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5 Selecting Separation Modes and Selectivities for Multi-Dimensional LC

Bob W.J. Pirok and Dwight R. Stoll

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5.1 INTRODUCTION TO THE THOUGHT PROCESS OF CHOOSING SEPARATION MODES AND SELECTIVITIES

Having addressed the fundamental principles of multi-dimensional chromatography in Chapter 3, we will now see how we can combine these ideas with information about the strengths and weakness of different LC separation modes on the way to developing actual 2D-LC methods. In this chapter, we focus on selecting and combining two separation modes or selectivities that will increase the likelihood of an effective 2D separation. We discuss how to identify appropriate retention modes, and refine separations using the properties of both the stationary and mobile phases.

5.1.1 The Concept of Sample Dimensionality

Before we consider specific retention modes in the development of a 2D-LC method, we first have to learn as much as we can about the analyte mixture. In Chapter 3 we discussed the importance of the orthogonality or complementarity of the two separations used in a 2D method. But, of course, two separations that will be highly complementary for one set of molecules may not be complementary

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for a different set of molecules. In other words, the extent to which two separations are complementary depends on both the properties of the stationary and mobile phases used, and the properties of the analyte mixture. With this in mind, it is often useful to identify the molecular descriptors that are likely to influence retention (if they are known). These are sometimes referred to as "molecular handles". In chromatography this concept has been described by Giddings as identifying the "sample dimensionality" associated with the analyte mixture at hand [1].

At the most fundamental level the sample dimensionality is directly related to the number of different types of intermolecular forces by which the various analytes interact with either or both the mobile and stationary phases. In this regard LC and especially 2D-LC have a significant advantage over GC and 2D-GC in that interactions in the mobile phase can be used to adjust selectivity. These forces include: Coulombic, London (dispersion), dipole-dipole, dipole-induced dipole and all the various types of acid-base (donor/acceptor) interactions such as hydrogen bonding, and Lewis acid-base processes. The toolbox of the LC analyst offers a wide array of retention modes that can be used to exploit these different types of intermolecular interactions between analytes and mobile and stationary phases. The different retention modes that have been used in 2D-LC, their selectivities, and examples of typical stationary phases are shown in Table 5.1.

5.1.2 Potential for Mixed-Mode and/or Unintended Interactions

In the case of a sample involving a mixture of components varying in hydrophobicity and charge, ideally we would have one dimension that separates exclusively on the basis of charge, whereas the other dimension would separate exclusively on the basis of analyte hydrophobicity. In practice, such an ideal distribution of selectivities is not very common. For example, in size exclusion (SEC) separations, chemical interactions can occur that influence the separation in ways that depend on analyte properties other than just size [2]. Another example of a single stationary phase exhibiting multiple selectivities is found in ion exchange (IEX) separations. In some cases, IEX stationary phase backbones may be quite low in polarity in nature [3, 4]. Such mixtures of retention mechanisms are sometimes deliberately exploited and the resulting phases and separations are typically referred to as "mixed-mode" (MM). Thus, depending on the stationary phase chemistry utilized, separation based on a pure retention mechanism can require adjustment of the mobile phase to counteract other interactions [5]. It is also possible for the mobile phase to either amplify or dampen the native selectivity of the stationary phase for a particular class of analytes. One example of such an effect is observed in the differential effect of the mobile phase modifiers acetonitrile and methanol on the retention of aromatic analytes by aromatic reversed-phase (RP) stationary phases (e.g., phenyl). The strongly dipolar and pi-electron rich acetonitrile dampens the selectivity of these phases for pi-electron rich analytes relative to that observed when using methanol [6, 7].

5.1.2 Suppression of Retention Mechanisms

Suppression of the retention mechanism that a stationary phase is designed for is also possible in extreme cases, such as the use of octadecylsilica columns for either RP or SEC separations of polymers. When used with aqueous-organic solvents as the mobile phase, polymers can be separated using solvent gradient elution, which reduces the dipolarity of the mobile phase as the fraction of organic solvent is increased. Yet, when the same column is used with the much stronger tetrahydrofuran as the mobile phase, analyte-stationary phase interactions are effectively suppressed, and the column essentially functions as a SEC column.

5.2 COMBINING SELECTIVITIES

The separation modes used in contemporary 2D-LC separations are summarized in Table 5.1, including acronyms, the chemical/physical basis of selectivity, and brief descriptions

