2
Basic LC Method Development and Optimization

Victoria F. Samanidou

2.1
Introduction

Chromatography is considered to be the main part of separation science that has been established over a century ago, and since then it has been catering to the needs of many scientific areas, including biological, pharmaceutical, food, forensic, environmental, and so on. High-pressure liquid chromatography (HPLC) is one of the most reliable analytical technologies used in any analytical laboratory. It is, beyond any doubt, the most powerful weapon in an analyst's arsenal and the most useful tool toward any analytical challenge, no matter what the sample matrix is or how complicated it appears to be.

In all chromatographic techniques, analytes are separated as they are distributed between two phases, the *mobile phase* and the *stationary phase*. It may sound an easy task, but an analyst knows that this is not true at all. The final chromatographic method is a jigsaw that has to be constructed by the analyst or the so-called "chromatographer." He or she has to choose the right column, the effective mobile phase, the proper detection technique, and all necessary operational parameters, summarized under the term "chromatographic conditions," which have to be used in order to achieve the desired target, that is, well-resolved peaks.

The whole procedure is called HPLC method development, and undoubtedly, it is a complicated process.

The physicochemical properties of the analytes to be separated are the driving force of the final goal, that is, *resolution*, and then the exciting journey to an optimum method starts.

In the beginning, the chromatographer has to decide where to start from. These are the "initial conditions," which are further optimized and the final method has to be validated before its application to real samples.

The *KISS* principle is the preferred approach during method development. This means "**K**eep **I**t **S**hort and **S**imple," in order to make a "chromatographer's

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life" easier. Simplicity should be a key goal while designing the method development strategy and unnecessary complexity should be avoided. For example, a gradient is not necessary, when analytes can be easily separated under isocratic conditions. Similarly, a ternary gradient is not the best choice, when a binary seems to be efficient.

Regardless of the final scope of the method application, whether it is routine analysis or a single-sample analysis, some steps in LC method development are common.

In this chapter, the principal factors that have to be investigated during LC method development and optimization are discussed.

2.2 Theoretical Aspects

The separation of target analytes in HPLC mixtures relies on the following properties: charge, hydrophobicity, affinity, solubility, and molecular weight. According to these properties, chromatographic separations are classified into five major separation modes:

1) Adsorption chromatography, based on adsorption/desorption procedures.
2) Partition chromatography, based on partitioning of analytes between two liquid phases.
3) Ion-exchange chromatography, based on exchange of ions between surface ionic groups and ions in mobile phase. Ionic interactions depend on charge, ion size, and polarization. The pH also affects the separation.
4) Size exclusion chromatography, also known as gel permeation or gel filtration chromatography, based on molecular size. According to their size, some molecules are included in the pores of the stationary phase and thus they are retained, while some are excluded, so they pass through the column unretained. In this case, the pore size is critical and affects the retention and as a result the retention time.
5) Affinity chromatography in which analytes (enzymes, antibodies, etc.) bind to a ligand bound on a substrate and are subsequently eluted by using a chaotropic agent, by changing pH, or by using a specific eluent.

As far as interactions that take place during chromatographic separation are concerned, electrostatic forces are stronger, while the polar interactions including hydrogen bonding (permanent dipoles), dipole-induced dipole, London dispersion forces, and van der Waals forces are the most universal interactions between molecules, although relatively weak [1–6].

Before delving deeper into the various aspects of the method development, it is necessary to describe briefly some fundamental terms [7].

2.2.1 Retention Factor *k*

The retention factor, k, describes the ability of the stationary phase to retain analytes and is given by Equation 2.1

$$k = (t_R - t_0)/t_0, \tag{2.1}$$

where t_R is the analyte's retention time and t_0 the column dead time, that is, the time that an unretained compound needs to pass through the column and reach the detector. Usually, uracil or nitrite and nitrate salts can be used in reversed phase HPLC for the determination of t_0.

2.2.2 Selectivity α

Selectivity α, also called the *separation factor*, is an indication of the separation degree of two adjacent peaks and is given by Equation 2.2.

$$\alpha = k_2/k_1 = (t_{R2} - t_0)/(t_{R1} - t_0). \tag{2.2}$$

2.2.3 Peak Asymmetry

Any chromatographer wishes to obtain perfectly symmetric peaks, which can be more accurately quantified, but in the real world of chromatography, most peaks are asymmetric to some degree. Peak tailing factor (T_f) and peak asymmetry factor (A_s) are two terms that are used to describe the same phenomenon, and they result in slightly different numeric values due to the different calculation approach. Peak asymmetry factor is given by the equation $A_s = b/a$, where a is the width of the front half of the peak and b is the width of the back half of the peak at 5 or 10% of the peak height from baseline to a line dropped perpendicularly from the peak apex.

