe some of the major decisions that must be made early on in the development process, and then finish this subsection with a case study that illustrates this decision-making process with concrete details.

## 7.1 SELECTION OF TWO-DIMENSIONAL SEPARATION MODE

A decision that has to be made early on in the development process is which mode of 2D-LC separation will be used. As described in [Section 6.1](#), this can range from simple heartcutting separations to more complex but more informative comprehensive separations. Readers are referred to [Section 6.1](#) for a detailed discussion of the different modes, their strengths and weaknesses, and the types of applications they are typically used for. This initial decision will have a significant impact on subsequent decisions, especially concerning choice of column dimensions and operating conditions.

**FIGURE 7.30**

Comparison of chromatograms obtained from LC  $\times$  LC separations of tristyrylphenol ethoxylate phosphate surfactants using an interface equipped with open loops (A) or packed traps (B).  $^1\text{D}$ , first dimension;  $^2\text{D}$ , second dimension.

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## 7.2 SELECTION OF COMPLEMENTARY SEPARATIONS

In [Section 5.1.3](#) I discussed some of the obvious possible combinations of separation modes and some of the potential disadvantages of these combinations. It is critically important at this stage of method development to understand the properties of the molecules in the sample at hand, and think about which separation mechanisms can be used most effectively to exploit the variation in two or more of the chemical characteristics of the analytes. For example, consider a 2D-LC separation of a surfactant sample where the molecules vary significantly in their charge or dipolarity (e.g., by end-group chemistry) and lipophilicity (e.g., by chain length). In a situation like this, it is chemically sensible to use an RP separation in one dimension, which will separate based on lipophilicity, and either IEX or HILIC in the other dimension, which will separate based on end-group charge or dipolarity.

As discussed in [Section 5.1.3](#), and as we ([Stoll et al., 2007](#)) and others ([Li et al., 2014b](#)) have noted, doing 2D-LC with RP columns in both dimensions is attractive for several reasons. A challenge in this case, however, is selecting two RP columns that will be different enough to be useful together in the context of a 2D-LC method. When the analytes of interest are weak acids and/or bases, then simply changing the pH enough to change the ionization state of the analytes in the two dimensions can bring about a large selectivity change ([Snyder et al., 2010b](#)). This means that it is even conceivable to use the same RP column in both dimensions, but with eluents buffered at different pH values. This kind of scheme has been used very effectively for RP  $\times$  RP separations of peptides ([François et al., 2009](#); [Gilar et al., 2005](#); [Vanhoenacker et al., 2015](#)).

In cases where changing the pH alone does not provide enough selectivity difference, significant differences in the stationary phase chemistry are required. The good news is that there are over 1000 commercially available RP phases to choose from today. However, this also presents a significant challenge because it is not feasible to screen them all experimentally, or even a significant fraction of them. Over the past two decades, several models of RP selectivity have been developed that can be leveraged to help identify phases that have complementary selectivities. These include procedures developed by the United States Pharmacopoeia, Katholieke Universiteit Leuven, and Euerby et al. ([Snyder et al., 2012](#)). In each case, selectivity data have been compiled for hundreds of phases. The largest single collection of freely available selectivity data has been compiled for the hydrophobic subtraction model (HSM), which was developed by [Snyder et al. \(2012\)](#). Interested readers are referred to their book chapter in *Advances in Chromatography* ([Snyder et al., 2012](#)), which contains a comprehensive description of the origins of the model, its strengths and weaknesses, and possible applications of the model and the database. At the time of this writing there are data for nearly 700 phases in the database ([www.hplccolumns.org](http://www.hplccolumns.org); [PQRI Approach for Selecting Columns of Equivalent Selectivity](#)). Briefly, the HSM approach enables a quantitative description of RP selectivity using Eq. (7.8)

$$\log\left(\frac{k_X}{k_{ref}}\right) = H\eta - S^*\sigma + A\beta + B\alpha + C\kappa \quad (7.8)$$

where  $k_X$  is the retention factor of any compound  $X$ , and  $k_{ref}$  is the retention factor of ethylbenzene. The parameters  $H$ ,  $S^*$ ,  $A$ ,  $B$ , and  $C$  are parameters that are measures of stationary phase characteristics: hydrophobicity ( $H$ ), steric hindrance ( $S^*$ ), hydrogen bond acidity ( $A$ ), hydrogen bond basicity ( $B$ ), and cation-exchange capacity ( $C$ ). The corresponding  $\eta$ ,  $\sigma$ ,  $\beta$ ,  $\alpha$ , and  $\kappa$  are the corresponding properties of the compound  $X$ : hydrophobicity ( $\eta$ ), bulkiness ( $\sigma$ ), hydrogen bond basicity ( $\beta$ ), hydrogen bond acidity ( $\alpha$ ), and cation-exchange capacity ( $\kappa$ ).

( $\alpha$ ), and cationic character ( $\kappa$ ). With the  $H$ ,  $S^*$ ,  $A$ ,  $B$ , and  $C$  values in hand for hundreds of phases, we can probe the data by asking about similarities or differences between phases. This can be done for any pair of columns using the so-called similarity factor ( $F_s$ ):

$$F_s = \left( [w_H(H_1 - H_2)]^2 + [w_{S^*}(S_1^* - S_2^*)]^2 + [w_A(A_1 - A_2)]^2 + [w_B(B_1 - B_2)]^2 + [w_C(C_1 - C_2)]^2 \right)^{1/2} \quad (7.9)$$

