CHAPTER-3

Fundamentals of High performance liquid chromatography

3.1 Adsorption High-Performance Liquid Chromatography

Liquid-solid chromatography was developed in 1906. Separation is carried out with a liquid mobile phase and a solid stationary phase that reversibly adsorbs solute molecules. The stationary phase is usually polar such as silica, porous glass, or alumina and the mobile phase is a relatively nonpolar organic solvent. Due to separation selectivity of adsorption HPLC, the compounds with different chemical structure are easily separated on silica and alumina. It can provide easy resolution of compounds with differing numbers of functional groups and provides maximum differentiation among isomeric mixtures. The retention of a solute requires displacement of an equivalent number of adsorbed solvent molecules. Polar functional groups are strongly attracted to the polar adsorbent surface, so that compounds with substituents of differing polarity are readily separated. Another feature of adsorption HPLC is the presence of discrete adsorption HPLC, the presence of discrete adsorption sites on the surface of the adsorbent. Optimum interaction between a solute molecule and adsorbent surface occurs when solute functional groups exactly overlap these adsorption sites.

Solvents in liquid chromatography have the following important properties. polarity, viscosity, boiling point, detector compatibility (DV adsorbance and cutoff) reactivity, miscibility, and safety. There are four major interactions between molecules of solvent and solute that are important in HPLC: dispersion, dipole, hydrogen bonding, and dielectricity. The larger the dispersion, dipole, hydrogen bonding, and dielectric interactions in combination, the stronger the attraction of solvent and solute molecules The ability of a sample or solvent molecule to interact in all four of these forces is referred to as the polarity of the compound. Thus the polar solvents preferentially attract and dissolve polar solute molecules. Solvent strength increases with solvent polarity in adsorption HPLC (The opposite is true in reversed-phase HPLC.)

Solvent polarity has been defined in various ways. Reichardt [31] provided a detailed summary of the empirical parameters of solvent polarity for 204 solvents. The

most widely applied solute polarity parameter (P') is based on experimental solubility data reported by Rohrscheider [32] and Snyder.

In general,. a change in P' by two units causes roughly a tenfold change in the retention expressed by the capacity ratio k'.

$$k' = (t_R - t_o) / t_o \qquad (60)$$

where t_R and t_o are retention time and dead time, respectively, as described by the following equation. .

$$k'_2 / k'_1 = 10^{(P'_1 - P'_2)/2} \qquad (61)$$

where k'_2 and k'_1 refer to the capacity ratios obtained by the first and second mobile phases, respectively, and P'_1 and P'_2 refer to the solvent polarity (P') of the first and second mobile phases. Since P' is based on actual solubility data, and the sample solubility in the mobile phase and stationary phases determines the k' values, it can be expected that P' will provide a reasonably accurate measure of solvent strength in liquid-liquid partition chromatography. A better index of solvent strength in this case is afforded by the experimental adsorption solvent strength parameter ε^0. In adsorption HPLC the solvent strength ε^0 does not vary linearly with solvent composition. Instead, ε^0 increases rapidly for small additions of a stronger solvent B to a weaker solvent A, then asymptotically approaches the value of pure B. In practice it means that we have to be very careful about the polar impurities of the organic mobile phase used for the retention determination. A very small change in the water concentration can cause a dramatic change in the adsorption chromatographic retention of a solute. When the mobile phase contains a few percent water on purpose, the various trace amounts of water in the organic solvents will not cause too much of a deviation in the retention. Solvent selectivity is the other very important characteristic of the mobile phase. By changing the selectivity without changing the solvent strength two overlapping peaks can be separated. The greatest change in the mobile phase selectivity results when' the importance of the various intermolecular interactions between solvent and sample molecules is markedly changed.

It is also important to check solvent miscibility, not only when preparing a mobile phase mixture, but also when the mobile phase is changed. It is also important to know what kind of mobile phase was used previously to avoid the formation of emulsion or precipitation in the column.

In adsorption liquid chromatography it is customary to add small amounts of water or a polar modifier such as methanol to the mobile phase. These polar compounds are preferentially adsorbed onto strong adsorption sites, leaving a more uniform population of weaker sites that serve to retain the sample molecules. This deactivation of the adsorbent leads to a number of improvements in subsequent separation. It causes less variation in sample retention from run to run, and we can observe higher column efficiency and reduced peak tailing in some cases. We must also consider that the equilibrium process in adsorption chromatography takes a minimum of 2 h or even more when we wish to equilibrate the column from a stronger mobile phase to a weaker one.

3.2 Reversed-Phase High-Performance Liquid Chromatography

The popularity of the technique rests with the reproducibility offered by use of the hydrocarbon bonded phase. Very efficient and high-speed separations can be achieved on chemically bonded hydrocarbon reversed-phase columns. The most popular RP columns are octyl and octadecyl silica, which are relatively stable with most aqueous eluents having a pH value less than 8. Both spherical and irregular silica particles are used as supports 1 and *n-octyl* or n-octadecyl functions are the most popular organic ligands attached covalently to the support surface. The inner diameter (ID) of the analytical column is usually 4 to 5 mm and the length varies between 50 and 300 mm. They are operated at eluent flow rates ranging from 0.5 to 3 ml/min.

The maximum coverage (ie., surface concentration) of organic materials reported for so-called monomolecular phases varies from 3 5 to 4 μmol/ m^2, with coverage for many commercial octadecyl supports well below these values. The coverage percentage of the available silanol groups usually varies from 10 to 60%. The coverage or the presence of free silanol groups can be tested by measuring

retention of some standard compounds (benzene, nitrobenzene, or some basic compounds). The extent of coverage affects the retention properties of both the stationary phase and the stability. At low coverage where a higher proportion of silanol is available, the stability in basic aqueous solution is lower than that for a higher coverage where the underlying siloxane bonds are protected by the hydrophobic ligand. In general, chromatographic retention increases with the degree of phase coverage. Selectivity of the support may be affected in this case of low coverage, due to a mixed retention mechanism (partition and adsorption on unreacted silanol groups). At higher coverage the change in selectivity is lower since solute retention is governed mostly by hydrophobic forces and even small polar molecules are unable to reach the free silanol groups.

In general, for reversed-phase packing of equal loading, the longer the chain length of the alkyl hydrocarbonaceous group, the greater the chromatographic retention when fixed conditions are used. The retention of polar and nonpolar samples was found to be influenced by the eluent, the length of the organic ligand, carbon content, pore size distribution of the silica support, and total porosity of the stationary phase. The authors described [34] that the rate of analysis increases with decreasing length of the alkyl chain. For lower pressure the spherical particles are more suitable: for higher separation efficiency, 3 to 5μ particle size stationary phases may be suggested

Eluents used in reversed-phase chromatography with bonded nonpolar stationary phases are generally polar solvents or mixtures of polar solvents, such as acetonitrile or methanol with water. The most significant properties of solvents that may affect the chromatography are surface tension, dielectric constant, viscosity, DV adsorbance, and elutropic value. In general, the lower the polarity, the higher the strength of eluent in RPHPLC Many samples contain ionogenic substances which may undergo ionization in aqueous eluent and as a result severe peak broadening may occur The effect is eliminated or reduced by use of buffer solutions. In practice, a buffer concentration between 10 to 100 mM seems to be sufficient in the pH region of peak buffering capacity.

3.3 Retention Determination

The capacity ratio (k') can be defined- by the following equation in both adsorption and reversed-phase chromatography:

$$k' = t_R - t_o / t_o \tag{62}$$

where the t_R is the retention time, which means the time passed from the injection to the appearance of the solute peak maximum, and to is the dead time. The exact dead time is one of the most important parameters in the accurate measurement of k'. The problem starts with the definition of dead time, i.e., the retention time (or if multiplied by the flow rate, retention volume) of the unretained solute. The dead volume can be the elution volume of a solvent disturbance peak obtained by injecting an eluent component, or the elution volume of an unionized solute that gives the lowest retention volume, or the elution volume of an isotopically labeled component of the eluent or an isotopically labeled water molecule (only in RPHPLC), or the elution volume of a salt ion, or the volume of the liquid the column contains, or the extrapolated elution volume of a zero number of a homolog series. Wells and Clark, [35] Berendsen et al.,[36] and Knox and Kaliszan [37] provided reviews of various techniques for experimental determination of dead volume in HPLC.

The most accurate method is to inject a UV active salt (potassium iodide or sodium nitrate) in reversed-phase chromatography together with the solute for which the capacity ratio has to be determined. The only objection to the method may be the interaction of salt with the solute or the mobile phase additives It may also happen that the dead time or dead volume vary with mobile phase composition. Especially when high concentrations of organic phase are applied in the mobile phase, the retention time of sodium nitrate or the potassium iodide may increase. In these cases the injection of the eluent component can provide a disturbance peak that can be used to calculate the capacity ratio.

In HPLC the retention of a solute is determined by the strength of solute-stationary phase and solute-mobile phase interactions; therefore, it is essential to reveal quantitatively how mobile phase composition affects solute retention. The dependence of the logarithm of the retention factor (log k') on the volume fraction of the organic component g, in the mobile phase can be described by the following equation:

$$\log k' = \text{slope } b + \log k'_0 \tag{63}$$

where the log k'_0 value (the intercept value of the straight line) refers to the extrapolated log k' value to neat water as mobile phase. The slope values shows the sensitivity of the change in the retention of a given solute caused by the change in organic phase concentration in the mobile phase. In practice the determination of these two parameters can be carried out by measuring the log k' values of the compounds by using a minimum of five organic phase concentrations in the mobile phase.

As we cannot expect linearity in a wide range of mobile phase compositions, the change of the organic phase concentration in 5% steps is advantageous. Then the log k' values obtained in five different concentrations will cover a concentration range of 25%. Compound retention should be within the range of 0 to 5 log k'. Retention in chromatography reflects solute distribution between the stationary and mobile phases as well as the temperature Melander et al. [38] described a quantitative dependence of the log k' values on the temperature and the mobile phase composition:

$$\log k' = A_1 X(1 - Tc/T) + A_2/T + A_3 + A_4 f(X,T) \tag{64}$$

where X is volume fraction of the organic cosolvent in the hydroorganic mobile phase; T is absolute temperature; and Tc is the so-called compensation temperature derived by taking advantage of the enthalpy-entropy compensation Linear relationships were found in RPHPLC between enthalpy and the entropy for reversible solute binding to hydrocarbonaceous stationary phases, as indicated by Melander et al. [38] The effect is called enthalpy-entropy compensation. Comparison of the compensation temperatures obtained from such data can be used to study the retention mechanism, whether they are identical or not in two different chromatographic systems.

3.4 Development of Column

During the last three decades the advancement of high-performance liquid chroma-tography (HPLC) column has paralleled, and sometimes even exceeded, the development of HPLC equipment. The rapid progress in HPLC techniques in the late 1960s was mainly due to the use of pellicular packings, a low-flow rate reciprocating pump and the flow through DV detector.

