CHAPTER

Solvent selection in liquid chromatography

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CHAPTER OUTLINE

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13.1 ELUTION STRENGTH

In liquid chromatography (LC), the elution strength is the ability of the mobile phase to sweep away the solutes retained on the stationary phase. This strength depends on the nature of the stationary phase and solutes, as well as on the mobile-phase composition (i.e., nature and concentration of the solvents and additives), pH, and column temperature. Therefore, for a given stationary phase, the elution strength is not a property exclusively related to the solvent, since solutes undergo different elution strengths depending on their particular molecular structures. The elution strength of the mobile phase is a very practical concept in LC, commonly used to adjust the overall retention for a group of solutes inside the target retention region, optimally within the $1 < k < 5$ range, or at least $0.2 < k < 20$, k being the retention factor or relative retention:

$$k = \frac{t_R - t_0}{t_0} \tag{13.1}$$

where t_R is the retention time and t_0 the dead time (i.e., retention time of an unretained solute). For a given stationary phase and set of solutes, if the elution strength is too high, retention times will be too short, and consequently, the resolution will be poor. Conversely, if the elution strength is too low, retention times will be excessive, and consequently, the analysis time will be too long and, due to excessive dilution, the signal-to-noise ratio at the peak maxima of the most retained analytes will decrease significantly. Once the elution strength has been adjusted, the selectivity (i.e., elution order and peak distribution) can be optimized without modifying significantly the overall retention [1]. The optimization criterion for selectivity is to resolve all the peak pairs of the target samples within a total analysis time as short as possible.

In addition to water, many organic solvents can be used to prepare the mobile phase (Table 13.1). Also, it is possible to use mixtures of solvents in different ratios to modify the solvent properties (e.g., the elution strength and selectivity). This can make solvent selection for a given purpose a puzzling task, unless suitable guidelines are followed. The purpose of this chapter is to summarize the most common strategies used by skilled chromatographers. Although mostly developed and used for reversed-phase liquid chromatography (RPLC) [8], the guidelines should be useful for normal-phase liquid chromatography (NPLC) as well [9], including the aqueous-compatible normal mode known as *hydrophilic interaction liquid chromatography* (HILIC) (see Chapter 6). The elution strength can be either maintained constant (isocratic elution) or gradually increased (gradient elution). In both approaches, the elution strength can be tuned to get the desired resolution and analysis time.

13.2 COLUMNS AND SOLVENTS IN RPLC, NPLC, AND HILIC

In RPLC, the stationary phase is nonpolar or weakly polar. The most common choice is octadecyl-silica (C18). The retention of highly hydrophobic solutes is reduced by using octyl- (C8) or butyl-silica (C4), and reversely, to increase the retention of some solutes, highly hydrophobic stationary phases such as triacontyl-silica (C30)

Table 13.1 Solvent Properties

Solvent	Normal Boiling Point (°C)[a]	Cut-Off Wavelength (nm)[a]	Viscosity at 20°C(mPa × s)[a]	Solubility Parameter (δ)[b]	Snyder's Global Polarity, (P)[c]
Isooctane	99.2	200–210	0.50	7.0	–0.4
Diisopropyl ether	68.0	380	0.33	7.1	1.8
n-Heptane	98.4	200	0.42	~7.5	0.0
n-Hexane	68.7	200	0.31	~7.5	0.0
Triethylamine	89.5	235	0.38	7.5	1.8
Cyclohexane	80.7	200	0.98	8.2	0.0
Carbon tetrachloride	76.8	263	0.97	8.6	1.7
Ethyl acetate	77.1	256	0.46	8.9	4.3
Toluene	110.6	284	0.59	8.9	2.3
Tetrahydrofuran	66.0	212	0.55	9.1	4.2
Chloroform	61.2	245	0.58	9.2	4.4
Dichloromethane	40.0	232	0.44	9.6	4.3
Methyl ethyl ketone	79.6	329	0.42 (15°C)	9.5	4.5
Acetone	56.3	330	0.30 (25°C)	9.6	5.4
Carbon disulfide	46.0	220	0.36	10.0	1.1
1,4-Dioxane	101.3	215	1.44 (15°C)	10.1	4.8
Pyridine	115.3	330	0.95	10.6	5.3
Isopropanol	82.3	205	2.86 (15°C)	11.4	4.3
1-Butanol	117.7	215	2.95	11.6	3.9
2-Methoxyethanol	124.6	210	1.72	11.7	5.7
Dimethylformamide	153.0	268	0.92	11.8	6.4
Ethanol	78.3	205–210	1.2	12.0	5.2
Dimethylsulfoxide	189.0	286	2.20	12.0	6.5
Acetonitrile	81.6	190	0.34	12.1	6.2
1-Propanol	97.2	210	2.26	12.2	3.9
Acetic acid	117.9	210	1.31 (15°C)	13.0	6.2
Methanol	64.7	205	0.55	14.5	6.6
Formamide	210.5	210	3.5	19.2	7.3
Water	100.0	<190	1.00	23.5	9.0

[a] *Refs. [2,3].*
[b] *According to Hildebrand, Refs. [2–5].*
[c] *Refs. [6,7].*

are used. Other bonded phases such as pentafluorophenylpropyl-silica or biphenyl-silica offer different selectivity. The mobile phase is prepared with water, to which a miscible organic solvent (the "modifier") is added to reduce the polarity and increase the elution strength. As the mixture progressively resembles the stationary phase, it competes better for desorption of nonpolar solutes, which are strongly associated with the stationary phase. In principle, a wide range of water-miscible organic solvents may be used as modifiers (Table 13.1); however, only three are usual in RPLC: acetonitrile (ACN), methanol (MeOH), and tetrahydrofuran (THF), especially the first. Solute elution occurs according to the decreasing polarities: The most hydrophilic solutes (which prefer the polar mobile phase) elute the first, while the most hydrophobic (which prefer the stationary phase) elute the last.

In NPLC, the stationary phase is polar. In order of increasing polarity, the most common stationary phases are cyanopropyl-silica, hydride silica, underivatized silica, diolpropyl-silica, and aminopropyl-silica. The mobile phase should be nonpolar and consists of an alkane mixed with a miscible polar solvent (the "modifier") to increase the elution strength. As the mixture more closely resembles the polar stationary phase, retention is reduced. Hexane is still largely used; however, because of concern about its long-term toxicity, it is being progressively substituted with isoheptane or the slightly more viscous *n*-heptane or cyclohexane. In addition, due to concern about the environmental impact of alkanes, sustainable or "green chemistry" solvents have been proposed as substitutes. These are mostly terpenes of vegetal origin as limonene, *p*-cymene, and α-pinene. Among the suitable modifiers (Table 13.1), the most common are chloroform (the worst choice from the viewpoint of green chemistry), ethyl acetate, dichloromethane, and isopropanol. Solutes elute in the order of increasing polarity: The most hydrophobic solutes elute the first, followed by the more polar solutes, which interact stronger with the stationary phase.

A water-rich layer adsorbed onto a polar stationary phase, such as underivatized silica or a silica-bonded polyol, ionic and zwitterionic stationary phase, is used in HILIC. Water-ACN mixtures (water is now the "modifier") are most frequently used as mobile phases. Instead of ACN, other water-miscible solvents used in HILIC are acetone, isopropanol, ethanol, 1,4-dioxane, dimethylformamide, and MeOH.

13.3 ASSESSMENT OF THE ELUTION STRENGTH

Two types of scales have been essentially used to estimate the capability of solvents to interact with their own and with other molecules: the solvatochromic scales, based solely on the solvent properties, and the eluotropic scales, which measure solvent properties in the presence of a reference stationary phase. For the first type, polarity scales based on spectroscopic measurements (spectral shifts in the absorption bands of some reference solutes), energy measurements, or theoretical descriptors have been proposed [5,10]. All these polarity scales can be used to estimate the elution strength of a solvent or a solvent mixture, and thus predict the retention for a given analyte.

Retention results from the many different intermolecular interaction mechanisms established between analytes and both the stationary and mobile phases. However, an extremely rough but rather useful simplification in LC is to refer to the elution strength of the mobile phase, independently from the nature of the solutes. This provides an idea about the global capability of the solvent mixture to push any heterogeneous group of analytes down the system. Fortunately, the elution strength is differently experienced by different analytes, which makes separation and selectivity tuning possible. Other decisions that should be taken in modeling or predicting retention are how many solvent interactions will be handled and how they will be measured. This is equivalent to selecting a polarity scale or a set of polarity descriptors, which estimate the interactions between the solvent molecules, while assuming that the strength of solute-solvent interactions for solutes of any kind is reasonably represented by the internal forces among the solvent molecules. As far as this assumption is true, any attempt of modeling and predicting retention on the sole basis of the descriptors of solvent properties will be successful. For instance, a highly associated solvent as water is assumed to strongly interact with polar solutes, whereas poorly associated solvents such as alkanes are assumed to weakly interact with all types of solutes.