However, according to US Pharmacopoeia, $T_f = (a + b)/2a$ is used to describe peak asymmetry as shown in Figure 2.1. In general, a 10% peak height is considered for the peak asymmetry factor and 5% peak height for the peak tailing factor [5,6].

2.2.4 Efficiency of Chromatographic Column and Theoretical Plates

The quality and subsequently the efficiency of a column are characterized by the separation ability and the separation efficiency, expressed by the height equivalent of theoretical plates (H = HETP) as given by Equation 2.3:

$$H = \frac{L}{N}, \tag{2.3}$$

where H and N are the height and the number of theoretical plates, respectively, and L is the length of the analytical column in centimeters.

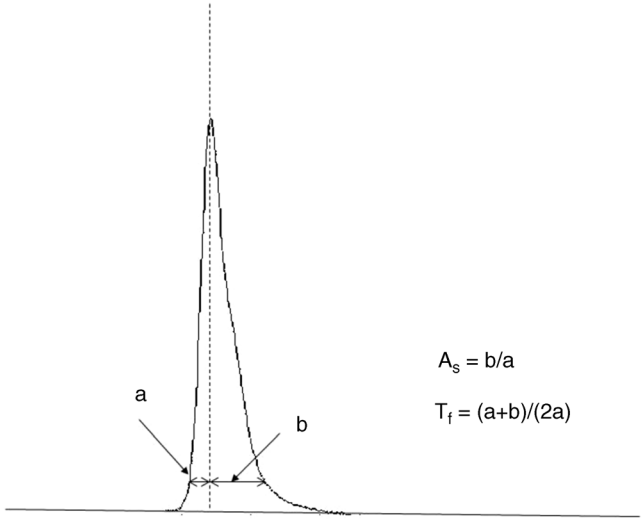

Figure 2.1 Peak asymmetry.

Actually, the efficiency of the stationary phase is described by the number of theoretical plates, N, and is calculated by Equation 2.4 or 2.5:

$$N = 16(t_R/t_b)^2 \tag{2.4}$$

$$N = 5.54(t_R/t_h)^2 \tag{2.5}$$

where t_b is the peak width at baseline and t_h is the half-height peak width, both expressed in time units.

2.2.5
Resolution R_s

Resolution describes the separation degree between a pair of adjacent peaks and is given by Equations 2.6 and 2.7

$$R_s = 2(t_{R2} - t_{R1})/(t_{b2} + t_{b1}) \tag{2.6}$$

or

$$R_s = 2(t_{R2} - t_{R1})/1.70(t_{h2} + t_{h1}) \tag{2.7}$$

depending on the method of peak width measurement used. In Equation 2.6, peak width is measured at the baseline, whereas in (2.7) it is measured at the half height of the peak.

According to the Purnell equation, the factors that regulate the resolution of a column are as follows:

$$R_s = \left(\frac{\sqrt{N}}{4}\right)\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k_2}{k_2 + 1}\right), \tag{2.8}$$

where N is the number of the theoretical plates, α is the selectivity, and k_2 is the retention factor for the compound, which is eluted last.

2.2.6 The Fundamental vanDeemter Equation

According to the fundamental *van Deemter* equation (2.9), the efficiency of a column is related to the linear velocity of mobile phase u:

$$H = A + \frac{B}{u} + Cu \tag{2.9}$$

where A is the eddy diffusion parameter, B is the diffusion coefficient that results in dispersion, and C is the resistance to mass transfer coefficient.

The comparison of the performance of different chromatographic columns is made by the H versus u plots, known as the van Deemter plots. The optimum linear velocity is achieved when the slope of the plot H versus u is zero ($dH/du = 0$):

$$u_{opt} = \frac{D_m}{d_p}\sqrt{\frac{B}{C}} \tag{2.10}$$

where d_p is the average particle size and D_m is the diffusion coefficient of the analyte in mobile phase.

The value of H at the optimum linear velocity is given by Equation 2.11:

$$H_{min} = d_p(A + \sqrt{B \cdot C}) \tag{2.11}$$

At this point of the curve, the performance of the column is the highest.

A theoretical van Deemter plot showing the relationship between the theoretical plate height and the mobile-phase velocity is presented in Figure 2.2.