In the early 1970s microparticulate packings (particle size <10μ) were predominant, which provided greater speed efficiency, and sample capacity benefits. Stable chemically bonded phases spurred the development of reversed-phase chromatography. The 1980s saw the introduction of biochromatography materials which provided better analytical and preparative problem-solving tools for life scientists

The solid-phase extraction cartridges enabled more convenient sample cleanup In the 1990s further refinements in column technology resulted in columns that provided large separation factors, more stable and durable reversed-phase columns, and special columns for solving specific chemical, environmental, and other speciality problems.

3.5 Direct (Normal)-Phase High-Performance Liquid Chromatography

3.5.1 Silica Supports

Currently more than 90% of column packings used in normal- and reversed-phase liquid chromatography are based on silica. Silica exists in various forms with the stoichi-ometric composition SiO_2 The atomic number of silicon is 14 and its atomic weight is 28 08. Silicon comprises 25 7% (w /w) of the earth's crust; it is the second most abundant element. Silicon is not found free in nature, but occurs mainly as oxide and silicates. Over 55% (w /w) of the earth's crust is made up of silicon oxide and silicates.

The silica used for liquid chromatography (LC) column packings is essentially porous and noncrystalline with the general formula $SiO_2.xH_2O$. Water is chemically bound in a nonstoichiometric amount, forming Si-OH. Silinol groups proved most useful for LC packing.[39]

Silanols are responsible for the polar character of silica packings used in normal phase LC to graft organic moieties This treatment changes the silica surface and allows the

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development of bonded phases used in reversed-phase and other modes of LC [40] The surface of silica forms is covered by hydroxylated silanol (Si-OH). Two hydroxyl groups on two vicinal silicon atoms are vicinal silanols .

When borne by the same silicon atom, the two groups are termed geminal silanols and the other type of silanols can be isolated as free silanol groups. The surface of a crystalline form of silica is covered chiefly by isolated silanol groups Crystalline forms of silica are found in nature. The unique amorphous silica source of biological origin is diatomaceous earth All other amorphous silicas used in the industry are produced by synthetic methods. There are two main methods for obtaining pure silica The liquid phase route and the gas phase route. Amorphous silica with poroud structure bears the above three types of silanol groups capable of hydrogen bonding with water molecules The more elevated the surface silanol concentration, the greater the hydrophilic character of the surface The role of silanols in LC selectivity is essential It is very important to know the concentration of surface silanols for a given silica-based LC stationary phase. There are three principal methods for the determination of surface hydroxyl groups. chemical, isotopic exchange, and spectroscopic methods.[41]

3.5.2 Chemical Methods

Water hydrogens are more acidic than silanol hydrogen's, thus all physisorbed water must be removed by heating the silica sample at 150°C, under vacuum, for several hours The most reliable estimate of surface silanol concentration was obtained by reaction of hydroxyl groups with methyl lithium under nitrogen atmosphere. Provided that the sample was carefully dried, the solution reacted with hydroxyl groups as follows:

$$Si\text{-}OH + LiCH_3 = Si\text{-}O^- + CH4 \qquad (65)$$

The amount of methane evolved was a direct measure of silanol surface concentration. If water molecules were present, methyl lithium reacted with these first to produce lithium oxide (LiP) and methane, then it reacted with silanols The methane was determined by gas chromatography. [42]

3.5.3. Isotopic Exchange Methods

Heavy water was used to determine surface silanol concentration by isotopic exchange

$$Si\text{-}OH + 2H_2O = Si\text{-}O^2H + H^2HO \tag{66}$$
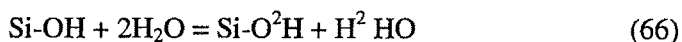
Surface silanol concentration was determined by weighing the dry sample before and after the exchange or by spectroscopic methods. Titrium-labeled water ^3HHO was preferably employed instead of heavy water, as this facilitates radioactive measurement of the exchanged sample.[43]

3.5.4 Spectroscopic Methods

Infrared (IR) spectroscopy was used to assess surface silanol concentration. Nuclear magnetic resonance (NMR) of ^{29}Si with cross-polarization and magic angle spinning (MAS) could also be applied to determine free and geminal silanol groups and silicon atoms with no silanols. Other methods such as thermo gravimetric analysis, diffuse reflectance, Fourier transform infrared spectroscopy, or pH measurements also proved to be suitable for silently determination.[44]

3.6. Chemical Properties of Silica

Most chemical properties of silica are due to surface saloons. Interactions of silica with water are silently dependent. Hydroxyl acidity is responsible for the acid-base properties of silica. Saloons were used to graft various organic moieties on the silica surface and to obtain the wide variety of bonded silica essential for LC packings.

3.6.1 Acid-Base Properties

Silica acid is a weak acid.

$$Is(OH)4 = Is(OH)P\text{-}+ H+ \tag{67}$$

The ionization constant of the reaction was 1.6×10^{-10} at 20°C, which corresponds to a pea value of 9.8. The pea value of the second ionization was 11.7. The hydroxyl groups on the silica surface have also an acidic character. The pe_a. value of the reaction was

about 6.8, which was 3 pH units lower than the free silica acid first ionization. There are several explanations for the higher mobility of silanol proton compared with that of silicic acid. The tendency for splitting off a proton from a particular silanol group was markedly promoted by its environment.[45]

$$Si(OH)O^- = Si(OH)^2O^-_2 + H+ \qquad (68)$$

$$SiOH = SiO^- + H^+ \qquad (69)$$
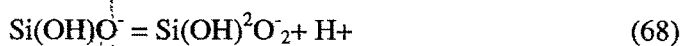
The acidic character of surface silica's conferred some ion-exchange properties on porous silica. The hydronium ions (protons) of the silanol groups were exchangeable by cations of the solution in buffer or electronic solution. The ion-exchange properties of silica were highly dependent on the pH of the solution, the surface area, and the silanol concentration. Silica bore negative charges in solution at pH 7.[46] The rapid dissolution of silica at mobile phase pH values was higher than 10 and / or temperature higher than 40°C. A slow and insidious solubilization occurred from pH 8. This may cause small voids in the column packing, inducing changes in solute retention and leading to lower efficiency. This could be reduced with the use of a guard column or a precolumn installed between the pump and the injection valve to saturate the mobile phase in soluble silica. It is important to improve the pH stability of silica.

3 6.2 Silanol Acidity

Silanol groups are acidic, although there has been some controversy over the origin of the acidity and the variability of this acidity between different silanol groups on the same silica surface and similar silanol groups on silica surfaces with different histories. There have been several attempts to define silica acidity as a measurable parameter for the selection and characterization of silicas. One method reported is the measurement of the pH values of an aqueous suspension of silica. [47] Such a measurement may lead to surprising results, e.g., pH values ranging from 3 to 9 5. One problem in the synthesis of silica is the removal of traces of acid or alkali used in the synthesis and washing of the packing. Silicas that have very high pH values in the tests generally have high residual content of sodium or other metal ions present within the matrix. The material (Zorbax

SIL) reported to have the lowest pH value involved extensive acid treatment for rehydroxylation and even though this is one of the more acidic silicas, it is highly probable that the pH measurement indicated the degree of acid removal after washing rather than a fundamental property of the silica. Because of the need for extremely careful pretreatment prior to pH measurement or titration of the silica, much of the measurement of acidity has relied upon physicochemical procedures such as IR, where the stretching frequency of the hydroxyl band is related to its acidity. Such infrared studies have suggested that the pKa of the silanol groups is around 7.1.[48] This result must be treated, however, with caution since these authors remarked that although the average pKa of the surface hydroxyls for a silica/ alumina catalyst was found to be 7.1, there were a few groups showing pKa values lower than -0.4. Thus, such measurements generally give average values and do not detect a small number of extremely active silanols.

3.6.3. Water Adsorption and Desorption

Silica hydroxyl groups are dissociated with water molecules by hydrogen bonding. This adsorbed water is tightly bound to the silica surface and it is removed by heating at 150°C under vacuum for several hours. Overheating of the silica sample led to dehydroxylation. Geminal silanols were reversibly dehydroxylated resulting in siloxane bridges. Between 200 and 800°C the temperature treatment under vacuum of a silica sample induces a monotonous decrease in the hydroxyl group concentration with siloxane bridge formation. If the calcination temperature does not exceed 600°C, the reactive siloxane group completely rehydroxylates when exposed to water.[49]

Complete dehydroxylation and sintering occur on heating above 1200°C. At this elevated temperature the amorphous character of silica slowly ceases. The hydrophilic surface is converted into a hydrophobic surface.[50]

The conversion of amorphous silica into crystalline form is dependent on the purity of silica and particularly on its sodium. At room temperature porous silica can adsorb water reversibly.

3.6.4 Water Solubility

Water solubility is very dependent on the crystalline or amorphous form of the sample and also on physicochemical properties, such as porosity, surface area, and particle size. The solubility of silica changes the effect of water temperature, pressure, and pH. The solubility of crystalline and glass form of silica is in the parts per million (ppm) range.

3.6.5 Particle Size

Silica packings are always obtained with particle size distribution, and 5 μ is generally considered as an average diameter. The average particle diameter can be defined as the average diameter in number d_n, the average particle diameter in surface d_s, or the average diameter in mass d_m, as follows.

$$d_n = \Sigma\, n_i d_i / N = \Sigma n_i d_i / \Sigma\, n_i \tag{70}$$

$$ds = (1/S)\, \Sigma\, n_i d_i^3\, \pi\, / 4 = \Sigma\, n_i d_i^3 / \Sigma\, n_i d_i^2 \tag{71}$$

$$dm = (4\,\pi\, p/3M)(Injd4) = \Sigma\, n_i d_i^4 / \Sigma\, n_i d^4 / S n_i d^3 \tag{72}$$

where n_i denotes the number of particles with diameter d_i; N is the total number of particles in the studied sample; S stands for the surface area of the sample; M designates the sample mass; and p is the density of the particles

Silica-based LC packings have a particle size ranging from 1 to 100 μ. Microscopy is the simplest method to determine particle size. Electronic counting scanners can give particle distribution, with few or no assumptions of the particle shape. Sedimentation is another widely used technique for particle size analysis.

The particle size is one of the most important parameters, greatly influencing the chromatography efficiency. The smaller the LC particle size, the higher the efficiency. The plate height (H) of a well-packed column can be as low as twice the mean particle diameter (100,000 plates per meter, H = 10 μ, dp = 5 μ). Unfortunately, particle size also affects column permeability P (cm^2) in the following way:

$$P= (d^2_p/Y)[\varepsilon^2_0/(1-\varepsilon_0)^2] \tag{73}$$

where Y is a dimensionless shape factor (Y = 180 for spherical particles) and ε_0 is the interstitial volume or external porosity (cm^3). Permeability is a parameter of the column pressure drop (P).

$$P = uvL / P \tag{74}$$

where u is the linear mobile phase velocity (cm/s); v is the mobile phase viscosity (g/cms); and L is the column length (cm). If the particle diameter is divided by a factor of two, the column driving pressure has to be four times as high in order to obtain the same flow rate. Optimum particle size with regard to the analysis time, plate number, and pressure drop is approximately 2 to 4μm. Packings with average particle diameter of 5 μm are the most commonly used for 10- to 25-cm analytical columns with I.D. of 4 to 4.6 mm. Packings of 3 μm are used to obtain high efficiency with short columns, 10 μm, 20 μm, or larger particle size packings are used when the pressure is an important factor, such as in preparative chromatography.