where the subscripts 1 and 2 on the column parameters indicate the columns being compared, and the terms  $w_i$  are weighting factors for each of the phase characteristics. Snyder et al. have asserted that two columns with  $F_s < 3$  are similar enough to be practically “equivalent,” and columns with  $F_s > 50$  are different enough to be useful in applications where large selectivity differences are desired, such as 2D-LC. For the purpose of identifying pairs of phases with complementary characteristics, one can simply choose one column of the pair (Column A), and then calculate  $F_s$  for all possible pairs of columns involving Column A and identify those phases that produce the largest  $F_s$  values. This is tedious, however, which has led groups to establish more visual ways of exploring the HSM database (Græsbøll et al., 2014; Johnson et al., 2012; Zhang and Carr, 2009). One of these that is very intuitive involves plotting the column parameters in a pseudo-three-dimensional triangular selectivity space (Zhang and Carr, 2009). The parameters  $S^*$ ,  $B$ ,  $A$ , and  $C$  are first normalized to the parameter  $H$ . Then, any combination of three of the parameters (e.g.,  $S^*/H$ ,  $B/H$ , and  $C/H$ ) can be plotted in a triangle. An example of such a plot is shown in Fig. 7.31. Each point in this space represents a single column. For phases that are very different, the distance between two points is a measure of how chemically different they are. The red, green, and yellow points provide an example of this. In this illustration, the Zorbax SB-C18 phase was chosen as the reference phase. The yellow point, which corresponds to a different Agilent C18 phase, is very close to the red point—indeed, the  $F_s$  value for this pair of phase is just 13. On the other hand, the red point for the Zorbax Bonus-RP phase is far away from the green point, and the  $F_s$  value for this pair of phases is 266. In this way, the selectivity triangles can be used to quickly identify pairs of phases that are likely to be different enough to be useful in 2D-LC separations.

### 7.3 SELECTION OF PARTICLE SIZES AND COLUMN DIMENSIONS

It is important to recognize that optimal combinations of column dimensions and particle sizes in the first and second dimensions of 2D systems are frequently specific to a given application. Challenges associated with specific groups of analytes and/or sample matrices and specific analytical goals (e.g., throughput, detection limits) can have a significant influence on these decisions. Nevertheless, it is useful to consider some general trends based on discussion of 2D-LC method development in the literature and our own practical experience.

#### 7.3.1 First Dimension

Benefits and Challenges Associated with Narrow Columns (1.0–2.0 mm i.d.)

- Narrow  $^1\text{D}$  columns allow one to work near the optimal velocity of these columns (i.e., in a van Deemter sense) without having to transfer very large volumes of  $^1\text{D}$  effluent to the  $^2\text{D}$  column.
- Using gradient elution with these columns requires the use of pumps with low gradient delay volumes (e.g., <200  $\mu\text{L}$ ), otherwise long gradient delay times will result in wasted analysis time (see Section 6.2.2 for more detail).

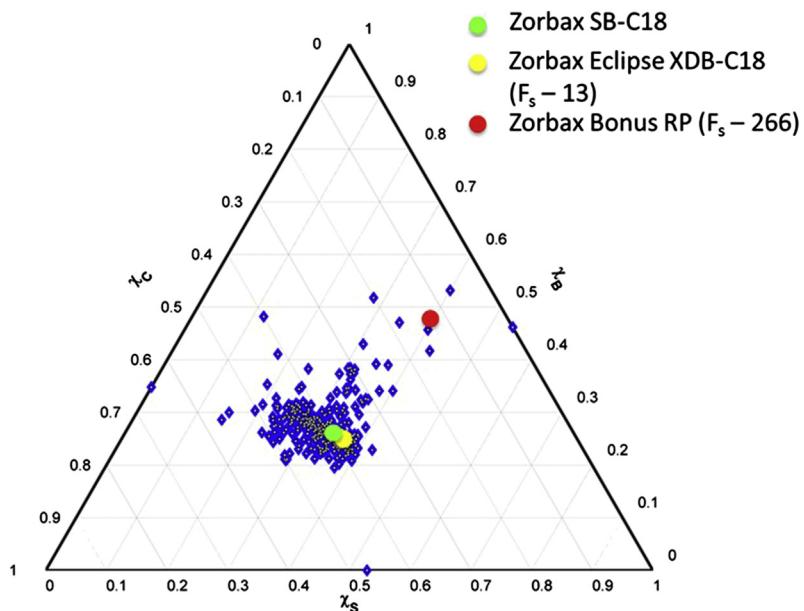


FIGURE 7.31

Selectivity classification of 632 reversed-phase columns using the S-B-C triangle with weighting factors inversely related to the range of each parameter (e.g.,  $S/H$ ), and normalized to the range of  $S^*$ . The coordinates of three specific phases are highlighted, along with the calculated  $F_s$  values for two of the phases in comparison to Zorbax SB-C18.

- Narrow  $^1D$  columns will have lower loadability, in both volume and mass terms, compared to wider columns.

Benefits and Challenges Associated with Wide Columns (3.0–4.6 mm i.d.)

- Wide  $^1D$  columns will have higher volume and mass loadability compared to narrow columns.
- Wide  $^1D$  columns are more compatible with pumping systems having a large gradient delay volume.
- Use of flow rates corresponding to the optimal velocities for these columns results in very large fractions of  $^1D$  effluent that must be transferred to the  $^2D$ . This in turn leads to challenges related to the mismatch between solvents used in the two dimensions (see [Section 6.3](#) for more detail).

### 7.3.2 Second Dimension

Benefits and Challenges Associated with Narrow Columns (1.0–2.0 mm i.d.)

- The primary potential benefit of using narrow  $^2D$  columns is improved detection sensitivity (see [Section 6.3](#) for details). Additional benefits may be derived from lower flow rates through these columns, including: less solvent cost and waste; and, ability to directly couple with mass spectrometry.

- Use of low flow rates and gradient elution conditions may lead to prohibitively long gradient delay and flush-out times—this is especially the case with 1 mm i.d. columns.
- These columns will be more sensitive to mismatch between the solvents used in the first and second dimensions because of their small dead volumes.

Benefits and Challenges Associated with Wide Columns (3.0–4.6 mm i.d.)

- Wide  $^2\text{D}$  columns allow the use of high  $^2\text{D}$  flow rates (e.g.,  $>3$  mL/min), which are helpful for achieving very fast  $^2\text{D}$  cycle times. However, this leads to high solvent consumption and waste generation costs.
- Wide  $^2\text{D}$  columns can mitigate the effects of mismatch between solvents used in the two dimensions; however, this comes at the cost of detection sensitivity.

