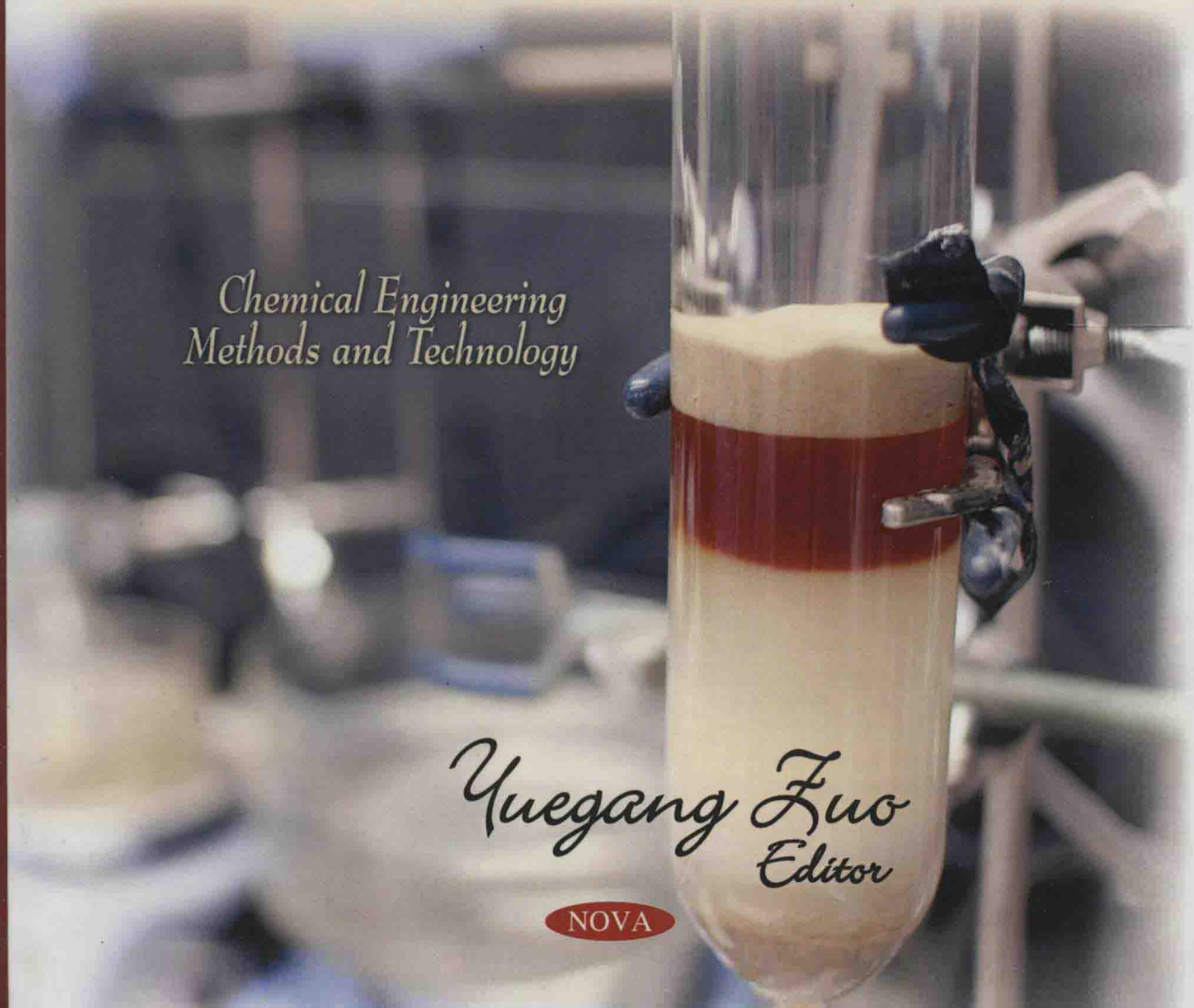
High-Performance Liquid Chromatography (HPLC)

Principles, Practices and Procedures



Chemical Engineering Methods and Technology

Yuegang Zuo
Editor

NOVA

CHEMICAL ENGINEERING METHODS AND TECHNOLOGY

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PRINCIPLES, PRACTICES AND PROCEDURES

