



Fundamental Investigations of Retention and Adsorption in LC with Emphasis on Charged Solutes

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Abstract

Reversed-phase liquid chromatography (RPLC) is widely used for the separation of organic solutes in pharmaceutical analysis. However, many drugs are weak organic acids or bases and exist in charged forms under typical RPLC conditions, which may lead to poor separation, low retention, and peak tailing. This thesis develops mechanistic, adsorption-based models to describe the retention of charged and ionizable solutes under various chromatographic conditions, using adsorption studies to elucidate the underlying retention mechanisms. These approaches can be used to address separation challenges in multivalent oligonucleotides and peptides, a growing class of therapeutics.

In **Paper I**, an analytical method was developed to quantify triethylamine and tributylamine in aqueous-organic mixtures by converting them to their uncharged volatile forms and measuring them by gas chromatography. This method was then used to obtain their adsorption isotherms on a RPLC stationary phase using a batch method.

In **Paper II**, an electrostatic retention model and a competitive adsorption model were developed to describe the *pH*-dependent retention and overloaded elution of charged and uncharged solutes in RPLC and mixed-mode chromatography. In **Paper III**, a mechanistic ion-pair RPLC model was developed by combining ion-pair reagent adsorption, surface potential, and surface ion-pair formation, enabling prediction of the retention of charged and ionizable solutes as a function of mobile phase *pH* and ion-pair reagent concentration.

In **Paper IV**, adsorption energy distribution (AED) analysis was extended from single-component to two-component, enabling visualization of competitive adsorption. **Paper V** identified key methodological limitations, including concentration range, choice of kernel function, and numerical convergence, while simultaneously demonstrating a clear relationship between peak tailing and AED.

Keywords: Reversed phase liquid chromatography, Ion-pair chromatography, Mixed-mode chromatography, Adsorption isotherm, Adsorption energy distribution.

Sammanfattning

Omvänd fasvätskekromatografi (RPLC) är en vanligt använd separationssteknik för organiska analysobjekt inom farmaceutisk analys. Många konventionella läkemedel är svaga organiska syror eller baser och förekommer därför i laddad form under typiska RPLC-betingelser, vilket kan leda till bristfällig separation, låg retention och toppsvansning. Denna avhandling utvecklar mekanistiska, adsorptionsbaserade modeller för att beskriva retentionen av laddade och joniserbara analysobjekt under varierande kromatografiska betingelser, med hjälp av adsorptionsstudier för att klarlägga de underliggande retentionsmekanismerna. Dessa angreppssätt kan användas för att hantera separationsutmaningar hos multivalenta oligonukleotider och peptider, en växande grupp av läkemedel.

I **Artikel I** utvecklades en analysmetod för kvantifiering av trietylamin och tributylamin i vatten-organiska blandningar genom omvandling till deras oladdade, flyktiga former och efterföljande analys med gaskromatografi. Metoden användes därefter för att bestämma deras adsorptionsisotermer på en RPLC-stationär fas.

I **Artikel II** utvecklades en elektrostatisk retentionsmodell och konkurrerande adsorption isoterm för att beskriva *pH*-beroende retention och överladdad eluering av laddade och oladdade analysobjekt i RPLC och multimodal kromatografi. I **Artikel III** utvecklades en mekanistisk jonpar-RPLC-modell genom att kombinera jonparreagensadsorptionen, ytpotentialen och jonparbildningen, vilket möjliggjorde förutsägelser av retentionen av laddade och joniserbara analysobjekt som en funktion av den mobila fasens *pH* och jonparreagenskoncentrationen.

I **Artikel IV** utvidgades analysen av adsorptionsenergifördelningar (AED) från ett- till tvåkomponentsystem, vilket möjliggör visualisering av konkurrerande adsorption. **Artikel V** identifierade centrala metodbegränsningar, inklusive koncentrationsintervall, val av kärnfunktion och numerisk konvergens och visade samtidigt ett tydligt samband mellan toppsvansning och AED.

List of Papers

This thesis is based on the following papers, hereafter referred to by their Roman numerals I–V.

- I. A. Haseeb, M. Rova, J. Samuelsson, Method development for the acquisition of adsorption isotherm of ion pair reagents Tributylamine and Triethylamine in ion pair chromatography, *J. Chromatogr. A.* 1687 (2023) 463687. <https://doi.org/10.1016/j.chroma.2022.463687>
- II. A. Haseeb, M.X. Fernandes, J. Samuelsson, Modelling the pH dependent retention and competitive adsorption of charged and ionizable solutes in mixed-mode and reversed-phase liquid chromatography, *J. Chromatogr. A.* 1730 (2024) 465058. <https://doi.org/10.1016/j.chroma.2024.465058>
- III. A. Haseeb, M. X. Fernandes, J. Samuelsson. Modeling the Combined Effect of pH and Ion-pair Reagent Concentration on the Retention of Charged and Ionizable Analytes in ion-pair RPLC. Manuscript
- IV. A. Haseeb, Y. Wondmagegne, M.X. Fernandes, J. Samuelsson, Introducing the Adsorption Energy Distribution Calculation for Two-Component Competitive Adsorption Isotherm Data, *Anal. Chem.* 97 (2025) 1966–1971. <https://doi.org/10.1021/acs.analchem.4c04663>
- V. A. Haseeb, Y. Wondmagegne, M.X. Fernandes, J. Samuelsson, Adsorption energy distributions: Theory and applications in liquid chromatography, *J. Chromatogr. Open.*(2025) 100252. <https://doi.org/10.1016/j.jcoa.2025.100252>

My contributions to the papers included in the thesis were:

- I. **Paper I:** I carried out most of the planning, performed all experiments, collected and organized the data, determined the selection, analysis, and presentation of the results, conducted most of the calculations, and wrote the paper together with the co-authors.
- II. **Paper II:** I did most of the planning, performed all the experiments, collected and organized the data, determined the selection, analysis, and presentation of the results, conducted part of the calculations, and wrote the paper with the co-authors.
- III. **Paper III:** I was responsible for most of the study planning, performed all the experiments, collected and organized the data, planned and designed the presentation of the results, conducted part of the calculations and wrote the paper with the co-authors.
- IV. **Paper IV:** I was part of the planning, collected and organized the data, interpreted the results and wrote the article with the co-authors.
- V. **Paper V:** I was part of the planning, interpreted the results and wrote the article with the co-authors.