In general, 2D-LC methods will involve  $^2\text{D}$  separations that are much faster than the  $^1\text{D}$  separation. With this in mind, it is generally true that smaller particles are more necessary in the second dimension than in the first dimension (Carr et al., 2009; Desmet et al., 2015; Matula and Carr, 2015). Many studies published in the past 5 years have involved  $^2\text{D}$  columns with particles in the sub-two- to sub-three-micron diameter range. For LC  $\times$  LC separations in particular, the first dimension need not be rigorously optimized because the effective performance of the  $^1\text{D}$  separation will be dominated by the effects of undersampling in most cases (Li et al., 2009). In cases where the  $^1\text{D}$  separation can be sampled very rapidly [e.g., one sample per second, as in sLC  $\times$  LC (Groskreutz et al., 2012a)] or where information from the  $^1\text{D}$  chromatogram obtained prior to sampling is used [e.g., as in 2D-assisted liquid chromatography (2DALC) (Cook et al., 2015)], the importance of optimizing the  $^1\text{D}$  separation is elevated significantly.

## 7.4 SELECTION OF OPERATING CONDITIONS

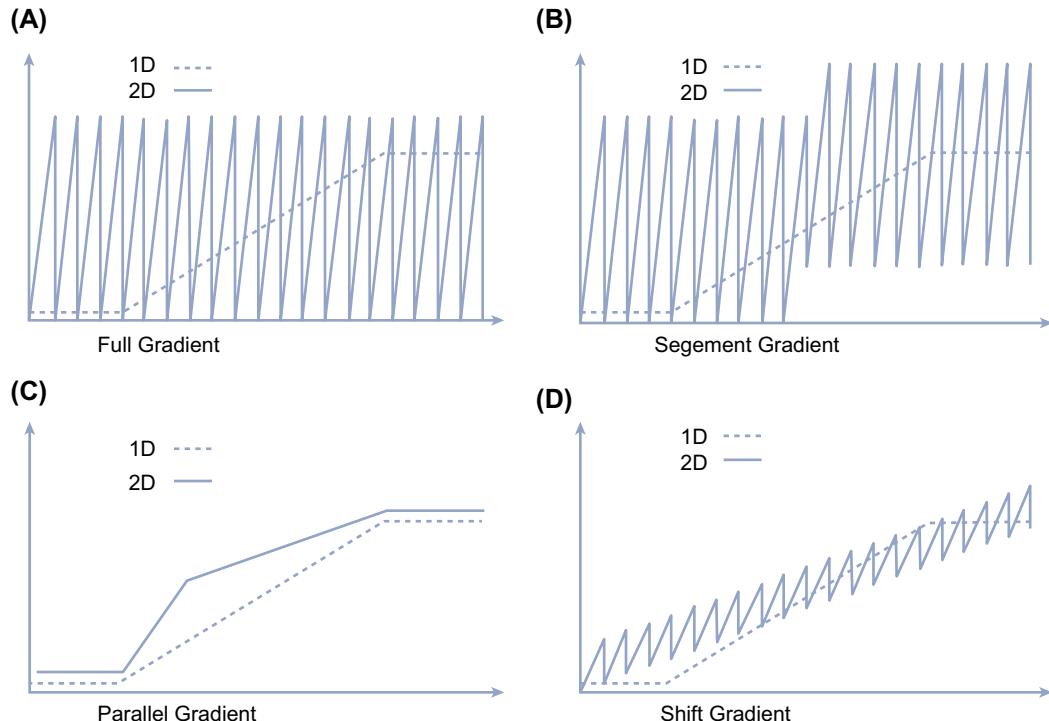
I suggest the following steps to make decisions about operating conditions in the method development process. As with the preceding section, these are general guidelines, and specific applications may demand a different approach.

1. Choose the mode of 2D separation that will be used (i.e., LC–LC, LC  $\times$  LC, etc.—see Section 6.1). This will have a significant impact on the following steps, primarily because different modes of 2D separation demand different levels of speed in the  $^2\text{D}$  separation.
2. Determine the speed level needed in each  $^2\text{D}$  separation. For LC  $\times$  LC separations this should be in the range of 12–60 s. At the other extreme,  $^2\text{D}$  separations for LC–LC may be 15 min or longer. For mLC–LC, sLC  $\times$  LC and stop-and-go modes, cycle times for  $^2\text{D}$  separations will be between these extremes. At this stage, think about whether isocratic or gradient elution conditions will be used in the  $^2\text{D}$ . For LC  $\times$  LC separations involving gradient elution in the second dimension, a  $^2\text{D}$  pump with a low gradient delay volume is an absolute requirement.
3. Given the desired  $^2\text{D}$  speed level, choose the  $^2\text{D}$  column diameter and length and particle size. Detailed considerations are described in Section 7.3. For the fastest  $^2\text{D}$  cycle times, narrow  $^2\text{D}$  columns (2.1 or 1.0 mm i.d.) are required. With longer  $^2\text{D}$  cycle times there is more flexibility. Larger diameters are less susceptible to peak broadening because of mismatch between the  $^1\text{D}$  and  $^2\text{D}$  eluents, but one must recognize that there is a trade-off with detection sensitivity here. For the fastest  $^2\text{D}$  separations, short (2–5 cm) columns with small (sub-two- to sub-three-micron) particles should be used. For longer  $^2\text{D}$  separations, longer lengths up to 15 cm will be

optimal; particle sizes in the 3  $\mu\text{m}$  range are preferred. There is good guidance in the literature for choosing optimal length and particle size once the column diameter has been selected (Carr et al., 2009; Desmet et al., 2015).