3.6.6 Surface Area.

The specific surface area of a porous solid material is equal to the sum of its internal and external surface areas. The external surface area corresponds to the geometric surface of particles per gram of sample. For spherical particles of equal size d the external surface area is obtained by

$$A= 6/d\sigma \tag{75}$$

where σ is the particle density, defined as the ratio of particle mass to particle volume. For nonporous particles σ is the solid silica density 2 2 g /cm^3. The external surface area is inversely proportional to the particle diameter. A mono disperse nonporous silica with particle size of 3 μm has a surface area of 0.91 m^2/g. Polydisperse nonporous silica with an average particle diameter of 3 μm has a surface area of 0.74 m^2/ g. Surface area is measured by the routine BET method. [51] The complete theory of gas adsorption and specific surface area determination is described in the book by Gregg and Sing.[52] The surface area of a silica sample is dependent on the porosity, which forms the internal

surface area. The specific surface area of silica used in high-performance liquid chromatography ranges from 10 to about 500 $m^2/$ g depending on the average pore diameter.

3 6.7 Porosity

A pore is a hole, a cavity, or a channel connected to the surface of solid. The spaces or interstices between particles are voids rather than pores. A cavity that does not communicate with the surface is called a closed pore or an internal void and will not contribute to porosity or specific surface area. The pore system and its characteristics are the topic of numerous papers.[53] The pore shapes shown has wide variety. The pore size covers a range of several orders of magnitude. A feature of special interest is the width Pd of the pores, which is the diameter of an equivalent cylindrical pore. According to IUPAC classification there are micropores with $P_d < 2$ nm, mesopores with $2 < P_d < 50$ nm, and macropores with $P_d > 50$ nm. The porosity of a sample ε_p is defined as the total pore volume V_p divided by the volume of the sample V_T.

$$V_T = V_p + V_s \qquad (76)$$

where V_s is the volume of solid silica, assuming that there are no closed pores. Pore volume and pore size distribution are estimated by gas adsorption-desorption isoterms.

The adsorption-desorption isotherms of gas onto solids can be divided in six different types. Types I, IV, and V isotherms show hysteresis; the desorption branch of the curve does not coincide with the adsorption branch. Both branches (desorption and adsorption) are used in pore shape and volume determination. Types I, IV, and V isotherms correspond to microporous, mesoporous, and polydisperse porosity, respectively. Types 11 and III correspond to nonporous samples with strong interaction and non- or macroporous samples with weak interaction, respectively. The type VI isotherm corresponds to nonporous material

[52,53]

3.6.8 Relationship between Surface Area and Porosity

The specific surface area of silica has an important impact on solute retention and selectivity The higher the specific surface area of silica, the higher the solute-stationary

phase exchange area Solute retention is roughly proportional to the specific surface area when all other parameters are constant (same kind of stationary phase, same mobile phase, same column length, same flow-rate, etc.)

Pore size, pore volume, and specific surface area are interrelated. Assuming that there are no closed pores, the specific pore volume, V_p (cm^3 /g) to P_a, the apparent density of the particle is defined as the ratio of the mass of the porous particle to its total volume (cm^3/g):

$$P_a = p / (1 + V_p p) = 2\ 2 / (1 + 2.2V_p) \qquad (77)$$

(2.2 g/cm^3 are the density of solid silica). If we assume a monodisperse distribution of perfectly cylindrical pores with diameter P_d the internal surface area due to the pores is expressed by

$$A_p = 4V_p/P_d \qquad (78)$$

where A_p is the internal surface area; V_p is the specific pore volume. The specific surface area is the sum of the external surface area ($A = 6/ d\sigma$) with the corrected density ($P_a=p/(1 + V_p p) = 2\ 2/(1 + 2.2V_p)$) and the internal surface area ($A_p = 4V_p/p_d$).
$$A_s = 6(1 + Vpp)/d\sigma' + (4V_p/p_d) \qquad (79)$$

Pore diameter on the surface area and the relative weight of the internal and external surface area are of great importance. For most samples more than 97% of the specific surface area is internal surface due to pores. The particle diameter has little importance with regard to the surface area, which depends mainly on the pore size and volume .

3.6.9 Surface Silanols and Polarity

Silanol groups are responsible for the polarity of the silica surface. A silica completely dehydroxylated by prolonged heating at 1300°C is hydrophobic The silanol concentration of a fully hydroxylated silica is about 9 µmol/m^2. The pretreatment temperature under vacuum may decrease the silanol concentration and adjust the hydrophilic character of a sample. [54] The selectivity of thermally treated silica used in normal phase LC greatly depends on the water content of the apolar mobile phase.[55]

Reproducible results with activated silicas and apolar solvents such as n-hexane are only obtained when the water content of the silica and the mobile phase is controlled and adjusted. Water molecules, always present in trace amounts in an apolar solvent, adsorb on most polar silanol sites, decreasing the retention of polar solutes and modifying the solute-stationary phase exchanges. Peak shape and efficiency are also affected by the adsorption of water on silica. Methanol and other polar solvents can be added to the apolar mobile phase to adjust the selectivity

Acid silanols have great affinity for basic groups The low efficiencies and peak tailings obtained with the amino-containing solutes are due to -SiOH. NH- acid-base interaction. In order to reduce the residual silanol concentration of bonded silica, the end-capping treatment was used The small trimethylchlorosilane molecule was used to react with silanols that were not accessible to the large octadecyl-containing reagent. An end-capped silica still bears unreacted silanols.The modem trend in manufacturing base-deactivated silica packings is to increase the ligand density.[56]

3.6.10 Carbon Content and Bonding Density

Two important parameters for the characterization of bonded silica are carbon content, in grams of C per 100 g of packing, and surface concentration or bonding density, in micromoles of bonding moiety per square meter of initial silica surface area. Although bonding density is almost never given by manufacturers, it is one of the most important parameters for a bonded silica-based stationary phase. High bonding density implies a low residual silanol concentration; this phase has better resistance to elevated pH mobile phases and higher capability to separate amino-containing compounds.

3.6.11 Chemical and Physical Chromatographic Requirements

The safe pH range for the mobile phase is 2 to 9. Below pH 2, Si-C bonds of derivatized LC packings can be split, above pH 9, silica is slowly solubilized into silicate. The working pH range increases when bonding density and/ or silica purity is high A small addition of sodium silicate to the mobile phase greatly reduces the dissolution of

silica at elevated pH [57] Zirconia cladding enhances the resistance of silica to alkaline solutions

The stationary phases must have some mechanical strength to withstand the column back-pressure, in the range 10 to 200 bar or 140 to 2900 psi. Silica pore size may be a critical parameter in bulky solute analysis For example, large-pore silica supports are required for biopolymer separation, Le , for proteins and peptides.[58] In these analyses, 30 and 50 nm pore size packings are commonly used to avoid internal interactions such as restricted and slow diffusion of biopolymers The large molecules can be trapped inside medium sized pores, inducing peak tails. A narrow pore size distribution is also required in size-exclusion chromatography to obtain sufficient efficiency.

3.6.12 Application of Normal-Phase Chromatography

Normal-phase (NP) chromatography is a powerful complement to the more popular reversed-phase HPLC method for separating nonionic compounds About one fifth of all HPLC separations are now performed by NPHPLC. The advantage of this method is that it can be performed with totally organic mobile phases of high solute solubility, which is important in preparative applications NPHPLC is also capable of extensive selectivity changes with the use of various mobile phase constituents. NPHPLC methods are especially useful for the separation of difficult mixtures containing positional isomers.[59]

Current NPHPLC applications commonly use columns containing polar bonded phases (cyano-, diol-, etc) Columns containing unmodified silicas are less popular, because of problems in maintaining a constant surface activity for repeatable separations However, retention with bonded-phase NPHPLC columns is generally more consistent, because of insensitivity to small concentrations of water in the mobile phase of samples. Chromatographic reproducibility of bonded-phase columns for NPHPLC is generally viewed as superior to the column of unmodified silica. The polar bonded-phase columns are less retentive in NP-HPLC than unmodified silica, often permitting the elution of highly polar compounds that are eluted with difficulty from the more retentive unmodified silica

A common problem with unmodified silica columns with totally organic solvents is that polar compounds often show broad, tailing peaks, especially for basic components. This condition can exist even if the mobile phase contains basic additives designed to minimize this effect. Such undesirable characteristics complicate the design of rugged and reproducible quantitative methods. As a result, unmodified silica columns are not widely accepted for quantitative analysis.

Unmodified silica columns present a special problem that must be solved for reproducible separations: the activity of the adsorbent surface must be constant. The most effective procedure to stabilize this surface activity is to control the level of water adsorbed to the silica surface This is often accomplished by fixing the concentration of water in the mobile phase, a procedure viewed as awkward by many users Thus polar, usually protic mobile phase modifiers such as methanol and propanol routinely are used instead of water to control surface activity The fundamental problem remains, however, that commercial unmodified chromatographic silica often exhibits an inhomogeneous adsorbing surface. This fact can result in broad, poorly shaped peaks when used for separating many polar solutes and basic drugs Previous studies have shown that chromatographic silicas can be arbitrarily classified into types to define their potential utility as supports in reversed-phase chromatography [44-61]

So-called type A silicas are generally more acidic and less purified. These materials apparently have less energy-homogeneous surfaces and are likely to exhibit tailing, misshapen peaks with more polar and basic compounds. Type B silicas are more highly purified and less acidic and are often preferred for many RPHPLC applications.

Kirkland et al.[62] determined the characteristics and chromatographic properties of a new porous silica microsphere, Zorbax RX-SIL. The chromatographic characteristics of this new type B silica were compared with conventional type A silica (Zorbax-SIL) to determine the type and level of mobile phase modifiers needed for good results. The effects of sample type and sample loading on retention column efficiency and peak shape were also measured.

Absolute differences in retention for the two silicas were found to be functions of differences in surface areas. It was assumed that the heterogeneous surface of type A silica led to isotherm nonlinearity and the related phenomena of large plate height and asymmetric tailing peaks. The effect of sample loading on column efficiency was similar for the two silica types. Sample loadability was essentially equivalent for the two silicas, with small variations dependent on differences in packing surface areas. As mentioned above, many users consider the necessity of maintaining a constant amount of water modifier in the organic mobile phase as experimentally awkward. Because of the convenience, other highly polar, organic-miscible modifiers such as methanol and propanol are often used in normal-phase separations to control and maintain the activity of the silica adsorbent.

These polar organic compounds strongly bind to the silica surface, although they do not bind as tightly as water. For the convenience of protic-solvent modifiers, a study was made with methanol and propanol to determine their effect on type A and B silicas. Similar results were found for the two silica types; both showed sharply increasing k' (capacity factors) vs. methanol characteristics at low methanol concentrations. The low absolute k' differences were due to different packing surface areas. These results suggest that methanol-modified silica is less homogeneous than water-modified silica. The new type B silica showed a constant plate height throughout the entire 0 to 0.4% methanol concentration range studied. These results again suggest superior surface-energy homogeneity of the new type B silica These studies confirm that deactivation with water usually gives the best chromatographic results with all silica types. The surface of water-deactivated silicas appear most homogeneous, producing more efficient columns and superior peak shapes. Still, methanol and 2-propanol are effective and more convenient deactivating agents when used at appropriate levels In some systems, however, these modifiers may cause misshapen peaks for some solutes, particularly when used at low concentrations.