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nova publishers
New York

Library of Congress Cataloging-in-Publication Data

High-performance liquid chromatography (HPLC) : principles, practices and procedures / editor, Yuegang Zuo (Department of Chemistry and Biochemistry, University of Massachusetts Dartmouth, North Dartmouth, MA, USA).
pages cm
Includes index.
ISBN 978-1-62948-854-7 (hardcover)
1. High performance liquid chromatography. 2. Liquid chromatography. 3. Chromatographic analysis. I. Zuo, Yuegang, editor of compilation.
QP519.9.H53H5356 2014
543'.84--dc23

2013047179

Published by Nova Science Publishers, Inc. † New York

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PREFACE

Since its appearance in the early 1970s, HPLC has shown its powerful ability to separate components in complex chemical mixtures. During the past four decades, various HPLC techniques, including normal phase, reversed phase, ion-exchange, and size-exclusion chromatography, have been developed and the related retention mechanisms have been explored. Today HPLC has become one of the most popular and versatile separation and analytical tools. Even though, new HPLC separation modes and applications have been continuously emerging. This book gathers leading research from all over the world dealing with new development in HPLC theories, stationary and mobile phases, separation mechanisms, methods and applications.

Hydrophilic interaction liquid chromatography (HILIC) is an effective alternative to conventional HPLC techniques for the separation and determination of polar and hydrophilic compounds. Since Alpert described it first in 1990, the popularity of HILIC has been growing exponentially as measured by the number of publications due to increasing demands for the analysis of polar components in complex matrices. HILIC is a chromatographic technique that uses aqueous-organic solvent mobile phases with a high percentage of organic solvent, and a polar stationary phase. So far, silica gels, amino, amide, cyano, carbamate-, diol- and zwitterionic-based stationary phases have been utilized in HILIC separation. Like other HPLC techniques, the understanding of HILIC retention mechanisms and theories has been behind the practice. This first chapter discusses the development, basic separation mechanisms, stationary and mobile phases of HILIC, and summarizes the applications of HILIC in several research fields such as bioanalysis, metabolomics, food, pharmaceutical, forensic and environmental sciences.

Characteristics and quality of the column is one of the most crucial factors in HPLC separation. In this chapter the authors present the advanced packing materials and technology currently used in HPLC. Column technology has recently led to the manufacture of sub-2 μm particle size columns used for Ultra High Performance Chromatography. However, these columns cannot be used with conventional chromatographic systems (with a pressure limit of 400 bar) due to pressure limitations. A new HPLC system, capable of withstanding pressures of over 1000 bar, is required. The ultrahigh pressure HPLC is too expensive for low budget laboratories. On the other hand, the columns using solid core particles can make ultra high performance separation possible under conventional pressure. In this chapter, sub-2μm porous particles, solid core particles and monolithic columns are compared with conventional

columns in terms of efficiency, speed, resolution and applications. The chapter also discusses the future development in column technology.

Carbohydrates are widely present in biological systems in both free states (e.g., starch, cellulose) and conjugated forms (e.g., proteoglycans, glycoproteins, glycolipids), providing energy storage as well as structural support functions. Carbohydrates participate in many biological processes including cell recognition, development, interaction, and inflammation. The analysis of carbohydrates is challenging due to their complex structure and heterogeneity. This chapter reviews current high performance liquid chromatography (HPLC) techniques for carbohydrate analysis. To analyze carbohydrate content by HPLC, the test sample must first be extracted and purified. Hydrolysis and derivatization techniques are used to release and label carbohydrates for sensitive and selective detection. Several HPLC modes are employed for carbohydrate separation including, reversed phase and normal phase chromatography, hydrophobic interaction chromatography, hydrophilic interaction liquid chromatography, anion exchange chromatography, and size exclusion chromatography. Common detection methods used for HPLC include refractive index, UV absorbance, fluorescence, evaporative or laser light scattering, and electrochemical detection. The authors have included a chitosan analysis method in this chapter. Chitosan, is a copolymer of glucosamine (2-amido-2-deoxy-D-glucose) and N-acetyl glucosamine via a β(1-4) linkage, a widely used food ingredient and additive. To quantify chitosan, it is first hydrolyzed to glucosamine with hydrochloric acid, then undergo derivatization with N-(9-fluorenylmethoxycarbonyloxy)succinimide (FMOC-OSu). The glucosamine-FMOC derivatives are then separated and quantified using reversed phase HPLC with UV detection.