Paper not included in the thesis.

- VI. A. Haseeb, B. Mesic, J. Samuelsson, Fundamental Investigation of the Adsorption of Antimicrobial Agents on Modified Calcium Carbonate and Silica and Its Potential Application in Food Packaging, *Adv. Mater.* 2024;13(1):1-9.
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Abbreviations

1D-AED	One-component AED
2D-AED	Two-component AED
AED	Adsorption energy distribution
BA	Benzoic acid
BAL	Benzyl alcohol
BTEAC	Benzyl triethylammonium chloride
GC	Gas chromatography
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High performance liquid chromatography
IPC	Ion-pair chromatography
IPR	Ion-pair reagent
IP-RPLC	Ion-pair reversed-phase liquid chromatography
IUPAC	International union of pure and applied chemistry
LC	Liquid chromatography
MBA	2-methylbenzyl alcohol
ME	Metoprolol
MMC	Mixed-mode chromatography
NPLC	Normal phase liquid chromatography
PA	Phenylbutyric acid
PE	2-phenylethanol
PP	3-phenyl-1-propanol
PROP	Propranolol
RPLC	Reversed phase liquid chromatography
SA	Salicylic acid
SFC	Supercritical-fluid chromatography
TBuA	Tributylamine
TEtA	Triethylamine
TPrA	Tripopylamine

Symbols

α	Fraction of solute or IPR present in charged form
B	A lump constant, depends on the dielectric constant of solvent, pore radius of the stationary phase and temperature
C	Solute concentration in the mobile phase
F	Faraday's constant
H	Henry's constant
K	Association equilibrium constant
k	Retention factor
q	Solute concentration in the stationary phase
q_s	Monolayer saturation capacity
R	Universal gas constant
T	Absolute temperature (K)
t_0	Void time
t_R	Retention time
z	Charge of solute or ion-pair reagent
ϕ	Phase ratio (the ratio between the volume of the stationary phase to the volume of the mobile phase)
Ψ	Surface potential

Table of contents

ABSTRACT	1
SAMMANFATTNING	2
LIST OF PAPERS	3
ABBREVIATIONS	5
SYMBOLS	6
TABLE OF CONTENTS	7
ACKNOWLEDGEMENTS	9
1. INTRODUCTION	12
1.1 Chromatography as a central separation technique	12
1.2 Challenges in the separation of charged and ionizable solutes..	14
1.3 Why charged and ionizable solutes matter	16
1.4 Adsorption and phase system heterogeneity	17
1.5 Aim of the study	19
2. ADSORPTION	20
2.1 Adsorption of charged solutes	23
2.2 Competitive adsorption of charged solutes	25
2.3 Competitive adsorption in ion-pair chromatography	27
2.3.1 Case A: Adsorption of charged solutes in the presence of a fully charged IPR.	27
2.3.2 Case B: Adsorption of charged solute when the IPR is fully uncharged	28
3. RETENTION INVESTIGATION AND MODELLING	29
3.1 Manipulation of mobile phase pH	29
3.1.1 Effect of organic solvents on pH and pK _a	29
3.1.2 Impact of pH on retention of ionizable solutes	31
3.2 Mixed mode chromatography	33
3.3 Ion-pair RPLC (IP-RPLC)	35
4. ADSORPTION ENERGY DISTRIBUTION (AED)	37
4.1 Theoretical framework	37
4.2 Numerical estimation	40
4.3 Competitive adsorption heterogeneity	41
5. DISCUSSION OF PAPERS	43
5.1 Paper I	43
5.2 Paper II	46

5. 3 Paper III..... 50
5.4 Paper IV54
5. 5 Paper V.....56
6. CONCLUDING REMARKS AND FUTURE PERSPECTIVES 60
REFERENCES 63

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1. Introduction

In both nature and human-made products, the analyte of interest is rarely found in a pure, isolated form and is typically present within a complex mixture of other components. For example, in medical testing, markers such as cholesterol, glucose, and hormones are measured in complex matrices like blood, plasma, or urine that also contain proteins, salts, lipids, and many metabolites [1]. Likewise, toxins in foods, pollutants in the environment, and active pharmaceutical ingredients in the final pharmaceutical products all sit within their own matrices alongside many other substances [2,3]. This is especially true for natural products from plants, microbes, and animals, where bioactive compounds occur within complex extracts and must be isolated before study or use [4].

Most common detectors used in analysis like UV–VIS, refractive index, conductivity, and fluorescence, do not read a molecule's unique identity; they measure general properties that several compounds can share in the mixture. As a result, signals from different species can overlap in a mixture. Even detectors that provide structural information, such as nuclear magnetic resonance spectroscopy and, to some extent, mass spectrometry, require pure compounds for reliable structure elucidation. Consequently, in real samples, direct identification and quantification are often not possible without prior separation. Separation is therefore a central and indispensable process in analytical science, including quality control, environmental monitoring, clinical diagnostics, and chemical manufacturing.

1.1 Chromatography as a central separation technique

Chromatography is one of the most widely used separation techniques in separation science [5–8]. International Union of Pure and Applied Chemistry (IUPAC) defines chromatography as “a physical method of separation in which components to be separated are distributed between two phases, one of which is stationary (the stationary phase) while the other (the mobile phase) moves in a definite direction” [9].

Chromatography separates mixture components because each component/solute distributes differently between the mobile and stationary

phase. As the mobile phase flows through the column (stationary phase), solutes interact differently with the stationary phase. Due to these differences in interaction, they travel at different average speeds depending on their affinity for the two phases. This variation in migration causes the components to separate along the column. As each component reaches the detector at the column outlet, it produces a distinct peak. The time at which a peak appears, its retention time, is characteristic of the compound under those conditions and can be used for its identification and quantification.