13.3.1 THE HILDEBRAND SOLUBILITY PARAMETER AND OTHER GLOBAL POLARITY ESTIMATORS

The simplest choice of using a single descriptor of polarity (i.e., a global polarity of a solvent or solvent mixture), will be discussed first. The Hildebrand solubility parameter is a global measurement of the interactions that hold the solvent molecules together and, thus, provides a quantitative polarity scale for solvents by handling a single parameter [10,11]:

$$\delta = \left(-\frac{E}{v} \right)^{1/2} \tag{13.2}$$

where E is the cohesive energy of a mole of solvent, and v the molar volume. The minus sign corresponds to the fact that the cohesion process is exothermic. As observed in Table 13.1, water is at the bottom of the scale, and its large δ-value is typical of a highly associated solvent. Other polar solvents occupy intermediate positions, and alkanes appear at the top of the scale, with a δ-value typical of solvents with weak internal interactions. From the data, it follows that, for mixtures containing the same amount of modifier, the elution strength increases in the following order: MeOH < ACN < isopropanol ≪ THF in RPLC, and ethyl acetate < chloroform < dichloromethane < isopropanol in NPLC. In RPLC, this order roughly coincides with the elution strength found for mixtures of water with a given amount of modifier. Similarly for NPLC, the order coincides with that observed using mixtures of an alkane with a given amount of a miscible modifier.

Obviously, not all solvent mixtures are possible. In RPLC and NPLC, only solvents that are miscible with water or heptane, respectively, are used. As a rule, solvents are completely miscible if they are in the same third of the Hildebrand polarity scale (Table 13.1). Therefore, all solvents in the upper-third, bottom-third, or center-third are completely miscible with each other. A particular case is the dichloromethane/1,4-dioxane pair. These solvents have the same global polarity parameter but are not miscible; dichloromethane is totally miscible with alkanes, whereas 1,4-dioxane mixes with water in all proportions. This reveals the limitations of global polarity parameters, where the contributions of the molecular interactions of different types are not individually considered. Thus, water is incapable of accepting protons from dichloromethane, but 1,4-dioxane readily accepts protons from water. Also, a few "universal" solvents, such as ACN, THF, and isopropanol, are miscible with almost all solvents including heptane and water.

The addition of surfactants at sufficiently high concentration increases the miscibility of certain solvents. This has been useful for the development of micellar LC, where some organic solvents, such as butanol and pentanol, are used at concentrations higher than those miscible in aqueous solution, expanding the range of possible mixtures in RPLC [12]. However, if surfactants are present in the mobile phase, and depending on the nature and proportion of the mixture components, either true solutions, thermodynamically stable and transparent microemulsions, or unstable translucent emulsions may result. In contrast, solvent immiscibility provides the basis for countercurrent chromatography. In this technique, the separation is based on the different relative solubilities of the solutes in two immiscible solvents, one playing the role of the stationary phase, and the other the role of the mobile phase [13].

Another way of globally measuring intermolecular interactions is the relative retention of solvents by adsorption on silica, ε°. On this strongly polar solid phase, alcohols show strong interaction ($\varepsilon^\circ = 0.6$–0.7), whereas alkanes interact weakly ($\varepsilon^\circ = 0.01$). This polarity descriptor is eluotropic, since it is established using a reference stationary phase. Other global eluotropic polarity scales are obtained by measuring adsorption on other solid surfaces, such as alumina. The discrepancies among the different solvatochromic and eluotropic scales are inevitable, due to the limitations inherent in the use of a single global polarity parameter or uniparametric approach; however, the discrepancies do not disappear by using a multiparametric approach relaying on a few solvent descriptors, as they also depend on the way they are defined and measured.

13.3.2 GLOBAL POLARITY FOR SOLVENT MIXTURES

In RPLC, the polarity of a mixture of solvents is usually estimated as follows:

$$\delta_M = \sum_j \delta_j \varphi_j \tag{13.3}$$

where δ_j and φ_j are the Hildebrand solubility parameter and volumetric fraction of solvent j in the mixture, respectively (of course, any other polarity scale, whether

solvatochromic or eluotropic based on a single descriptor, can be used for these calculations). For instance, for the MeOH-water mixtures used in RPLC:

$$\delta_M = 14.5\,\varphi_{MeOH} + 23.5\left(1-\varphi_{MeOH}\right) \tag{13.4}$$

The variation of the global polarity of a mixture (and, consequently, of the elution strength) with mobile-phase composition is approximately linear for RPLC using modifier concentrations below 30% (v/v). Nonlinear relationships, as those provided later in this work, should be expected outside this limit. In NPLC, nonlinearity begins at lower modifier contents. Thus, the effect of minute amounts of a polar solvent in an alkane can be much larger than the effect of further adding larger amounts. However, keeping in mind these limitations, Eq. (13.3) is useful to estimate the composition of isoeluotropic mixtures in RPLC, as will be next explained.

13.3.3 APPLICATION FIELD OF THE CHROMATOGRAPHIC MODES AS DEDUCED FROM THE SCHOENMAKERS' RULE

Two conditions should be fulfilled to elute solutes within the target retention region:

1. Ideally, the solute polarity (δ_X) should be not far from the mean value of the stationary-phase (δ_S) and mobile-phase (δ_M) polarities:

$$\delta_X \approx \frac{\delta_S + \delta_M}{2} \tag{13.5}$$

   Otherwise, solutes will show an excessive preference for one of the phases. With gradient elution, δ_M changes with time. This means that each solute should fulfill Eq. (13.5) during its main elution stage, when the analyte is progressing along the column.
2. The polarities of both phases should differ significantly, which is required for a group of solutes of a wide polarity range to fulfill Eq. (13.5). If $\delta_M \approx \delta_S$, then δ_X for most solutes would not be in between δ_M and δ_S.

These two conditions are summarized in the rule proposed by Schoenmakers et al. [11], which states that the retention factors are within the optimal target region when

$$\left(\delta_M + \delta_S - 2\delta_X\right)\left(\delta_M - \delta_S\right) \approx 0 \tag{13.6}$$

The second parenthesis should be as large as possible, so that all solutes in a mixture can fulfill Eq. (13.5).

Assuming a linear behavior, the rule can be expressed graphically as shown in Fig. 13.1. According to the scheme in Fig. 13.1A, solutes with $\delta_X \approx 15.5$ (rather polar) are properly eluted with water ($\delta_M = 23.5$) on a C18 stationary phase ($\delta_S = 7.0$), and a miscible organic solvent should be added to elute less polar solutes. With 100% ACN, solutes with $\delta_X \approx 10$ (rather low polarity) are properly eluted. Therefore, within the limits of the predictions based on the Hildebrand solubility parameter and the assumption of linearity, solutes in the $10 > \delta_X > 15.5$ range are properly eluted using

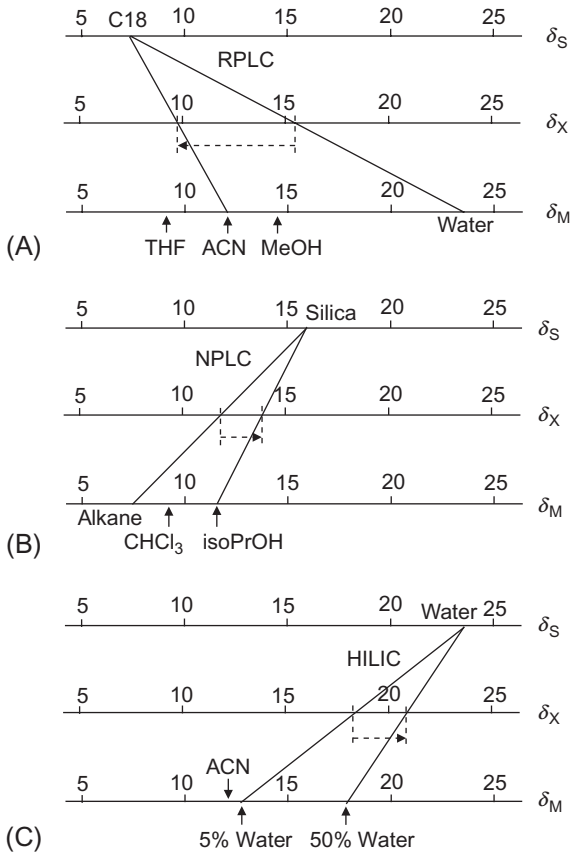

FIG. 13.1

Graphical expression of the Schoenmakers' rule. Within the limits of predictions based on the Hildebrand solubility parameter, range of global polarity of solutes that are properly eluted when a wide elution gradient is applied for: (A) RPLC with C18 and ACN-water; (B) NPLC with underivatized silica and isopropanol-heptane; and (C) HILIC with a water layer and water-ACN. The Hildebrand global polarity of the stationary phase, solute, and mobile phase are represented on the δ_S, δ_X, and δ_M scales, respectively.

a 0%–100% ACN gradient. Less polar solutes, going down to $\delta_X \approx 8.5$, are eluted by substituting ACN with THF.