Infections with the human immunodeficiency virus (HIV) are typically treated with drug combinations consisting of at least three different antiretroviral drugs. A non-nucleoside reverse transcriptase inhibitor (NNRTI) is an essential component of this highly active antiretroviral therapy (HAART). NNRTIs are orally administered and subjected to large interindividual, and sometimes intraindividual, variability in their plasma levels. HPLC constitutes a rapid, specific and sensitive technique for determining NNRTI plasma concentrations in routine clinical practice, and evaluating the purity of the active substance and the quality of pharmaceutical products containing NNRTIs. This chapter reviews the HPLC analyses of non-nucleoside reverse transcriptase inhibitors, particularly the sample preparation, chromatographic conditions and detection methods for biological fluids and pharmaceutical products.

The most commonly used medication for the treatment of high cholesterol levels is statins such as atorvastatin, simvastatin and lovastatin. Although safe and effective, statins can cause muscle problems, lung and liver disorders as well as kidney damage. Therefore, many patients seek alternative therapies to control their cholesterol levels. Many natural cholesterol-lowering agents are widely available to the public as dietary supplements. Dietary supplements are usually used by patients at their own discretion, in an unmonitored setting and without the input of their physicians. Dietary supplements are readily available, not classified as over-the-counter medications, and not regulated as such. Health practitioners and patients often consider these products safe and probably effective. Unfortunately, information on dietary supplements in nonmedical literature and even in scientific literature is usually unreliable. Moreover, numerous studies have used products that were not well characterized. There is no uniform legislation in the dietary supplements area despite their ever growing popularity and presence on the market. Health safety, nutritional value and laboratory control

of declared content is very rare. Therefore, greater attention has recently been given to quality control of dietary supplements. Due to its superior precision, high resolution and capacity to analyze thermally labile and non-volatile compounds, high performance liquid chromatography is applied for the quality control of dietary supplements. In this chapter a critical review on chromatographic methods for quality control of most frequently used natural cholesterol-lowering dietary supplements such as red fermented rice, artichoke, phytosterols, omega-3 fatty acids, green tea, soybean, gugulipid, coenzyme Q10, taurine, flax seeds and policosanol is given. Special attention is paid to determination of active ingredients as well as toxic compounds. Additionally, sample preparation procedures and chromatographic methods used for determination of active ingredients in biological fluids are also discussed.

The relationship between lipoprotein profile in blood and progress of atherosclerosis is investigated in a lot of studies. A low cholesterol level of high-density lipoprotein (HDL), and high cholesterol levels of low-density lipoprotein (LDL), intermediate-density lipoprotein (IDL), and very-low-density lipoprotein (VLDL) are well known to be the risk markers for atherosclerotic disease such as coronary heart disease. The changes of lipoprotein levels are seen in patients with the impaired lipoprotein metabolism. Therefore, the development of the analysis method for estimation of lipoprotein profile is important for clinical diagnosis and medical care of the diseases. The methods for analyzing lipoprotein profiles by using ultracentrifugation, electrophoresis, gel-permeation chromatography, and anion-exchange chromatography were previously reported. All major lipoprotein classes (HDL, LDL, IDL, VLDL, chylomicron, etc.) can be separated by ultracentrifugation which is a standard method for separation of lipoprotein classes, but it is laborious and time-consuming. HDL, LDL, and VLDL can be separated by electrophoresis and gel-permeation-chromatography, however, LDL and VLDL in patients with diabetes or hyperlipidemia sometimes cannot be well separated. This chapter reported an anion-exchange (AEX) HPLC method using a column containing diethylaminoethyl-ligand nonporous polymer-based gel with a step gradient of sodium perchlorate concentration, by which HDL, LDL, IDL, VLDL, chylomicron, and lipoprotein(a) can be separated, even in blood from the patients with diabetes or hyperlipidemia. Additionally, the subfractions in HDL and LDL in human blood and the major five lipoprotein classes (HDL, LDL, IDL, VLDL, and chylomicron) in rabbit blood can be separated by the AEX-HPLC method. The AEX-HPLC method described is suitable for the convenient and accurate assay of lipoprotein profile in clinical studies.