TABLE 5.1
Overview of Separation Modes

Mode	Acronym	Selectivity	Common stationary phase (SP) selectors
Reversed-phase	RP	Hydrophobicity, Chain length, carbon skeleton	Alkyl (hydrocarbon: C1 to C30; most commonly C18), cyano (π-π)*, phenyl (π-π)*, carbon-clad zirconia (or graphitized carbon), PEG
Ion pairing	IP	Hydrophobicity, suppression of analyte ionization (acid/ bases)	Alkyl (hydrocarbon)
Hydrophobic interaction	HIC	Hydrophobicity	Short-chain alkyl hydrocarbons (C4 to C8)
Normal phase	NP	Polarity, Functional groups	Bare silica, Amino-propyl, diol, cyano
Argentation	AgLC	Degree of saturation, cis-trans isomers	IEX columns (*e.g.* sulfonic acid) or bare silica loaded with silver ions
Hydrophilic interaction	HILIC	Hydrophilicity, Polar character	Zwitterionic: Sulfobetaine, Phosphocoline; Basic: Amino propyl; Neutral: Diol, Amide
Ion exchange	IEX	Charge, Ionic interactions	Strong Cation Exchangers (SAX): Sulfonic Acid; Weak Cation Exchangers (WCX): Carboxylic Acid; Weak Anion Exchangers (WAX): Triethyl amine; Strong Anion Exchangers (SAX): Quaternary Amine
Size exclusion	SEC	Molecular size, Molecular weight	Crosslinked poly(styrene – divinyl-benzene) or methacrylate porous beads (SEC organic solvents); Polar-functionalized porous silica (SEC aqueous)
Mixed mode	MM	Combination of retention mechanisms	Anion-exchange/reversed-phase (AEX/RP), Cation exchange/reversed-phase (CEX/RP), Anion-exchange/cation-exchange/reversed-phase (AEX/CEX/RP); AEX/HILIC, CEX/HILIC, AEX/CEX/HILIC
Chiral	Chiral	Selector-specific chirality	Variety of selectors depending on the application. Most common are based on polysaccharide derivatives, antibiotics, and Pirkle phases
Affinity	Affinity	Selector-specific affinity	Stationary phases prepared for specific phase-analyte interactions (e.g., boronate-cis-diol; antibody-antigen)

Source: Adapted from [8].

of commonly-used stationary phase chemistries. Just because two different selectivities can be identified as ones that will both provide selectivity for analytes in the sample, and be complementary to each other, does not necessarily mean it will be easy to combine them in a 2D separation format. For many applications the number of possible combinations of stationary phase chemistries and mobile phase compositions is large, however some combinations have clear benefits and/or drawbacks compared to others. Thus, the challenge for the analyst is to narrow the list of possible combinations of selectivities to a small number to be evaluated experimentally. A list of factors for consideration is summarized in Table 5.2.

Some of the aspects listed in Table 5.2 may be surprising, simply because we do not have to consider them when developing 1D-LC methods. For example, slow column re-equilibration of columns

TABLE 5.2
Overview of Symbol Definitions as Used in Table 5.3

Symbol	Meaning	Relevant to	Description
A	Adsorption	^{1}D, ^{2}D	Lengthening of elution time due to injection solvent. Applies exclusively to SEC.
B	Breakthrough/Peak distortion	^{1}D, ^{2}D	Anomalous early elution in the second dimension. See Section 4.4.4 for more information.
E	Easy to modulate	2D-LC	Ease of developing active-modulation methods (e.g., trap columns or solvent dilution).
F	Fast separation	^{2}D	Method with short analysis times (e.g. <1 min).
H	High-resolution separation	^{1}D, ^{2}D	Method capable of high peak capacity.
I	Isocratic	^{1}D, ^{2}D	Possibility of (easily) running isocratic methods, reducing the complexity of the setup.
M	MS compatible	^{2}D	Possibility of using volatile mobile-phase additives and achieving good MS sensitivity.
O	Orthogonal/Complementary	2D-LC	Degree of independence of two separation mechanisms, assuming that the analyte mixture exhibits sample dimensions targeted by the two dimensions.
P	Applicability	2D-LC	Usefulness of the resulting separation.
Q	Column re-equilibration	^{2}D	Speed of column re-equilibration.
	Reversed-order recommended	LC×LC	Recommended to consider the reversed order of the modes.
S	Selectivity/Specificity	^{1}D, ^{2}D	Capability of the separation method to separate based on chemical characteristics of sample components (e.g. shape, orientation, composition/ sequence).
X	Solvent compatibility	LC×LC	Extent of (in)compatibility of ^{1}D effluent and ^{2}D eluent.
	Polymers		Suitable/Unsuitable for separations of polymers.
	Proteins		Suitable/Unsuitable for separations of proteins.

Source: Adapted from [8].

following gradient elution is typically viewed as an inconvenience in 1D-LC, but not a factor that threatens the viability of the method altogether. However, in 2D-LC – and particularly LC×LC where fast ^{2}D separations are required – use of a separation that requires long re-equilibration times is simply not viable in the second dimension. Thus, fast separations (indicated in Table 5.2 with *F*) and short column re-equilibration times (indicated by *Q*) are useful in the second dimension of 2D-LC methods. This also explains why isocratic experiments may be desired in the second dimension in some cases (indicated in Table 5.2 by *I*). It is also good to realize that ultimately, all ^{1}D effluent components will enter the ^{1}D and ^{2}D detectors unless appropriate measures have been taken to avoid this when it is expected to be a problem. This is most important is when mass spectrometric (MS) detection is used in the second dimension.

Taking into account all of the potential benefits and drawbacks of each separation type, we can assess the potential effectiveness of each possible combination of separation modes. This information is organized in Table 5.3, which provides an overview of the strengths and weaknesses of possible combinations, utilizing the symbols listed in Table 5.2 to communicate where the strengths and weaknesses lie. While this table provides a one-stop overview, we cannot emphasize strongly enough

TABLE 5.3
Overview of the Possible Combinations of Separation Modes for Online 2D-LC