4. Choose a reasonable sample loop size for the valve interface. This is the volume of  $^1\text{D}$  effluent that will be injected into the  $^2\text{D}$  column in each  $^2\text{D}$  separation cycle. At this point one must decide if the  $^1\text{D}$  effluent will be diluted (Stoll et al., 2014) or split (Filgueira et al., 2011) prior to entering the loop. Larger loop volumes are preferred for high detection sensitivity in the  $^2\text{D}$ , but one has to be careful not to get into a volume overload situation because this will compromise resolution of the  $^2\text{D}$  column. Currently, commonly used loop volumes are in the 10–100  $\mu\text{L}$  range.
5. Choose the flow rate through the  $^1\text{D}$  column. This is dictated by the sampling time ( $t_s$ ) and the fraction volume that will be injected into the  $^2\text{D}$  column. Two examples are useful here. Suppose we use a sampling time of 20 s, a loop volume of 40  $\mu\text{L}$ , and there is no dilution or splitting of the  $^1\text{D}$  effluent. In this case the  $^1\text{D}$  flow rate must be 120  $\mu\text{L}/\text{min}$  if we fill the loop 100%. In practice, we generally fill the interface loop 80%. If the  $^1\text{D}$  effluent is diluted 1:1 with weak solvent prior to filling the loop, then the  $^1\text{D}$  flow rate must be decreased to 60  $\mu\text{L}/\text{min}$ .
6. Choose the diameter, length, and particle size for the  $^1\text{D}$  column. Given the flow rates arrived at in the preceding step, it is clear that in most cases a 2.1 mm i.d. column is preferred. In cases where even lower flow rates are needed, one may consider using 1.0 mm i.d. columns. It is important to consider again the characteristics of the pumping system used in the first dimension, particularly if gradient elution will be used. If a pump with a large gradient delay volume cannot be avoided (e.g., >200  $\mu\text{L}$ ), one should consider using a higher  $^1\text{D}$  flow rate and then split the effluent prior to transfer to the second dimension (Filgueira et al., 2011). As in Step 2, there is good guidance in the literature for choosing good combinations of column length and particle size, once the diameter has been chosen.
7. Finally, once the  $^1\text{D}$  column diameter has been selected, the injection volume for the  $^1\text{D}$  column can be set. Obviously larger injection volumes will generally lead to better detection limits, but one has to avoid mass and/or volume overload conditions.

Once one has made selections for the parameters outlined in the steps above, the 2D separation can be optimized according to the goals of the analysis, with a focus on performance metrics such as analysis time, detection limits, peak capacity, or resolution of specific pairs of peaks. One very powerful step in this optimization process is adjustment of elution conditions in the second dimension over the course of the 2D separation. In other words, the  $^2\text{D}$  elution conditions used in the first 2 min of a 2D separation need not be the same as those used in the rest of the separation, and changing these dynamically can lead to significant improvements in one or more of the performance metrics mentioned here. The potential for this improvement is best illustrated by way of example. Fig. 7.32, adapted from the work of Li and Schmitz (Li and Schmitz, 2013), shows examples of the types of elution schemes that can be used in the second dimension, ranging from a consistent, repeating  $^2\text{D}$  gradient (Panel A) to a so-called shifted gradient (Panel D) where the beginning and ending compositions used in each  $^2\text{D}$  gradient are slightly different. Good examples of the impact of optimized  $^2\text{D}$  elution conditions on usage of the available 2D separation space are shown in Fig. 7.33, from the work of the Jandera group (Jandera et al., 2015). Adopting dynamically adjusted  $^2\text{D}$  elution conditions roughly doubles the fraction of the separation space that is actually used (compare Panels A and C). An additional benefit of using the so-called parallel gradients in Fig. 7.32C is that the

**FIGURE 7.32**

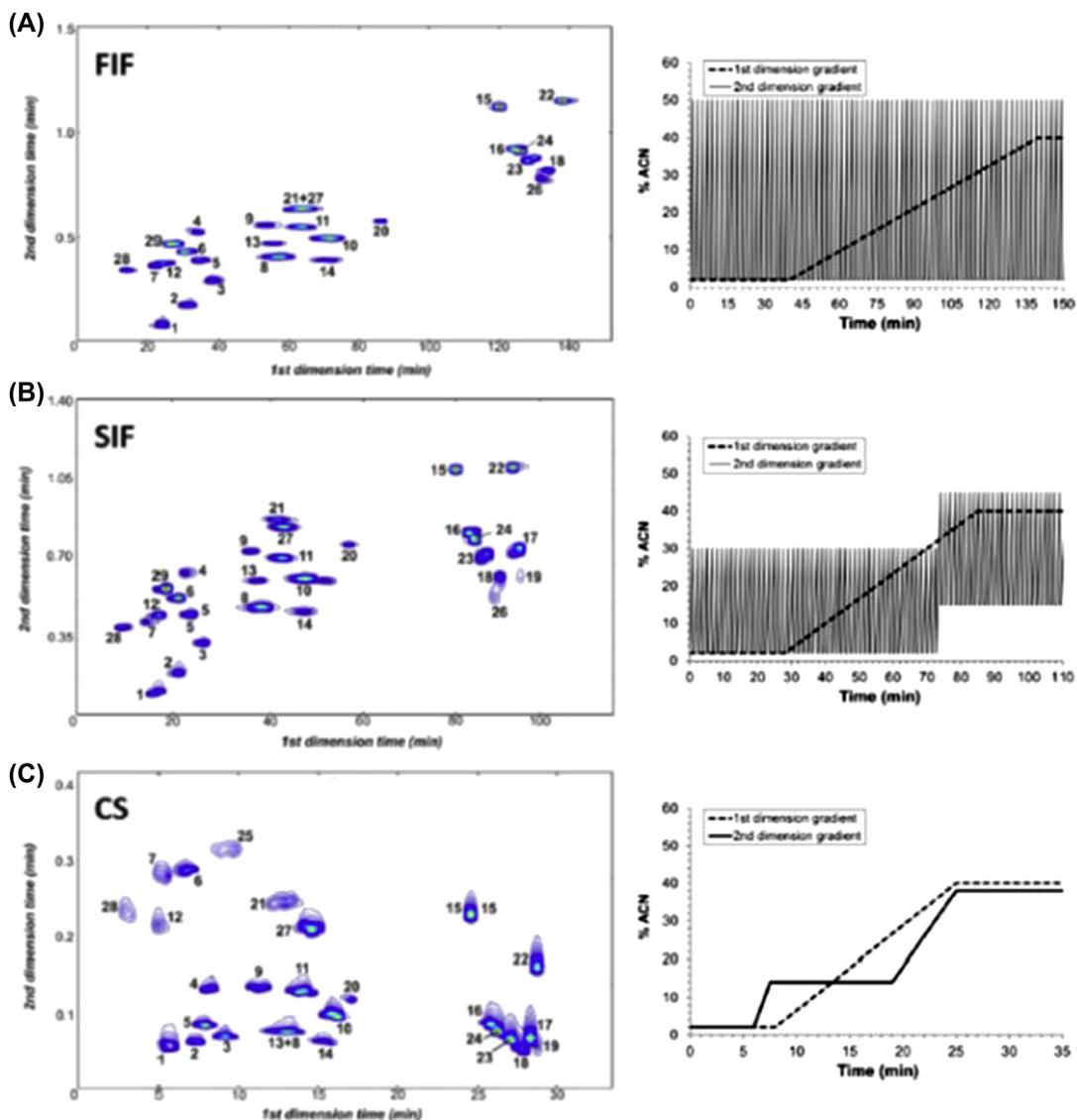
Examples of dynamic elution profiles that can be used in the second dimension of two-dimensional liquid chromatography systems. In each plot the *dashed trace* represents the eluent composition used in the first dimension, and the *solid trace* represents the eluent composition used in the second dimension.