Benzodiazepines belong to the most commonly self-administered group of sedative, antianxiety and anticonvulsant drugs. Generally, they are known as safe drugs because of their high therapeutic index. However, the use of benzodiazepines together with other drugs or substances (e.g. alcohol) may result in serious consequences. Some benzodiazepines (e.g. flunitrazepam) are used for criminal purposes as date rape drugs. Therefore, a variety of biological materials, both conventional (plasma, serum, urine) and alternative (hair, saliva, vitreous humor, nails), have been investigated for these drugs. In the last decade, liquid chromatographic methods, especially LC/MS(MS), have played a dominant role in the determination of benzodiazepine drugs in biological samples. Analysis of biological material by LC methods usually should be preceded by sample preparation using extraction techniques such as SPE, LLE or MAE. This chapter critically reviews the developments of LC methods for benzodiazepine determination and their applications in pharmacological and toxicological studies in the last five years.

Triethylene glycol dimethacrylate (TEGDMA) and 2-hydroxyethyl methacrylate (HEMA) are methacrylic monomers present in dental auto- and photo-polymerizable resins. Many in vitro studies have shown that the polymerization of the above mentioned compounds is never complete and the uncured monomers are released in the oral environment, causing possible local adverse effects. The evaluation of the amounts of the methacrylic monomers released from composite resins is therefore very important and HPLC technique is particularly suitable to this purpose. HPLC can be also applied to the study of the metabolites of methacrylic monomers. This chapter summarizes the recent studies about the detection of TEGDMA and HEMA in complex systems like cells and culture media by HPLC, evidencing its key role in the investigations of the substances involved in cytotoxic processes. Moreover the great specificity and sensitivity of the used method allow to relate the concentration values of methacrylic monomers to their cytotoxic effects, in particular when such molecules are used in presence of N-acetyl cysteine, helping to clarify its mechanism of detoxification.

Various methods have been described for the analysis of vitamins in food matrices, with more and more of these including the use of HPLC to measure the levels of these micronutrients in foodstuffs. The renewed interest in rapid and accurate quantification of micronutrients in foodstuffs is due to more stringent requirements by food regulatory agencies around the world. Legislation now demands that the nutrition information displayed on food labels be backed up by reliable results obtained using validated analyses. Three common challenges in quantifying vitamins in food matrices are to: 1) efficiently extract the various forms of the vitamin from each unique matrix, 2) ensure that labile forms of the vitamin are protected against degeneration by light and/or air (oxygen) for a sufficiently long period to afford accurate quantification and 3) obtain an analytical method with sufficient sensitivity, selectivity, accuracy and precision, with cost and time also being considerations. This chapter deals with these aspects concerning vitamin B1, B2, B3, B6, B9, B12 and vitamin C. Extraction procedures are described, as well as typical HPLC methods and recent improvements in this field.

This chapter describes the development and optimization of three chromatographic methods based on ultra-performance liquid chromatography (UPLC) coupled to electrospray ionization (ESI) – tandem mass spectrometry (MS/MS) and ultraviolet visible (UV-vis) detectors for the identification and quantification of seven organic acids (trans-aconitic acid, cis-aconitic acid, tartaric acid, succinic acid, L(+)-lactic acid, L(-)-malic acid and citric acid) and sixty-four wine polyphenols of which five were hydroxybenzoic acid and derivatives, eight hydroxycinamic acid and derivatives, two stilbenes, nine flavan-3-ols, twelve flavonols and twenty-six anthocyanins. The identification and quantification of organic acids in wine was performed using the UPLC-ESI-MS/MS under isocratic conditions and with the electrospray ionization source operating in negative mode. A second method was developed for the determination of cis/trans aconitic acid and non-pigmented polyphenols, while UPLC was accomplished using ESI-MS/MS and UV-vis detectors. Finally, UPLC coupled to ESI-MS/MS and UV-vis detection results in effective and fast screening of the anthocyanin pigments. Separation was achieved with an Acquity BEH C18 (100 mm x 2.1 mm, 1.7 μm) column. The relative standard deviation (RSD) for the repeatability test (n=5) of peak area and retention time were all below 1.47% and 0.03% for ESI-MS/MS and UV-vis detection respectively. A good separation of organic acids and phenolic compounds was achieved in 12.5 min. The applicability of this analytical approach was confirmed by the successful analysis of red wine samples.