3.6.13 Silanophilic Interaction

The silanophilic theory was developed by Horvcith and co-workers.[63] Irregular retention behavior of crown ethers in reversed-phase chromatography with silica-bonded

hydrocarbonaceous stationary phases is interpreted by using the dual binding model. It has been assumed that retention is caused not only by the usual solvophobic interactions, but also by silanophilic interactions between the eluate and the accessible silanol groups at the surface of various alkyl-silica bonded phases. Such behavior has also been documented for peptides.[64]

Both normal- and reversed-phase behavior have been observed for a wide range of species when separated under reversed-phase conditions. At low concentrations of the organic solvent system, the behavior is predominantly reversed-phase, but at higher concentrations the solutes show increasing normal-phase behavior. This results in typical U-shaped plots of log k' vs. organic solvent concentration. In the absence of silanophilic interactions, these plots are linear with a negative slope.

3.6.14 Reduction of the Effects of Silanol Groups

The reaction of silica with long-chain alkylsilyl halides does not cover all the available silanol groups. The effects of the silanol groups can thus be eliminated by reacting them with some other smaller reagent, which covers as many of the remaining groups as possible. The usual procedure is to replace the hydrogen atom of silanol with a trimethylsilyl group .[65]

3.6.15 Ionic Interactions

In the course of the reversed-phase chromatography of basic compounds peak shapes were found to be poor and efficiencies low. Some of the difficulties in the course of chromatography were established by Jane, [66] who employed the method of using bare silica rather than reversed-phase packings with methanol mobile phase containing a small percentage of water buffered to high pH. In the development of reversed-phase analytical methods for quaternary ammonium compounds used as animal feed additives, it has been observed that the retention of the components was strongly influenced by pH and buffer concentration Surprisingly, the substitution of a bare silica column gave an almost identical separation. The result prompted a study of the chromatography of basic compounds on silica [67] using methanol-water mobile phases buffered to less extreme pH values. It can be concluded from the above studies that in contrast to the silanophilic effects noted for crownethers, the principle mechanism of retention was ion exchange chromatography on the acidic silanol groups. By studying the separations of several

probes of different basicity using packings of different surface coverages of bonded phase groups, retention mechanisms were observed comprising a number of competing interactions, which included hydrophobic interactions with siloxane bridges and silanophilic interactions in addition to ion exchange. A method for the assessment of the ion exchange character of retention was described by Stout et al.[68]

It has been shown that the predominant retention mechanism of basic, ionogenic solutes on unbonded silica in buffered aqueous organic solvents was one of ion exchange. Under such a retention mechanism, the plots of capacity factor against the inverse of competing ion concentration are linear. If ion exchange is the sole mechanism, such a plot will pass through the origin. When the separation mechanisms that are not influenced by ionic species are present,

there is an intercept, corresponding to the retention that should be observed at an infinite competing ion concentration. Such an intercept is typical where silanophilic or reversed-phase retention mechanisms occur.

The slope of the capacity factor of an ionic solute vs. the inverse of ionic strength was shown to be directly related to the ion exchange distribution coefficient. For silicas of similar surface area, the slope of this plot acted as an indicator of the ion exchange character of the material. It has been shown that silicas with properties favorable for the reversed-phase chromatography of basic compounds had low values of the slope, while those of silicas unfavorable for the chromatography of bases had high slope values.

Different authors have shown that physicochemical properties, e.g., pore volume, mean pore diameter, specific surface area, concentration of accessible groups, and the structure of the siliceous support matrix, significantly affect the efficiency of the chemical process.[69-71] Moreover, purity of the support surface is one of the major factors influencing structural and physicochemical properties of the basic silica materials used in the preparation of packings with high coverage density of chemically bonded phases.[72,73] The effect of the surface purity of silica gel supports on the coverage density of chemically bonded phases was studied by Buszewski.[74] The silicas were washed with 20% v/v HCl before chemical modification. Different physicochemical

methods - porosimetry, pH measurement, secondary ion mass spectrometry (SIMS), CP *IMAS* NMR and chromatography were used for the characterization of these materials. Application of SIMS shows the presence of eight different elements, forming strong specific adsorption centers on the surface of unmodified silica gels. Purification of the surface of silica gels with 20% HCl solution caused partial removal of these elements and a decrease in concentration. Surface chemical modification with C18 ligands of adsorbent after washing caused approximately a 15% increase in coverage density values, which led to a decrease in retention times as well as an increase in the peak symmetry of substances by HPLC methodology.

It is very important to know the distribution, organization, and orientation of the alkyl chain ligands on the silica surface Most silicas applied in chromatography are not pure. The effect of the metal ions may gave very poor peaks how ever purified silica gave acceptable performance. Other researchers also noted the effects of metal ions that are incorporated in the silica matrix It has been calculated that aluminum present in the Structure confers high acidity upon adjacent silanol groups [75] while Sadek and co-workers [76] have shown that the removal of metal impurities from silicas leads to a fundamentally different retention behavior of hydrogen bond acceptor solutes, which was attributed to a lower number of metal hydroxides and the reduced influence of metal ions upon surface silanol groups. These data, together with observations on the gas chromatographic adsorption of amines, led Nawrocki [77] to the conclusion that the metals play an all important role in enhancing the acidity of surface silanol groups in chromatographic silicas.

3.6.16 Reversed-Phase Character of Silica

Besides polar silanol groups, silica possesses nonpolar siloxane bonds. When these are at the surface, they are expected to reduce polarity on the surface This reduction has been observed for heat treated silicas It should also be possible, under appropriate conditions, to observe retention of non polar molecules on these silanol bridges. Plots of logarithm capacity factor for both propylbenzene [78] and toluene [79] against methanol concentration were linear, as expected for a reversed-phase mechanism. The retention was, however, small compared with that found for an alkyl bonded-phase packing,

although the contribution of reversed-phase mechanism to the separations of basic compounds on bare silica with predominantly aqueous mobile phases was shown to be significant. The reversed-phase component of the retention mechanism was a function of the degree of silica hydroxylation with the more highly rehydroxylated silicas showing a significantly lower reversed-phase character. These facts confirm the assumption that the siloxane groups are the site of reversed-phase interactions on bare silica.

3.7 Reversed phase Chromatography

Reversed-phase chromatography has become the dominant branch of high-performance liquid chromatography over the last few years. The name *reversed-phase chromatography* was a rational choice at a time when chromatography was practiced almost exclusively by using polar stationary phase and a nonpolar eluent. However, today an estimated 80 to 90% of chromatographic systems used in HPLC work consist of non polar stationary phases and polar eluents. The popularity of the technique rests with the reproducibility offered by the use of hydrocarbon bonded phases. The aqueous eluents have high optical transparency at low UV wavelength and are cheaper, less toxic, and less flammable

The first attempt to bind an organic moiety to the surface of silica gel was made by Halasz and Sebestian in 1969, who attached aliphatic hydrocarbon chains to the silica gel surface by means of silicon-oxygen-carbon linkage. The original synthesis by Halasz and Sebestian involved refluxing the silica gel with an aliphatic alcohol, but, unfortunately, the silicon-oxygen-carbon bonds were very weak and the bonded hydrocarbon chain rapidly hydrolyzed from the surface, generating the original silica gel. Nevertheless, the material was sufficiently stable to allow Halasz to identify the highly desirable chromatographic properties of the bonded phase Gilpin and Burke [80] described in 1973 the use of chlorosilanes as bonding reagents. When the hydroxyl group of silica gel reacted with a chlorosilane, hydrogen chloride was released and the organic moiety was attached by means of the silicon-oxygen-silicon bond The silicon-oxygen-silicon bond was much stronger than the silicon-oxygen-carbon bond and such bonded phases could be used satisfactorily in a liquid chromatography column over long periods of time, provided the extremes of pH were avoided.

This type of bond was the basis for the synthesis of the vast majority of contemporary bonded phases. There are basically three types of bonded phases: the "brush" phase, the "bulk" phase, and the oligomeric phase.

The brush phase is created by using monofunctional silanes such as dimethyloctychlorosilane that react directly with surface silanol groups with the elimination of hydrogen chloride. As a result the surface is covered with dimethyloctyl chains like bristles on a brush, hence the term *brush phase* evolved. The brush phase, synthesized under carefully controlled conditions, is the most reproducible, and, consequently, it is the most commonly used phase in LC analysis.

When bifunctional silanes such as methyl-octyldichlorosilan were used in the synthesis it was possible to produce an oligomeric phase. Dichlorosilane reacted with a silanol group producing a methyloctylmonochlorosyl group on the surface with the evolution of hydrogen chloride. When this monochlorosilane reacted with water more hydrogen chloride was generated and the bonded moiety became a methyloctyl-monohydroxysilyl group. The bonded silica containing the hydroxyl groups could then be again treated sequentially with the dichlorosilane and then water, each time building another methyloctylsilyl group onto the surface. In this way a layer of hydrocarbon chains can be laid down on the silica surface as an oligomer, producing a very stable type of reversed phase. Employing a fluidized bed technique of synthesis [81] and their properties was reported. [82]

Trifunctional silanes such as octyltrichlorosilane could produce the third type of bonded phase, the bulk phase. When the surface of silica gel was saturated with water and treated with octyltrichlorosilane, the reaction occurred between both hydroxyl groups and adsorbed water. The water caused the formation of octylsilyl polymer, which is crosslinked, and consequently the stationary phase assumed a multilayer character. The synthesis could also be accomplished by applying a procedure similar to that used in the preparation of the oligomeric phases, that is, by employing a sequence of two-stage reactions involving first treatment with water and then with trichlorosilane. In this way the polymeric layer could be increased to whatever thickness was desired. After the last stage the product was treated with water and finally end capped. The multilayer character of this type of bonded phase evoked the term *bulk* or *polymeric bonded phase.*

Akopo et al. [81] carried out some retention measurements on samples of bulk and brush phases using methanol-water mixtures as mobile phases. It has been established that at very low methanol concentrations the bulk phase performed in the expected manner and the retention of a solute decreased with the increase of methanol concentration. However, solute retention on the brush phase first increased with methanol concentration until a maximum was reached. At higher concentrations solute retention started to decrease and subsequently gradually became reduced as methanol concentration continued to increase. It can be seen that the retention volume of ethanol decreases continuously from 0 to 10% (w /w) of methanol on the bulk phase ODS-3. In contrast to this, in the brush phase the retention volume of ethanol reaches a maximum at about 2.5% (w /w) methanol in the mobile phase and subsequently falls; the graph of retention volume against methanol concentration finally becomes parallel to the curve of the bulk phase. [83]

Other chromatographers described the same phenomenon [84] and suggested that it was due to dispersive forces between the hydrocarbon chains themselves, which are greater than the dispersive forces between hydrocarbon chains and the mobile phase. As methanol concentration increased the dispersive interactions of the hydrocarbon with the mobile phase eventually became sufficiently large to allow chains to disengage from one other and, consequently, to increase the effective surface area of the stationary phase.