	^{2}RP ($F^{+}H^{+}Q^{+}M^{+}$)	^{2}NP ($F^{-}Q^{-}$)	^{2}HILIC ($M^{+}Q^{-}$)	^{2}HIC ($F^{-}H^{-}M^{-}Q^{-}$)	^{2}IEX ($M^{-}Q^{-}S^{+}$)	^{2}SEC-Aq ($F^{+}H^{-}I$)	^{2}SEC-Or ($F^{+}H^{-}I$)	^{2}Ag ($F^{-}Q^{-}S^{+}$)	^{2}Chiral ($F^{+}IS^{+}$)	^{2}Affinity ($H^{-}Q^{-}S^{+}$)	^{2}SFC ($F^{+}H^{+}M^{+}$)
^{1}RP (H^{2+})	E O^{+} P^{+} X^{+}	B O^{2+} X^{2-}	B O^{2+} X^{+}	B E O^{-} P^{-} X^{+}	O^{+}	A E O^{+} P^{+} X^{+}	A E O^{+}	B O^{2+} X^{-}	O^{2+}	O^{2+} X^{+}	B O^{2+} X^{-}
^{1}NP (H^{+})	B O^{2+} X^{2-}	O^{-} P^{-} X^{+}	O^{-} P^{-} X^{-}	B O^{2+} P^{-} X^{2-}	O^{2+}	O^{2+} X^{2-}	O^{2+} P^{+} X^{+}	O^{+} X^{+}	O^{2+}	O^{+} X^{2-}	O^{-} X^{2+}
^{1}HILIC (H^{+})	B O^{2+} P^{+} X^{+}	B O^{-} X^{-}	O^{-} X^{+}	B O^{2+} P^{-} X^{-}	O^{+} X^{+}	O^{2+} P^{+}	A O^{+} X^{+}	B O^{+} X^{-}	O^{2+}	X^{-}	X^{+}
^{1}HIC (H^{-})	E O^{-} X^{2+}	B O^{2+} P^{-} X^{2-}	B O^{2+} X^{-}	O^{2-} P^{2-}	B O^{+} P^{-} X^{2+}	O^{2+} P^{-} X^{2+}	A O^{+} P^{-} X^{-}	B O^{2+} P^{-} X^{2-}	O^{2+} P^{2-}	O^{+} X^{+}	O^{+} P^{2-} X^{2-}
^{1}IEX ($H^{-}S^{+}$)	E O^{+} P^{+} X^{2+}	B O^{2+} X^{2-}	B O^{+} X^{-}	B O^{+} P^{-} X^{2+}	B X^{-}	O^{+} X^{2+}	A O^{+} P^{-} X^{-}	B O^{+} X^{-}	O^{2+}	O^{+} X^{+}	O^{+} X^{2-}
^{1}SEC-Aq (H^{2-})	E O^{+} P^{+} X^{2+}	B O^{2+} X^{2-}	B O^{2+} X^{-}	B O^{+} P^{-}	O^{+} X^{2+}	O^{2-} P^{2-}	A O^{2-} P^{2-} X^{2-}	O^{2-} X^{2-}	O^{2+} P^{-}	O^{2+} X^{+}	E O^{2+} P^{-} X^{-}
^{1}SEC-Or (H^{2-})	B^{2-} O^{+} X^{-}	B O^{2+} X^{+}	O^{+} X^{+}	B O^{+} P^{-} X^{2-}	B O^{+} P^{-} X^{-}	O^{2-} P^{2-} X^{-}	O^{2-} P^{2-}	O^{2+} X^{+}	O^{2-} P^{-}	O^{2+} P^{2-} X^{-}	O^{+} P^{-} X^{+}
^{1}Ag ($H^{+}S^{+}$)	B O^{2+}	O^{+} X^{+}	O^{+} X^{+}	B O^{2+} P^{-} X^{-}	O^{2+} X^{-}	O^{2+} X^{-}	O^{2+} X^{-}	O^{2-} P^{2-}	O^{2+}	O^{2+} X^{2-}	O^{+} X^{+}
^{1}Chiral (IS^{+})	O^{2+}	O^{2+}	O^{2+}	O^{2+} P^{2-}	O^{2+}	O^{2+} P^{-}	O^{2+} P^{-}	O^{2+}	O^{2-} P^{2-}	O^{2+}	O^{2+}
^{1}Affinity ($H^{-}S^{++}$)	O^{2+} P^{-} X^{+}	B O^{2+} P^{-} X^{-}	B O^{2+} P^{-}	O^{2+} P^{-}	O^{+} P^{-} X^{+}	O^{2+} P^{-} X^{+}	A O^{2+} P^{2-} X^{2-}	B O^{2+} P^{-} X^{-}	O^{2+} P^{-}	O^{-} P^{2-}	O^{+} P^{-} X^{2-}
^{1}SFC (H^{+})	E O^{2+} X^{+}	O^{-} X^{+}	E O^{-}	O^{2+} P^{3-}	O^{2+} X^{+}	O^{2+} P^{2-} X^{2+}	O^{2+} X^{2+}	O^{+} X^{+}	O^{2+}	O^{2+} X^{-}	E O^{-} X^{2+}

Source: Reprinted from [8].

that the strengths and weaknesses of each combination of selectivities depends strongly on the application at hand and the detector used. As such the table should be viewed as a first approximation – a starting point with which to begin thinking about which combinations should be considered first in method development, and then refine the perspective of the table based on nuances related to the specific application. Moreover, many of the challenges highlighted in Table 5.3 are not as serious when developing non-comprehensive 2D-LC separations – in short, more time in the second dimension helps mitigate many problems. It is also good to realize that the color does not indicate usefulness. Indeed, seemingly challenging combinations may be highly useful for certain applications. This has driven researchers to explore the limits of these challenges to develop practically useful methods.