In the last chapter, HPLC coupled with spectrophotometric (UV-Vis) and electrospray mass spectrometric (MS) detection has been applied to examination of natural colorants used for dyeing of historical textiles. Five examined red fibers were taken from Italian velvet brocades (parts of chasubles) dated at the 15^{th} and 16^{th} century, belonging to the collection of the Wawel Cathedral treasury (Cracow, Poland). In extracts from these fibers carminic, kermesic, flavokermesic and laccaic acids, alizarin, purpurin and munjistin were found. Successful identification of natural preparations used for dyeing examined textiles: dyes of animal origin – lac dye, Polish and Mexican cochineal, and also those produced from plants – various madder species, has confirmed unique potential of liquid chromatography in analyses of objects of historical value.

CONTENTS

In: High-Performance Liquid Chromatography (HPLC) ISBN: 978-1-62948-854-7
Editor: Yuegang Zuo

Chapter 1

HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY: FUNDAMENTALS AND APPLICATIONS

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ABSTRACT

Hydrophilic interaction liquid chromatography (HILIC) is an effective alternative to conventional HPLC techniques for the separation and determination of polar and hydrophilic compounds. Since Alpert described it first in 1990, the popularity of HILIC has been growing exponentially as measured by the number of publications due to an increasing demand for the analysis of polar components in complex matrices. HILIC is a chromatographic technique that uses aqueous-organic solvent mobile phases with a high percentage of organic solvent, and a polar stationary phase. So far, silica gels, amino, amide, cyano, carbamate-, diol- and zwitterionic-based stationary phases have been utilized in HILIC separation. Like other HPLC techniques, the understanding of HILIC retention mechanisms and theories has been behind the practice. In this chapter, we will discuss the development, basic separation mechanisms, stationary and mobile phases of HILIC, and summarize the applications of HILIC in several research fields such as bioanalysis, metabolomics, food, pharmaceutical, forensic and environmental sciences.

Keywords: Hydrophilic interaction liquid chromatography, HPLC, RP-HPLC, NP-HPLC, ion-exchange, silica, cyano, diol, amide, amino, zwitterionic, creatinine, uric acid, ascorbic acid, cocaine, benzoylecgonine, urine, orange juice, banknote

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INTRODUCTION AND BACKGROUND

The Russian botanist Mikhail Tswett invented chromatography, now one of the most powerful and popular chemical separation techniques, at the beginning of the twentieth century. In his original work, Tswett separated plant pigments by passing an extract of the plant leaves through a column of chalk powder (calcium carbonate), or alumina with an eluent of petroleum oil (hydrocarbon) (Tswett, 1906; Berezkin, 1990). For historic reasons, this classical mode of chromatography, using a polar stationary phase and a nonpolar mobile phase, is now called normal phase liquid chromatography (NP-LC). In the early days of liquid chromatography silica gel was used as stationary phase because it was cheap to produce, relatively inert and has a high surface area. Prior to 1965, conventional liquid chromatography was primarily accomplished in long and large columns with large particles ($\geq$100 μm) under gravity feed. In the early 1970s, the technology of producing small spherical silica particles ($\leq$10 μm) allowed the use of small-volume columns with largely increased packing density and homogeneity, and a more regular flow of the mobile phase. This tremendously enhanced the separation efficiency of the columns but also back pressure. Even with the increased separation efficiency, the application of NP-liquid chromatography is still limited in the separation of polar compounds due to the site heterogeneity of normal stationary phase on naked inorganic oxides and its slow equilibration with eluents, which resulted in nonlinear adsorption isotherms, peak tailing and fronting, and poor reproducibility. In addition, it is difficult to dissolve hydrophilic compounds in a nonpolar mobile phase, e.g., n-hexane, ethyl ether, methylene chloride, or chloroform.