The A, B, and C terms of the van Deemter equation are given by (2.12)–(2.14) relationships.

$$A = 2\lambda d_p \tag{2.12}$$

$$B = 2\gamma(D_m) \tag{2.13}$$

$$C = \frac{w d_p^2}{D_m} \tag{2.14}$$

where d_p is the average particle diameter and λ is a constant almost close to 1, D_m is the analyte diffusion coefficient in the mobile phase, γ is a factor related to the diffusion restriction by column packing, and w is a coefficient determined by the pore size distribution, pore shape, and particle size distribution [1,4,8,9].

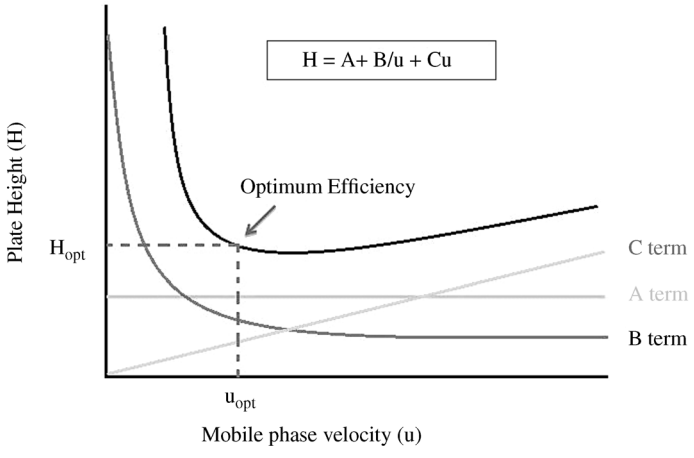

Figure 2.2 A theoretical van Deemter plot showing the relationship between theoretical plate height and mobile-phase velocity (http://www.restek.com/Technical-Resources/Technical-Library/Pharmaceutical/pharm_A016).

2.3 Controlling Resolution

Resolution is governed by different physicochemical phenomena. Physics (band spreading) and chemistry in terms of selectivity and retention lead to well-resolved or not so well-resolved peaks. It is up to the chromatographer to obtain the desired resolution.

Resolution can be influenced by changing one of the three parameters, selectivity, efficiency, and retention, as described by the Purnell equation (Equation 2.8), which determines the method development strategy. As shown in Figure 2.3, the factors that control resolution are N, k, and α. So, in order to reach the desired separation, the chromatographer has to

- increase N (either by increasing column length or by using more efficient column),
- increase k-values (by increasing the retention of analytes), and
- increase α (by using a more selective column or mobile phase).

As expected by the square root sign, efficiency (N) has a relatively smaller effect on resolution, but it can significantly influence run time. The number of theoretical plates can be increased by increasing the length of the analytical column, according to the van Deemter equation $H = L/N$. Resolution is improved as the peaks become narrower.

The retention term $k/(1+k)$ can never exceed unity and, of course, an increase in k-values increases the total run time, while generally contributing to resolution to a lesser extent.

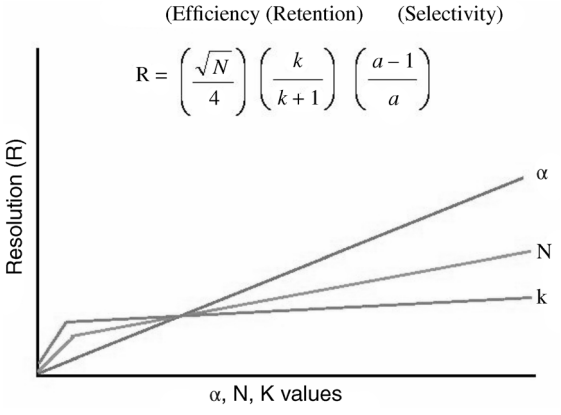

Figure 2.3 Resolution versus selectivity, retention, and efficiency.

Obviously, selectivity (α) is the most important parameter as far as resolution is concerned, while it is hard to change. By changing selectivity, the peaks are resolved and k' is often also changed as shown in Figure 2.4 [1,10].