In the following sub-sections we briefly discuss each of the different separation modes. In each case we briefly discuss the basis for separation, strengths, and weaknesses in the context of 2D-LC separations, and provide a few examples of 2D-LC applications involving that particular separation mode. Our intent is not to list all 2D-LC applications in this chapter; readers interested in a comprehensive list of all 2D-LC applications are referred to our free, online database (www.multidlc.org/literature/2DLC-Applications). Reversed-phase LC is frequently used in 2D-LC methods in a number of application areas, and thus the final section of this chapter is entirely devoted to the selection of RP phases for 2D-LC.

5.2.1 Normal-Phase Liquid Chromatography

The oldest separation mode in our toolkit, normal-phase liquid chromatography (NPLC) utilizes a sorbent that is more polar than the eluent as the stationary phase. In combination with a low polarity mobile phase, compounds can be separated based on differences between their dipolarity and hydrogen bonding characteristics, and locations of the polar groups within the analyte structure. Retention is decreased by increasing the fraction of the polar component of the mobile phase. Whereas NPLC is usually thought of as involving mobile phases containing only organic solvents (or very little water), hydrophilic interaction chromatography (HILIC) is somewhat similar to NPLC but usually involves 1–30% (v/v) water in the mobile phase.

Strengths – Selectivity for regioisomers and amenable to separation of analytes that are only soluble in 100% organic solvent mobile phases.

Weaknesses – Application of NPLC within two-dimensional separation systems can be challenging due to incompatibility of the non-polar organic solvents employed in NPLC and the polar (especially water) solvents frequently used with other separation modes. This is the single biggest reason that combinations involving NPLC in Table 5.3 receive low scores. For example, in the case of RPLC×NPLC, the amount of water in the RPLC mobile phase typically makes it immiscible with many NPLC mobile phase. One approach to deal with this is to remove the volatile organic solvent from the ^{1}D NPLC effluent by evaporation [9] (see Section 4.4.7). When used in combination with RPLC, it is preferred to use the NPLC separation in the first dimension because active modulation techniques are more effective with this configuration compared to the opposite one (i.e., RPLC×NPLC). This can be avoided by using RPLC in the non-aqueous mode (NARP), but this is not useful for many classes of compounds. One exception to the poor compatibility of NPLC with other separation modes is the combination NPLC with SEC separations that use mobile phases composed of a solvent (i.e., “organic SEC”).

The other significant weakness of NPLC is that equilibration of the stationary phase following a change in mobile phase conditions (e.g., as in gradient elution) is perceived to be slow. To minimize the impact of this limitation, most applications involving NPLC use it either in the first dimension, or under isocratic [10, 11] or pseudo-critical conditions [12] in the second dimension.

Representative applications – SEC×NPLC has been applied to the analysis of complex polymer mixtures [13]. NPLC×RPLC separations have been described for the analysis of cold-pressed lemon oil [14], alcohol ethoxylates [15], and oligomers [16].

5.2.1.1 Argentation (Silver-Ion) Normal-Phase Chromatography

A special form of NPLC is argentation (silver-ion) chromatography (AgLC). This separation mode utilizes a silica-based stationary phase that is treated with an aqueous solution of silver nitrate. When used in conjunction with an organic mobile phase, this mechanism is quite selective for π-π interactions between the double bonds of unsaturated analytes and the silver ions. This gives rise to selectivity that is based on differences in the extent and location(s) of unsaturation within different molecules, and thus AgLC has been mainly applied to the analysis of lipids. The silver ions bound to the stationary phase can easily be disturbed by the presence of small quantities of non-suitable solvents, and the relative instability of AgLC systems renders them mainly useful as ^{1}D separation. Example applications of AgLC×RP include the determination of lipids in samples of rice oil [17], soybean oil [18], peanut oil and mouse tissue [19].

5.2.1.2 Hydrophilic-Interaction Liquid Chromatography (HILIC)

Although there is ongoing study of the retention mechanism of HILIC separations [20] they can be thought of conceptually as a specific form of NPLC. The use of mobile phases rich in organic solvents and a small amount of water is thought to promote the development of a water-rich layer at the surface of the highly polar stationary-phase sorbents used for HILIC [21]. Hydrophilic analytes can interact with, and partition into, this aqueous layer. A wide range of stationary phase chemistries have been commercialized for HILIC separations, which sometimes incorporate ionogenic elements (e.g., carboxylate or amino groups). Thus, the ability to choose from very different HILIC selectivities for applications involving different analyte mixtures has attracted a lot of interest for its use in 2D-LC. The latest developments in HILIC research have been summarized in recent review articles [22, 23].

Strengths – The ability of HILIC to separate molecules with subtle differences in dipolarity and structure can be remarkable, and highly complementary to other separation modes such as RPLC. For example, we found that glycosylated proteins that coeluted under RPLC conditions were separated by tens of minutes in a HILIC separation [24, 25].

Weaknesses – HILIC has most of the same weaknesses as conventional NPLC, however they are not quite as serious. Several groups have demonstrated that active modulation techniques can be used to effectively couple HILIC and RPLC separations [26], and it has been shown that re-equilibration of HILIC phases is sufficiently repeatable even with short re-equilibration times (e.g., 3 s) to allow their use in the second dimension of 2D-LC even with gradient elution [27, 28]. A number of authors have employed HILIC in the second dimension. D'Attoma and Heinisch compared RPLC×RPLC to RPLC×HILIC and found the latter combination to suffer from injection effects [29]. Holčapek *et al.* analyzed lipidomic samples using RPLC×HILIC-MS [30].