Also in the early 1970s, bonded phases appeared and hydrophobic groups were attached to the silica surface to provide reverse phase columns. During the same period of time, HPLC (high pressure liquid chromatograph, now preferred as high performance liquid chromatograph) was built and high-pressure pumps were used to deliver a desired flow of the mobile phase. With the development of reverse phase (RP) columns, the popularity of RP-HPLC, using a nonpolar stationary phase and a polar mobile phase, usually aqueous solutions of water-miscible organic solvents such as acetonitrile, methanol or tetrahydrofuran, has been growing rapidly and become a predominant HPLC technique because of its high reproducibility and suitability for a wide range of molecules, including small organic and inorganic compounds as well as high molecular weight species such as proteins, and peptides. Today, approximately 80-90% of HPLC separations are conducted in the RP mode. However, RP-HPLC has a major limitation, lack of adequate retention of very polar and hydrophilic compounds. To achieve some retention of extremely polar compounds on RP-HPLC stationary phases, several approaches, such as utilization of a highly aqueous mobile phase, addition of ion pairing agents in the mobile phase and derivatization of the polar functional groups into hydrophobic groups in analytes, have been successfully explored in some applications for the determination of polar and ionized molecules (Jiao, 2007; Cecchi, 2008; Zuo et al., 2003, 2006; 2007; 2008; 2013). However, all these approaches have certain drawbacks that affect their wide utilizations. For example, a high percentage of water in the mobile phase is preferred in the framework of green chromatography, but it results in dewetting and nonreproducibility problems. Ion pairing reagents reduce the sensitivity and selectivity of electro spray ionization mass spectrometric (ESI-MS) detectors and the lifetime

of the column. Derivatization is not only time-consuming, but also increases the uncertainty in quantitative analysis, and decreases the separation between closely related molecules.

Hydrophilic interaction chromatography (HILIC) is an appealing alternative HPLC mode for separating polar compounds with limited retention on RP columns. HILIC, like NP-HPLC, utilizes a polar stationary phase such as silica, amino or cyano, but with a relatively hydrophobic aqueous organic solvent, commonly acetonitrile, mobile phase similar to those used in the RP-HPLC (Alpert, 1990). As early as in 1975, Jane (1975), Palmer (1975), Linden and Lawhead (1975) had already employed this chromatographic technique to successfully separate mono- and oligo-saccharides, and a wide range of abused drugs. But it was Alpert who first named this chromatographic mode as "HILIC" in 1990. Since then, HILIC has gained a great deal of attention as demonstrated by the exponential growth of the number of published papers on this technique (Figure 1).

Several factors seem to contribute to the increasing interest on HILIC. First, HILIC nicely complements RP-HPLC, NP-HPLC and ion chromatography (IC) for separating hydrophilic compounds, thus solves many of the previously difficult separation problems and meets the increasing need to determine polar components in various complicated matrices. Figure 2 illustrates the relationship between HILIC and other three major HPLC techniques. Second, HILIC separations are easy to combine with electrospray ionization-mass spectrometry, which is becoming a common detector in many analytical fields such as clinical and bioanalytical applications. The ACN–rich eluents typical of HILIC lower surface tension, thereby facilitating spray formation and improving ionization efficiencies leading to a gain in detection sensitivity. The third attractive feature of HILIC is its orthogonality to RPLC, which make them an excellent combination for 2-dimensional HPLC separation.