The polarity range of solutes properly eluted from a silica column with alkane-isopropanol mixtures in NPLC is depicted in Fig. 13.1B. As observed, the solute polarity range is approximately $11.5 < \delta_X < 13.5$, which is inscribed within the range covered by RPLC. Therefore, all analytes eluted by NPLC can be also eluted with optimal retention factors using RPLC. However, this does not mean that NPLC and RPLC have the same or a similar chromatographic value. Thus, hydrophobic samples as mineral and vegetable oils that can be directly injected on an NPLC system are

not compatible with most RPLC mobile phases. Furthermore, NPLC and RPLC can provide rather different values of selectivity and efficiency depending on the nature of the solutes.

Finally, in HILIC, where solutes are retained on a water layer ($\delta_S \approx 23.5$, Fig. 13.1C), highly polar solutes in the $18 < \delta_X < 21$ range (mainly ions, polyions, or zwitterions) are eluted with water-ACN mixtures by increasing water from 5% to 50%. However, a problem with HILIC is that the samples and polar analytes should be soluble in the organic-rich mobile phases that are required, mainly at the beginning of the gradient.

13.4 ISOELUOTROPIC MIXTURES

Fine tuning of the polarity through discrete or continuous changes of the mobile-phase composition in the isocratic and gradient elution modes, respectively, is mainly achieved by adjusting the modifier concentration in the solvent mixture. On the other hand, the selectivity is controlled by changing the solvent nature, and for some solutes, by also modifying the mobile-phase pH [14], or column temperature [15,16]. For ionic analytes, the concentration of an ion-pairing salt is also an important factor. The selectivity depends mainly on the specific interactions of solutes with the stationary and mobile phases [17,18], that is, on the profile of the contributions to the global polarity of solutes and phases.

A basic question in selectivity optimization is how to modify the nature of a solvent mixture without altering the selected elution strength. Mixtures with the same elution strength but prepared with different modifiers are called *isoeluotropic mixtures*. For binary mixtures of MeOH, ACN, or THF with water, from Eq. (13.3), and using the Hildebrandt parameter as a measure of global polarity assuming a linear behavior,

$$\delta_{MeOH}\varphi_{MeOH} + \delta_{H2O}\left(1-\varphi_{MeOH}\right) = \delta_{ACN}\varphi_{ACN} + \delta_{H2O}\left(1-\varphi_{ACN}\right) = \delta_{THF}\varphi_{THF} + \delta_{H2O}\left(1-\varphi_{THF}\right) \tag{13.7}$$

By substituting the polarity values given in Table 13.1,

$$\varphi_{MeOH} = 1.27\varphi_{ACN} = 1.60\varphi_{THF} \tag{13.8}$$

Hence, the elution strength of an aqueous mobile phase with 20% MeOH is approximately the same as for 15.7% ACN or 12.5% THF. Since THF is the most hydrophobic solvent, the same elution strength is achieved with a smaller percentage of organic solvent. As indicated previously, the predictions of elution strength depart from linearity at large modifier concentrations. To address this problem, nonlinear relationships and nomograms, such as that shown in Fig. 13.2, can be used. On this nomogram, all possible isoeluotropic binary mixtures constituted by water and either ACN, MeOH, or THF can be estimated. ACN is generally stronger than MeOH, and THF appreciably stronger than ACN. Note that the scale for ACN is linear, making it necessary to draw nonlinear scales for MeOH and THF. However, due to the

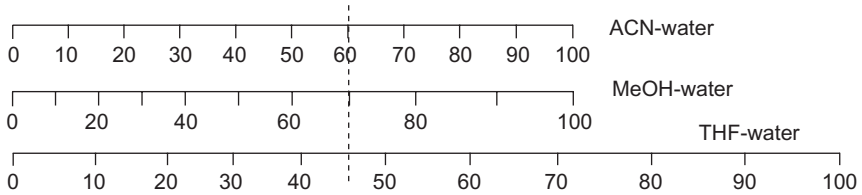

FIG. 13.2

Nomogram showing isoeluotropic binary mixtures in RPLC. The compositions are obtained by connecting the solvent scales with a *vertical line*. The example indicates that aqueous binary mixtures having 60% ACN, 70% MeOH, or 46% THF are isoeluotropic.

Adapted from Sigma-Aldrich.com/Supelco 2009–10 chromatography products catalog, p. 38.

limitations inherent in the global polarity parameters, predictions are rough and depend largely on the solute properties.

13.5 SOLVENT-SELECTIVITY TRIANGLES

13.5.1 THE SNYDER'S SOLVENT-SELECTIVITY TRIANGLE

Mobile-phase selectivity is understood as a consequence of the particular profile of the contributions of solvent-solvent intermolecular interactions to the global polarity. Six types of interactions are considered to contribute to the Hildebrand solubility parameter [10]: interactions between permanent dipoles, between induced dipoles, between permanent and induced dipoles, hydrogen ion donation (acidity), hydrogen ion acceptance (basicity), and electrostatic interactions. However, as commented below, these are not the only possible interactions. Owing to the different contributions, if solutes with exactly the same global polarity but structural differences are separated by chromatography, retention times will be close but still different. We could add "fortunately different," because otherwise selectivity optimization would not be possible.

To deal with more than three parameters, multivariate statistics is required, where the solvents in the multivariate space are projected on the reduced space of the first principal components [2]. However, in the strategy proposed by Snyder in 1974 [6,19], electrostatic interactions are neglected and some of the most akin interactions (among permanent and induced dipoles) are summarized in a single property called *dipolarity* (i.e., polarity and polarizability). Accordingly, mobile-phase selectivity was characterized by only three parameters: acidity, basicity, and dipolarity. This made possible plotting solvent properties on a triangular diagram, called the Snyder's *solvent-selectivity triangle* (SST), where each corner represents one of the properties (Fig. 13.3) [20].

The solvent properties were estimated using three probes: ethanol (e), 1,4-dioxane (d), and nitromethane (n), which is a simplification of the six-probe system formerly proposed by Rohrschneider to represent solvent properties. By using these three

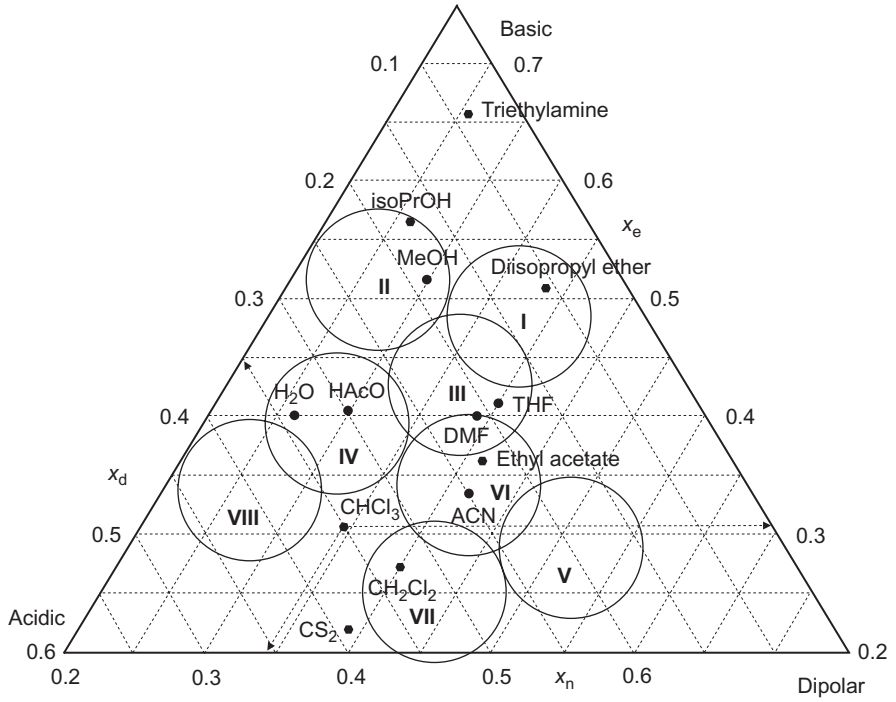

FIG. 13.3

Snyder's solvent-selectivity triangle, indicating the eight solvent families (*large circles*). The location of several solvents, including those most commonly used in RPLC and NPLC, is indicated (*DMF*, dimethylformamide; *HAcO*, acetic acid; *isoPrOH*, isopropanol). The *arrows* starting from chloroform illustrate how to read the scales.

probes, the intended properties are: "hydrogen ion donor" (ethanol), "hydrogen ion acceptor" (1,4-dioxane), and "polar or polarizable" (nitromethane). In fact, none of the three probes represents these characteristics uniquely: Ethanol is predominantly a hydrogen ion donor but also a weak acceptor and is moderately dipolar; 1,4-dioxane is a good hydrogen ion acceptor, weakly dipolar and a nonhydrogen ion donor; and nitromethane is strongly dipolar but also both weakly acidic and weakly basic. Although far from ideal, the selected probes led to a useful classification of solvents.

