Liquid Chromatography Separation Mechanism

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Contributor: Handajaya Rusli

Separation is a critical process to isolate a particular compound, whether it is a natural product or a synthetic product. Studies of a compound's characteristics and elucidation structure provides reliable results for pure compounds because there is no interference from other compounds. The primary source of difficulty in a separation process is the high similarity between two or more compounds, such as racemic and homologous mixtures. Liquid chromatography has proven to be an effective solution to those problems. The key to liquid chromatography separation is a sustainable retention and elution process. Stationary phases are essential for separating compounds in liquid chromatography. Various liquid chromatography columns of both preparative and quantitative types have been used and continue to develop. This research will discuss the separation mechanism in liquid chromatography.

liquid chromatography | stationary phase | separation mechanism

1. Introduction

Separation is a critical process to isolate a particular compound, whether it is a natural product or a synthetic product. Studies of a compound's characteristics and elucidation structure provides reliable results for pure compounds because there is no interference from other compounds. The primary source of difficulty in a separation process is the high similarity between two or more compounds, such as racemic and homologous mixtures. Liquid chromatography has proven to be an effective solution to those problems. The key to liquid chromatography separation is a sustainable retention and elution process. Stationary phases essential for separating compounds in liquid chromatography. Various liquid chromatography columns of both preparative and quantitative types have been used and continue to develop. For this reason, multiple studies and publications related to liquid chromatography can be found and accessed easily.

2. The Principle of Separation of Compounds in Liquid Chromatography

Separation using liquid chromatography is possible because of the different interactions between the compounds present in the sample with the stationary and the mobile phases in the liquid chromatography system (**Figure 1**). Stationary phases can be developed to enable compound separation based on several modes, such as (A) differences in the affinity to the compounds, (B) differences in the strength of electrostatic forces with the target

compounds, and (C) size differences of target compounds. One or more of these modes of interaction will result in compound separation using liquid chromatography. Choosing the appropriate columns, whether commercial or under development, could be somewhat confusing for a user. Understanding the modes of interaction will be helpful to assist users in selecting the appropriate columns for a particular type of analyte.

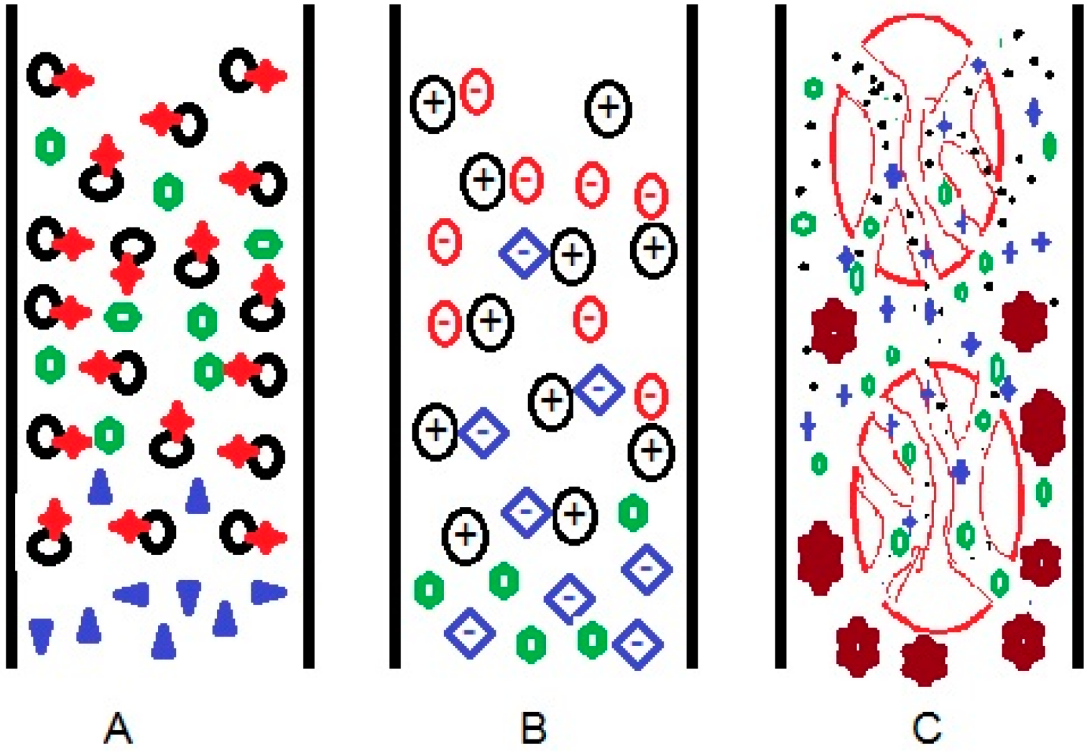

Figure 1. Scheme of physical interactions between column and target molecules, based on (**A**) affinity, (**B**) electrostatic forces, and (**C**) size difference in liquid chromatography systems.