The behavior of the oligomeric phase when in contact with water has apparently not been studied so far. However, as the polymer is linear and not cross-linked, it is likely to have properties similar to those of the brush phase. However, the oligomeric phase differs in one important aspect from the brush and bulk phases: in its stability to mobile phases with very low pH values.

3.7.1 Solvent-Stationary Phase Interactions In Reversed-Phase Liquid Chromatography

The mechanism of interaction between solvent molecules and a reversed-phase surface is similar to the complementary interactions of solvent molecules with a silica gel

surface. A layer of solvent is built up on the surface by absorption. However, the interactive forces between the solvent and reversed phase are dispersive in nature, as opposed to those with silica gel, which are mainly polar.

The adsorption isotherm can be described by the Langmuir equation. However, since the interactions with reversed phase are almost exclusively dispersive and are not a mixture of dispersive and polar interactions as in the case of silica, a detailed study of the adsorption isotherm can provide a more exact understanding of the surface than can be attained by silica gel

3.7.1.1 Molecular Interactions and Retention in Liquid Chromatography

The first major contribution to the molecular interaction theory of solutes was discovered in gas chromatography where retention is solely controlled by interactions in the stationary phase. Laub et al. [85,86] studied the effect of mixed phases on solute retention and arrived at the surprising conclusion that the corrected retention volume of a solute was linearly related to the volume fraction of either one of the two phases.

$$V'_{ab} = \alpha V'_a + (1 - \alpha) V'_b \qquad (80)$$

where V'_{ab} is the corrected retention volume of a solute on the mixture of phases; V'_a is the corrected retention volume of the solute in phase a, V'_b is the corrected retention volume of the solute in phase b; and α is the volume fraction of phase a. Rearranging this equation, we have:

$$V'_{ab} = \alpha(V'_a - V'_b) + V'_b \qquad (81)$$

Laub and Purnell [85] confirmed the above relationship in a number of interesting ways. They showed that the following alternative chromatography systems all provided the same value for the corrected retention volume of a substance. A given α fraction of phase a could be mixed with a fraction $(1 - \alpha)$ of phase b, and coated on a support and packed in a column. Also, the two fractions could individually be coated on some support and the coated supports mixed and packed in a column. Finally, each fraction could be coated on a support and packed into separate columns and the columns joined in series. It has been

demonstrated that all three columns gave exactly the same corrected retention volume for a given solute. The effect of a stationary phase volume was determined. More importantly, it shows that for the distribution systems examined, the distribution coefficients but not their logarithms can be summed. It is now of interest to see if the same relationship can be obtained in liquid chromatography.

3.7.1.2 Stationary Phase Effects in Reversed-Phase Liquid Chromatography

A retention mechanism universally operative in reversed-phase liquid chromatography is hydrophobic interaction between a solute and a stationary phase in the presence of an aqueous mobile phase. The simplest model for the retention mechanism involves the intermolecular association between the hydrophobic moieties, one in the solute and the other in the stationary phase. A similar interpretation has been provided by taking into account the solvation of these components. [87,88]

It is possible to explain most observations in reversed-phase liquid chromatography based on these mechanisms, especially when the results were obtained for compounds with a similar skeleton under a limited range of Conditions. Good correlation has been observed between log k' values in RPLC under such conditions and log P values in 1-octanol-water two-phase systems. [89]

As is well known, a methylene group in a molecular structure contributes to the relative increase in k' values as in the distribution coefficients of liquid-liquid partitioning. The free-energy changes associated with transfer of one methylene group from water to the organic phase is 3421 to 3682 J/mol in liquid-liquid partitioning [90] and about 3389 J/mol in RPLC with a C18 phase and water. [91] These results imply the partitioning mechanism in a primitive sense to be operative between an aqueous mobile phase and stationary phase alkyl groups, the latter not contributing to the selectivity but merely controlling k' values via phase ratios. However, there are numerous results that clearly indicate the contribution of additional effects of the stationary phase to solute retention and selectivity. Here secondary retention processes caused by the participation of silanols and metal impurities on the silica support are omitted. The effect of stationary

phase (alkyl groups and solvent molecules existing in the stationary phase under elution conditions) on retention is not very substantial with the alkyl type bonded phases, but is of great significance for at least two reasons. First, only a minor change in selectivity is needed to effect separation owing to the high efficiency of reversed-phase HPLC. Second, hydrophobic interaction alone is inadequate as a means of varying the selectivity - changes in the composition of the alkyl-bonded phase and in the organic solvent can provide additional selectivity effects. Although several mechanisms have been proposed to account for the retention process in reversed-phase liquid chromatography, most of these were based on or could be applied to the retention behavior of a limited range of solute and mobile phase compositions. It is desirable to have a uniform understanding of the retention process to explain each retention behavior, including that caused by the stationary phase effects. Important information can be gained from the stationary phase effects in terms of the retention mechanism, which points to an active role of the stationary phase. The participation of not only bonded moieties, but also organic solvents residing in the stationary phase is important to effect separation in practice. In the case of an electron donor or acceptor bonded silica phase, the stationary phase effect may predominate in the retention and separation.

3.7.2 Alkyl-Bonded Silica Phase

3.7.2.1 Effect of Alkyl Chain Length of Bonded Phase on Selectivity

A simple model of reversed-phase retention as a means of predicting the effect of alkyl chain length on retention is presented. We assume that the primary factors in determining retention are

1.   Unfavorable interaction between the hydrophobic portion of a solute and aqueous solvent, and

2   Association of a solute with the individual alkyl chains so as to reduce the hydrophobic surface area, referred to as hydrophobic or solvophobic interactions. [92] Here absolute retention is dependent on the phase ratio determined by alkyl chain length and the surface density, but the selectivity will not be affected by solute structures in terms of shape and polar properties.

Early studies dealt with the linear relationships between k' values and surface coverage and the alkyl chain length of the stationary phase or phase ratios, assuming that the retention is determined by the area of contact between the hydrophobic moiety of the solute and the stationary phase. This argument, however, does not take into account how the alkyl groups are arranged and solvated in the stationary phase The k' values are not necessarily proportional to the phase ratios determined by the length of alkyl chains and surface coverages. This means that the selectivities based on steric and polar characteristics of solutes were clearly affected by these factors beyond the predictions based on the ratios.

3.7.2.2 Steric Selectivity of Alkyl-Bonded Stationary Phase

A C18 stationary phase is known to be relatively tangled especially for mobile phases with low organic solvent contents. As organic solvent content increases, the alkyl chains sorb organic solvents, swell, and become more ordered in their conformation. As a result, PAHs can more readily penetrate the stationary phase and their retention relative to the more bulky or flexible solutes increases.[93]

The steric discrimination of planar from nonplanar compounds was also given with the polymeric C18 phase in comparison to the monomeric C18 phase. [94-97] It has also been demonstrated that the steric selectivity and the difference based on the chain length are affected by the mobile phase organic solvent. [98,99] The authors established that the three stationary phases (C 1, C8 and C18) gave different selectivities between aromatic and saturated compounds in methanol and acetonitrile. The results preclude the possibility that the difference in selectivity was caused by the difference between aromatic and saturated compounds in the mobile phase interaction, as no such difference was observed with the C1 phase The difference could not be due to the steric effect either, as planarity of the solutes did not influence the results. It can only be explained by the difference in the solvation of solutes in stationary phase between C1 and the longer alkyl-bonded phases

It should be emphasized that the effect of solute-solvent interaction on the CIS phase was found to be much greater than on the C1 phase, and even larger with higher

organic solvent contents. These results imply that the hydrocarbon solute molecules in the stationary phase can realize the orderliness of bonded chains determined by the chain length,surface density, and mobile phase composition, and simultaneously associate with the solvent molecules in the C 18 phase. The effects are much lower with C8 and negligible in the C1 phase. The results suggest a mechanism based on partitioning of solutes between the mobile phase and the effective stationary phase, namely alkyl chains associated with solvent molecules.

˜ However, the effective stationary phase should not be taken as a simple mixed solvent, as C 1 or C 8 and C 18 phases showed considerable differences in their response to the change in the mobile phase. Insensitivity of the C 8 phase toward the change in mobile phase implies that the chain overlap is much less than with the C 18 phase in this range of mobile phase, as would be the case with the C 1 phase.

The effect of organic solvent in stationary phase on polar group selectivity has been studied. [99] The results indicated a notable difference in polar group selectivity between tetrahydrofurane (THF)-water and methanol-water systems, which is higher than that between acetonitrile-water and methanol-water. Thus THF and methanol would constitute an interesting set-up pair to be used with water in a ternary mobile phase for controlling the separation of substances with different functional groups.

The k' values for these benzene derivatives in 50% methanol showed good correlation with log P values, but not with the k' values in THF-water. The retention in THF vs. methanol decreases in the order: phenols = nitro compounds > hydrocarbons, chlorobenzenes > esters = alcohols Alcohols, especially alkanols , were preferentially retained in methanol-water compared with THF or acetonitrile systems. The difference between phenols and alcohols can be explained by the difference in their ability to stabilize the partial negative charge upon hydrogen bonding as indicated by their pK_a values

The stationary phase effects are compatible with a mechanism based on the partitioning of solutes between the mobile phase and the effective stationary phase,

anchored alkyl chains associated with solvent molecules. Simple solvated alkyl chains or simple mixed solvents, however, do not give an adequate description of the alkyl-bonded silica stationary phase. The same mobile phase composition results in a difference in the selectivity for different stationary phases. The difference in alkyl chain length or in surface coverage can be envisaged to produce the difference in steric requirement and hydrophilic-hydrophobic properties, which in turn determine the chromatographic properties. In this sense, the properties of the C18 phase are primarily determined by the extent of surface coverage with alkyl groups.

3.8 *Polymer-Based Packing Materials in Reversed-Phase Liquid Chromatography*

3.8.1 Selectivity of Polymer-Based Packings

Several high-efficiency polymer gel packings are available, and according to reports their selectivities are difficult to understand, or are considerably different from those of silica-based phases. The pore structures of polymer gels are also different from those of silica. [100,101]

Molecular mass-elution volume curves obtained for polymer gels are always associated with a second plateau in a molecular mass range below 500, corresponding to the micropores, whereas no such compounds are present in ordinary silica particles. Thus in the biporous structure of cross-linked polymer gels, macroporous particles are composed of microporous materials. Lightly cross-linked polymer gel chains on the surface of solid cores of polymer gels might be responsible for the microporosity. The micropores play a major role in determining the selectivity of the size-exclusion effect, which represents the characteristics of selectivity of all polymer gels. [102]

3.8.2 Steric Selectivity of Polymer Gels

The characteristics of steric selectivity of polymer gels are in fact the result of the size-exclusion effect of the micropores, and that of the micropore structure, and hence, the selectivity between bulky, flexible and rigid, compact solutes can be controlled by the choice of the diluents in suspension polymerization or in multistep swelling polymerization.