Chromatographic techniques are commonly classified according to the physical state of the mobile phase into liquid chromatography (LC), gas chromatography (GC), and supercritical-fluid chromatography (SFC), as described in IUPAC analytical nomenclature and classification guidelines [10,11]. In LC, the mobile phase is a liquid while the stationary phase is a solid adsorbent (liquid–solid chromatography) or a liquid film immobilized on a solid support (liquid–liquid chromatography) [12–14]. (B) In GC, the mobile phase is an inert carrier gas, while the stationary phase is either a solid adsorbent (gas–solid chromatography) or a liquid film coated on a solid support (gas–liquid chromatography) [12,15]. (C) In SFC, the mobile phase is generally a combination of gas and liquid, most commonly carbon dioxide modified with small amounts of organic solvents, while the stationary phase is either a solid adsorbent or a liquid film coated on a solid support, similar to GC and LC [16,17].

Among these techniques, LC, commonly referred to as high-performance liquid chromatography (HPLC), is the most widely used for analytical separations and is also routinely employed in preparative applications requiring high-purity compounds. Its widespread use is attributed to its high resolution and efficiency, versatility, and broad applicability across pharmaceuticals, biological fluids, food analysis, environmental samples, and industrial products. In addition, HPLC is relatively easy to operate, and the instrumentation is widely available, reliable, robust, and cost-effective [18,19].

In HPLC, reversed-phase liquid chromatography (RPLC) is by far the most widely used mode, accounting for more than 90% of all HPLC separations, especially in pharmaceutical and food industries,

biomedical laboratories, and life sciences [18,20–22]. There are several reasons for its popularity and widespread use. First, RPLC is a versatile technique which can separate a wide range of compounds from small organic molecules to large peptides and proteins [8]. Second, the stationary phases (RPLC columns especially C18 phases) are highly stable, reproducible, universally available and regulated in pharmacopeias. Third, methods developed in RPLC are robust, transferable and can be easily validated for quality control in pharmaceuticals. Fourth, the mobile phases used are generally compatible with the commonly used detectors such as UV, fluorescence, refractive index, and mass spectrometric detection. Fifth, mobile phases are typically simple (water–organic mixtures), and gradient elution is straightforward, allowing retention and selectivity to be easily adjusted making the methods simple and highly flexible. Sixth, Samples in pharmaceutical formulations, biological fluids, food and environment are generally aqueous and are compatible with the mobile phase in RPLC. There are other advantages such as availability of stationary phases in different chemistries (e.g., C18, C8, phenyl, polar-embedded) which gives it more flexibility. Importantly, it can be easily scaled from analytical to preparative mode for purification of intermediate and pharmaceuticals [23].

1.2 Challenges in the separation of charged and ionizable solutes

Despite the advantages mentioned above, RPLC is not well suited for the analysis of charged and ionizable solutes or very polar solutes such as monosaccharides and oligosaccharides. In RPLC the stationary phase is nonpolar (typically C18 alkyl chains bonded to silica), while the mobile phase is polar (water–organic mixtures). Charged and ionizable solutes interact weakly with the stationary phase and are therefore carried by the mobile phase, eluting near the void volume. This results in low retention, poor resolution (with peaks eluting close together), and often reduced efficiency (broader peaks). In addition, secondary interactions with residual silanol groups on silica, especially for basic solutes can lead to peak distortions such as tailing or fronting, which further degrades the column performance [24–26]. Large-molecule drugs such as oligonucleotides and many peptides, which carry multiple charges and are strongly hydrated, exhibit limited hydrophobic (solvophobic) partitioning, leading to low retention and limited selectivity under conventional RPLC conditions [27,28].

To improve their separation, two general approaches can be considered. The first is to employ alternative chromatographic modes more suitable for polar or ionic compounds, such as ion-exchange chromatography, hydrophilic interaction liquid chromatography (HILIC), or normal-phase liquid chromatography (NPLC) [29]. While these methods can be effective in specific cases, they are often less robust and reproducible than RPLC, and their stationary phases may lack stability over a broad pH range or be sensitive to moisture and small variations in mobile phase composition.

A second, more practical approach is to retain the advantages of RPLC described above while adopting strategies that enhance the retention and separation of charged and ionizable solutes within this framework. In this thesis, three different approaches are presented to investigate and model the retention of charged and ionizable solutes in RPLC (**Papers I–III**). Mixed-mode chromatography (MMC) is employed in **Paper II**, while ion-pair chromatography (IPC) is used in **paper III**. In both **Paper II** and **III**, the mobile phase pH adjustment approach was also used to investigate and model the retention of these solutes in RPLC, serving as a reference.

While these approaches can substantially improve retention and separation, their effective application depends critically on an understanding of the underlying retention mechanisms. Consequently, a mechanistic understanding of retention in these systems is essential for rational method development and predictive modeling [30].

Several mechanisms have been proposed to explain retention in ion-pair reversed-phase liquid chromatography (IP-RPLC). Early work introduced the dynamic ion-exchange model [31], which assumes that ion-pair reagents (IPRs) adsorb onto the stationary phase, causing the column to act as a dynamic ion exchanger and enabling retention through an ion-exchange mechanism. Horváth later proposed the ion-pair formation model [32], in which charged solute ions and IPRs form ion pairs in the mobile phase that subsequently adsorb as an uncharged complex onto the reversed-phase stationary phase. Schill and co-workers proposed the ion-pair adsorption model [33,34], in which ion pairs are adsorbed at the stationary phase surface without specifying whether pairing occurs in the mobile phase or at the surface. In

contrast, electrostatic models based on Stern–Gouy–Chapman theory, pioneered by Cantwell and subsequently extended by other researchers [35,36], describe retention in terms of an electrical double layer formed by adsorbed IPRs and counterions, generating a surface potential that governs the electrostatic interaction with charged solutes.