In affinity-based separation (**Figure 1**A), stationary particles (black) with specific functional groups (red) interact with the appropriate compounds (green) through specific binding via a network of interactions, resulting in retention in the column. The stationary phase does not suspend noninteracting compounds (purple), and the compounds flow without retention. The common interactions in this mode of separation are hydrogen bonding, dipole–dipoles interaction, London forces, and complex formation. The combination of hydrogen and dipole-dipole interaction facilitates a hydrophilic interaction, while the London force is hydrophobic interaction.

Complex formation commonly occurs in chromatography where the analytes are proteins [1][2][3], DNA [4], and RNA [5][6]. The stationary phase is modified so that it has a metal-complex binding site that can accept ligands from macromolecules. Stronger interaction between compounds and the stationary phase increases retention time, while a weaker interaction decreases retention time. It is almost impossible to find compounds that have no affinity interactions at all. Even though stationary particles and compounds have very different polarities and no functional groups can interact, London forces always exist. Each molecule interacts differently due to its different functional groups, atomic numbers, and 3D structures. This difference allows for the separation of analog [7] and homologous compounds [8][9].

Affinity chromatography is commonly employed in the separation of biomacromolecules. Further, reverse-phase chromatography (RPC) and hydrophilic interaction liquid chromatography (HILIC) are common for the separation of smaller organic compounds, in which affinity-based separation occurs as a result of interactions based on hydrophobicity and hydrophilicity.

The mode of separation that is based solely on electrostatic separation requires coulombic forces to occur between cationic species and anionic species. Stationary particles can function either as cations or anions, depending on the type of functional groups and the experimental conditions. As exemplified in **Figure 1**B, the blue-colored anion, which binds to the stationary cation (black) via electrostatic forces, is replaced by the analytes (red) due to a stronger interaction. This process is an ion-exchange mechanism. Green-colored compound, which is neither charged nor negatively charged, is eluted from the column without being retained, as no coulombic interaction occurs between the green-colored compound and the stationary cation. The same principle applies to the separation of cationic compounds using anionic stationary phases. A greater charge density of molecules will produce a stronger electrostatic force, resulting in a longer retention time.

Such electrostatic forces are widely used in ion-exchange chromatography (IEC) or ion chromatography (IC) systems. The charge of the stationary phase and analyte can be changed by adjusting the pH or composition of the mobile phase system so that separation can be carried out. The pH setting affects molecules that are weak acid or weak base. The new temporary molecules or the changes of 3D conformation can result from the changes of the mobile phase. The variables that can change in a mobile phase system are salinity, buffer type, and complexing ligand. The types of samples are small ions [10][11] and macromolecules [12].

Separation based on size difference is better known as size exclusion chromatography (SEC) or gel permeation chromatography (GPC). In this type of chromatography, compounds are separated based on the possibility of the analyte being trapped in stationary particle pores. Commonly, the smaller-sized analyte is retained for a longer time in the stationary pores, so the retention time is increased compared to bigger compounds, as shown in **Figure 1**C. Generally, this system is used to separate compounds with large molecular masses between 2000 and 20,000,000 amu, such as proteins [13][14], carbohydrates [15], surfactants [16], and polymers [17].

Combining two or three of the above modes of separation could result in more effective compound separation compared to applying only one mode of separation. For instance, chiral chromatography combines the principles of size exclusion and affinity-based separation. Electrochromatography (EC) [18][19][20], widely used to separate proteins and DNA, combines size and electrostatic separation principles. Separation in EC is based on the ratio of the charge density of the compounds. Larger charge density compounds elute faster than the low ones. Mixed-mode chromatography (MMC) [8][21][22][23][24][25][26][27][28][29][30][31][32][33][34][35][36][37] is a system that can be used for at least two types of chromatography depending on the measurement conditions, especially in the mobile phase. Multi-column chromatography (MCC) is used to separate compounds from complex matrices utilizing a combination of the three separation principles above.

Table 1 summarizes several examples of the types of chromatography that have been developed in the last 10 years. Most of the developed stationary phases are based on silica and organic polymers. The samples analyzed are also extensive, ranging from simple ions to uncharged macromolecules, from polar to nonpolar molecules, and from water-based to organic-based solvents. Various isomers (functional group, optical, and structural isomers) or molecule derivatives can be separated using an appropriate liquid chromatographic system.

Table 1. Types of liquid chromatography, separation principles, and new developments in the field of liquid chromatography.