The difference in selectivity of two polymer gel was investigated by Hosoya et al. [103] The authors described the polymer gel packings prepared from methyl methahacrylate and ethylene dimethacrylate in two diluents, cyclohexanol and 2-octanol. Despite the similarity in meso- and macropore sizes, the two gels showed different selectivities for hydrocarbons with different planarity and size. This is due to the smaller micropores of the polymer gel prepared in 2-octanol. Similar results were obtained with aliphatic compounds. Shodex DE-613 poly(alkyl methacrylate) gel, having short alkyl groups, showed a clear preference for aromatic compounds having more than one phenyl group compared with alkyl benzenes, and for cycloalkanes compared with linear alkanes Esterified Asahipak ODP-50 poly(vinyl alcohol) gel (PVA) showed a clear preference for planar PAHs compared with bulky aromatic compounds.[104] The general preference by polymer gels decreases in the order PAHs > polyphenylalkanes (Ar-Ar) > alkylbenzenes > cycloalkanes > linear alkanes Such a preference was observed for all polymer-based stationary phases, regardless of the alkyl chain length or the size of the macropores. Shodex DE-613 with short alkyl-bonded polymer gel showed higher selectivity for different types of hydrocarbons than Asahipak GDP-50 and TSK C1s-4PW. C1s-bonded types for the effect of polymer network structure is predominant with Shodex DE-613 owing to the smaller extent of hydrophobic interaction. Polymer gels with alkyl backbones, DE-613 and TSK C1s-4PW, showed preferential retention of aromatic compounds in spite of their saturated structures, possibly owing to dipole-π interactions involving ester groups. The preference toward rigid, compact compounds over bulky, flexible compounds was also seen with saturated compounds having no functional groups such as cyclohexane and adamantane, which are expected to undergo minimum specific interactions with the stationary phase except hydrophobic and steric interactions. Therefore the shape selectivity associated with polymer gels can be attributed, at least in part, to the structural matching between solute and the rigid polymer network structure, or the micropores. The C18 type polymer packings, TSK C18-4PW, alkylated poly(hydroxylalkyl acrylate or methacrylate), and Asahipak aDP-50 esterified poly(vinyl alcohol), showed greater retention of alkyl compounds than did DE-613, having short alkyl groups. The preference for compact solutes compared with bulky solutes increases in the order silica C18 < DE-613 < TSK C18-4PW < aDP-50 < PLRP -5 300. The results

indicate a greater population of the relatively large micropores of the DE-613 than aDP-50 or PLRP-S 300, probably due to the type of diluent used in preparation.

3.8.3 Effect of Mobile Phase on Steric Selectivity

The difference in the effect of solute structure on column efficiency can be explained on the basis of the difference in the structures of the polymer gels. DE-613 and TSK C18-4PW are alkyl type gels containing no aromatic functionality, whereas PLRP-S 300 and ODP-50 contain aromatic groups in monomer and/ or cross-linking reagents. These functionalities can provide interactions with aromatic compounds, especially when the solutes are planar. The fact that the retention of bulky compounds on ODP-50 and PLRP-S 300 is relatively weaker than those of PAHs compared with DE-613 and TSK C18-4PW in similar mobile phases indicates that the former possess tighter network structures than the latter. With better solvation in THF and more so in acetonitrile with a closer solubility parameter to the PVA backbone, only PAH showed slightly higher h (reduced plate height) values, whereas excellent efficiencies were observed for the other solutes with ODP.

3.8.4 The Effect of Biporous Structure on Chromatographic Properties

Polymer-based packings showed excellent performance for polypeptides including bovine serum albumin (BSA) owing to the favorable macropore structure. Although the retention times for polypeptides are comparable on all packings, those for 1-napthalenemethanol are considerably different. Whereas the retention and separation of large molecules indicate an abundance of macropores, the retention of a small molecule indicates an abundance of micropores in each packing.Large molecules such as polypeptides are chromatographed by using macropores, and the small molecules are chromatographed by using micropores of polymer gels. Such difference cannot be seen with silica-based phases.

3.8.5 The Effect of Polymer Structure on Polar Group Selectivity

In addition to size-exclusion effect of macropores, some polymer gels possess another factor, the presence of dipolar carboxylate groups in methacrylate and esterified

poly(vinyl alcohols) gels, to show unique selectivity. These polymer gels in methanol show a somewhat similar selectivity to cyanoalkyl-bonded phases on silica or to alkyl-bonded phases in dipolar solvents. Preferential retention of aromatic compounds, especially π-acids, is notable. A stronger mobile phase for aromatic compounds with about 10% more methanol, and a weaker mobile phase for aliphatic compounds with about 10% less methanol, are recommended with polymer gels in comparison with an ordinary silica C18 phase. The results obtained with polymer gels suggest the applicability of these packings in RPLC in wider mobile phase conditions than for silica C18 although the full scope of application is yet to be explored. A basic understanding of polymer gel structures and resultant selectivity will help with the selection of separation conditions. [105]

Silica and polymer-based packings are frequently compared and should complement each other in the separation of small molecules, because silica-based packings are not as chemically stable as polymer gels, and polymer gels are generally less efficient. One area of RPLC where silica- and polymer-based packings can be in serious competition is the separation of polypeptides, where micropores play no role, compared to packings on widepore silicas and those based on polymer gels Silica C18 phases prepared from wide-pore silicas showed poorer performance than short alkyl-bonded phases owing to smaller pore sizes, which are much less stable than C18 in trifluoroacetic acid (TFA) solution. Polymer gels provided equal or better recoveries and performance and much greater stability in TFA solution compared to C18 phases. The easy control of pore size in both macro- and micropore size ranges is an additional advantage of polymer-based packings.[106]

3.9 Correlation of Retention and Selectivity of Separation in Reversed-Phase High-Performance Liquid Chromatography with Interaction Indices and with Lipophilic and Polar Structural Indices

Various models have been proposed to describe the retention in reversed-phase systems, for example, a competitive adsorption model with a modified scale of solvent strength, [107,108] a model based on probability of interactions of the solute in stationary

and the mobile phases, [109,110] modified models of the distribution of a solute between two liquid phases such as the model using the Hildebrand solubility parameter theory,[111,112] or the model supported by the concept of molecular connectivity, [113] and rigorous models based on the solvophobic theory, [92,114] or on the molecular statistical theory. Unfortunately, sophisticated models introduce a number of physicochemical constants that are often not known or difficult and time-consuming to determine, so that such models are not very suitable for rapid prediction of the retention data. The most characteristic feature of reversed-phase chromatography is lower polarity of the stationary phase in comparison with the mobile phase. Theoretically, alkyl phases chemically bonded on a suitable support such as octadecylsilica or octylsilica materials commonly used in contemporary practice of reversed-phase HPLC should behave as almost ideally nonpolar materials with properties of long-chain aliphatic hydrocarbons. The bonded alkyl chains differ from the free molecules of hydrocarbons in liquid phase in their limited mobility Futhermore, for steric reasons it is virtually impossible to modify all available silanol groups on the surface of silica gel by chemical reaction with the silanization reagent and the unreacted silanol groups may affect the retention by specific interactions, especially with basic solutes. Finally, organic solvents used as the components of the mobile phases in reversed-phase systems are preferentially adsorbed by the stationary phase and can significantly modify its properties [115,116]

The solvophobic theory emphasizes the importance of mobile phase interactions in the control of the retention mechanism The solvophobic interactions are considered as the driving force of the formation of associates of the solutes with the nonpolar stationary phase The replacement of weaker interactions between the moderately polar solute and polar mobile phase by mutual interactions between the strongly polar molecules of the mobile phase in the space element of the mobile phase occupied by a solute molecule before transition results in an overall energy decrease in the system, which is the driving force of retention in the absence of strong interactions between the solute and the stationary phase. The theoretical background of this retention model is useful as the starting point in the derivation of a simplified semiempirical description of reversed-phase systems, making it possible to characterize and predict the retention and selectivity

within a series of compounds to be separated. [117] Due to simplifications concerning the effects of the stationary phase accepted in the derivation, this approach can be applied to relative rather than absolute predictions of retention and selectivity and a suitable set of standard reference compounds is necessary for the calibration of the retention (or selectivity) scale.

3.9.1 Structural Correlations of Lipophilic and Polar Indices

In accordance with expected potentials for characterizing the lipophilicities of solutes by the indices nce or fule, good agreement was found of the relative indices fule with Hansch and Leo hydrophobic constants π (correlation coefficient was 0.991), [118] but no correlation could be detected between n_{ce} or Δn_c and Snyder's polarity indices P'. [119]

It has been shown that the relative polar index Δq is directly proportional to the difference between the interaction indices of the solute and the reference standard compound after subtraction of the lipophilic contributions from the alkyl substituents, $I_{ox,i}$ and $I_{ox,s}$ respectively:

$$\Delta q = (C_M (I_{H2O} - I_{org}/ 2.3 \, RT)(c_x V_{ox,i} I_{ox,i} - c_x V_{ox}. I_{ox,s}) = constant(I_{ox,i} - I_{ox,s}) \quad (82)$$

where $V_{ox,i} = V_{ox}$ is the molar volume of the zeroth member of the calibration homologous series. Therefore, Δq is expected to be directly proportional to the polarity of the functional groups in the solute. Good correlations of the relative indices q with Snyder's polarity indices P' was found experimentally (correlation coefficient = 0.988),[341] which seems to confirm that the indices q_i or Δq characterize the polarity of a solute. The relative polar indices Δq were found to increase with the polarities of the functional groups approximately in the order n-alkanes < polycyclic aromatic hydrocarbons < n-alkylbenzenes < benzene, styrene, biphenyl < halogenated benzenes < dialkyl ethers < alkyl aryl ethers, diaryl ethers < aromatic nitriles < aromatic ketones and aldehydes < aromatic amines < aromatic alcohols < phenols, alkylphenols < chlorophenols. For a given class of compounds, the Δq values were found within a relatively narrow range.

The interaction indices can be used for the prediction of retention and selectivity in reversed-phase systems with binary or ternary mobile phases, but the precision of prediction over a wide range of mobile phase composition is improved by introducing two structural indices for each solute, which is characteristic of the lipophilicity of the hydrocarbon moiety of the molecule and the polarity of functional groups(s). The structural contributions to these indices have proved to be additive in the classes of compounds studied so far. Combination of the two indices of the solutes controls the selectivity of their separation in binary and ternary mobile phases. Based on the lipophilic and polar indices, conditions for optimum isocratic or gradient separation can be predicted.

The prediction of retention on one column from the indices determined on another column is possible in principle, but the columns must possess the same length as the bonded alkyl chains. Limitations to this approach may originate in a limited precision of experimental data such as those caused by nonlinearities of the log k' vs. concentration of the organic solvent in aqueous-organic mobile phase, or in possible specific interactions with the stationary phase such as interactions between unreacted silanol groups in alkyl-bonded silicas and polar solutes, mainly basic compounds.[120]

3.10 Quantitative Structure-Retention Relationships in Reversed-Phase High-Performance Liquid Chromatography

Quantitative structure-retention relationships (QSRRs) are some of the most extensively studied manifestations of linear free-energy relationships. [121] Linear free-energy relationships are extrathermodynamic relationships, and are not necessarily due to thermodynamics. Extrathermodynamic approaches combine detailed models of processes with certain concepts of thermodynamics The thermodynamic properties of a given system are bulk properties reflecting just the net interactive effects in that system. The magnitude of thermodynamic parameters represents the combination of individual interactions that may take place at the molecular level. Thus, from chemical aspects classical thermodynamics is inadequate [122]

The presence of linear free-energy relationships suggests a real connection between some correlated quantities, and that the nature of this connection can subsequently be

identified. It is also assumed that the correlations between specific quantities can be attributed to some physicochemical relationships. Statistically derived correlations encourage attempts to determine also the relationships in the background.