*Reprinted with permission of Springer from Li, D., Schmitz, O.J., 2013. Use of shift gradient in the second dimension to improve the separation space in comprehensive two-dimensional liquid chromatography. Anal. Bioanal. Chem. 405, 6511–6517. <http://dx.doi.org/10.1007/s00216-013-7089-5>.*

use of isocratic conditions in each <sup>2</sup>D separation enables much faster sampling of the <sup>1</sup>D separation. On the other hand, this also results in wider <sup>2</sup>D peak widths because there is no compression of peak widths under isocratic conditions.

## 8. DETECTION

Many of the guidelines used for making decisions about detection in 1D chromatography apply in 2D-LC as well. However, there are some important characteristics of 2D-LC that change things a bit—in this section I will focus on those aspects that are different and deserve attention. In the case of coupling 2D-LC to MS detection in particular, interested readers are referred to the thorough review of [Donato et al. \(2012\)](#) that is focused on this topic.

**FIGURE 7.33**

Examples of the effect of  $^2$ D elution conditions on the utilization of the 2D separation space in LC  $\times$  LC separations of phenolic acids and flavones. The separation in Panel (A) is obtained when the same  $^2$ D gradient is used throughout the 2D separation. The separation in Panel (B) is obtained when the 2D separation is split into two segments, and different gradient elution programs are used in each segment. Finally, Panel (C) shows that the peaks can be further spread out by using isocratic conditions in the second dimension that are optimized over the course of the 2D analysis time to maximize use of the available separation space.

Reprinted with permission of Springer from Jandera, P., Hájek, T., Staňková, M., 2015. Monolithic and core–shell columns in comprehensive two-dimensional HPLC: a review. *Anal. Bioanal. Chem.* 407, 139–151. <http://dx.doi.org/10.1007/s00216-014-8147-3>.

## 8.1 ACQUISITION SPEED

As was discussed in [Section 7.3](#),  $^2\text{D}$  separations are generally much faster than  $^1\text{D}$  ones in 2D-LC. This means that one need not be so concerned with acquisition speed for detectors employed between the  $^1\text{D}$  column and the sampling interface. Indeed, slower acquisition is preferred at this point to allow smoothing of the signal to improve signal-to-noise ratios. In the second dimension, however, acquisition speed is often very important, especially in LC  $\times$  LC work where peak widths less than 1 s are not uncommon. This is not a problem for state-of-the-art spectroscopic detectors (e.g., UV absorbance, fluorescence) that are capable of acquisition rates exceeding 100 Hz. Some types of mass analyzers are more suitable than others for MS detection with high acquisition rates. Time-of-flight and quadrupole-time-of-flight are especially well suited to fast separations, and this has been borne out in the recent literature describing coupling of 2D-LC and MS detection ([Donato et al., 2012](#); [Stoll et al., 2016](#)).

## 8.2 EXTRA-COLUMN DISPERSION

Although dispersion in the tubing and connections between the  $^1\text{D}$  column and the  $^1\text{D}$  detector are important to the  $^1\text{D}$  peak width, dispersion in the  $^1\text{D}$  detection element itself (e.g., UV flow cell, MS ionization source) are usually not that important because relatively large fractions of the  $^1\text{D}$  effluent are transferred to the  $^2\text{D}$  column, which results in some amount of remixing of  $^1\text{D}$  peaks anyway. Exceptions to this exist in the case of noncomprehensive 2D separations, where fractions of  $^1\text{D}$  effluent can be very narrow in time (<5 s), and may be small in volume as well (e.g., <5  $\mu\text{L}$ ), depending on the conditions of the experiment. In these cases, one should carefully consider the contributions of flow-through detectors to the variance of the  $^1\text{D}$  peaks as they travel to the sampling interface. Dispersion in the  $^2\text{D}$  detector, on the other hand, can be much more detrimental to the resolving power of the 2D system. As we approach  $^2\text{D}$  peak variances on the order of 10  $\mu\text{L}^2$  ([Haidar Ahmad et al., 2015](#)), it is becoming increasingly important to choose detection elements that are designed with low dispersion in mind.

## 8.3 BACKGROUND CHARACTERISTICS OF SECOND DIMENSION DETECTION

In comparison to typical conditions used in 1D-LC, several aspects of  $^2\text{D}$  separations in 2D-LC are extreme. For example, it is common to have injection volumes of  $^1\text{D}$  effluent that are a significant fraction of the  $^2\text{D}$  column dead volume. It is usually the case that the composition of the  $^1\text{D}$  effluent that is injected is significantly different from the  $^2\text{D}$  eluent, with differences in organic solvent type, and buffer type and pH. Finally, when gradient elution is used, the gradients are usually quite fast in absolute time units (e.g., 10–60 s). These factors can lead to significant detector background disturbances ([Stoll et al., 2014](#)) and/or relatively steep baseline slopes in the case of gradient elution ([Filgueira et al., 2012](#)). These features of the detector background can, in turn, have a significant impact on signal-to-noise ratios at the  $^2\text{D}$  detector and have negative effects on the implementation of peak detection ([Filgueira et al., 2012](#)) and chemometric data analysis tools ([Bailey and Rutan, 2011](#)).