Mode of Liquid Chromatography	Separation Principle	Stationary Phase	Analyte	Mobile Phase
Reverse-phase chromatography	Affinity	Silica modified with octadecyl acrylate and 2-vinyl-4,6-diamino-1,3,5-triazine [38]	PAHs	Methanol
	Affinity	Silica modified with octadecyl acrylate and *N*-methylmaleimide [39]	PAHs and tocopherols	Mixed of methanol and water
	Affinity	Silica modified with *N*-Boc-phenylalanine and cyclohexylamine [40]	Phytohormones	Mixed of phosphate buffer and acetonitrile
	Affinity	$Zr_6O_4(OH)_4$ MOF modified with 2-amino-terephthalic acid or 4,4′-biphenyl-dicarboxylic acid [41]	PAHs and aromatics compound	Mixed of methanol and water
Hydrophilic interaction liquid chromatography	Ionic	Silica modified with (2-(methacryloyloxy)-ethyl)dimethyl-(3-sulfopropyl)ammonium hydroxide or 2-methacryloyloxyethyl phosphorylcholine [42]	Mixed of toluene, formamide, dimethylformamide, and thiourea	Mixed of water and acetonitrile
	Affinity	Amino silica modified with polyhedral oligomeric silsesquioxane and acrylamide derivatives [34]	Nucleosides, organic acids, and β-agonists	Mixed of acetonitrile and ammonium formate solution
	Affinity	Silica modified with EGDMA and maltose [32]	Nucleobases and nucleotides	Mixed of water and acetonitrile
	Affinity	Silica modified with vinyl silsesquioxane and		

Mode of Liquid Chromatography	Separation Principle	Stationary Phase	Analyte	Mobile Phase
		dithiothreitol [43]		
	Ionic and affinity	Silica modified with pyrazinedicarboxylic anhydrate [44]	Oligosaccharides, alkaloid, and organic acid groups	Mixed of acetonitrile and ammonium formate solution
	Ionic and affinity	Silica modified with 2-methacryloyloxyethyl phosphorylcholine [35]	Protein and lysozyme	Mixed of acetonitrile, ammonium formate solution, KH_2PO_4 solution, NaCl solution
	Ionic and affinity	Silica modified with octadecyl and diol groups [8]	Aristolochic acid and derivatives	Mixed of formic acid and acetonitrile
Mixed-mode chromatography	Ionic and affinity	Silica modified with glutathione [26]	Protein	Mixed of water, formic acid, acetonitrile
	Ionic and affinity	poly(12-methacryloyl dodecylphosphatidic acid-co- ethylene glycol dimethacrylate) [45]	Ketone aromatic, phenol and derivatives, small organic compounds	Mixed of ammonium formate solution and acetonitrile
	Affinity	Amino silica modified with octadecyl and carbon dots [31]	PAHs, nucleosides, and nucleobases	Mixed of water and methanol, acetonitrile, and ammonium acetate solution
Affinity chromatography	Ionic and affinity	Agarose modified with 2-Mercapto-1-methylimidazole [46]	Protein	NaOH solution
	Ionic and affinity	Sepharose modified with ligand complex [3]	Protein with histidine	Mixed of Tris buffer, sodium chloride, and imidazole
	Ionic and affinity	Silica modified with N-methylimidazolium ionic liquid [28]	Protein	Mixed of acetonitrile, trifluoroacetic acid, $NaClO_4$ solution, KH_2PO_4 solution, and NaCl solution

Mode of Liquid Chromatography	Separation Principle	Stationary Phase	Analyte	Mobile Phase
	Ionic and affinity	Amino silica modified with glutaraldehyde [47]	Protein	Phosphate buffer
Ionic chromatography	Ionic	Bentonite modified with chitosan and cetyltrimethylammonium bromide (CTAB) [11]	Cr(III) and Cr(VI) solution	Nitric acid solution for Cr(III) and ammonia solution for Cr(VI)
	Ionic	Polystyrene-methacrylate derivatives modified with poly(amidoamine) [10]	Small anions like nitrate, sulfates, bromide, etc.	NaOH solution
Chiral chromatography	Size and affinity	Polysaccharide modified with 3-chloro-4-methylphenylcarbamate [48]	Paroxetine hydrochloride groups	Mixed of supercritical CO_2, methanol, and ammonium acetate solution
	Affinity	Isopropylcarbamate cyclofructan 6 groups [49]	Methionine groups	Mixed of methanol, acetonitrile, acetic acid, and triethylamine
	Size	Silica modified with 3,3′-phenyl-1,1′-binaphthyl-18-crown-6-ether [50]	Amino acids and peptides	Mixed of perchloric acid solution, acetonitrile, and methanol
Electrochromatography	Affinity	Poly(styrene-divinylbenzene) coated with chitosan [9]	Benzoin	Mixed of water and acetonitrile
	Size and ionic	Poly(POSS-co-META-co-DMMSA) [18]	Benzoic acid, nucleosides, bases, glycopeptides	Mixed of phosphate buffer, triethylamine, and acetonitrile
	Affinity	Silica modified with a metal-organic framework (MOF) [20]	Benzenes and derivatives	Mixed of phosphate buffer and acetonitrile
Size exclusion chromatography	Size	Poly(methacrylic acid-co-ethylene glycol dimethacrylate) [51]	Protein	Mixed of water and acetonitrile

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