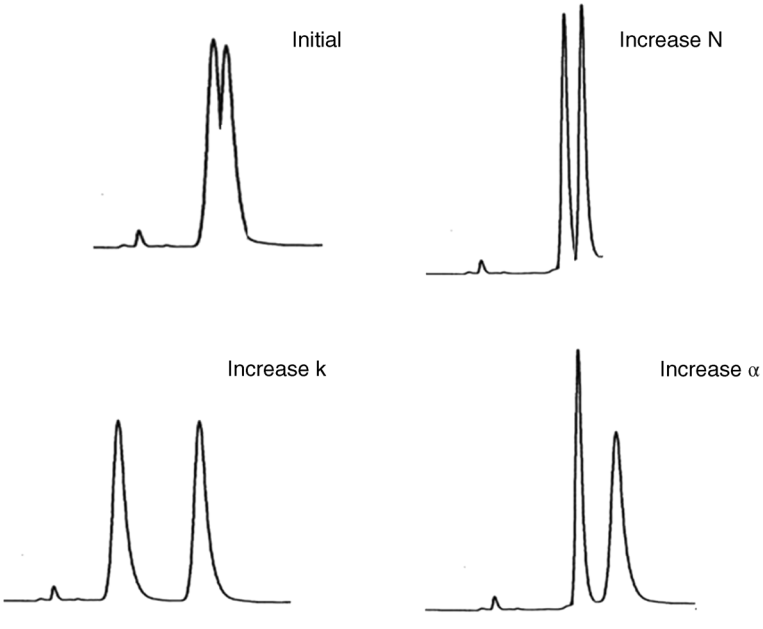

Figure 2.4 The factors that control resolution.

2.3.1 How to Improve N

While choosing the right packing material of the analytical column, the most common choice is the almost universal solution of octadecylsilane (C_{18} or ODS), which is very nonpolar. In this case, retention is based on London (dispersion) interactions with hydrophobic compounds.

Octyl C_8-bonded phases are also common, and are less hydrophobic than C_{18}; therefore, retention times for hydrophobic compounds are typically shorter, with to some extent different selectivity. Phenyl-bonded phases are also nonpolar with retention being based on a mixed mechanism of hydrophobic and p – p interactions. Exceptional selectivity results in p – p interaction of the bonded phase with electron-deficient functional moieties of analytes.

Cyanopropyl phases are of intermediate polarity, where retention is a mixed mechanism, resulting from both hydrophobic interactions and dipole interactions of the bonded phase C≡N group with solute amino groups or p – p interactions with unsaturated sites. This phase is the best for polar organic compounds and is flexible enough to be used in both normal- and reversed phase modes.

2.3.1.1 Physical Characteristics of Packing Material

When considering the influence of the physical properties of the packing material, it is also important to take into account the influence of the column size, the particle size and shape, the surface area, the pore size, the carbon load, the bonding type, and the base material.

Column dimensions: Refer to the length of column and the internal diameter of the packing bed within the column. The use of short columns (from 30 to 50 mm) results in short run times, faster equilibration, and low back pressure. On the other hand, the use of long columns (250–300 mm) results in higher resolution, higher back pressure, longer analysis times, and higher consumption of solvents used. Narrow columns produce narrower and taller peaks and a lower limit of detection.

Particle shape and size: Chromatographic packing materials may be spherical particles or irregular in shape. Spherical particles offer reduced back pressures.

Particle size: Refers to the average diameter of the particles. Although the manufacturers give a nominal size, this is typically the average size and some bigger or smaller particles are also included. Standard particle sizes range from 3 μm, which exhibit high efficiency, to 10 μm, although the latter are not so common in recent applications. Smaller particles allow less diffusion of analytes and thus they result in narrower and sharper peaks. However, smaller particles cause higher back pressures. Therefore, particles of 5 μm prevail in conventional systems and offer a good compromise between efficiency and back pressure.

Recently, smaller porous particles, with a diameter of less than 2 μm, have gained significant attention. These particles, which are named sub-2 μm particles, offer both faster separations, without any loss of column efficiency, and lower limits of detection.

The main drawback is that columns packed with such particles require special chromatographic systems, the so-called ultrahigh-pressure liquid chromatography (UHPLC) in order to overcome the high column pressure drop [4,9,11–14].

Surface area: The total surface area of a particle is the sum of the outer particle surface and the internal pore surface (expressed in m^2/g). Packing materials with high surface area ($300\,m^2/g$) result in longer retention, greater capacity, and higher resolution than those with low surface area ($200\,m^2/g$).

Pore size: it refers to the average size of the pores in porous packing materials. Larger pores allow larger solute molecules to be retained longer through maximum exposure to the surface area of the particles.

A pore size of 150 Å or less is chosen for a sample with molecular weight (MW) ≤ 2000 and a pore size of 300 Å or greater is usually chosen for a sample with MW>2000.