In contrast to IP-RPLC, MMC employs stationary phases that combine reversed-phase and ion-exchange functionalities, leading to retention governed by the concurrent contribution of multiple interaction types [8,22,37]. Accordingly, retention in MMC has been described using mechanistic models that incorporate the combined contributions of these interactions and is discussed in **Paper II**. Adjustment of the mobile phase *pH* represents a more conventional and widely applied strategy to modulate solute ionization in RPLC and is therefore treated here as a complementary tool rather than a distinct chromatographic mode.

1.3 Why charged and ionizable solutes matter

Charged and ionizable solutes are of critical importance across a wide range of scientific and applied fields. A vast majority of approved small-molecule drugs are either weak acids or bases and are at least partially present in charged form at physiological *pH* [38,39]. The ionization status of these drug molecules depends on their pK_a values and the *pH* of the surrounding medium and influences key properties such as solubility, permeability, stability and other biopharmaceutical characteristics [40,41].

Similarly, large-molecule drugs such as oligonucleotide- and peptide-based therapeutics carry multiple charges, making their separation particularly challenging under conventional RPLC conditions. Beyond pharmaceuticals, many other important substances are charged or ionizable including amino acids, metabolites, peptides, and electrolytes across diverse matrices ranging from bacterial and plant extracts to mammalian tissues and human body fluids [42]. Because these solutes are often present at low concentrations and are sensitive to *pH*, ionic strength, and the organic modifier content of the mobile phase, their reliable analysis requires dedicated and mechanistically informed separation strategies.

In this thesis, three such strategies for addressing the challenges associated with charged and ionizable solutes in RPLC are investigated and modeled (**Papers I–III**).

1.4 Adsorption and phase system heterogeneity

In LC, retention and separation are governed by the distribution of solutes between the stationary and mobile phases which depend on their affinities for the two phases. Efficient separation is achieved when solutes are well resolved within a reasonable analysis time, producing sharp and symmetrical peaks. While adjustment of mobile phase composition and operating conditions are central to method development, such empirical optimization provides limited insight into the fundamental mechanisms governing retention.

A mechanistic understanding of chromatographic behavior requires explicit consideration of adsorption processes [43]. Under typical analytical conditions, solutes often occupy only a small fraction of the available adsorption sites, preferentially interacting with the high energy sites on the stationary phase, while low energy sites remain largely unoccupied. As a result, analytical scale separations probe only a narrow portion of the interaction landscape [44,45]. Adsorption isotherm studies conducted over a broad concentration range are therefore necessary to characterize both strong and weak interactions, providing deeper insight into the properties of the separation system and the mechanisms underlying retention [14,45,46].

Adsorption is inherently a heterogeneous process that depends on the combined properties of the solute, stationary phase, and mobile phase, i.e., the chromatographic phase system [43,47,48]. Chromatographic stationary phases are intrinsically heterogeneous and contain multiple types of interaction sites with different adsorption energies. This heterogeneity arises from factors such as residual silanol groups, elemental impurities (e.g., boron and iron), strained surface atoms with unsatisfied valences, non-uniform ligand coverage and orientation, structural cavities within the bonded layer, irregular pore geometry, and batch-to-batch differences in ligand density [48–53]. A single solute can interact with different sites on the stationary phase surface in multiple ways, each interaction characterized by a different adsorption

strength (adsorption energy). This phenomenon is referred to as adsorption heterogeneity [49,54].

Importantly, adsorption heterogeneity is not solely a property of the stationary phase surface but rather a characteristic of the entire chromatographic phase system, encompassing the solute the mobile phase, and the stationary phase [52,55]. A given stationary phase may exhibit homogeneous adsorption behavior for one solute while displaying heterogeneous adsorption for another [56]. Moreover, the same stationary phase can exhibit different types of adsorption heterogeneity for different solutes. Even for a single solute, changes in mobile phase composition can modify the apparent heterogeneity of the same stationary phase surface [57,58]. This indicates that adsorption heterogeneity is governed by the combined influence of solute chemistry, surface characteristics, and mobile-phase conditions.

From a chromatographic perspective, adsorption heterogeneity manifests as peak tailing, reduced efficiency and resolution, excessive retention of cationic solutes, unpredictable retention behavior, limited column loading capacity, and decreased sensitivity in analytical chromatography [52,54,59,60]. In preparative chromatography, heterogeneity produces broad and asymmetric elution profiles with long tails, thereby reducing process productivity [14].

Adsorption processes are generally modeled using adsorption isotherms, and a variety of isotherm models are available to describe equilibrium adsorption [14,61]. However, achieving a good fit to a particular isotherm model does not necessarily provide reliable information about heterogeneity within the chromatographic phase system. To address this limitation, adsorption energy distribution (AED) analysis can be employed as a dedicated tool to directly characterize heterogeneity in adsorption energetics. In this thesis, the theoretical foundation, mathematical formulation, and physicochemical interpretation of AED are presented, together with its role in visualizing adsorption heterogeneity, retention mechanisms, and peak asymmetry in LC (**Paper V**). In addition, the one-component AED (1D-AED) framework is extended to competitive adsorption processes to enable visualization of heterogeneity under competitive conditions (**Paper IV**).

1.5 Aim of the study

The overall aim of this thesis is to develop a fundamental understanding of retention and separation of charged and ionizable solutes in LC by investigating the physicochemical principles governing their adsorption. The work progresses from experimental characterization of adsorption processes, through mechanistic retention modelling, and finally, to energetic interpretation of adsorption heterogeneity using AED analysis.

A key objective is to develop mechanistic models capable of predicting the retention of charged and ionizable solutes in IP-RPLC and MMC (**Papers I–III**). An important component of this work is the development of a practical method for obtaining adsorption isotherms of the IPRs that lack chromophores and are difficult to study using conventional chromatographic methods with UV detection (**Papers I, III**), thereby providing a necessary experimental basis for subsequent modelling.

The thesis further develops an electrostatically modified competitive adsorption model to describe competitive adsorption between two charged solutes or one charged solute and one uncharged solute and applies it to simulate overloaded elution in reversed-phase and MMC (**Paper II**). Building on these competitive adsorption models, AED analysis is extended to competitive systems to enable simultaneous characterization of the energy distributions of two components (**Paper IV**). Finally, the thesis explores the theoretical foundation and mathematical formulation of AED and demonstrates its use in interpreting adsorption heterogeneity, retention mechanisms and peak tailing in LC (**Paper V**).