Solvents were characterized according to their capacity to interact with the three probes, which was estimated from gas-liquid partition equilibria. Snyder's global polarity, P' (Table 13.1), was defined as the sum of the three contributions:

$$P' = \log k_e' + \log k_d' + \log k_n' \tag{13.9}$$

where k_e', k_d', and k_n' are the gas-liquid partition coefficients for the probes, which were determined from their equilibrium concentrations in a sealed vial, containing a fixed volume of the solvent to be characterized. The partition coefficients were

defined as the ratio of the solute concentration in the solvent and in the vial void volume, after making two corrections to eliminate the effect of the solvent volume and the nonspecific contributions (C–H weak permanent or induced dipole interactions, obtained with *n*-octane). Finally, to eliminate the differences among the global polarities of the solvents, normalization was performed:

$$1 = \frac{\log k'_e}{P'} + \frac{\log k'_m}{P'} + \frac{\log k'_n}{P'} = x_e + x_d + x_n \tag{13.10}$$

where x_e represents the basic character, x_d is the acidic character, and x_n is the dipolar character of the solvent (Table 13.2). Using this approach, the character of a solvent is defined by the balance or profile of these three normalized parameters, independently from its global polarity. It is therefore assumed that a solvent that preferably retains ethanol or 1,4-dioxane rather than nitromethane should have a predominantly basic and acidic character, respectively; and a solvent that preferably retains nitromethane rather than the other two probes has a polar character or is readily polarizable rather than a proton donor or acceptor.

The x_e, x_d, and x_n data for a large number of solvents are plotted on the SST (Fig. 13.3). Solvents are grouped according to their properties in eight families: (I) aliphatic ethers and amines; (II) aliphatic alcohols; (III) pyridine and THF; (IV) glycols and acetic acid; (V) dichloromethane and dichloroethane; (VI) aliphatic ketones, esters, 1,4-dioxane, and nitriles; (VII) aromatic hydrocarbons and nitrocompounds; and (VIII) phenols and water. The scales should be read counterclockwise: x_e is represented on the right side (the higher on the scale, the stronger is the basic character of the solvent), x_d is on the left side (the lower on the scale, the stronger is the acidic character), and x_n is on its base (with the solvent dipolarity increasing to the right).

The diagram shows that the most common solvents in RPLC provide different selectivity, since they have rather different profiles of the three properties defined in the SST. Thus, water is a strong hydrogen ion donor and acceptor (it is situated at half-height in the SST), but a weak dipole (it is on the left). ACN is less acidic than water but appreciably more dipolar. MeOH is appreciably more basic (higher in the diagram), more dipolar than water, and less dipolar than ACN. Finally, THF has both acidic and basic character, but it is more dipolar than water.

The SST scales should not be interpreted as "percentages" of the intended properties, since solvent properties were obtained from solutes with a mixed character, and therefore, the vertices do not represent "pure" properties. For example, a strongly basic solvent such as triethylamine is not located close to the upper vertex due to its basicity but because it strongly retains ethanol and weakly retains 1,4-dioxane and nitromethane. Ideally, if the SST scales would correspond to pure properties (each vertex representing 100% acidity, 100% basicity, and 100% dipolarity), mixtures of three hypothetical solvents, each one located at each vertex, would provide a whole universe of possibilities. However, such solvents do not exist. Furthermore, real solvents located close to the SST vertices are not mutually miscible or are not compatible with common stationary phases. ACN, MeOH, and THF are at intermediate locations in the SST, being excellent choices to achieve a wide range of properties in RPLC. Not surprisingly, these solvents were already popular by the time the SST was developed.

Table 13.2 Normalized Selectivity Factors

Solvent[a]	Derived from Gas-Liquid Partition Data of Rohrschneider's Probes[b]			Derived from Kamlet-Taft Solvatochromic Parameters[c]		
	x_d	x_e	x_n	α	β	π^*
Diisopropyl ether	0.10	0.51	0.39	0.00	0.64	0.36
Hexane	–[d]	–[d]	–[d]	0.00	0.00	0.00
Carbon disulfide	0.39	0.22	0.39	0.00	0.10	0.90
Triethylamine	0.07	0.61	0.32	0.00	0.84	0.16
Carbon tetrachloride	0.38	0.30	0.32	0.00	–[d]	0.59
Ethyl acetate	0.23	0.34	0.43	0.00	0.45	0.55
Toluene	0.28	0.25	0.47	0.00	0.17	0.83
Tetrahydrofuran	0.20	0.38	0.42	0.00	0.49	0.51
Chloroform	0.35	0.31	0.34	0.43	0.00	0.57
Dichloromethane	0.33	0.27	0.40	0.20	0.00	0.82
Methyl ethyl ketone	0.22	0.35	0.43	–[d]	–[d]	–[d]
Acetone	0.23	0.35	0.42	0.06	0.38	0.56
Carbon disulfide	0.39	0.22	0.39	0.00	0.10	0.90
1,4-Dioxane	0.24	0.36	0.40	0.00	0.40	0.60
Pyridine	0.22	0.41	0.36	0.00	0.42	0.58
Isopropanol	0.19	0.55	0.27	0.35	0.43	0.22
1-Butanol	0.19	0.59	0.25	0.37	0.41	0.22
2-Methoxyethanol	0.24	0.38	0.38	–[d]	–[d]	–[d]
Dimethylformamide	0.21	0.39	0.40	0.00	0.44	0.56
Ethanol	0.19	0.52	0.29	0.39	0.36	0.25
Dimethylsulfoxide	0.27	0.35	0.38	0.00	0.43	0.57
Acetonitrile	0.27	0.31	0.42	0.15	0.25	0.60
1-Propanol	0.19	0.54	0.27	0.36	0.40	0.24
Acetic acid	0.31	0.39	0.30	0.54	0.15	0.31
Methanol	0.22	0.48	0.31	0.43	0.29	0.28
Formamide	0.33	0.38	0.30	0.33	0.21	0.46
Water	0.37	0.37	0.25	0.43	0.18	0.45

[a] *Solvents ordered according to Table 13.1.*
[b] *Large values of x_d, x_e, and x_n denote good hydrogen ion donor, good hydrogen ion acceptor, and large permanent or induced dipole moments, respectively [5–7].*
[c] *α, β, and π^* represent solvent ability to interact as hydrogen ion donor, hydrogen ion acceptor, and by polar and polarization effects, respectively [21,22].*
[d] *Not available.*

13.5.2 PREDICTION OF THE CHARACTER OF SOLVENT MIXTURES

The SST allows predicting whether the elution strength will increase or decrease for certain solutes when one modifier is replaced by another. For example, substituting a MeOH-water mixture with an isoeluotropic ACN-water mixture will reduce the ability of the mobile phase to accept hydrogen ions; so the elution strength will be reduced for acidic solutes. Simultaneously, the dipolar character of the mobile phase will increase so that dipolar and polarizable compounds will elute earlier. This reasoning can be of help in solute identification. Thus, if a solute elutes earlier when a MeOH-water mixture is substituted with an isoeluotropic ACN-water mixture, then the solute should have a basic or a dipolar character or both.

As shown in the SST of Fig. 13.4, the character of all possible mixtures of water, ACN, MeOH, and THF is delimited by straight lines connecting the four solvents. This figure illustrates how wide the selectivity range in RPLC is. The character of isoeluotropic mixtures of the four solvents, at increasing elution strength, is indicated by the three small *a*, *b*, and *c* triangles. The location of these isoeluotropic mixtures on the SST was established according to their compositions obtained from the nomogram of Fig. 13.2. A linear variation of the properties with modifier concentration was also assumed. The small triangles *a*, *b*, and *c* of Fig. 13.4 illustrate how

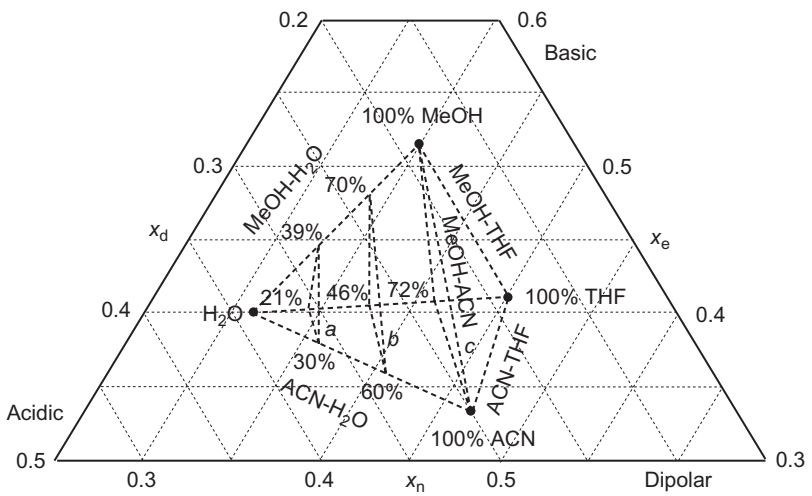

FIG. 13.4

Snyder's solvent-selectivity triangle indicating the character of mixtures of water, ACN, MeOH, and THF. The small triangles *a*, *b*, and *c* describe isoeluotropic mixtures at increasing elution strength. In *a*, the lowest vertex corresponds to 30:70 ACN-water, the upper vertex to 39:61 MeOH-water, and the left vertex to 21:79 THF-water. Other points on the sides of the small triangle *a* correspond to ternary mixtures, and points inscribed in triangle *a* correspond to quaternary mixtures. Similarly, the small triangles *b* and *c* correspond to isoeluotropic mixtures with respect to 60:40 ACN-water and 100% ACN, respectively.

the character of a mixture of solvents is modified by varying its composition, while maintaining a constant elution strength, as estimated by the Hildebrand solubility parameter, δ.