Bonding type: It refers to how the bonded phase is attached to the substrate. Monomeric bonding offers increased mass transfer rates, higher column efficiency, and faster column equilibration, while polymeric bonding results in increased column stability, especially with highly aqueous mobile phases.

Carbon load: It is a good indicator of hydrophobic retention and refers to the amount of bonded phase attached to the base material.

Endcapping: Important in reversed phase chromatography, endcapping is the process of bonding short hydrocarbon chains to free silanols remaining after the primary bonded phase has been added to the silica base. Endcapping reduces peak tailing of polar analytes that interact to a great extent with the most acidic silanols.

2.3.2 Increase of *k*

The retention factor k is increased by decreasing the percentage of organic modifier in the mobile phase.

The pH of mobile phase also affects k, in the case of weak organic acids and amines, depending on their pK_a value. In the range of $pK_a \pm 1.5$, a linear relationship between k and pH is observed.

2.3.3 Factors Influencing Selectivity or How to Improve α?

Selectivity α can be influenced by changing the mobile-phase type and composition, the pH, the concentration of buffer, the column temperature, and the packing material of analytical column.

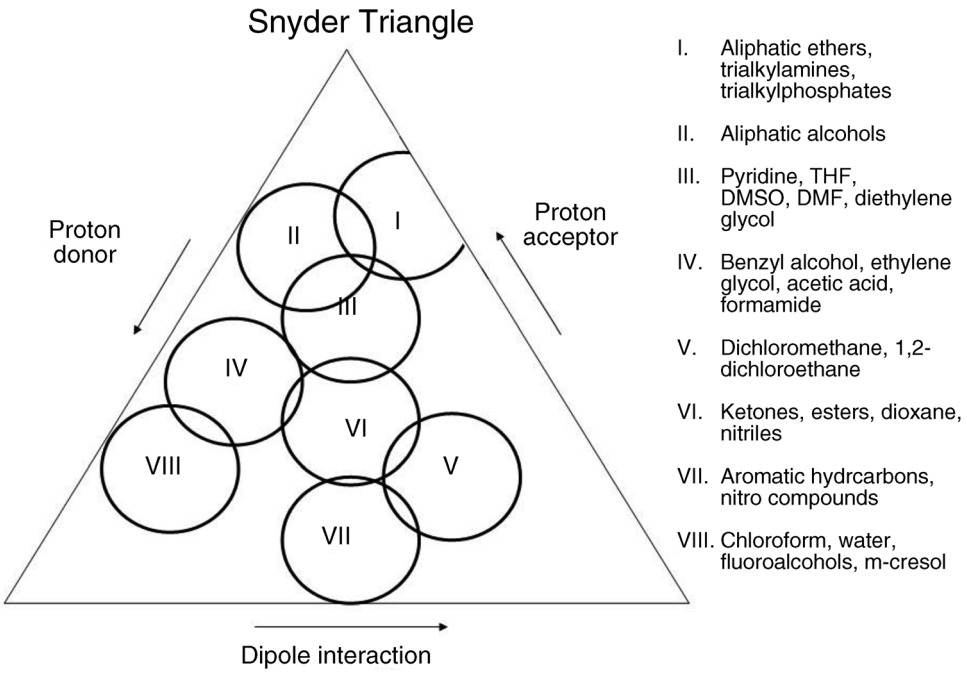

Figure 2.5 Snyder triangle.

2.3.3.1 **Optimization of Mobile-Phase Composition**

The solvent type (e.g., acetonitrile, methanol, and THF) is beyond any doubt the best approach to control selectivity. Kirkland and Snyder introduced "solvent triangle" (Figure 2.5), which describes the effect of organic modifier on the separation of analytes.

A change in the percentage of organic modifier, the application of gradient elution, the type of gradient (e.g., binary and ternary), and the gradient profile (e.g., linear and multistep) are the main factors for altering selectivity.

The Snyder triangle helps in finding the optimum mobile phase. The idea behind the triangle is that solvents in the same group will provide a comparable chromatographic selectivity. Therefore, switching from one solvent to another within the same group would not yield a spectacular change in selectivity as expected by switching to a solvent in a group with different characteristics, the same way as it happens by switching from group I to group VII, for example.