Together, these contributions link experimental adsorption measurements, mechanistic retention modeling, and energetic analysis, thereby advancing the understanding of the chromatographic behavior of charged and ionizable solutes in LC.

2. Adsorption

As discussed in the introduction, retention in chromatography depends on the interaction of solutes with the stationary phase surface. Since adsorption governs how strongly a solute interacts with the surface, it directly affects retention and selectivity. Understanding and characterizing adsorption behavior are therefore essential for predicting and controlling chromatographic performance [14].

The adsorption process for a given system is generally characterized by measuring adsorption isotherms and fitting the resulting data to appropriate isotherm models. An adsorption isotherm describes the relationship between the concentration of a solute in the mobile phase and the amount adsorbed on the stationary phase at equilibrium at a constant temperature. Several isotherm models have been proposed to describe adsorption behavior; among them, the simplest and most commonly used is the Langmuir model, which is given as [62]:

$$q(C) = q_s \frac{KC}{1 + KC} \quad (1)$$

where q is the concentration of solute in the stationary phase, C the concentration of solute in the mobile phase, K , the association equilibrium constant, and q_s , the monolayer saturation capacity. According to the Langmuir model, adsorption increases proportionally with mobile phase concentration at low coverage. As molecules progressively occupy the stationary phase surface and adsorption sites become limited, the rate of adsorption slows until a saturation plateau is reached (see Figure 1a). The model assumes that all adsorption sites are identical, there are no interactions between adsorbed molecules, and adsorption occurs in a single layer (monolayer).

The adsorption isotherm is related to the retention time of the solute as [63]:

$$t_R = t_0 \left(1 + \phi \frac{dq}{dC} \right) \quad (2)$$

Where t_R is the retention time of the solute, t_0 is the retention time of an unretained solute (void time), ϕ is the phase ratio (the ratio between the volume of the stationary phase to the volume of the mobile phase)

and dq/dC is the slope of the isotherm. At infinite dilution, adsorption is linear and the slope become constant. Under these conditions, Eq. (2) reduces to:

$$t_R = t_0 \left(1 + \phi \frac{dq}{dC} \right)_{c=0} = t_0 (1 + \phi q_s K) = t_0 (1 + \phi H) \quad (3a)$$

Eq. (3a) in terms of retention factor (k) can be expressed as:

$$k = \phi \left(\frac{dq}{dC} \right)_{c=0} = \phi q_s K = \phi H \quad (3b)$$

Where H is the Henry constant, representing the initial slope of the adsorption isotherm. This condition generally applies in analytical chromatography, where solute concentrations are low and injected volumes are very small. Under analytical conditions, all terms in Eq. (3 a-b) remain constant; therefore, the retention is independent of concentration as long as the isotherm remains in its linear range. This behavior is illustrated in the insets of subplots (b) and (d) in Figure 1, where retention remains constant and increasing concentration results only in higher peak heights and larger peak areas, irrespective of the isotherm type. In contrast, in preparative chromatography or under overloaded injection conditions, local solute concentrations are high, the isotherm slope varies with concentration, and both retention and peak shape become concentration dependent.

In nonlinear chromatography, both the retention and the shape of the elution profile depend on how the slope of the adsorption isotherm varies with concentration. For a type I isotherm, the slope is high at low concentrations and decreases as concentration increases. According to Eq. (2), this decrease in the slope leads to reduced retention at higher concentrations. Under overloaded conditions, this behavior produces a characteristic elution profile with a sharp front and a diffuse rear. This effect is illustrated in Figure 1a–b, where retention decreases with increasing concentration as the isotherm slope decreases.

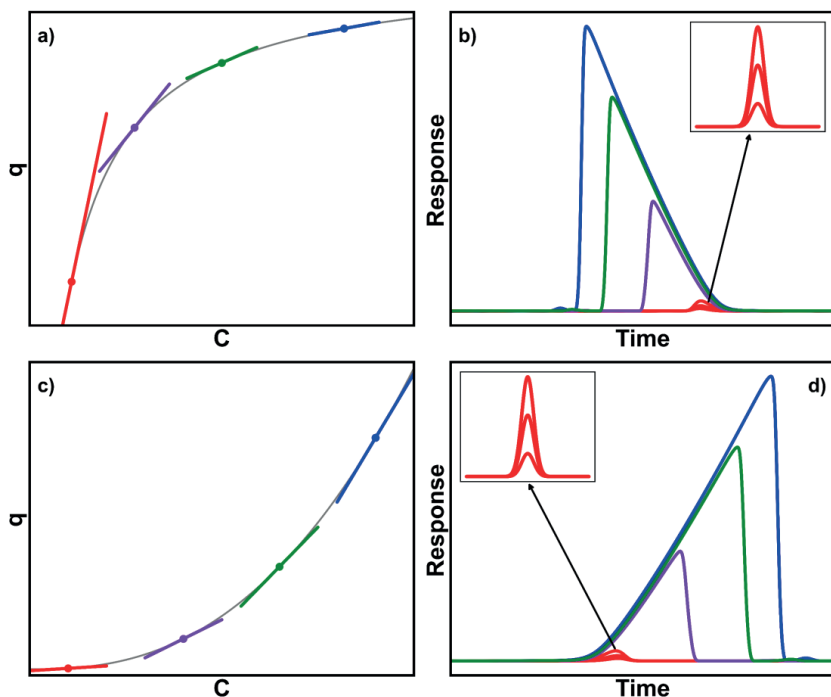


Figure 1. Influence of isotherm shape and slope on retention time and shape of elution profile. a) Type I isotherm showing slopes corresponding to different solute concentrations. b) Corresponding elution profiles and retention times for the slopes shown in a). c) Type III isotherm with slopes corresponding to different solute concentrations. d) Corresponding elution profiles and retention times for the slopes shown in c). The insets in b) and d) illustrate peak shapes and retention times when the isotherm remains in its linear region.