Representative applications – Most 2D-LC applications involving HILIC use it in the first dimension, along with RPLC in the second dimension. This configuration leverages the strengths of these separations, and minimizes their weaknesses. Examples include separations of cocoa procyanidins [31], anthocyanins in red wine [32], phosphatidylcholine isomers [33], and surfactants [34]. Finally, the assortment of commercially available HILIC selectivities is making it increasingly practical to use two HILIC phases in a 2D-LC system [28]. For example, Wang *et al.* developed an online HILIC×HILIC method and applied it to separate the saponins from *Quillaja saponaria* [35].

5.2.2 Ion-Exchange Chromatography

Ion-exchange separations employ stationary phases functionalized with ionogenic groups that enable the separation of analytes in the charge state that is opposite of that of the stationary phase. Elution is facilitated by increasing the concentration of counterions in the mobile phase. So-called "strong" ion-exchange phases contain functional groups that remain charged independent of the mobile phase pH (e.g., sulfonate groups for cation-exchange, and quaternary ammonium groups for

anion-exchange). In contrast, so-called “weak” ion-exchange phases carry functional groups whose ionization state depends strongly on the mobile phase pH *e.g.* diethylaminoethyl groups for anion-exchange (WAX), or carboxylate groups for weak cation-exchange (WCX).

Strengths – IEX is a very powerful approach for separation of analytes that have different charges in solution. This is particularly evident for polyvalent ions such as oligonucleotides, peptides, and proteins where IEX is an indispensible LC separation mode. IEX can also be highly complementary to other commonly used LC modes (e.g., RPLC). Yet, especially for small molecules or polymers with charged moieties as end-groups, much attention is required to achieve good usage of the separation space in IEX×RPLC systems. When the ^{1}D IEX separation is based primarily on the charge state of the analyte, ideally the charge should play no role in retention in the ^{2}D RPLC separation. This is difficult to realize because the charged groups increase solubility in the RPLC mobile phase. One way to address this is to use ion-pairing conditions in the ^{2}D separation. Pirok and coworkers used this strategy in their development of IEX×RPLC methods for the characterization of synthetic [5] and natural dyes in cultural heritage samples [36]. Since multi-valent analytes require a great deal more ion-pairing agent to fully suppress the charged character of the compound, the strategy appeared to be useful only when the number of ionogenic groups on the analytes was limited [5].

Weaknesses – Elution of ionogenic analytes from IEX phases requires “salty” mobile phases, which is a serious limitation when considering coupling with some detectors including MS and ELSD. In the area of protein separation by IEX this situation is improving slowly as several groups develop methods (i.e., stationary phases and mobile phase conditions) that are effective even with modest concentrations of volatile mobile phase buffers [37]. Nevertheless, the need for salty mobile phases in IEX separations is the major reason that IEX is primarily used as a first dimension in 2D-LC. This allows diversion of salts in the ^{1}D effluent to waste as they elute in the dead volume when RPLC is used in the second dimension, for example. Finally, IEX mobile phases are usually entirely aqueous, which makes coupling with LC modes running with organic-rich mobile phases challenging due to mobile phase mismatch, and the potential for precipitation of buffer salts from the mobile phase.

Representative applications – One of the most widely used applications of 2D-LC combines IEX and RPLC separations for the characterization of peptides using MS. One well-known variant of this approach is the so-called multi-dimensional protein-identification technology (MudPIT) [38]. Readers interested in this and other 2D-LC applications involving IEX are referred to Chapters 9, 10, and 13, which are focused on separations of small and large molecules in the pharmaceutical context, and chiral separations.

5.2.3 Size-Exclusion Chromatography and Hydrodynamic Chromatography

Out of all the LC separation types discussed in this book only size-exclusion chromatography (SEC) does not involve any chemical interaction between the analytes and stationary phase. In principle, separation by SEC is entirely entropic in nature, however it is not uncommon to observe non-specific analyte-phase interactions that influence the observed elution volume, and the mobile phase conditions are typically chosen to minimize these effects (e.g., tetrahydrofuran for organic SEC, and pH buffered aqueous solution for aqueous SEC of proteins and other bio-macromolecules). In the ideal case, SEC elution volume is entirely determined by steric exclusion of analytes from the pore volume. Molecules that are too large to enter the pores will only explore the interparticular volume, and thus elute faster through the column relative to smaller molecules that can (partially) permeate the pores. In some cases, large analytes excluded by the pores may be separated according to the mechanism of hydrodynamic chromatography [39]. Within the context of polymer separations, another interesting alternative is critical chromatography, formally known as “liquid chromatography at the critical conditions” (LCCC). In LCCC, the mobile-phase conditions are chosen to

decouple retention from the molecular weight of the polymers. This mechanism can be regarded as a niche form of RPLC or NPLC and is not featured in Table 5.3.

Strengths – While the resolution obtained in SEC is rather limited relative to other modes of LC, calibration using standards allows estimation of the distribution of analyte molecular weights based on the elution volume. SEC is by its nature an isocratic technique, which makes it somewhat attractive as a second dimension because no re-equilibration is needed after elution, and overlapped injections can be used in the second dimension to make more efficient use of the available ^{2}D time [40].