Snyder's triangle helps the chromatographer to choose wisely from among a selection of various solvents. For example, if methanol in the mobile phase is ineffective, a similar result is expected from the use of another alcohol such as ethanol or propanol. Choosing a solvent from an entirely different part of the triangle is more likely to yield the desired separation. On the other hand, larger

alcohols, such as propanol, tend to be less denaturing to biomolecules than methanol; so, it may be the solvent of choice in some cases.

Another useful application of the solvent triangle is to identify alternative mobile-phase solvents in terms of cost or availability [15,16].

2.3.3.2 pH Control, Ion-Pair Reagents, and Other Additives

Controlling the pH can also play a significant role in changing the selectivity, when the analytes are weak acids or bases. Ionized forms are strongly retained in ion chromatography but are retained less in reversed phase, while nonionized forms exhibit the opposite behavior.

Buffer type and buffer concentration are among the factors that need to be taken into consideration during method development.

Ion-pair reagents, for example, hexane sulfonate, and other additives like tailing suppression reagents, such as triethylamine, can also affect resolution directly or indirectly.

Optimization process can also be performed by the prediction of elution times, t_R, of the analytes under examination using mathematical models in an attempt to minimize both the solvent consumption and the trial time [17].

2.3.3.3 Temperature

Temperature is a significant factor that is often neglected or underestimated in liquid chromatography because it is erroneously related to gas chromatography (GC). However, it can also be powerful in resolution control and can be a critical parameter in HPLC, but in a narrower range and to a lesser extent than in GC.

Resistance in mass transfer (the *C* term in van Deemter equation) is significantly reduced in elevated temperatures.

In nearly all separations, an increase in temperature will also cause a decrease in retention. Moreover, a decreased solvent viscosity at elevated temperatures leads to lower back pressure. This allows the use of higher flow rates using standard equipment. Since high temperature leads to a flatter van Deemter curve, it enables the use of higher flow rates without sacrificing efficiency and thus optimizing the resolution (Figure 2.6).

The temperature-programmed liquid chromatography (TPLC) is a favorable technique in many applications. However, disadvantages of elevated temperatures in HPLC should also be mentioned. For example, there are inconveniences related to instrumentation or type of columns. Undoubtedly, more research in this field is required [18].

2.3.3.4 Stationary Phase and Column Selection

The stationary phase is another significant factor, which also plays an important role in selectivity control.

A radical change of the sorbent type is more effective with regard to its polarity; however, a smaller effect is also expected among similar sorbents from different companies.

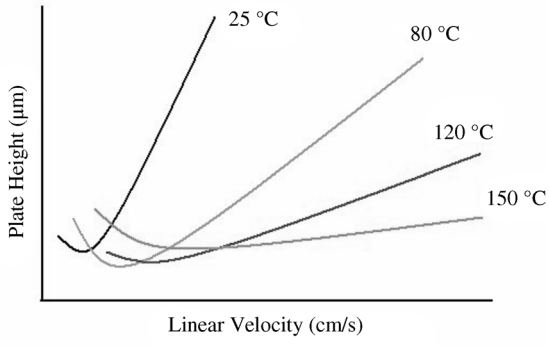

Figure 2.6 Effect of temperature on column efficiency. (Reproduced from http://www.richrom.com/application/v2/public/upload/0/default/157.pdf.)

Obviously, all parameters exerting an influence on selectivity also have a significant influence on the retention factor.

The particle size is an important parameter in separation control as is already mentioned by van Deemter equation and the *A*, *B*, and *C* terms as shown in Equations 2.12–2.14.

By taking a closer look, one can see that *A* term (eddy diffusion) is independent of linear velocity of the mobile phase. Concerning the effect on efficiency, *A* is smaller for small particle size and for spherical particles or even better for no particles at all. However, a small particle size leads to a high back pressure in conventional HPLC, although the recent development in technology has solved this problem, either by using suitable instrumentation or by using modern fused core silica particles, with low pressure drop.

The *B* term due to molecular diffusion (longitudinal) is caused by random motions of molecules and is large for smaller molecules. Dispersion increases when the analyte spends more time in mobile phase like it happens at slow flow rates.

The *C* term due to mass transfer to and within the stationary phase yields a higher dispersion for high flow rates and less dispersion for small particles.

Clearly, there are contradictory conditions, so the optimum one has to be chosen.

2.3.3.5 Stationary Phase and Packing Material Composition

Silica-based packing material is the most commonly used material available in a wide variety of bonded substrates. The main disadvantage is the pH range stability, which is limited from pH 2 to pH 8.