## 8.4 DETECTION SENSITIVITY

As was discussed in [Section 6.3](#), poor detection sensitivity at the  $^2\text{D}$  column outlet has historically been a weakness of 2D-LC methods. This often results from a mismatch between the eluent compositions used in the two dimensions, which limits the volume of  $^1\text{D}$  effluent that can be injected into the  $^2\text{D}$

column without compromising the performance of the  $^2\text{D}$  separation. Aside from the strategies discussed in [Section 6.3](#) to overcome these limitations, detection sensitivity can also be improved by increasing the signal-to-noise ratio at the point of detection following the  $^2\text{D}$  separation. The most straightforward example of this is increasing the path length in a UV detector flow cell ([Pursch and Buckenmaier, 2015](#)). In principle, detection limit should improve in proportion to the increase in the detection path length. This is not strictly observed in practice with commercial instruments because an increase in path length is accompanied by an increase in cell volume, which in turn causes some peak dispersion and loss of peak height. The anticipated improvement also assumes that detector noise is independent of path length, which may not always be observed.

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## 9. DATA ANALYSIS, SOFTWARE, AND QUANTITATION

Data handling and analysis is more complex in 2D-LC compared to 1D-LC. This presents both challenges that require the development of new analysis strategies and tools, and opportunities for the application of sophisticated chemometric methods that benefit from the high dimensionality of the data. As with instrumentation for 2D-LC, which a decade ago was largely “home-built” for most users, so too were many algorithms reported in the literature as “written in-house” for the analysis of data from 2D-LC experiments. Currently, there are good commercially available packages that provide functionalities for at least the most essential data processing and analysis steps in 2D-LC. A thorough discussion of these aspects is beyond the scope of this contribution. Readers interested in these topics are encouraged to study the review of Matos, Duarte, and Duarte ([Matos et al., 2012](#)). Unfortunately, our understanding of the fundamentals of quantitation and the development of software tools are not as advanced in 2D-LC as they are in 1D-LC—this is not surprising given the head start in 1D-LC of 30 years or so. But, the 2D-LC community is catching up quickly, and the future is promising in this area.

### 9.1 DATA STRUCTURES AND HANDLING

[Fig. 7.34](#) shows one of the often-cited figures ([Adahchour et al., 2006](#)) that clearly illustrates how data from 2D-LC experiments are handled to produce 2D chromatograms.

In the absence of software dedicated to the analysis of 2D-LC data, these steps can be accomplished by first exporting raw data from the chromatography data system, and then manipulating those data using any of a number of programming and analysis packages such as MatLab or Mathematica. While this process is not onerous for spectroscopic data (e.g., UV absorbance, or fluorescence detection), it becomes practically untenable for high-resolution MS data because of the large volumes of data involved (e.g., >1 GB of data per analysis) and proprietary MS data file formats. Thus, the continued evolution of dedicated software for analysis of 2D-LC data is very important. From an analyst’s point of view, it is far better to dedicate time to optimization of methods and data collection, than to development of software for data analysis.

### 9.2 DESIRABLE FEATURES OF SOFTWARE SUPPORTING TWO-DIMENSIONAL LIQUID CHROMATOGRAPHY

I have written this section to serve as a series of suggestions to new users considering getting involved in 2D-LC, either through shared equipment or a new equipment purchase. Following are several

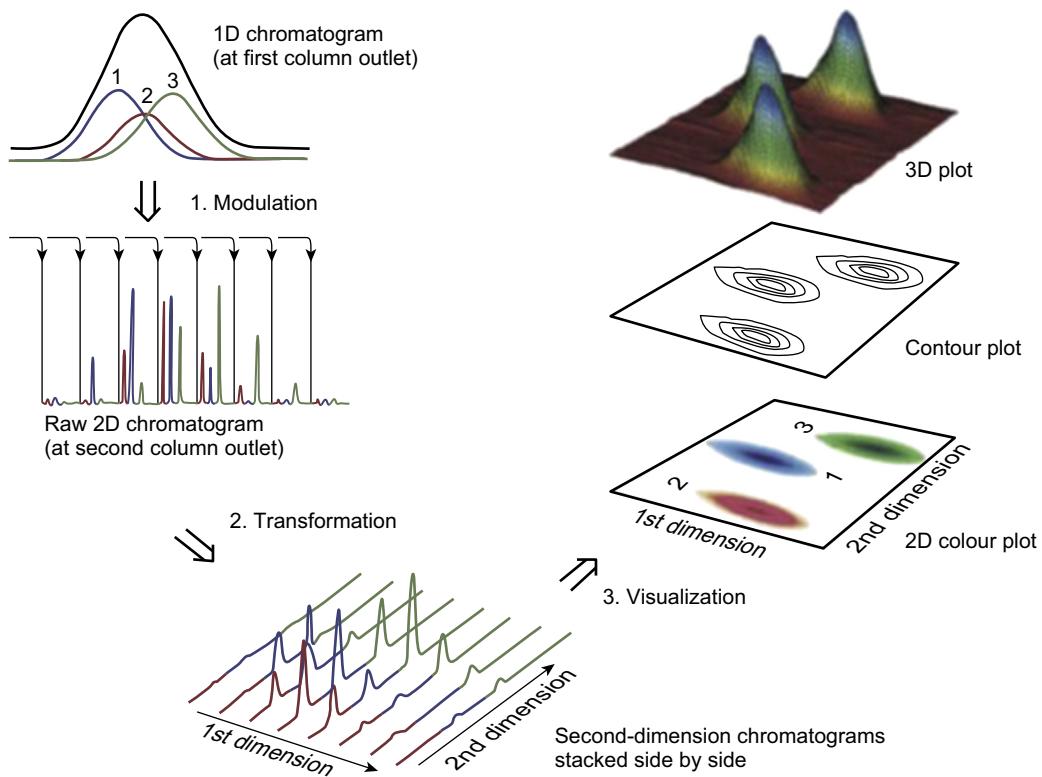
**FIGURE 7.34**

Illustration of the flow of information in a two-dimensional liquid chromatography experiment. The scenario shown here is precisely applicable to LC  $\times$  LC, but can be extended to other implementations of 2D separation. (1) Peaks eluting from the  $^1\text{D}$  column are sampled multiple times, which leads to the appearance of a particular compound in multiple  $^2\text{D}$  chromatograms. (2) The data from these  $^2\text{D}$  separations are then transformed into a 2D array of data where the two dimensions are  $^1\text{D}$  and  $^2\text{D}$  time. This array can then be visualized as a contour plot or a 2D topographic map, both of which can be colored in various ways to aid visualization.