In contrast, Type III isotherms are characterized by low adsorption at low concentrations, followed by a progressively increasing isotherm slope as concentration rises. Under these conditions, retention increases with concentration. As a result, overloaded elution profiles exhibit a diffuse front and a sharp rear, which is markedly different from the behavior observed for Type I systems. This behavior is illustrated in Figure 1c–d.

2.1 Adsorption of charged solutes

When describing the adsorption of charged solutes, the assumptions underlying the Langmuir model are often inadequate [43]. In particular, as charged molecules adsorb onto the stationary phase surface, they repel incoming charged molecules through electrostatic interactions, resulting in reduced adsorption of incoming species. According to the electrostatic theory, charged molecules adsorbed on the surface form a charged layer that generates a surface potential. As more charged molecules adsorb, the surface potential increases, leading to stronger electrostatic repulsion for incoming charged solutes [64].

As a consequence, adsorption of charged solutes exhibits two sources of nonlinearity. First, adsorption is limited by the finite number of available sites, as described by the Langmuir isotherm. Second, electrostatic repulsion increases with increasing surface coverage due to the development of surface potential. Incorporating this electrostatic contribution into the Langmuir model leads to the electrostatically modified (or potential-modified) Langmuir isotherm, which is given by [65]:

$$q = q_s \frac{K C \exp\left(\frac{-zF\psi}{RT}\right)}{1 + K C \exp\left(\frac{-zF\psi}{RT}\right)} \quad (4)$$

Where ψ is the surface potential, T is the absolute temperature, R is the gas constant, F is Faraday's constant, z is the charge of the solute. The term $\exp\left(\frac{-zF\psi}{RT}\right)$ describes the electrostatic contribution to adsorption. In the absence of any external charges, electrostatic effects often lead to lower apparent affinity and a reduced slope for charged form of the solute under a given set of conditions. This behavior is illustrated in Figure 2 for phenylbutyric acid (PA) and metoprolol (ME) on an XBridge C18 column using aqueous buffers-methanol as mobile phase [44].

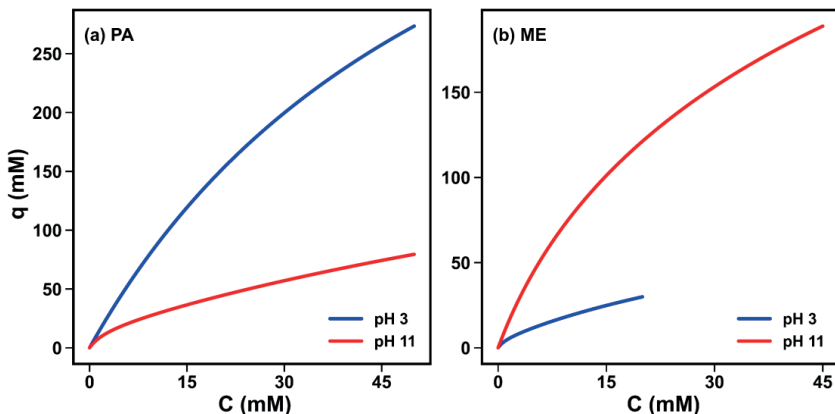


Figure 2. Adsorption isotherm of a) Phenylbutyric acid (PA) and b) Metoprolol at pH 3 and pH 11 on X-bridge C18 column using aqueous buffer-methanol as mobile phase

For PA, adsorption is higher at pH 3, where the compound is predominantly uncharged, than at pH 11, where it is charged, even though the pH 3 isotherm was measured at a higher methanol fraction (50%), which generally reduces adsorption, compared with 24% methanol at pH 11. Conversely, ME exhibits higher adsorption at pH 11, where it is predominantly uncharged, than at pH 3, where it is charged, despite the adsorption isotherm at pH 11 being obtained at a higher fraction of organic modifier [44]. These trends indicate that charged solute species exhibit reduced adsorption relative to their uncharged counterparts, primarily due to electrostatic effects, as discussed above.

In the adsorption isotherm given by Eq. (4), the surface potential ψ itself depends on the concentration of the adsorbed ions on the surface [66–68]. The exponential factor in Eq. (4) can be expressed by a simple expression, as employed by Ståhlberg [65,66]:

$$\exp\left(\frac{-zF\psi}{RT}\right) = \exp(-z^2Bq) \quad (5)$$

where B is a constant that depends on the dielectric constant of the eluent, pore radius of the stationary phase, and temperature [66]. Substituting Eq. (5) into Eq. (4) yields an implicit isotherm in which q depends on q through the exponential term. The implicit adsorption isotherm can be converted to an explicit form by considering the inverse of the adsorption isotherm (i.e., expressing C as a function of q).

$$C = \frac{q}{K(q_s - q)} \exp(z^2 Bq) \quad (6)$$

In **Paper II**, the adsorption isotherm of charged solutes propranolol (PROP), ME and benzyl triethylammonium chloride (BTEAC) were modeled using Eq. (6) while the adsorption isotherm for uncharged solute 3-phenyl-1-propanol (PP) was modeled using the Langmuir form (Eq. (1)). Similarly in **Paper III** is the adsorption isotherm of TBuA at pH 3 was modeled using Eq. (6) and its adsorption isotherm at pH 12 was modeled using Eq. (1).

2.2 Competitive adsorption of charged solutes

In chromatographic separations, adsorption is inherently a competitive process, as at least two components are always present and interact simultaneously with the stationary phase. Because the number of adsorption sites is limited, different molecules must compete for these sites based on their affinities for the stationary phase and their concentrations. As a result, the adsorption behavior of any single compound is influenced not only by its own properties but also by the presence of other solutes. This competition becomes especially important under overloaded conditions, where surface sites approach saturation.