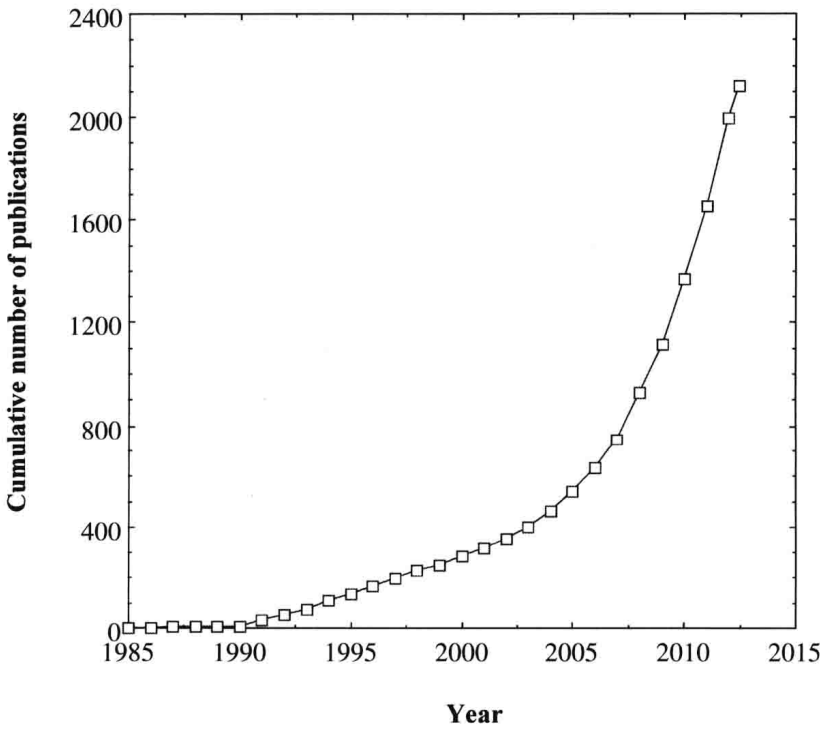

Figure 1. Cumulative number of publications in hydrophilic interaction chromatography indexed on Web of Science.

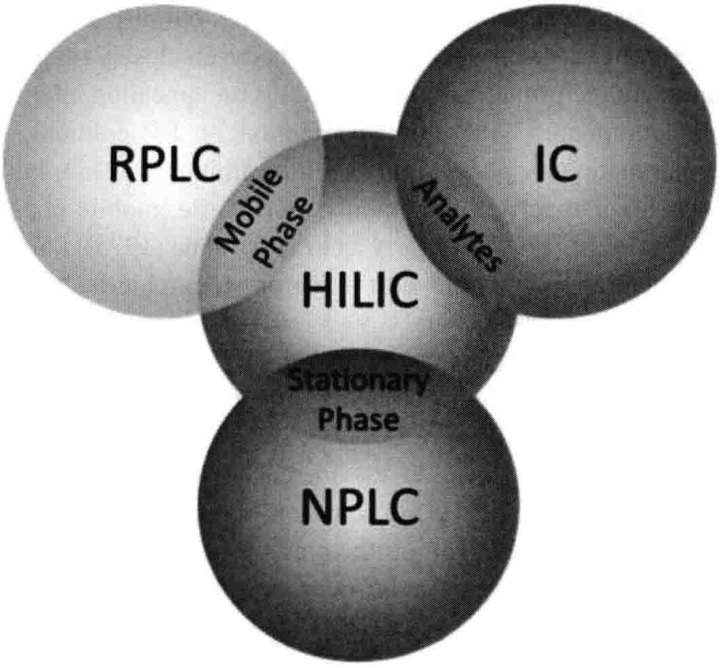

Figure 2. Relationship between HILIC and other three major liquid chromatographic techniques.

The orthogonality between HILIC and RPLC also renders advantages in sample preparation using solid phase extraction (SPE) or liquid extraction. The eluates from RPLC-SPE, as well as organic extracts and supernatants from protein precipitation and liquid-liquid extraction, can be directly injected onto an HILIC column without the solvent mismatching problem with the mobile phase and resulting in chromatographic peak shape deterioration. Similarly aqueous eluates from HILIC-SPE can be directly injected onto a RPLC column. Thus, the time-consuming evaporation and reconstitution steps during the sample pretreatment are eliminated. This chapter discusses the principle, stationary phases, mobile phases, and retention mechanisms of HILIC first, and then presents a few case studies to demonstrate the applications of HILIC in bioanalytical, food, forensic, environmental, metabolomics and pharmaceutical research.

PRINCIPLE AND RETENTION MECHANISMS OF HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY

Like in other HPLC techniques, the understanding of HILIC retention mechanisms and theories has been behind the practice. HILIC uses a polar stationary phase such as silica, cyano, or amino and an aqueous organic mobile phase. In this regard, HILIC may be viewed as a variant of normal-phase liquid chromatography, occasionally called aqueous normal phase chromatography, but uses reversed-phase type mobile phases, commonly consisting of acetonitrile and water or an aqueous buffer. In the 1970s and the early 1980s, the retention mechanism in HILIC was considered mainly due to the adsorption of analytes on the polar surface functional groups of the stationary phase, like in the NP-HPLC (Palmer, 1975; Majors, 1980, Boumahraz et al., 1982). However, in the 1980s, experimental evidences,