Weaknesses – Generally speaking, large volume columns (e.g., 300 mm x 7.8 mm i.d.) are used for SEC. If these columns are used in the first dimension of a 2D-LC system this can lead to very large fraction volumes that must be transferred to the second dimension for high sensitivity. The generally poor resolving power of SEC limits its utility in LC×LC separations, however recent advances in SEC carried out under UHPLC conditions [41, 42], or with superficially porous particles [43, 44], are changing this situation. Aqueous SEC can work well when coupled with other separations such as RPLC and IEX. Coupling organic SEC with other LC separations can be more difficult due to the use of organic solvents for the mobile phase such as tetrahydrofuran, and the potential mobile phase mismatch that can arise. Organic SEC can also be quite sensitive to small amounts of water that lead to adverse adsorption effects, so care must be taken when coupling organic SEC as a second dimension with another LC mode that uses water in the mobile phase.

Representative applications – 2D-LC separations involving SEC are focused nearly exclusively on the analysis of large molecules such as polymers or proteins. Readers interested in these applications are referred to Chapter 11 (polymers) and Chapter 10 (proteins). In both cases one can find examples where SEC is used in the first dimension, the second dimension, or even both dimensions.

5.2.4 Hydrophobic Interaction Chromatography

Also known as salting-out chromatography, hydrophobic interaction chromatography (HIC) is exclusively applied to the separation of proteins [45]. High concentrations of salt (e.g. 2 M ammonium sulphate) in the mobile phase promote adsorption of the protein onto a moderately hydrophobic stationary phase [46]. Retention is decreased by decreasing the mobile phase salt concentration (i.e., the opposite of IEX), without the need for organic solvents (i.e., different from RPLC).

Strengths – Given that mobile phases used for HIC are almost always entirely aqueous, the native structure of protein analytes is preserved in the mobile phase environment, and the separation selectivity is different from that obtained with RPLC [47,48]. This in turn makes the coupling of these two separations attractive.

Weaknesses – Use of mobile phase salt concentrations in excess of 1 M is typically a pre-requisite for adequation retention in HIC, which prevents its use in the second dimension when MS is used as the 2D detector.

Representative applications – To date, the most active application area for 2D-LC separations involving HIC has been the characterization of antibody-drug conjugates (ADCs). In this case the HIC separation resolves different protein species according to the number of hydrophobic small molecule drugs bonded to the protein, and the ^{2}D RPLC separation effectively separates the protein analytes from the salt present in the ^{1}D effluent prior to MS detection [49, 50]. Interested readers are referred to Section 10.4 for further discussion of these applications.

5.2.5 Chiral Chromatography

The entirety of Chapter 13 of this book is devoted to the use of chiral separations in one or both dimensions of 2D-LC systems. Readers interested in this topic are directed to Chapter 13, and the

strengths and weaknesses of this separation mode in the context of 2D-LC are only discussed briefly here. Chiral chromatography utilizes an array of column chemistries in which chiral selectors (e.g., small molecules, oligosaccharides, and proteins) are immobilized on a porous substrate (e.g., silica) to separate chemical compounds on the basis of their stereoconfigurations. Naturally, this separation mechanism is particularly useful for the separation of molecules having one or more chiral centers, including pharmaceuticals and their metabolites, agrochemical compounds, and amino acids. Method development for chiral separations typically involves lengthy studies to screen for chiral selectors that exhibit stereoselectivity for particular analytes of interest.

Strengths – The primary strength of chiral separations in the context of 2D-LC is that they can separate enantiomers and other structurally similar molecules that are difficult to separate any other way. As such, they are highly complementary to all other LC separation modes. Historically, much of the development of chiral separations was focused on NPLC conditions. However, in recent years there has been tremendous growth in the development and use of chiral separations that rely on other separation modes including HILIC, IEX, RPLC, and SFC [51,52]. These developments have created numerous opportunities to couple chiral separations with other achiral separation modes in 2D-LC formats.

Weaknesses – The two biggest weaknesses of chiral separations in the context of 2D-LC are the slow kinetics of desorption from the chiral support [53, 54], and unpredictable selectivity. The slow separation kinetics have hindered the use of chiral separations in the second dimension of LC×LC separations in particular, though this is beginning to change as discussed in Chapter 13. The inability to predict which chiral stationary phase is best suited for the separation of a particular pair of enantiomers means that many phases are often evaluated experimentally, and in some cases more than one chiral stationary phase is used in a 2D-LC separation (i.e., one phase is used in the second dimension to separate particular pairs of enantiomers that coelute in the first dimension, while other pairs are separated in the second dimension with a different phase; see Section 9.5.2).

Representative applications – Currently the most active application areas for 2D-LC involving one or more chiral separation are pharmaceutical analysis (e.g., analysis of molecules with one or more chiral centers, and their metabolites) and bioanalysis (e.g., separation of D-and L- amino acids). Please see Chapter 13 for a complete discussion of these and other applications.

5.2.6 Affinity Chromatography

Affinity chromatography comprises a group of separations that on one hand covers a wide array of chemistries, and on the other hand can offer incredibly high selectivity. Stationary phases are often prepared by immobilizing proteins that exhibit strong interactions with a small number of molecules with particular three-dimensional structures. Prevalent examples include antibodies and protein receptors that are ubiquitous in biological systems [55–58]. Other stationary phases used for this purpose include phenylboronate [59, 60].