13.5.3 A SOLVATOCHROMIC SOLVENT-SELECTIVITY TRIANGLE

The essential conclusion of the Snyder's SST and other alternative diagrams also based on solvatochromic properties, independently from the approach used to construct them, is that, to explore the full range of possibilities during mobile-phase selectivity optimization, solvents having both mutual miscibility and, at the same time, maximal differences in their properties should be selected. Another application of the diagrams is the visualization of the possibility of substituting a solvent by an equivalent one with improved non-chromatographic characteristics, such as price, availability, or better conformation to the principles of green chemistry. Finally, the diagrams are also useful to predict the miscibility of solvents and the solubility of the solutes in a number of alternative solvents with similar properties. In addition to the Snyder's pioneering work, other solvent descriptors and the diagrams derived from them could be also useful in providing more clarifying and complementary criteria for solvent classification, comparison, and selection.

According to the "mixed" character of the probes used to construct the SST, x_e reflects, in fact, a composite of hydrogen bond basicity, hydrogen bond acidity, and dipolarity; x_d reflects a composite of solvent acidity and dipolarity; and x_n reflects predominantly solvent dipolarity with small contributions from hydrogen bond basicity and acidity. In 1989, Rutan and Carr [7,20,23] substituted the gas-liquid partition coefficients obtained with Rohrschneider's probes by the Kamlet-Taft "solvatochromic parameters" (Table 13.2). These parameters, mainly derived from spectroscopic measurements, separately estimate the hydrogen bond donor (α), hydrogen bond acceptor (β), and dipolarity/polarizability (π^*) properties of solvents as contributors to the global solvent polarity. Solvatochromic parameters are averages over results obtained with several probes. Thus, it is normally assumed that they provide more "pure" measurements of the addressed properties than gas-liquid partition coefficients derived from only three probes. However, reconstruction of the SST using normalized solvatochromic parameters was rather disappointing, since many solvents laid on a line joining the basic and dipolar summits of the triangle, and thus, solvent discrimination was rather poor [20].

13.5.4 OTHER SOLVENT DESCRIPTORS AND ALTERNATIVE DIAGRAMS FOR SOLVENT CLASSIFICATION AND COMPARISON

An alternative to the use of the Snyder probes and the Kamlet-Taft solvatochromic parameters are the Hansen parameters [24,25]. These are derived from the Hildebrand solubility parameter, which is split into three contributions:

$$\delta^2 = \delta_d^{\,2} + \delta_p^{\,2} + \delta_h^{\,2} \tag{13.11}$$

each one representing the dispersive forces (δ_d), the polarity (δ_p), and the hydrogen bonding (δ_h) (both donor and acceptor). By using the Hansen parameters, an alternative SST to that of Snyder, also showing a good dispersion of solvents according to its character, was constructed.

A somewhat more complex but widely accepted solvent classification system is that based on the five linear solvation energy relationships (LSERs) or Abraham descriptors [26–32]. The solvation parameter model describes five interactions by means of five descriptors related to the compound properties: *E* (the excess molar refraction, related to the presence of *n*- and π-electrons resulting in charge transfer, π-π interactions and dipole-induced dipole interactions); *S* (standing for the presence of dipoles and polarizability); *A* and *B* (describing hydrogen bond acidity and basicity, respectively); and *V* (the McGowan's volume, related to dispersive interaction and cavity energy formation). Representation procedures other than triangles should be used to deal with five descriptors. A possibility is to use projections after a principal component rotation. However, by using principal components, the chemical significance of the axes is lost. An alternative is the use of spider diagrams [33], as that given in Fig. 13.5. With this representation technique, a number of parameters above three can be projected on a plane with little loss of information. Careful selection of the order of the axes is essential to minimize the loss of information due to the reduction of the number of dimensions. Thus, those descriptors that are the most positively correlated (for instance *E* and *S* for the LSERs descriptors) should be juxtaposed, in opposition to those that are negatively correlated, while the least correlated ones should be placed as orthogonal as possible. However, as in any other projection technique, compensation of descriptors making rather different solvents to lie in close positions on the spider diagram is possible.

On the spider diagram of Fig. 13.5, obtained from the LSER descriptors, water is located at the bottom right, showing its high acidity (*A* is large) and weak hydrophobicity (*V* is low). Alcohols, acetic acid, and formamide are located close to water. Nitriles (like ACN) display higher dipole interactions and are located at the right-hand side of the plot, above the alcohols. Alkanes, with high hydrophobicity, are naturally at the opposite of the figure, on the left, close to the *V* axis. Aromatic solvents are at the top of the diagram, around the *E* axis. THF, 1,4-dioxane, acetone, and ethyl acetate are located in the same group, at the center of the diagram.

The Abraham descriptors are very useful in explaining the selectivity differences between the three solvents more frequently used in RPLC. Thus, MeOH is the best donor and acceptor of hydrogen bonds, ACN displays the greatest dipolar interactions, and THF, having the greatest McGowan's volume, favors the solubility of most organic compounds through dispersive interactions, explaining its high eluting strength in RPLC.

Finally, the Abraham descriptors also provide a useful global polarity scale defined as

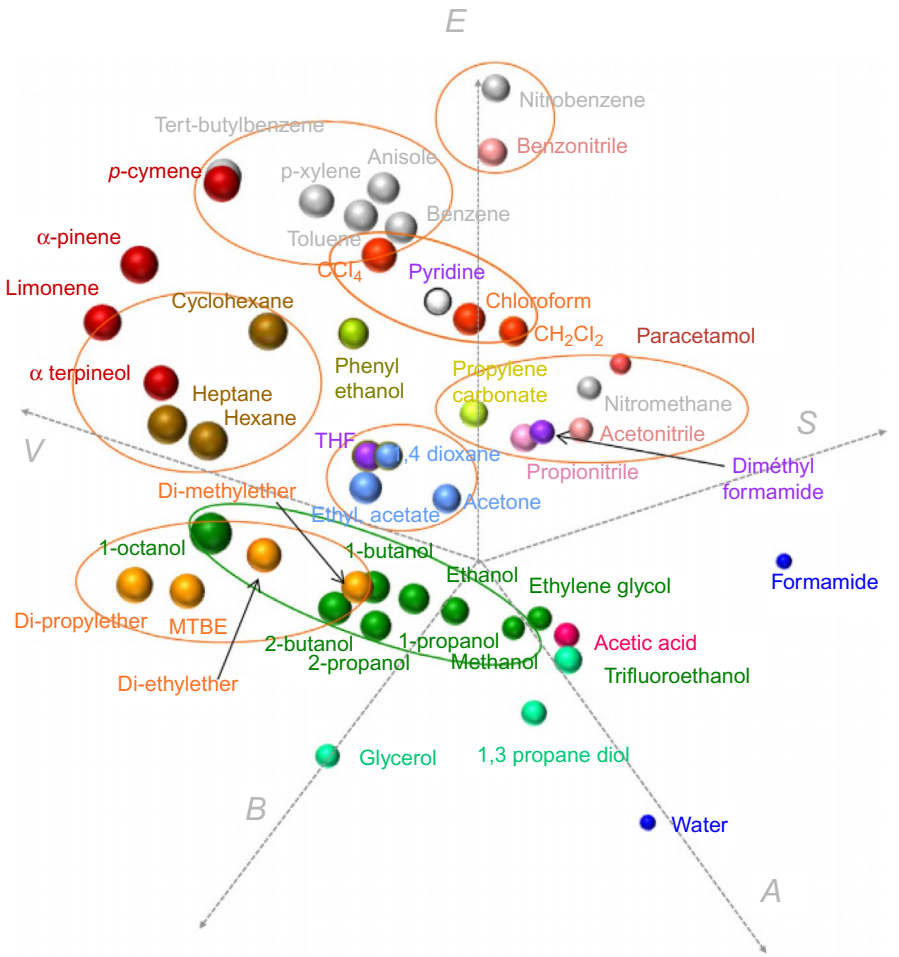

FIG. 13.5

Spider diagram based on the Abraham descriptors *E, S, A, B, V*. The point size is proportional to the *V/U* ratio.

Reproduced with permission from Lessellier E. ∑pider diagram: a universal and versatile approach for system comparison and classification. Application to solvent properties. J Chromatogr A 2015;1389:49–64.

$$U_X = \left(E_X^2 + S_X^2 + A_X^2 + B_X^2 + V_X^2\right)^{1/2} \tag{13.12}$$

where the equation is written for a given solute, X. This global parameter can be used to estimate the elution strength of solvent mixtures, as done above in Eq. (13.3) using the Hildebrand parameter. In Fig. 13.5, the size of the symbol representing each solvent was made proportional to *V/U*.