*Adapted with permission from Adahchour, M., Beens, J., Vreuls, R.J.J., Brinkman, U.A.T., 2006. Recent developments in comprehensive two-dimensional gas chromatography (GC  $\times$  GC) – introduction and instrumental setup. TrAC Trends Anal. Chem. 25, 438–454. <http://dx.doi.org/10.1016/j.trac.2006.03.002>. Copyright 2006, Elsevier.*

desirable features of software one might use for 2D-LC, not only for data analysis, but also for instrument control and data acquisition.

### 9.2.1 Instrument Control and Acquisition

As implementations of 2D-LC separations have become more creative and sophisticated, the need for dedicated software for instrument control and data acquisition has increased. For example, the concept of shifted gradients discussed in [Section 7.4](#) is straightforward in principle, but is very tedious to

implement if gradient tables have to be entered on a line-by-line basis. Likewise, the concepts associated with the implementation of mLC–LC and sLC  $\times$  LC discussed in [Section 6.1](#) are straightforward, but leaving it to the user to keep track of all of the timed events (e.g., valve switches and gradient starts) is tedious and prone to error. Finally, software that “knows” which detector signals belong to which detector in the system facilitates efficient and effective use of all of the data that results from a single 2D-LC separation. Using software that is designed with these 2D-LC-specific operational features in mind again allows the user to focus more on the chromatography concepts and less on the actual implementation.

### **9.2.2 Data Processing—Peak Finding and Integration**

The strategies used for peak finding and integration in 1D chromatography are not directly applicable to 2D-LC data. This has motivated a number of groups to develop and study alternatives that are more effective in the 2D case ([Allen et al., 2012](#); [Latha et al., 2011](#); [Vivó-Truyols and Janssen, 2010](#)). Peak finding is complicated by the fact that in 2D chromatograms we are interested in the evolution of the detector signal in two dimensions rather than a single dimension. Furthermore, the characteristics of the detector background (i.e., baseline) are different in the two dimensions ([Filgueira et al., 2012](#)), often more pronounced in the second dimension of 2D-LC compared to 1D-LC, and different enough from those in GC  $\times$  GC that algorithms cannot be directly employed with LC  $\times$  LC data. One of the most pronounced challenges, which is present in 1D chromatography but only in one dimension, is variability in  $^2\text{D}$  retention time—both between 2D-LC analyses and between  $^2\text{D}$  separations within a single 2D-LC analysis ([Reichenbach et al., 2009](#)). For compounds that are present in multiple fractions of  $^1\text{D}$  effluent transferred to the  $^2\text{D}$  and thus appear in multiple adjacent  $^2\text{D}$  separations, this variability in  $^2\text{D}$  retention time can make the assignment of multiple  $^2\text{D}$  peaks to a single  $^1\text{D}$  peak difficult. This is especially true in cases where unsupervised peak detection is used, and can lead to artificial peak splitting.

### **9.2.3 Advanced Processing—Application of Chemometric Methods**

The higher dimensionality of data obtained from 2D-LC experiments compared to 1D-LC lends itself to processing using advanced chemometric methods. These methods enable extraction of chemically meaningful information by mathematical means that would remain obscured if we only relied on the physical separation of compounds as represented by 2D chromatograms ([Stoll et al., 2007](#)). The most powerful approaches involve the use of multichannel detectors along with two dimensions of chromatographic separation (e.g., multiple wavelength UV absorbance, or MS detection at multiple masses). So long as the UV or mass spectra of two overlapping chromatographic peaks are slightly different, they can be resolved by chemometric means even with chromatographic resolution in each dimension as little as 0.2 ([Cook et al., 2015](#)). This is remarkable considering that a resolution of about 1.5 is generally required for accurate quantitation in 1D-LC, and the fact that resolution is very expensive in terms of analysis time (see [Section 5.3.1](#)).

## **9.3 QUANTITATION**

When comparing the quantitative performance of 2D-LC to that of 1D-LC it is convenient to use metrics such as accuracy and precision. 2D-LC has the potential to improve the accuracy of quantitative determinations, especially for complex samples that lead to crowded chromatograms.

When relatively nonselective detectors are used (e.g., UV absorbance), the potential for additional peak capacity and/or selectivity provided by a 2D separation compared to a 1D one simply reduces the degree of peak overlap in chromatograms. This reduces the likelihood that an interfering peak, whether known or unknown, will be overlapped with the peak for a target analyte that is being quantified, which would lead to an overestimation of the concentration of the target analyte (Bailey et al., 2012). On the other hand, even when highly selective detection is used (e.g., high-resolution MS or tandem MS) the potential for additional separation and/or selectivity provided by a 2D method can improve accuracy by reducing matrix effects (Donato et al., 2012; Groskreutz et al., 2012b; Pascoe et al., 2001). This is especially valuable in cases where isotope-labeled standards are either not available or prohibitively expensive, as the elimination of matrix effects enables achievement of good accuracy when using external standards for signal calibration (Simpkins et al., 2010).

The precision of quantitation in 2D-LC, however, is quite a different matter. Whereas in 1D-LC we are accustomed to percent relative standard deviations (%RSDs) for peak areas on the order of 1% or less for replicate injections, the data we see in the 2D-LC literature are generally not this precise, particularly for LC  $\times$  LC separations. A quick survey of papers reporting peak area/volume precision reveals %RSDs in a range of about 1%–10% (Elsner et al., 2014; Mondello et al., 2008; Stevenson and Guiochon, 2013; Stoll et al., 2014, 2008; Vanhoenacker et al., 2015). In our own experimental work, we have measured the precision of peak areas obtained using detectors placed at the outlets of both the  $^1\text{D}$  and  $^2\text{D}$  columns in LC  $\times$  LC systems to help understand the contributions of each part of the system to the total variance in peak area (Stoll et al., 2014, 2008). In a theoretical study of quantitative precision in LC  $\times$  LC, Thekkudan et al. (2010) used simulations in an attempt to identify the factors that have the greatest influence on  $^2\text{D}$  peak area variation. After considering factors including the modulation ratio (i.e., the number of samples of a  $^1\text{D}$  peak that are transferred to the  $^2\text{D}$ ) and sampling phase (i.e., the time at which sampling a  $^1\text{D}$  peak starts relative to its native centroid) they concluded that the poorer precision of peak areas observed in LC  $\times$  LC experiments (4%–5%) compared to simulations ( $\sim 2\%$ ) may be due to imprecision in sampling the effluent from the  $^1\text{D}$  column. The general observation that  $^2\text{D}$  area is less precise than  $^1\text{D}$  area, even in the same LC  $\times$  LC system, in turn led us to test the idea that data from the  $^2\text{D}$  detector could be used to inform the data analysis, but that quantitation would ultimately be based on data from the  $^1\text{D}$  detector—we refer to this approach as (2DALC) (Cook et al., 2015).