One of the simplest descriptions of competitive adsorption is provided by the two-component Langmuir model, given by [14]:

$$q_i(C_1, C_2) = q_{s,i} \frac{K_i C_i}{1 + K_1 C_1 + K_2 C_2}, \quad i = 1, 2 \quad (7)$$

Where q_i is the concentration of solute 1 or 2 adsorbed on the stationary phase and C_1 and C_2 are their corresponding concentration in the mobile phase, K_1 and K_2 are the corresponding association equilibrium constants and q_s is the saturation capacity. Eq. (7) accounts for direct competition for a shared pool of adsorption sites but does not include electrostatic interactions and is therefore limited to uncharged solutes.

In systems involving charged solutes, adsorption becomes more complex because the interaction of each solute with the stationary phase depends not only on its own concentration and adsorbed amount, but also on the amount and charge of co-adsorbing species. In such

systems, adsorption of charged molecules generates an electrostatic surface potential that couples the adsorption behavior of all components.

In **Paper II**, a two-component competitive adsorption model for charged solutes was developed using a thermodynamic framework based on chemical potentials, in which electrostatic effects are incorporated through the surface potential of the stationary phase. The resulting adsorption isotherm model is given by:

$$q_i = q_s \frac{K_i \exp\left(\frac{-z_i F \psi}{RT}\right) C_i}{1 + K_1 \exp\left(\frac{-z_1 F \psi}{RT}\right) C_1 + K_2 \exp\left(\frac{-z_2 F \psi}{RT}\right) C_2}, \quad i = 1, 2 \quad (8)$$

Although this model is derived independently but is similar to the competitive Langmuir model with the addition that the electrostatic contributions of charged solutes are explicitly included. One advantage of the model is its flexibility: in the case where both components (1 and 2) are charged, Eq. (8) applies directly; if only one component is charged, the charge of the uncharged solute is simply set to zero; and if both components are uncharged ($z_1 = 0$, $z_2 = 0$) Eq. (8) reduces to the classical two-component competitive Langmuir isotherm given in Eq. (7).

As in the case of a single component isotherm, the surface potential (ψ) depends on the amount of charged solutes adsorbed to the stationary phase. One of the simplest descriptions of the exponential factors in Eq. (8) is as follows [64]:

$$\exp\left(\frac{-z_i F \psi}{RT}\right) = \exp(-B z_i \sum z_j q_j) \quad (9)$$

Inserting Eq. (9) into Eq. (8), the isotherm becomes implicit. Unlike the single-component electrostatically modified Langmuir isotherm, which can be inverted to obtain an explicit expression, this competitive form cannot be analytically inverted and therefore must be solved numerically. In **Paper II**, the competitive isotherm was used to simulate overloaded elution profiles of charged and uncharged solutes.

2.3 Competitive adsorption in ion-pair chromatography

In IPC an amphiphilic or lipophilic ion (an ion containing both a hydrophobic alkyl moiety and a charged group) is added to the mobile phase to enhance the retention of oppositely charged solute ions in RPLC [32,69,70]. The ion added to the mobile phase is called IPR and the technique is also referred to as IP-RPLC.

In IP-RPLC, solute retention is governed not only by the adsorption of the solute itself but also by the adsorption behavior of the IPR and its influence on the stationary phase surface. In **Paper III**, the adsorption and retention of charged solutes were investigated in the presence of tributylamine (TBUA) as the IPR. Because the ionization state of TBUA depends on the mobile phase pH , its adsorption varies with pH , thereby modulating both the surface potential and solute adsorption. To describe this behavior mechanistically, two limiting cases were considered: (A) fully ionized IPR and (B) fully uncharged IPR.

2.3.1 Case A: Adsorption of charged solutes in the presence of a fully charged IPR.

This case is conceptually related to the competitive adsorption of two charged solutes discussed in Section 2.2 (Eq. 8), but with important distinctions specific to IP-RPLC. In competitive adsorption of charged solutes without IPRs, the surface potential arises solely from the adsorption of the solute ions themselves. In contrast, in IP-RPLC the dominant contribution to the surface potential originates from the adsorption of the charged IPR, which effectively imposes an external charge on the stationary phase and promotes the adsorption of oppositely charged solutes.

In addition to electrostatic attraction between the solute and the charged surface, solute adsorption in IP-RPLC involves multiple concurrent interactions. These include the direct adsorption of the solute onto the stationary phase, as well as the formation of surface-associated ion pairs between the solute ions and adsorbed IPR molecules. These processes are represented through their respective chemical equilibria in the adsorption model.

The resulting competitive adsorption isotherm for a charged solute A in the presence of a fully charged IPR is given by:

$$q_A = q_{s,HB} \frac{K_A C_A \exp\left(-\frac{z_A F}{RT} \psi\right) + K_{HB} K_{HBA} C_A C_{HB} \exp\left(-\frac{z_{HB} F}{RT} \psi\right)}{1 + K_A C_A \exp\left(-\frac{z_A F}{RT} \psi\right) + K_{HB} C_{HB} \exp\left(-\frac{z_{HB} F}{RT} \psi\right) + K_{HB} K_{HBA} C_A C_{HB} \exp\left(-\frac{z_{HB} F}{RT} \psi\right)} \quad (10)$$

q_A is the concentration of solute A adsorbed on the stationary phase, K_A and K_{HB} are the association constants of solute A and IPR, respectively, while C_A and C_{HB} are their concentrations in the mobile phase. z_A and z_{HB} are the charges of the solute and IPR and $q_{s,HB}$ is saturation capacity of the solute or IPR. The saturation capacity for both species is kept the same for thermodynamic consistency.

Eq. (10) represents a competitive adsorption isotherm in which electrostatic interactions and surface ion-pair formation are explicitly incorporated. As in Section 2.2, the surface potential ψ depends on the total amount of charged species adsorbed and can be expressed using Eq. (9), rendering the isotherm implicit. Eq. (10) was used in **Paper III** to model the retention of sulfonate ions and charged carboxylic acids in IP-RPLC under conditions where the IPR is fully ionized.