Polymeric packing materials are more stable in the total pH range but have a poorer performance. These are the most often used materials in ion chromatography since anion- or cation-exchange sites are embedded in the styrene–divinylbenzene backbone.

Other packing materials include zirconia-based columns as an alternative to the silica-based ones, which offer a unique interaction mechanism based on Lewis acid–base theory. In addition to the main hydrophobic interactions with the modified surface of the zirconia substrate, ion-exchange and ligand-exchange

interactions also take place. Porous graphite carbon is a highly stable packing material mostly used for the resolution of geometrical isomers, and hybrids of silica core and polymers between silica and bonded phase form a combination that improves silica stability [19].

Recently, the use of superficially porous or solid core particles has been shown to have resulted in a better resolution.

The terms superficially porous, core–shell, fused core, or solid core particles refer to particles that consist of a solid core and a porous outer shell. These particles provide faster separations compared to the large porous particles [20–23].

Monolithic columns where the stationary phase is a continuous porous material lead to low back pressures, and thus higher flow rates can be applied minimizing the analysis time without sacrificing resolution. These columns have several advantages over particulate columns [24], some of which are as follows:

- The porous polymeric rod improves both mass transfer and separation efficiency.
- They allow higher mobile phase flow rates with lower back pressure.
- They exhibit stability over a wide pH range.

2.4 Method Development Strategy

During method development, the chromatographer has to take into consideration the following main parameters as shown in Figure 2.7:

1) Which column is the most suitable one?
2) Should a buffer be used in a mobile phase?
3) Which is the best organic modifier? For example, should acetonitrile or methanol be preferred? Obviously, each one has its own benefits and drawbacks.
4) Should the use of a mixture of organic solvents be considered, since sometimes the combination leads to a better resolution?
5) Which mixtures should be chosen: binary or ternary?
6) Is isocratic elution sufficient or should a gradient system be followed?
7) In case gradient elution is applied, is a binary sufficient or should a ternary one be followed?
8) In the case of gradient, what is the expected gradient time? How many steps are required?
9) What is the optimum temperature?
10) What is the optimum flow rate?

Reasonably, changes start with those variables that are most possible to alter separation. The easier one to manipulate should be preferred and then one should turn to more complicated approaches.

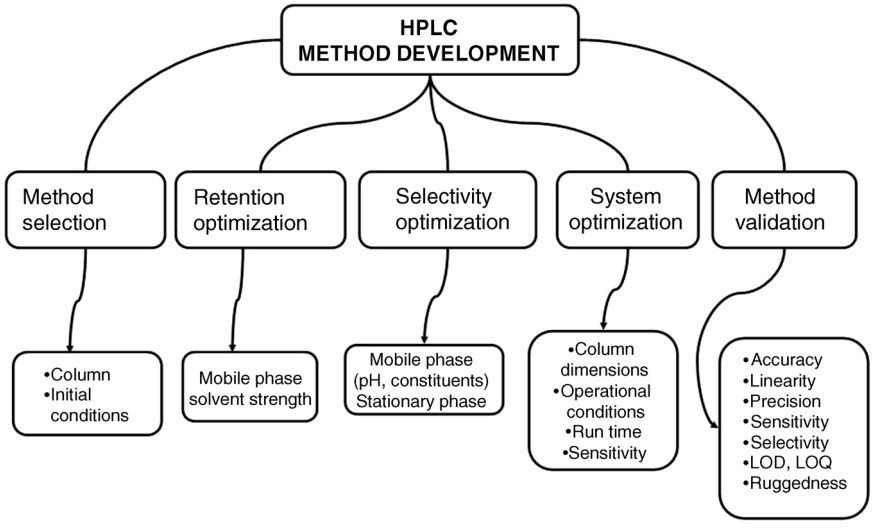

Figure 2.7 HPLC method development strategy.

2.4.1 Gradient Elution versus Isocratic

When two adjacent peaks cannot be well resolved by using isocratic elution, then gradient elution may help that also leads to a faster analysis.

But still optimization is needed with regard to total gradient time, type of gradient, steepness, and so on. However, gradient elution has its own set of problems, such as ghost peaks, peak asymmetry, baseline drift, and so on.

2.4.2 Other Parameters in LC Method Development

Needless to say that additional parameters with regard to sample preparation, detection, and quantification also require optimization.

External standard calibration is simpler, but injection errors may lead to false results. Internal standards are essential especially in the case of significant matrix effects. In this case, the difficulty of finding the most suitable internal standards is the main concern that arises. This compound has to fulfill some requirements, such as being absent from real samples, having similar properties but being differentiated and separated from analytes.