Over a period of 15 years QSRR studies have been conducted with the following aims in view:

- Prediction of retention for a new solute
- Identification of the most informative structural descriptions
- Elucidation of the molecular mechanism of separation operating in a given chromatographic system
- Evaluation of complex physicochemical properties of solutes (other than chromatographic)
- Estimation of relative biological activities within a set of Solute xenobiotic compounds

Chromatographic retention data must be some function of the temperature, chemical structure of solute, stationary phase, and mobile phase, with all their mutual interactions. However, there is no general, strict, unequivocally verifiable canonical equation relating retention to the four main chromatographic variables, that is, the temperature, the structure of the solute, the stationary phase, and the mobile phase. Even if the stationary and mobile phases applied in a given chromatographic system remain constant, precise, quanitative description of the retention of a series of solutes may involve difficulties, the more so the more diverse the solutes considered, although the problem is by no means trivial for homologues. The plots of log k' vs. carbon number of a homologue are usually linear, but only for some limited range of aliphatic chain length.

In general, relationships between empirical or theoretically calculated parameters of molecular structure require statistical evaluation in order to check the significance of the resulting correlations. Basically, the research strategy now applied in QSRR studies was transferred from studies initiated in the 1960s on quantitative structure (biological) activity relationships (QSARs).[123] The advantage of QSRRs over other quanitative structure-property relationship studies is that chromatography can readily yield a great

amount of relatively precise and reproducible data. In a chromatographic process all conditions may be kept constant. Thus, solute structure becomes the single independent variable in the system.

The first reported QSRRs were derived by multiple regression analysis of retention data against a set of structural descriptions. Another early approach to QSRRs was based on the assumption of additive substituent effects on retention analogously to the new nonparametric method of correlation analysis applied by Free and Wilson in medical chemistry. [124] One can attempt to derive QSRRs making no mention of any existing chromatographic theory. A typical strategy is to generate a multitude of solute descriptions that are next regressed against retention data. Observing all the statistical rules, one selects the minimum number of descriptors needed to produce an equation yielding the calculated retention data in satisfactory agreement with the observed values. The number of descriptors that can be assigned to an individual solute is virtually unlimited. The most commonly used structural descriptors in QSRRs are given in below .

3.10.1 Structural Descriptors in QSRR

- *Bulkiness-Related (Nonspecific) Parameters*

    Molecular mass Refractivity Molecular volume Total energy Solvent-accessible area Taft constant

- *Geometry-Related (Shape) Parameters*

    Moments of inertia Length-to-breadth ratio Sterimol width parameters Angle strain energy

- *Physicochemical Parameters*

    Hydrophobic constant (1t) Hammett constant Solubility parameters Boiling points Solvatochromic parameters

- *Polarity-Related (Electronic) Parameters*

    Swain-Lupton's constants Dipole moments

    Atomic excess charges Orbital energies

    Su per delocalizabilities Partially charged surfaces

- *Molecular Graph-Derived (Topological) Parameters*

Adjacency matrix indices

Distance matrix indices

Information content indices

However, also numerous rare, sometimes ad hoc designed solute descriptors are reported. It is often difficult to assign any physical sense to such parameters and to interpret QSRR equations consisting of terms produced by various transformations and combinations of such descriptors, their square roots, cubes, reciprocals, or products. If QSRRs result from the analysis of tens or hundreds of descriptors, then most likely several equations with similar predictive abilities but consisting of different sets of variables can be derived. QSRRs that are not interpretable in physical terms are not very informative regarding the mechanism of retention. A more promising QSRR is to start from the existing theories of chromatographic separations and to attempt to quantify the abilities of solutes to take part in the postulated intermolecular interactions.[125] These fundamental intermolecular interactions involving solute molecules, molecules forming mobile phase, and molecules of stationary phase are as follows:

- dipole-dipole (Keesom)

- dipole-induced dipole (Debye)

- instantaneous dipole-induced dipole (London) . hydrogen bonding

- electron pair donor-electron pair acceptor

- solvophobic interactions

The potential energy (E) of the first three types is approximated by

$$E = -W^2 \varepsilon^{-1} r^{-6} [2\mu^2_1 \mu^2_2 / 3kT + \alpha_2 \mu_{12} + \alpha_1 \mu_{22} + 3I_1 I_2 \alpha_1 \alpha_2 / 2(I_1 + I_2) \qquad (83)$$

where W and k are constants; ε is relative electric permittivity of the medium; r is the distance between interacting molecules; T is the absolute temperature, and μ, α and I are the dipole moments, polarizabilities, and ionization potentials, respectively, of the interacting molecules. Equation 83 substantiates the assumption that, within a set of solutes of similar hydrogen-bonding and charge-transfer properties, chromatographed under identical conditions, the retention parameters can be approximated by a combination of polarizabilities, ionization potentials, and squares of dipole moments. In pre-QSRR stud-

ies, attempts were made to select solutes either with similar dipole moments and varying polarizability [109] or with similar polarizability and varying dipole moments and to relate retention to the variable.

Tijssen and his co-workers [126]considered three types of interactions: dispersion, orientation, and so-called acid-base interactions. The ability of an individual compound to take part in the respective interactions is reflected by its specific partial solubility parameter. The problem encountered when testing the predictive potency of the approach was to determine precisely the solubility parameters. Similarly, Horvath et al.'s Martine and Boehmn's [127] molecular statistical theory and several other early theoretical approaches to RPHPLC required a knowledge of a number of physicochemical parameters that Were not available for individual solutes. The individual solute properties affecting retention were identified, which, in turn, suggested the choice of the most informative structural descriptions for QSRRs. Carr and his co-workers, [128,129] in studies on the nature of RPHPLC separations, proposed an approach based on the solvotochromic comparison method and linear solvation energy relationships (LSERs). The chemical and physical characteristics of the solute that determines the retention can be described by the following equation:

$$
\log k' = \text{constant} + M \, (\delta^2_m - \delta^2_s) \, V_2/100 + S(\pi^*_s - \pi^*_m) \, \pi^*_2
$$
$$
+ A(\beta_s - \beta_m)\alpha_2 + B \, (\alpha_s - \alpha_m) \, \beta_2 \tag{84}
$$

where the subscript 2 designates a solute property such as molar volume (V_2) polarizability–dipolarity (π^*_2, hydrogen bond acidity (α_2), and. hydrogen bond basicity (β_2) Each solute property is multiplied by a term that represents the difference in complementary "solvent" properties of the mobile (see subscript m) and the stationary (see subscript s) phases. Thus α_m and α_s are the abilities of the phases (bulk or bonded) to donate a hydrogen bond. These properties complement the solute ability to accept a hydrogen bond (β_2)' Similarly, σ^2_m and σ^2_s, the squares of the Hildebrand solubility parameter or cohesive energies of the two phases, complement the solute molar volume.

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Another recent theory that had an impact on QSRR is studies in the mean-field statistical theory of Dill, [130] applied to RPHPLC by Dorsey and his co-workers.[131,132] According to this theory, two main forces dominate the retention:

1. The free-energy change resulting from contact interactions of the solute and neighboring   molecules of the stationary and mobile phases
2. Ordering of the stationary phase hydrocarbon chains leading (at higher hydrocarbon   bonding density) to an entropic exclusion of the solute from the stationary phase relative to that expected in an amorphous hydrocarbon-water partition system

However, there are two important consequences of this theory for retention prediction and other QSRR studies. One is that the retention in RPHPLC increases with the grafted stationary phase chain density up to a density value of about 3 0 μmol/m, where the retention reaches a plateau Another conclusion derived from the theory concerns the nature of the slope and intercept of the rectilinear relationship between logarithm of capacity factor, log k', and composition of binary organic-water eluent. The slope was postulated [133,134] to be directly proportional to the size of the solutes, although measures of solute size such as vander Waals volume and molecular connectivity indices did not confirm the expectations. It has been argued that the RPHPLC distribution coefficient could be calculated from known values of the activity coefficients of the substance of interest in both chromatographic phases. A means for the assessment of activity coefficients is the UNIFAC group contribution method according to Fredeslund and co-workers. [135] The UNIFAC method transforms a solution of molecules into a solution of groups. The magnitude of a given group contribution to the activity coefficient depends on the vander Waals group volume and surface area. The number of distinct groups is limited, but is not so small as to neglect significant effects of molecular structure on physical properties.

There are theoretical approaches aimed at the prediction of RPHPLC retention using the substituent and or fragmental contribution to retention parameters. Some of the purely predictive applications of these methods (which form the basis of expert systems) are interesting attempts to identify and quantify mutual interactions between substituents

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or fragments. Smith and Burr [136] described the RPHPLC retention parameter I of disubstituted (X, Y) aromatic solutes employing the following equation:

$$I = I_p + I_{S,R} + \Sigma I_{s,Al-X} + \Sigma I_{s,Ar-X} + \Sigma I_{I,X-Y} \qquad (85)$$

where I_p represents the retention parameter of a parent unsubstituted compound; $I_{S,R}$ is a contribution for saturated alkyl chains; $I_{S,Al-X}$ are contributions for substituents on saturated aliphatic carbons; $I_{s,Ar-X}$ are contributions for aromatic substituents; and $I_{I,X-Y}$ are terms accounting for any interaction between substituents caused by electronic, hydrogen bonding, and steric effects. The interaction terms are calculated by the following equation:

$$I_{I,X-Y} = (\sigma_x P^*_y + \sigma_y P^*_y) + F^*_{HB} + F^*_0 \qquad (86)$$

where P^*, F^*_{HB} and F^*_0 are expressed in units of the retention parameter; P^* are the susceptibilities of X and Y to the modifying effects of Y and X on the Hammett constants of the substituents σ_x and σ_y. F^*_{HB} is a term accounting for hydrogen bonding; and F^*_0 is a term reflecting the ortho effect. It should be mentioned that the σ/p correction values, along with ortho effects, were demonstrated by Tsantili-Kakoulıdou and his co-workers. [137]

3.11 Hydrophobicity Concept in Chromatography

Boyce and Millborrow[138] extrapolated retention parameters determined at various organic-water eluent compositions to a pure water eluent. It later became common practice to employ extrapolated data as measures of hydrophobicity. [138] The extrapolation based on the assumption of the lınear Soczewinski-Wachtmeister relationship[139] between log k' and the volume fraction of the organic modifier in a binary aqueous eluent. It has been demonstrated that the rectilinear relationship in RPHPLC applies only over a limited solvent composıtion range that varıes depending on the solute and the chromatographic system employed. [112,140] In effect, the values of the logarithm of the capacity factor extrapolated to a pure aqueous eluent - which is the intercepts in the Soczewinski-Wachtmeister equation denoted commonly by log k'$_w$ - are

usually different from those determined experimentally and depend on the organic modifier employed. Owing to this observation, some workers are inclined to believe that the extrapolation of capacity factors to 0% organic modifier is a manipulation and the values of log k'_w itself has no physical meaning. [141] Interpretation of log k'_w as the logarithm of the capacity factor corresponding to a pure water (buffer) eluent might be misleading, especially if the extrapolation is carried out over a considerable eluent composition range with a fitting function that is basically unreliable. [142] The parameter log k'_w is not devoid of merits, however, as it may be regarded as a means of normalizing retention. [143]