Noncomprehensive 2D-LC separations present different challenges and opportunities relevant to quantitative performance. On one hand, simple heartcutting approaches require that a fraction of  $^1\text{D}$  effluent much larger than the volume of the  $^1\text{D}$  peak itself is transferred to the  $^2\text{D}$  column to ensure that the peak is transferred quantitatively. If this is not done, then any shifts in  $^1\text{D}$  retention of the peak will lead to significant variation in peak area measured at the  $^2\text{D}$  detector. On the other hand, hybrid approaches such as sLC  $\times$  LC effectively break several of the linkages between  $^1\text{D}$  and  $^2\text{D}$  parameters used in LC  $\times$  LC separations, allowing more flexibility in the sampling process. For example, the ability to transfer a  $^1\text{D}$  peak to the  $^2\text{D}$  column as a series of small volumes enables quantitative transfer without the use of very large sample loop volumes. Initial reports of the quantitative precision of sLC  $\times$  LC separations have been impressive—typically less than 1% RSD (Groskreutz et al., 2012a; Pursch and Buckenmaier, 2015).

## 10. FURTHER READING—SELECTED REVIEW ARTICLES

Much of this chapter is written with a general view of 2D-LC in mind. Of course, in every application area there are important details associated with the characteristics of analytes and sample matrices encountered, and typical analytical objectives that can significantly influence one or more of the aspects of the guidance discussed here (e.g., column selection). For this reason, readers interested in particular application areas are strongly encouraged to seek out review articles on the implementation of 2D-LC in these specific areas to increase their understanding of the nuances associated with the use of 2D-LC in these areas. Whereas 10 years ago most reviews on 2D-LC were more general in nature, currently several reviews are appearing that are more focused on the use of 2D-LC in specific areas. **Table 7.4** provides a summary of a selection of these reviews, as well as some important general reviews.

**Table 7.4 Summary of a Selection of Important Review Articles on Two-Dimensional Liquid Chromatography (2D-LC)**

Application Area	Application Focus	Research Group	Year	References
General	Comprehensive review; focus on practical issues	Carr et al.	2007	<a href="#">Stoll et al. (2007)</a>
General	Comprehensive review; focus on theoretical issues	Guiochon et al.	2008	<a href="#">Guiochon et al. (2008)</a>
Proteomics	Comprehensive 2D-LC separations of peptides	Mondello et al.	2011	<a href="#">Donato et al. (2011)</a>
Pharmaceutical analysis	Potential for 2D-LC in pharmaceutical analysis	Zhang et al.	2013	<a href="#">Zhang et al. (2013b)</a>
Polymers	Characterization of synthetic polymers	Schoenmakers	2014	<a href="#">Schoenmakers and Aarnoutse (2014)</a>
General	Separations with RP in both dimensions	Schmitz	2014	<a href="#">Li et al. (2014b)</a>
Polymers	Characterization of polymers and biopolymers	Kilz and Radke	2015	<a href="#">Kilz and Radke (2015)</a>
Bioanalysis	Overview of 2D-LC used for analysis of biological samples	Stoll	2015	<a href="#">Stoll (2015)</a>
Traditional Chinese medicines (TCMs)	Characterization of TCMs	Li et al.	2016	<a href="#">Li et al. (2016)</a>
Food analysis	Analysis of polyphenols in food	Mondello et al.	2016	<a href="#">Cacciola et al. (2016)</a>
Pharmaceutical analysis	Characterization of biotherapeutic proteins	Stoll et al.	2016	<a href="#">Stoll et al. (2016)</a>

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## 11. FUTURE OUTLOOK

I believe that in the next 5 years we will see the most impactful gains in the following three areas.

1. Improvements in interfacing between the two dimensions of 2D-LC systems—The current challenges in this aspect of 2D-LC were described in considerable detail in [Section 6](#). This is a very active research area in a number of research groups, and future developments will positively impact detection sensitivity, ease of use, the types of separation that can be coupled effectively, as well as the manner in which 2D-LC separations are carried out (e.g., mLC–LC vs. LC × LC).
2. Method development—A number of research groups are currently engaged in the development of strategies to improve the efficiency of method development for 2D-LC. Currently, a lot of experience is required to understand the interconnectivity between different variables encountered in the method development process. In addition to strategy, perhaps we will observe the emergence of software tools to simplify the process as well. I hope that the long sought after concept of “universal methods” develops in a way that is accessible to many users. That is, the development of a small number of powerful, but broadly applicable methods for 2D-LC, such that successful separations can be obtained with little method development.
3. Continuation of the rapid adoption of 2D-LC in the pharmaceutical industry—The most rapidly growing application area for 2D-LC is in the analysis of biopharmaceutical materials. Most of this effort is currently focused on the analysis of proteins, such as monoclonal antibodies. This is likely to expand to include analyses of therapeutic peptides in significant ways.

As we look further into the future, it will be interesting to see what comes of the resurgent interest in 3D-LC ([Vonk et al., 2015](#)). The potential for dramatically increased separation power is unquestionable. It remains to be seen whether or not the technological barriers to practical implementation of 3D-LC can be overcome in ways that are cost-effective.

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## ACKNOWLEDGMENTS

I want to thank Peter Carr, Claudia Seidl, and Gabriel Leme for helpful discussions during the writing of this contribution, Joe Davis for the contribution of [Fig. 7.11](#), and Eli Larson for his careful review of the manuscript.

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