13.6 PRACTICAL GUIDELINES FOR OPTIMIZATION OF MOBILE-PHASE COMPOSITION

13.6.1 SELECTION OF THE CHROMATOGRAPHIC MODE

The optimization of the modifier type and volume fraction in the mobile phase is frequently performed on a trial-and-error basis. Next, some guidelines to rationalize and speed up this process are given. After selecting the chromatographic mode (e.g., RPLC, NPLC, or HILIC), and deciding between isocratic or gradient elution, the elution strength should be adjusted, and finally, the selectivity optimized until all peak pairs of interest are resolved. To select the chromatographic mode, two criteria are attended:

1. *Solute nature*. If the solute molecules contain extensive hydrophobic regions in "external" structural parts, they are retained on the hydrophobic RPLC stationary phases. In contrast, if the influence of ionic or polar groups (e.g., −COOH, −OH, or $-NH_2$) predominates, the solute experiences poor retention and requires polar stationary phases typical in NPLC. A good solution to increase retention of permanent ionic analytes is ion pairing [34]. In this technique, a salt is added to the mobile phase. Retention is enhanced by mixed mechanisms involving association of ions of opposite charge in the hydro-organic mobile phase, and by ion exchange on the surface of the stationary phase, where the added salt is adsorbed. Since permanent ions and other highly polar solutes are not compatible with NPLC mobile phases, HILIC could be another correct choice. However, a frequent limitation in HILIC is the poor solubility of ionic analytes in the rich organic solvent mobile phases that are required.
2. *Sample compatibility with the mobile phase.* Direct injection of samples soluble in water or in hydro-organic mixtures (e.g., serum, urine, and other aqueous samples or aqueous extracts) require RPLC or HILIC. If HILIC is selected, the elution strength should be decreased by evaporation of water in the sample, followed by redissolution in a rich ACN mixture, or by dilution with ACN at the cost of a poorer limit of detection. For hydrophobic samples (oils, greases, hydrocarbons, or extracts in heptane, dichloromethane, or other hydrophobic solvents), NPLC is needed. Extracts in solvents that provide high elution strength, such as ethyl acetate in NPLC, or isopropanol in both RPLC and NPLC, should be avoided. It is often possible to change the solvent initially used to extract the sample. For instance, an aqueous sample can be extracted with heptane or dichloromethane, a vegetable oil can be extracted with an aqueous buffer or MeOH, and compounds of interest in an environmental aqueous sample can be concentrated on a solid phase, followed by elution with an appropriate solvent. Within the limits of the analyte's solubility or stability, it is possible to change the solvent nature by evaporation and dilution to make the medium compatible with a given chromatographic mode. Within this context, centrifugal evaporators that allow the removal and substitution of the solvent using vacuum but without boiling thus to prevent analyte losses, are most useful.

13.6.2 DESCRIPTION OF THE RETENTION USING THE MODIFIER CONTENT AS A FACTOR

Solute retention is most commonly controlled by the modifier concentration in the mobile phase. In order to predict the optimal chromatographic conditions, it is convenient to know the retention behavior as the organic solvent content is varied. In RPLC, the retention for a solute X can be expressed in terms of the solubility parameters according to [35]:

$$\ln k_X = \frac{\nu_X}{RT}\left[\left(\delta_M - \delta_X\right)^2 - \left(\delta_S - \delta_X\right)^2\right] + \ln \frac{n_S}{n_M} \tag{13.13}$$

where R is the gas constant, T the absolute temperature, ν_X the solute molar volume, and n_M and n_S the moles of mobile phase and stationary phase in the column, respectively. For a binary mixture of water (w) and organic solvent (o), the mobile-phase polarity can be calculated as a function of the modifier volume fraction. By substituting Eq. (13.13) in Eq. (13.3), for binary mixtures, a general-purpose parametric equation is obtained, which is commonly used to characterize the retention [36]:

$$\log k = c_0 + c_1\varphi + c_2\varphi^2 \tag{13.14}$$

In narrow modifier concentration ranges, the quadratic relationship can be simplified to a linear one, which is very often used.

Surface adsorption in NPLC is better described by nonlogarithmic and logarithmic empirical models [37]:

$$\frac{1}{k} = \left(c_0 + c_1\varphi\right)^n \tag{13.15}$$

$$\log k = c_0 + c_1 \log\varphi \tag{13.16}$$

where φ is again the concentration of the stronger solvent (here the more polar) in a binary mobile phase. Eq. (13.15) has been also found highly satisfactory for RPLC (where φ would be the less polar solvent).

Retention in HILIC is more complex. Equations that combine both partitioning and adsorption phenomena have been suggested [38], such as

$$\log k = c_0 + c_1 x + c_2 \log x \tag{13.17}$$

where x is the fraction of water in the mobile phase. The applicability of the model can be expanded to higher solvent strength regions as follows:

$$\log k = c_0 + c_1 \log x + c_2 \left(\log x\right)^2 \tag{13.18}$$

$$\log k = c_0 + c_1 x + c_2 \log\left(1 + c_3 x\right) \tag{13.19}$$

13.6.3 SYSTEMATIC TRIAL-AND-ERROR MOBILE-PHASE OPTIMIZATION FOR ISOCRATIC ELUTION

Isocratic elution can be selected if the polarities of the compounds in the sample are similar. In contrast, if the polarities span a wide range then, gradient elution is needed. For an unknown problem, it is preferable to start the optimization in the gradient elution mode. However, we focus first on the simpler development of an isocratic method.

Usually, in RPLC, a C18 stationary phase is tried first. If no previous information about solute polarities is available, starting with a mobile phase of high elution strength, such as 95% ACN, is advisable. This ensures elution of most compounds in the sample, although many may elute close to the dead time. If the retention of one or more solutes is still too high ($k > 20$), NPLC is probably preferable. Other options are changing the C18 column for C8 or C4 columns, or using a higher column temperature. Less retentive stationary phases, such as C2 or C1, are not recommended, owing to their low stability. Next, the retention of solutes eluting close to the dead time should be increased by using progressively smaller modifier concentrations (e.g., 60%, 40%, and 20%). At this stage, gradient elution is probably necessary if the solutes of interest cannot be moved to the target range of the retention factor, with any of the modifier concentrations tried.

An analogous strategy can be followed by using NPLC: Initially, a polar column (e.g., bare silica or propyl-cyan silica) and a mobile phase with high elution strength are selected. However, the chromatographer should be aware that, in NPLC, a few parts percent of a polar modifier added to the alkane in the mobile phase can cause dramatic effects on retention. For instance, a smaller increase in retention can be produced by decreasing the ethyl acetate concentration from 40% to 2% than from 2% to 0%. This is because, contrary to RPLC where the "strong" solvent is water and not the modifier, in NPLC, the "strong" solvent, which mainly determines the solvating properties of the mixture, is the modifier. Therefore, in NPLC with moderate modifier concentrations, most solutes probably elute close to the dead time. In the absence of excessively retained solutes, the elution strength should be progressively reduced by decreasing the amount of modifier until appropriate retention times are obtained. Similarly, for HILIC, aqueous mixtures containing up to 50% water can be initially tried, followed by the stepwise reduction of the water concentration. The retention mechanism is rather different with hydride silica columns, where the solutes are mainly retained by accepting protons from those covering the stationary phase surface. Elution is promoted by substituting a weak solvent, as ACN, by MeOH, which is a much stronger proton acceptor. Thus, MeOH displaces the analytes from their union sites on the hydride silica stationary phase.

In the three most usual chromatographic modes (i.e., RPLC, NPLC, and HILIC), the selectivity can be further optimized to improve the resolution between all peak pairs. For this purpose, solvent mixtures of similar elution strength, another pH or column temperature, or if necessary, a different stationary phase, can be tried. Here, we will discuss the selection of an isoeluotropic mixture. This may be based on solute properties guided by the polarity scales described above with the help of any

of the triangular or spider diagrams that can be derived. For example, in the RPLC elution of two solutes with the same retention but with different acidity, the more acidic solute elutes earlier if ACN is replaced by MeOH. However, often solute properties are not known or the interpretation of the possible solute-solvent interactions in multifunctional solutes is not straightforward. Therefore, the selectivity is most frequently optimized in an empirical fashion.

In RPLC, by following an empirical experimental scheme, the first modifier to be tested is ACN, due to its low viscosity and short ultraviolet (UV) cut-off wavelength (190 nm) (Table 13.1), which allow a low backpressure and a UV detection window capable of detecting many absorbing compounds, even if they are poorly conjugated. If the separation is not satisfactory, the second option is MeOH. The viscosity of MeOH-water mixtures is much higher than for ACN-water mixtures, with a maximum at 40% MeOH, which due to the large backpressures, makes them unsuitable for working at high flow rates with long packed columns, or small particle sizes. Also, the cut-off wavelength of MeOH is higher (205 nm). The third option, THF, has a still higher viscosity, a cut-off wavelength of 212 nm, and requires long equilibration times. Therefore, not surprisingly, these solvents are always tried in the same order: ACN, MeOH, and THF. This is indicated by the A-B-C vertices of the method development triangle (Fig. 13.6).