Isocratic capacity factors determined with various organic modifiers naturally depend on the properties of the modifier. One could expect the values extrapolated to pure water (log k'_w), however, to be independent of the organic modifier used. Different modifiers yield different chromatographic measures of solute hydrophobicity. There is no reason to assume that anyone modifier provides a better measure of hydrophobicity than others. When the reference hydrophobicity scale is that of the 1-octanol-water partition system (log P), then individual organic modifiers appear to be advantageous. Braumann et al. strongly advocated the view that a general relationship between log P and log k'_w can only be expected for capacity factors determined in methanol-water eluents. [144] According to these authors, similar solute-solvent interactions operate in methanol-water and 1-octanol-water systems, whereas other organic modifiers (acetonitrile or tetrahydrofuran) introduce interactions that are not present in the 1-octanol-water system. Although Braumann et al. postulated the identity of log k'_w and log P with non polar solutes, such as chlorobiphenyls and alkylbenzenes, separate regression of log P vs log k'_w has to be developed for each class of compound [145]

3.12 Correlations between RPHPLC Retention Parameters and 1-Octanol-Water Partition Coefficients

One can certainly expect close correlation between log P and RPHPLC retention parameters if the chromatographic system closely resembles the conventional 1-octanol-water partition system. Several authors have reported the procedures based on

dynamically coating a stationary phase with 1-octanol and using a 1-octanol-saturated aqueous eluent. [146] The correlations were satisfactory but serious technical problems made the approach impractical. To achieve high correlations between retention parameters determined in a stable RPHPLC system and log P, various researchers have recommended specific treatment of the stationary phase before use and the presence of various additives in the mobile phase. [147]

The correlations of such chromatographic data with log P were good only as long as the solutes analyzed were more or less closely related (congeneric). In contrast to earlier trends in recent publications on relationships between log P and reversed-phase liquid chromatographic parameters (from both HPLC and TLC), only moderate correlations were reported. [148] For a series of congeneric solutes the correlation between log k'w and log P reported by Clark et al , [145] r = 0.953, appears reliable and realistic. However, poor correlations were reported even for pyrazine and oxazoline derivatives. [149]

To retain log P as the RPHPLC retention descriptor, some authors have introduced empirical correlations to log P or the hydrophobic substituent (fragmental) constant. [150,151]Although such correlations may be of some use for the prediction of retention, they did not offer much help with understanding the mechanism of separations. The same holds true if the correlation between log k' and log P is improved by the introduction of indicator variables or molecular refractivity into the regression equations.[152,153]

In a QSRR study Patel and co-workers [154] employed 1-octanol-water partition coefficients to account simultaneously for changes in solute structure and mobile phase composition. The In k' was modeled by the following equation:

$$\text{Ln } k' = A + B(\log P / P_{sm}) + C(1/P_{sm}^2) \qquad (87)$$
or
$$\text{Ln } k' = A' + B'(\log P / P_{sm}) + C'(1/P_{sm}^2) \qquad (88)$$

where A, B, A', B', and C' are regression coefficients; P is the octanol-water partition coefficients of the solute; and P_{sm} stands for calculated 1-octanol-water partition coeffi-

cients of a solvent mixture containing water and methanol or acetonitrile or tetrahydrofuran. P_{sm} is calculated from the following equation:

$$\log P_{sm} = \Sigma (X_i \log P_{s,i}) \tag{89}$$

where X_i is the mole fraction of the i-th solvent component; $P_{s,i}$ is the 1-octanol-water partition coefficient of the i-th solvent component. [154]

The limited performance of log P, determined in a liquid-liquid partition system, in modeling RPHPLC retention suggests differences in the two types of partition process. There is evidence that with hydrocarbonaceous stationary phases the solute molecule can penetrate vertically into the bonded hydrocarbon layer. [155] In addition, retention in such phases is affected by the surface density of the bonded alkyl chains. In this situation the chromatographic process can not be directly modeled by the bulk organic-water partitioning process, because the non polar stationary phase is an interphase (immobilized at one end) and not a bulk medium.

3.13 Correlation Analysis in Liquid Chromatography of Metal Chelates

Timerbaev et al. [156] discuss the choice of the most significant parameters to be used as variables for the multiple linear regression analysis. The fist selection principle utilized is to reject some less important parameters based on results obtained earlier in our one-dimensional correlations. Then, to facilitate the development of multivariate models, the set of the parameters were further reduced using one-dimensional correlation analysis for particular chelates and the separation system studied in this work. Finally, the remaining valuable parameters were termed solvophobic and hydrogen bonding groups. The molecular descriptors applied by the authors is listed below.

Structural Parameters of Metal Chelates Applicable to the Description Of Retention Behavior in RPHPLC:

3.13.1 Molecular Parameters

- Distribution constant ($\log K_D$)
- Stability constant ($\log \beta_n$)
- Molecular connectivity index Molar volume (V_m)

- Solubility in the mobile phase

3.13.2 Parameters of Metal Atom

- Effective charge

- Electronegativity

- Ratio of electronegativity to ionic radius Orbital electronegativity

- Distribution coefficient

- Metal increment in the distribution constant

3.13.3 Ligand Parameters

- Carbon number of alkyl homologies (n_c)

- Induction constant (σ^*)

- Steric constant (E_s)

- Hydrophobic constant, π and f

- Molecular connectivity index of the ligand

- Molar volume of the ligand

The authors did not consider molar volume, which show a poor correlation with log k' values owing to inaccuracy in the evaluation of the metal increment and solubility, which requires special experiments to be performed. The retention values of metal dithiophosphates (as the chelates of the S,S type) in RPHPLC did not correlate with the effective charge or electronegativity of a metal atom, or with the ratio of electronegativity to the metal ion radius. The application of the electronic and steric constants of alkyl substituents, characterizing the hydrogen bonding, capability of donor atoms, is limited by the comparatively small number of carbon atoms in a ligand (not more than four atoms). Both types of hydrophobic constants well describe the hydrophobic nature of a chelate molecule in RPHPLC.

The authors have stated that the solvent polarity parameter E_t was sensitive only to the proton-donating properties of the mobile phase and consequently was linearly correlated with log k' values only for water-alcohol eluents Of the parameters reflecting the ability of the phase to participate in intermolecular interactions of different types, n* and proton-donating ability by Kosower will be considered below as those that are characterized by higher correlation factors. The list of eluent macroscopic parameters has been reduced to the volume concentration of the organic modifier and surface tension of

the mobile phase based on the same principle.Following the separation mechanism, the authors subdivided selected solute and eluent parameters into solvophobic and hydrogen bonding terms and then composed a number of multi parametric retention models given by

$$\log k' = a_o + a_1x_1 + a_2x_2 + a_3x_3 + a_4x_4 \qquad (90)$$

The parameters X_1 and X_2 characterize the hydrophobicity and hydrogen bond accepting ability of the chelates, respectively; X_3 and X_4 are the corresponding terms of the mobile phase (the eluent property complementary to solute hydrogen bond accepting ability is hydrogen bond donating ability).

3.14 Functional Group Contributions to the Retention of Analytes in Reversed-Phase High-Performance Liquid Chromatography

The functional group contribution (τ_x) to the retention of a compound can be determined from the difference of two solutes, which differ by the presence and absence of X, the functional group of interest. It can be defined the following equation:

$$\tau_x = \log k'_{R-X} - \log k'_{R-H} = \log k' (k'_{R-X}/ K'_{R-H}) \qquad (91)$$

Early studies in HPLC were reported by Rileyet al., [157] who showed that the τ values were directly related to Hansch partition constants (π values) and Rekker f constants, and the solvophobic theory provided a general framework for rationalization of many observations. For standardized functional group contribution, which is independent of the mobile phase composition, the τ values in mixed organic-water eluents are often extrapolated to give τ_w or τ_{water} values in 100% water.

Hafkenscheid and Tomlinson [158] examined the retention of a number 1,4-disubstituted benzenes and measured the extrapolated τ_w values for combinations of methyl, chlora, nitro, amino, and carboxylic acid groups using methanol-water eluents on an Hypersil ODS column. These investigations showed a close correlation with the corresponding 11 values. The τ_w values for the mono substituted groups (methyl = 0.54, chloro = 0.66, nitro = -0.14, hydroxyl = -0.70, amino = -1.19 and carboxylic acid = -0.13) compared well with those obtained in other studies. 4-Nitrophenol and 4-nitroaniline showed great differences and nitro group contributions were 0 40 and 0 45, respectively,

with similar significant changes in the values of the hydroxyl and amino groups, pointing to the presence of strong interaction.

In order to obtain a comparison between retentions and octanol-water partitions coefficients, Unger et al. [159] determined a number of log k'_w values using an ODS column and a mobile phase of pH 7.0 buffer saturated with octanol The corresponding τ_w values derived from monofunctional solutes can be calculated and show good comparison with the values obtained earlier.

Riley et al. [157] found good correlations between τ values and π values for a range of substituents on phenylazapurines, phenyltriazines, and benzoic acids, although the correlation was poorer in the case of 2-substituents, which could undergo hydrogen bonding. The τ_w values obtained by Altomare et al. [160] for substituents on benzenesulfonamides were correlated with 1t values and significant correlation was found (r = 0.953) No significant differences were found, however, between 3- or 4-substituents, which suggested that no significant crossing interactions were taking place Gago et al. [161] used measured log k' values for 3- and 4-substituted pyridines to calculate log P values and found that these correlated well with literature log $P_{o/w}$ values, suggesting that the τ values and group contributions to log P were closely related Lurie and Allen [162] examined τ values for methyl, alkyl, and fluoro groups on fentanyl homologues and analogues and correlated changes with calculated connectivity values. Good correlation (r = 0 9710) was found between the values of the aqueous group contributions (τ_w) from three modifiers determined by Smith [163] and the Hansch contribution. The values for group contributions for the individual eluents, which would give similar capacity factors, were also compared with the Hansch contributions Correlations for the methanol-buffer (1:1) were good (r = 0.9850), but there were greater deviations for acetonitrile-buffer (4 6) (r= 0.9645) and tetrahydrofuran-buffer (3·7) (r = 0 9537) and most of the changes seemed to be occurring between the more hydrophilic groups. In both acetonitrile and tetrahydrofuran, the noticeable groups, which had a relatively more negative group contribution than other groups with similar π values, were the carboxamide ($CONH2$), N-acetyl ($NHCOCH3$), hydroxyl (OH), and methylenehydroxyl (CH_2OH) groups. Amino and sulfonamide groups were apparently unaffected