Matrix effects are often a limited factor in HPLC. Therefore, the optimum sample preparation technique should be chosen.

Finally, once desired resolution has been reached and the optimum LC method has been developed, this has to be validated before it is applied to the routine analysis. Method validation is performed in terms of precision, accuracy,

limits of detection and quantitation, specificity, selectivity, linearity, range, robustness, and system suitability [25].

2.5 Current and Future Trends

The "three S" model describes the current status in HPLC analysis requirements: **s**eparation (resolution), **s**ample capacity, and **s**peed are the principal demands.

Advances in column and instrumentation technology are the key factors in present and future liquid chromatography. The combination of analytical columns with smaller particle size and the use of more sophisticated instrumentation with reduced dead volume, low sample carryover, and better pump and detector specifications ensure high speed and optimum separations.

The van Deemter plot shows that smaller particles provide not only increased efficiency but also the ability to take advantage of this efficiency over an extended flow range. However, small particles cause increased back pressures. Moreover in conventional HPLC, faster chromatography reduces resolution. By increasing the flow rate, compression is observed. In ultra performance LC, efficiency is maintained over large areas of linear velocity (flow), which means that the flow can be increased without losing efficiency and this is the key for faster chromatography. Sub-2 μm particles offer the required improvement in column performance [11].

Numerous significant developments in materials science have been given by core–shell particle technology and monolithic columns, both of which have led to improved separation efficiency using relatively low pressures.

Columns with core–shell particles provide excellent mass transfer kinetics and a lower C term, compared to totally porous particles, in the separation of both large and small molecules, due to the lower A and B terms of the van Deemter equation. The most common size of shell particles used nowadays is 2.5–2.7 μm. These particles compete with the sub-2 μm particles, as they provide faster separations with the same efficiency but with only half the back pressure. Therefore, the most important advantage of these particles is that they do not require a special LC system [12–14,26].

Monolithic columns consist of a single rod of a highly porous material and are prepared as a single-piece block into a tube. Silica-based or polymer-based monolithic columns offer many advantages compared to particle columns; for instance, since they have a higher column permeability, they yield faster separations and low column back pressure at high flow rates of the mobile phase [27,28].

2.5.1 Two-Dimensional Chromatography

In some cases where a higher degree of selectivity is required, one-dimensional separation alone is not enough and multidimensional chromatography is required. In this case, parts of the separated sample components can be subjected to additional separation procedures, and the procedure is called two-dimensional

HPLC according to IUPAC definition, 1997. A fraction eluting from the column can pass into another column with different separation characteristics. Instrumentation is based on a normal LC instrument equipped with an extra pump and a switching valve. The two columns are connected together via a multiport switching valve. The effluent from the first column can be directed, by switching the valve, to waste, to detector, or to the second column. The two-dimensional systems can be divided into two categories, namely, heart-cut and comprehensive techniques.

In the *comprehensive* two-dimensional liquid chromatography (LC/LC) mode, the entire sample is subjected to two different separations, representative of the entire sample. It is used when information is needed from all sample components.

In *heart-cut* approach, only one or a few fractions of the first separation are collected and then transferred to the second column for further separation (second dimension). These techniques are applied when only some components are analyzed from a complex matrix. A typical example of heart-cut LC (LC–LC) is the analysis of drugs in a biological sample (e.g., urine) and the first column is used mainly for selective cleanup and concentration [29].

2.6 Conclusions

Separation science is an analytical tool of utmost importance. High-pressure liquid chromatography is one of the most reliable analytical technologies in any analytical laboratory. One of the most difficult tasks is to develop selective and fast separations in manual and/or automated approaches. No matter how sophisticated the instrumental design developments are, and no matter what improved sorbent materials are synthesized, the HPLC method development will always need the expertise of the chromatographer to arrive at an optimum final analytical method.

There are various factors affecting resolution and method developments, each one to a different extent. Practically, a combination of solvent strength (% B or gradient type) and temperature provides the most powerful control in peak resolution.

In the immediate future, new approaches will need to be explored. The ability to separate more and more species in complex matrices will remain a critical challenge. Advances in instrumentation and column technology, as well as in multidimensional separation approaches, will be the cornerstone of the chromatographic separation science. Although HPLC method development will continue to be based on chromatographer's experience, software and mathematical models in method prediction may save a lot of the laboratory budget for organic solvents, not to mention the greener chemistry that will be achieved.

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