If one of the three isoeluotropic mixtures is successful, the problem is over. If some peaks remain unresolved, ternary or even quaternary isoeluotropic mixtures may be tried. For this purpose, the order of the D-G mixtures in Fig. 13.6 is usually followed. After selecting the optimal isoeluotropic mixture, its composition can be slightly changed until all the peaks of interest are satisfactorily resolved. Let us consider a 70:30 ACN-water mixture, for which all peaks for a given sample are in the

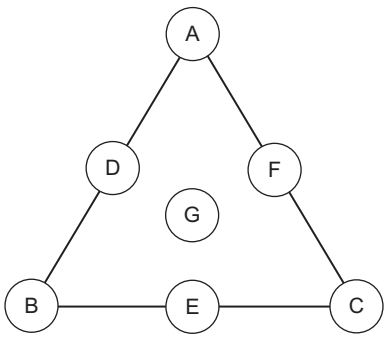

FIG. 13.6

Method development triangle. A, B, and C represent isoeluotropic binary mixtures of water with ACN, MeOH, and THF, respectively; D–F are isoeluotropic ternary mixtures (e.g., point D is an ACN-MeOH-water mixture, where half of the first modifier has been substituted by an isoeluotropic amount of the second modifier). The central point G is the ACN-MeOH-THF-water isoeluotropic quaternary mixture, where two-thirds of the first modifier have been substituted by isoeluotropic amounts of the two other modifiers.

target range of k values. If the resolution between some peak pairs is unsatisfactory, following the scheme in Fig. 13.6 and the nomogram in Fig. 13.2, the mobile phase to try next is 78:22 MeOH-water (point B in Fig. 13.6). If required, we continue with 52:48 THF-water (point C), 35:39:26 ACN-MeOH-water (point D), 39:26:35 MeOH-THF-water (point E), and so on. Mixtures D and E were calculated by substituting half of the ACN content of the A mixture by its equivalent amount of MeOH or THF, respectively. This trial-and-error method is more common in practice than the use of considerations based on polarity descriptors, owing to its simplicity, and because it requires no knowledge of solute properties. However, when the problem remains unresolved, either the polarity descriptors or a computer-assisted interpretive optimization (see Section 13.6.5) is of help. Similarly, selectivity optimization in NPLC and HILIC can be conveniently carried out by systematically substituting the modifier by other miscible solvents exhibiting a different profile of its descriptors, thus, laying down in a different location on any SST or selectivity spider diagram.

13.6.4 SYSTEMATIC TRIAL-AND-ERROR MOBILE-PHASE OPTIMIZATION FOR GRADIENT ELUTION

When analyzing samples with solutes covering a wide range of polarities, a gradient of elution strength is needed to get both an adequate retention of the first peaks in the chromatogram, and progressively expedite the elution of the most retained solutes. For this purpose, at least two solvent mixtures with different elution strength (mixtures A and B, with B stronger) should be combined. The gradient is normally started at the time of sample injection, although full control on the actual gradient conditions is lost if the delay time, or time required for the gradient to arrive to the column, is not taken into consideration. During the gradient time, t_G (the time the gradient is run), the flow of B and A are increased and decreased, respectively, keeping the sum of the two flows constant, until only B is pumped. To reduce the baseline noise due to fluctuations in the mixture composition, which can be particularly large with quaternary pumps, A and B mixtures containing at least 5% of the minor solvent, should be used.

In gradient elution, starting with mobile phases with low elution strength, strongly retained analytes migrate very slowly, so that this range of mobile-phase compositions does not contribute significantly to their elution. As the elution strength increases along the gradient, the analytes are "accelerated" through the column. A graphical image of the effect is described by: "a solute sits at the head of a column until a strong enough solvent comes along to push it through the column leaving the other solutes behind, then it travels to the column outlet fairly quickly" [39]. The point at which this occurs depends on the strength of solute interaction with the mobile phase and stationary phase. Therefore, solutes in gradient RPLC seldom experience the whole range of mobile-phase compositions. The fraction of the solvent composition range that actually affects solute migration has been called "significant solvent concentration range" [40]. Thus, in addition to the chromatographic separation mechanisms,

gradient elution also works as a fractional extraction, making the analytes to progress along the column when they are extracted from the stationary phase. In this sense, the elution strength plays in LC an analogous role as temperature in gas chromatography where fractional distillation is a significant separation mechanism.

For the first trial on an unknown sample, a broad gradient with a small slope is recommended to ensure the elution of all solutes (e.g., in RPLC, from 5% to 100% ACN). The ratio $\Delta t/t_G$, where Δt is the difference between the retention times of the first and last peaks of interest in the chromatogram, provides a criterion for deciding whether the sample can be separated isocratically or gradient elution is required. If $\Delta t/t_G < 0.25$, the sample can be isocratically eluted within the k target region by using a mobile-phase composition close to that running when the midpoint in Δt was reached. In contrast, $\Delta t/t_G > 0.25$ means that the solutes elute in a wide k range and isocratic elution is not practical. In this case, the new gradient should be focused between the mobile-phase composition at the time of the first eluting peak (start of Δt; new mixture A) and the time for the last peak (end of Δt, new phase B). If the sample contains other components that are more retained than the analytes, then, a final gradient step at a high elution strength should be executed thus to clean up the column. This will prevent cross-contamination between successive injections.

If some peak pairs remain unresolved, the composition of mixtures A and B should be modified without altering significantly their respective elution strengths. In RPLC, this can be achieved by substituting ACN with MeOH or THF, or by using isoeluotropic ternary or quaternary mixtures, as discussed for isocratic elution. When all solutes are satisfactorily resolved, the gradient time can be further reduced without losing resolution. The easiest way is to increase the gradient slope as much as tolerated by the resolution of the least resolved peak pair. Another option is using a segmented or multi-linear gradient, that is, a gradient whose slope changes according to the peak distribution: The slope is smaller in time regions of poorly resolved peaks and steeper in regions without peaks. Nonlinear gradients with concave or convex profiles are also occasionally applied when dealing with multicomponent samples requiring extra resolution. Gradients include often isocratic hold periods, at the beginning and/or the end of the runs, or inserted between linear or nonlinear gradient segments. Reverse gradients (with decreasing modifier concentration) can be useful in some cases (e.g., to elute amphiphilic analytes whose solubility increases by increasing both the polar and the less polar component of the mobile phase).

In addition to elution strength gradients, it is possible to establish selectivity gradients by increasing the mobile-phase acidity, basicity, dipolarity, or any other polarity descriptor, at either constant or increasing elution strength. Therefore, in principle, there are four possibilities:

1. Isocratic isoselective elution where the mobile-phase composition is constant.
2. Isocratic elution with a selectivity gradient, obtained by modifying the solvent mixture in such a way that the polarity descriptors, for instance acidity, basicity, or dipolarity are varied while a global polarity descriptor is maintained invariable. This entails the continuous modification of the coordinates of the

mixtures used on an SST or a selectivity spider diagram, with the restriction of not modifying δ_X (Hildebrand solubility) or U_X (Abraham global polarity, see Eq. (13.12)). For example, on the Snyder's SST a selectivity gradient is obtained by following any line along the sides of the *a*, *b*, or *c* small triangles in Fig. 13.4 that correspond to isoeluotropic mixtures. Obviously, any translation along the triangle surface implies a change in selectivity.

3. Isoselective gradient elution where the elution strength is increased but the selectivity is not modified. Isoselective gradients are implemented by using A and B mixtures corresponding to the same profile of normalized polarity descriptors (e.g., to the same point on a given selectivity diagram), but where solvent mixture B has a higher global polarity than solvent mixture A. Then, as the B/A ratio increases, the global polarity of the mixture increases but without a substantial modification in selectivity.
4. Double-gradient elution where both elution strength and selectivity are modified. These are the most common gradients: When the ACN or MeOH content is increased in a mixture with water, not only the global elution strength increases, but also the polarity descriptors are varied, thus making the coordinates in any SST or selectivity spider diagram also to change. Double RPLC gradients can be programmed by progressively decreasing the water flow while simultaneously increasing the flow for one or even two modifiers at different rates. In this way, the elution strength is increased, and simultaneously, the selectivity is continuously modified in the desired direction (higher acidity, basicity, dipolarity, etc.).

13.6.5 COMPUTER-ASSISTED INTERPRETIVE OPTIMIZATION

Finding the best mobile-phase composition or gradient to obtain good peak resolution within a short analysis time is not easy. In spite of being particularly slow and inefficient, the trial-and-error strategies explained previously (or other less systematic ones) are still frequent. Many solute mixtures, however, are so complex that the protocol can be too long and, often, the best (or at least acceptable) conditions are not found. Fortunately, method development can be expedited with more reliable results by applying computer-assisted interpretive strategies [41–45].