Strengths – Generally speaking, the selectivities of affinity-based separations are unparalleled. With sufficient effort, a stationary phase bearing a selector ligand can be made to be highly selective for almost any target analyte. Most affinity-based separations function well in highly aqueous mobile phases, making them highly compatible with most other LC separation modes.

Weaknesses – The extremely high selectivity (approaching absolute specificity in some specific instances) of affinity-based phases is also their greatest weakness. Any given stationary phase is only applicable to small number of target analytes. Desorption of the analyte from the stationary phase can also be slow [61], which limits their potential for use in ^{2}D separations. Sometimes affinity separations require elution conditions that can be detrimental to the following ^{2}D separation. This appears to be the case when separating antibodies using an ^{1}D affinity separation based on Protein A and a ^{2}D SEC separation (i.e., ProA-SEC). In this case the acidic mobile phase required for elution of the protein from the Protein A may induce protein aggregation during the SEC separation [62].

This is an area that should be looked at in more detail using class-selective affinity ligands such as boronates, lectins, and more general affinity ligands such as co-factor emulators.

Representative applications – While the number of published applications is small, they are significant. The use of ProA-SEC for the determination of titer and purity of therapeutical monoclonal antibodies (mAbs) has been demonstrated by several groups [57, 62, 63]. Hu *et al.* have used affinity chromatography and RPLC in the first and second dimensions of a LC×LC system for the characterization of traditional Chinese medicines [64].

5.2.7 Supercritical-Fluid Chromatography

While technically not part of the LC portfolio, supercritical-fluid chromatography (SFC) is increasingly being used in multi-dimensional separation systems. In SFC, the mobile phase is generally composed of carbon dioxide (CO_2) at sub- or supercritical conditions. Organic solvent modifiers are used to change retention and selectivity in ways similar to those used in NPLC under high pressure conditions [65].

Strengths – The advantages of SFC over LC are lower mobile-phase viscosity (and correspondingly lower pressure drops and higher diffusion coefficients of analytes) [66, 67], and NPLC-like selectivities without large quantities of hazardous organic solvents characteristic of NPLC [65]. The latter has been particularly important for chiral separations. The low viscosity of SFC mobile phases may enable faster ^{2}D separations than what is achievable with RPLC, however this has not been demonstrated in practice to date.

Weaknesses – When SFC is used in the second dimension, it is very susceptible to mobile phase mismatch effects when a first dimension is used that relies on a significant concentration of water in the mobile phase. Also, the scope of applications is relatively limited compared to RPLC (e.g., SFC is not broadly applicable to separation of biopolymers).

Representative applications – SFC is routinely applied in a variety of areas ranging from hydrocarbon analysis to lipids and pharmaceutical analysis. Several groups have demonstrated the use of SFC in the second dimension in 2D separations where RPLC is used in the first dimension [68, 69]. SFC is also potentially interesting as ^{1}D separation, because the mobile phase is compatible with ^{2}D RPLC, as demonstrated by François *et al.* [70, 71]. SFC×SFC using packed (capillary) columns has been demonstrated by Hirata [72, 73]. Open-tubular SFC×SFC [74] is an amazing technological achievement, but not a robust approach ready for routine practical use with current technology. Readers interested in these applications are also referred to Chapters 9 and 13.

5.3 CHOOSING REVERSED-PHASE SELECTIVITIES

Before discussing strategies for selecting RP stationary phases for use in 2D-LC, it is worth noting that in some situations adjusting mobile phase conditions can be just as effective as changing stationary phases. This point is illustrated in detail in Chapter 6 by way of a method development case study. Some contemporary applications, such as RP×RP separations of peptides where varying the mobile phase pH is particularly effective for inducing a selectivity difference between two similar RP phases [75, 76], have exploited the power of the mobile phase in 2D-LC to great effect.

5.3.1 Selecting an RP Column for One Dimension of a 2D-LC Separation

When considering an RP separation for use in one dimension of a 2D-LC system, usually choosing the RP column is relatively straightforward. In most cases the particular RP selectivity (e.g., C8 vs. C18 vs. phenyl) that is used is not so important, because of the large differences in selectivity between the RP mode and other modes, in a general sense. For example, an ion-exchange separation will typically be highly complementary to a RP separation, so long as there is no strong correlation

between the charge state of analytes in solution and their hydrophobicites. In our work in situations like this, we prioritize other characteristics of RP columns when choosing what to use in a 2D-LC system. These include chemical (i.e., pH and temperature) and physical (i.e., robustness of the particle bed) stability of the column, commercial availability of the phase in multiple column dimensions, and peak shape in simple mobile phases (e.g., dilute formic acid for MS detection). In other words, we prioritize ease-of-use over selectivity of the RP column in these situations.

5.3.2 Selecting RP Columns for Both Dimensions of a 2D-LC Separation

Using two RP columns in a 2D-LC method is attractive from the point of view of compatibility of the ^{1}D and ^{2}D separations. Note that the upper-left corner of Table 5.3 is very green, indicating many benefits of the ways that RP separations work well together when used in both dimensions. However, one of the most prominent criticisms of this pairing is that it is not immediately obvious how the selectivities of two RP phases could be as complementary as RP paired with a very different separation mode such as SEC or HILIC. The good news is that there are more than 1,000 commercially available RP phases, which provides ample opportunity to discover RP phases that might be complementary enough to solve a given separation problem – the challenge, of course, is finding them quickly.

This challenge has inspired a number of groups to explore different strategies to narrow the number of phases that should be tried when screening different phase combinations. The Hydrophobic Subtraction Model (HSM) of RP selectivity developed by Snyder, Dolan, Carr, and coworkers has been used more than any other framework for addressing this problem. Readers interested in how the model was formulated and its uses are referred to other resources [77, 78]. In addition, a recent, comprehensive review article on modern models of RP selectivity is also a valuable resource [79]. Briefly, the HSM asserts that the selectivity of a given stationary phase (defined as the ratio of the retention factor of an analyte [k_x] to the retention factor of ethylbenzene [k_{EB}]) can be quantified using a linear combination of five stationary phase – analyte property pairs as shown in Eq. 5.1:

$$\ln\left(\frac{k_x}{k_{EB}}\right) = H \cdot \eta - S^* \cdot \sigma + A \cdot \beta + B \cdot \alpha + C \cdot \kappa \tag{5.1}$$

Here, the H, S*, A, B, and C parameters quantify the contributions of the stationary phase to selectivity and represent hydrophobicity (H), resistance of the phase to penetration by bulky analytes (S*), hydrogen bond acidity (A) or basicity (B), and ionic character of the stationary phase (C), respectively, and the corresponding η, σ, β, α, κ parameters represent the conjugate characteristics of analytes. The column parameters have been measured for about 760 columns and are freely available through an interactive web-based database (www.hplccolumns.org). Although the initial motivation for the development of the HSM was focused on a strategy for identifying columns with similar selectivities, the data can also be used to identify columns that exhibit very different, or even complementary selectivities. All models of RP selectivity have strengths and weaknesses. One of the primary strengths of the HSM in the context of column selection for 2D-LC is the depth of the available data.

The HSM data has been used by a number of different groups, in a number of ways, for the purpose of identifying phases with selectivities different enough to be useful in 2D-LC separations. Zhang and Carr adapted Snyder's ternary plots [80] to visualize which columns lie at the periphery of the multi-dimensional space defined by the five parameters of the HSM [81]. We have further adapted this concept and developed an interactive tool for exploration of a three-dimensional space defined by any combination of three of the HSM parameters (www.hplccolumns.org). Lindsey,

Siepmann, and coworkers [82] used numerical simulations to predict RP×RP separations of a huge number of hypothetical small molecules using all possible combinations of RP columns in the HSM database, and then rank the resulting separations to identify the combinations of RP phases that looked most promising in a general sense. Interestingly, in spite of their very different approaches, both of these theoretical studies identified a common set of phases that appear to be good candidates for use in at least one of the two dimensions of 2D-LC separations that use RP in both dimensions. Moreover, it is interesting to note that none of these five phases is a traditional C18.

- Zorbax Bonus RP (polar embedded group)
- EC Nucleosil 100-5 Protect 1 (polar embedded group)
- BetaMax Acid (polar embedded group)
- Hypersil Prism C18 RP (polar embedded group)
- ZirChrom-PS (zirconia substrate with polystyrene coating)

It is important to recognize here that these conclusions represent the average selectivities of these phases toward small molecules of all kinds. As pointed out by Lindsey *et al.*, the identification of highly complementary column pairs is strongly dependent on the analyte set that is considered. It is entirely possible that for a given application the optimal column pair may not involve any of the members of the list above. So, the real value of this list is that it provides a rational starting point in method development; it does not guarantee that the final method must include one of these phases.

While the paragraphs above describe a theoretical basis for selecting RP phases for 2D-LC, there have also been some systematic experimental studies that provide support for the predictions from theory. Allen *et al.* used different combinations of six RP phases predicted by HSM to be highly complementary for RP×RP separations of a variety of small molecule extracts including those from corn seed, St. John's Wort, ginko biloba, and valerian root [83]. The stationary phases considered were C3, CN, PFP, C18, Bonus RP, and carbon-clad zirconia. Based on the experimental chromatograms the authors found that it was most important to have either the carbon-clad zirconia or the Bonus RP column in one of the two dimensions to obtain a good distribution of peaks across the 2D separation space. The carbon-clad zirconia column was not considered in the study of Lindsey *et al*, however this experimental work confirms the finding from the previous theoretical work that the Bonus RP phase is particularly attractive as a first choice to consider in method development.

The discussion in the preceding paragraphs was focused on RP selectivity selection for 2D-LC separations of small molecules. Over the past few years, Field, Euerby, and Petersson have initiated and continued a massive effort to characterize the selectivities of RP phases for (non-proteomic) separations of peptides [84–87]. Although it is still early in the development of this framework, and the knowledge has not yet been applied to 2D-LC separations, the framework looks very promising as a potential tool for selectivity selection in the case where a 2D-LC separation of peptides involving RP separation in both dimensions is desirable.

5.4 CLOSING REMARKS

Although much has been learned in recent years concerning the benefits and pitfalls associated with pairing different separation modes for 2D-LC methods (i.e., as summarized in Table 5.3), narrowing the list of viable options and selecting specific stationary phases is still a major impediment to 2D-LC method development. In some ways the same challenge is encountered in 1D-LC, but in 2D-LC it is considerably worse because there are two separations instead of one. Moreover, both must work well together, which is not always easy to arrange. In the years to come it will be important to develop more sophisticated frameworks to provide guidance in making these decisions that do not depend so strongly on user experience.

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