The optimization process includes two steps: system modeling using data from experimental chromatograms, and resolution prediction through computer-simulated chromatograms. In the first step, to fit equations or train algorithms that allow the prediction of retention, a number of experiments as reduced and informative as possible are carried out. Incidentally, in addition to relative retention times, other properties that summarize a chromatogram, such as peak width and asymmetry, are also inferred from the experiments. The aim is to develop models capable of predicting the separation at any new arbitrary condition [46]. Next, based on the models, the separation quality is predicted for a large number of separation conditions, to find that giving the maximal (or at least an appropriate) resolution of all the peak pairs. In practice, this is done by simulating the sample separation inside a prefixed factorial

space, and calculating a numerical value that qualifies the chromatograms, ideally according to the analyst's appraisal of resolution. In addition to resolution, properties such as short analysis time, minimal solvent consumption, or desirable peak profiles (i.e., high efficiencies and low asymmetries) can be optimized.

To assist an interpretive optimization, several software packages, such as DryLab [47], ChromSword [48], Osiris [49], PREOPT-W [50], and MICHROM [51], have been commercialized. The user can also develop his or her own software with the aid of a spreadsheet or a high-efficiency programming environment, such as MATLAB or R. More information on computer-assisted method development can be found in Chapters 14 and 15.

13.6.6 USE OF COMBINED MOBILE PHASES OR GRADIENTS TO ACHIEVE FULL RESOLUTION

Conventional HPLC presents major challenges in the analysis of complex samples. When a separation fails, the usual choice is introducing a drastic change in the chromatographic system (column, solvent, pH, temperature, and/or use of additives). However, the possibilities of HPLC may be also expanded through other strategies that combine mobile phases or gradients.

Thus, the use of one or more pulses of a weak eluent (e.g., 200 µL water or 500 µL buffer solution on an RPLC system), strategically inserted to alter abruptly the local mobile-phase composition, may improve the resolution between poorly separated peaks but with little or no effect on the already resolved neighboring peaks [52]. This may be very practical when full resolution has been achieved for most analytes. Another approach, termed solvent modulation, consists of introducing individual solvent zones of constant composition (usually two, A and B, such as 90% and 100% MeOH, or 75% MeOH and 60% ACN), in a varying or repeating sequence into the LC column [53]. The applied sequence is established by the length ratio of the solvent zones A and B within one cycle, and the number of cycles carried out along the elution. Because the solvent zones are separated from one another spatially and temporally, nonideal solvent-solvent interactions are effectively eliminated, and the overall solute retention is just a linear combination of the retention times in the individual solvent zones. The advantage is that the effect on the chromatogram of changing the length of the zones is easy and accurately predicted. The approach has also been applied in gradient elution, in the so called "relay gradients," which is a special type of segmented gradients where the nature of the modifiers is abruptly changed between segments.

On the other hand, it is not rare to analyze a sample using two different columns or the same column, and two different isocratic or gradient conditions, to separate different target analytes. The possibilities of this approach can be maximally exploited if the two solvent systems are optimized to be complementary [54]: a separation condition focuses on the resolution of some compounds in the sample, while the other analytes remain unresolved, but are optimally resolved in a second (or subsequent) condition(s). When the results of the optimal complementary separation conditions are considered altogether, all analytes are maximally resolved.

The approach using parallel columns may involve different separation modes, such as RPLC and HILIC, to deal with samples comprising analytes in a wide range of polarities. However, for high throughput analyses, performing separate chromatographic runs with different columns is unpractical, thereby the interest in coupling in series RPLC and HILIC columns. However, despite both chromatographic modes use the same solvents, diametrically opposed concentrations are needed: HILIC needs a high organic solvent content, while RPLC needs a high amount of water. The solvent strength incompatibility between RPLC and HILIC is, however, solved by increasing the ACN content in the eluate from the RPLC column (aimed to separate low polarity solutes) by on-line mixing with ACN to meet the solvent requirements of the HILIC column (aimed to separate highly polar solutes) [55]. Another option is the direct connection of RPLC and HILIC, using a single gradient program starting at a high organic solvent content compatible with both RPLC and HILIC [56].

More sophisticated configurations connect the two columns through valve setups and involve two chromatographic pumps that allow the operation with different solvent systems in a two-dimensional (2D) fashion [57]. The principle of operation is to carry out the off-line or on-line transference of specific fractions of the eluent from the outlet of the first column (which represents the first dimension) to the inlet of the second column (the second dimension). In comprehensive 2DLC (LC×LC), the whole eluate from the first dimension is chopped into small segments that are continuously separated in the second dimension. Instead of this, in heart-cutting (LC-LC), only selected segments of the first dimension eluate, presumably those containing target unresolved analytes, are transferred to the second dimension for further separation. This is technically much simpler than LC×LC, since the segments can be parked for a time on the head of the column or different columns, until the system is ready to proceed with the elution in the second dimension. Optimization of the elution conditions and data treatment is also much simpler in LC-LC than in LC×LC. For both approaches, the advantage of exploiting different retention mechanisms, and the freedom to manipulate independently the mobile-phase gradient in each column, yield a considerable increase in peak capacity. Chromatographic optimisation of 2DLC is nontrivial, but can open enormously the range of resolutions.

13.7 ADDITIONAL CONSIDERATIONS FOR SOLVENT SELECTION

There may be several reasons to choose a given solvent other than the elution strength and selectivity, or the limits established by solvent viscosity and cut-off wavelength (Table 13.1) [58,59]. Thus, below 220 nm, the baseline drift caused by the differential solvent absorbance can be sufficient to prevent the practical use of certain solvents, such as MeOH or THF. In its turn, MeOH is less expensive and less toxic than ACN, and its higher polarity reduces the risk of buffer precipitation.

In general, solvents producing high backgrounds or baseline drift with the selected detector cannot be used. In this regard, the continuous modification of the concentration of a minor component in the mobile phase might be far more significant in gradient methods than in isocratic approaches. This occurs, for instance, when an absorbing solvent is used with UV detection or when one of the components of the mixture contains a conducting buffer with conductimetric detection, and in all instances with refractometric detection. Also, lot-to-lot variability of solvents can affect UV detection, particularly when working near the cut-off wavelength. A wider range of solvents is compatible with evaporative light scattering, corona-charged aerosol, mass spectrometric and ion-mobility spectrometric detectors; however, nonvolatile buffers and low volatility solvents cannot be used with these detectors.

Other desired features are solvent stability, reduced reactivity, and ability to dissolve a wide range of solutes. Thus, THF has the drawback of its relative instability. However, using other ethers instead of THF can be problematic, due to their limited miscibility with water. Analytes can also be affected by reactivity with certain solvents. For example, higher alcohols (e.g., isopropanol) tend to be less denaturing to biomolecules than MeOH. In fact, one of the reasons that made ACN a popular choice for LC is its ability to dissolve a wide range of compounds with minimal chemical change. Care should be also taken with bacterial growth, which is a source of unexpected and unexplained chromatographic peaks, promoted by certain reagents added to aqueous mobile phases.

Unavailability or legal restrictions should be also attended. For instance, from late 2008 to early 2009, the production of ACN came down, giving rise to an important increase in its price. There is also a concern that many volatile organic solvents are toxic or hazardous to human health or the environment (e.g., chlorinated solvents deplete the ozone layer). Therefore, legislation restricting the use of certain solvents can affect their choice or impel finding alternatives for established methods in analytical laboratories.

To reduce solvent consumption and its environmental impact, columns with a narrower internal diameter and/or smaller particle size can be used. Also, solvent recycling technologies can be a solution. All these reduced consumption patterns are supported by commitments to "greener" strategies in an effort to minimize pollution and wastes and increase sustainability. As commented above, several "green" solvents of vegetal origin, mainly terpenes, have been recommended to substitute alkanes. Ethanol and solketal are green alternatives to ACN and MeOH, but with the drawback of their larger viscosity. Also, ethanol is subjected to restrictions in some countries to avoid illegal diversion to human consumption. Acetone is a good green alternative, but the cut-off for UV-Vis detection is large, ca. 330 nm.

The organic solvent required in RPLC for a given separation can be reduced by using high column temperatures. Commercial equipment for control and programming of column temperature up to 200°C, with mobile-phase preheating and post-column cooling, as well as bonded-silica columns capable of routinely

supporting high temperatures are now available [60]. Preheating is necessary to avoid the loss of efficiency produced by radial gradients within the column. Post-column cooling is also required to prevent boiling of the mobile phase when pressure falls down.

Water becomes less polar at high temperature. This increases its elution strength. From room temperature to 200°C, a 5°C increase is equivalent to approximately a 1% and 1.3% increase in ACN and MeOH, respectively. This allows the development of water-based greener, environmentally friendly RPLC methods, although at the cost of the additional energy needed to maintain the oven temperatures and preheating and cooling systems [61,62]. Selectivity changes achieved by increasing the temperature are complementary with respect to those produced by modifying the mobile-phase composition. These changes are mainly due to a different polarity of the solvent mixture, also depending largely on the solute molecules (derived from entropic, steric, conformational, and ionization effects) [16]. Unfortunately, the elution strength of water is still relatively low below 200°C, which in most cases hinders total replacement of organic solvents by water. Further reduction of water polarity can be achieved at temperatures over 200°C, but commercial equipment is not available and the choice of suitable stationary phases, capable of standing the harsh conditions, is rapidly reduced.

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