2.3.2 Case B: Adsorption of charged solute when the IPR is fully uncharged

At sufficiently high pH, the IPR becomes uncharged and no longer contributes to the surface potential. Under these conditions, the adsorption behavior reduces to that of a charged solute competing with an uncharged solute, analogous to the case in which one solute is charged and the other is uncharged in Eq. (8) of Section 2.2. The corresponding adsorption isotherm is given by:

$$q_A = q_{s,B} \frac{K_A C_A \exp\left(-\frac{z_A F}{RT} \psi\right)}{1 + K_A C_A \exp\left(-\frac{z_A F}{RT} \psi\right) + K_B C_B} \quad (11)$$

Where $q_{s,B}$ is the saturation capacity IPR or solute. The saturation capacity is the same for thermodynamic consistency. Eq. (11) was used in **Paper III** in modelling the retention of sulfonates and acids at higher pH (≥ 10) when the IPR is fully uncharged.

3. Retention Investigation and Modelling

As discussed in the introduction, charged and ionizable solutes generally exhibit low retention and limited selectivity in RPLC. To improve the retention and separation of such solutes within the RPLC framework, several strategies can be employed, including manipulation of the mobile phase pH , adding charged functionality to reversed-phase system (MMC), and the use of IPRs as mobile phase additives (IP-RPLC). In this section, the theoretical background and modeling approaches used to describe retention of charged and ionizable solutes using these strategies are presented.

3.1 Manipulation of mobile phase pH

According to IUPAC, pH is defined as the negative logarithm of the activity of hydrogen ions in solution [9]. Since this definition depends on the activity of a single ion, which cannot be determined directly, it is considered an operational rather than an absolute quantity [71,72]. In practice, pH is determined potentiometrically using a pH -sensitive electrode (typically glass) in combination with a reference electrode (e.g., Ag/AgCl), following calibration with standard buffer solutions of known pH .

In LC, pH is a critical parameter because it governs the ionization state of weak acids and bases and, in the case of IPC, also affects the ionization of common IPRs such as TBuA and triethylamine (TEtA). Changes in ionization directly influence adsorption, retention, and selectivity.

3.1.1 Effect of organic solvents on pH and pK_a

In RPLC, mobile phases are typically prepared by mixing an aqueous buffer with an organic modifier such as methanol or acetonitrile. The addition of organic solvent alters both the pH of the buffer and the pK_a values of ionizable solutes and buffer components. These changes arise primarily from a decrease in the dielectric constant of the medium, which affects solvation and electrostatic interactions.

For an acidic buffer in aqueous solution, the dissociation equilibrium is:



Upon addition of organic solvent, solvation of charged species becomes less favorable due to the lower dielectric constant of the medium, destabilizing the dissociated ions (H^+ and A^-). As a result, the equilibrium shifts toward the undissociated acid, leading to a decrease in the acid dissociation constant, an increase in apparent pK_a , and a higher apparent pH in aqueous–organic mixtures compared to pure aqueous solutions. In **Paper II**, it was observed that a 50% (v/v) methanol–buffer mixture exhibited a pH at least one unit higher than the corresponding aqueous buffer. Similar effects were observed for acidic solutes: in **Paper III**, the pK_a values of three acids measured in 15% (w/w) acetonitrile were consistently higher than their aqueous pK_a values.

In case of a basic buffer, the equilibrium in aqueous system is:



Addition of organic solvent destabilizes the charged base (BH^+), shifting the equilibrium toward the unprotonated form and lowering the apparent pK_a of the conjugate acid. Consequently, basic buffers and solutes typically exhibit lower apparent pK_a values with increasing organic modifier content. In **Paper III**, the pK_a of TBuA in 15% (w/w) acetonitrile was determined to be 9.62, compared to 10.89 in water. Evidence further suggested that the effective pK_a at the stationary phase surface was even lower, possibly due to adsorption of acetonitrile within the bonded phase, altering the local solvent environment. Similar reductions in pK_a were observed for basic solutes PROP and ME in 50% (v/v) methanol in **Paper II**.

In order to take this into account, three different approaches are used to measure the pH of aqueous-organic mixtures (mobile phases). In the first approach, the electrodes are calibrated in standard aqueous buffer solution and then the pH of the aqueous buffer is measured using this electrode before mixing it with organic solvent. This system of pH measurement is referred to as ${}^w_w pH$ scale. However, because addition of

organic solvent alters the pH , this value does not represent the true pH of the final mobile phase.

In the second approach, the electrode is calibrated in the same solvent composition in which the mobile phase is prepared and then the pH is measured in this solvent composition. This method of pH measurement is referred to as $^s pH$ scale. While this best reflects the true pH , it is difficult to implement in practice due to challenges in buffer preparation, electrode stability, and the lack of commercially available standards for mixed solvents.

In the third approach, the electrode is calibrated in aqueous standards buffers and the pH is measured in the aqueous-organic mixture referred to as $^w pH$ scale. This is a more practical approach as the standards are widely available, and the pH approximately reflects the true pH of the mobile phase.

Numerous studies have shown that using the pH of the final mobile phase rather than the aqueous buffer pH leads to more reliable chromatographic results. Therefore, the $^s pH$ scale was used consistently in this thesis (**Papers II–III**). In **Paper I**, this effect was discussed in relation to ionization of the IPRs, TBuA and TEtA, at higher acetonitrile fractions, although the mobile phase pH was not explicitly controlled.

3.1.2 Impact of pH on retention of ionizable solutes

In RPLC, buffered mobile phases are used to control solute ionization and thereby modulate retention [22]. Depending on the pH of the mobile phase, a weak acid or base may be present in a fully charged, fully uncharged, or partially ionized form. A weak acid is predominantly uncharged when the pH is at least two units below its pK_a and predominantly charged when the pH is at least two units above its pK_a . When pH of the mobile phase is between these two limits an acid is partially charged. Now the retention of the acid as a function of pH can be formulated as follows:

$$k_{\text{acid}} = k_{\text{HA}}\alpha_{\text{HA}} + k_{\text{A}}\alpha_{\text{A}} \quad (14)$$

Where k_{acid} is the retention factor of the acid in the given pH , k_{HA} is the retention of the acid when it is fully uncharged, α_{HA} is the fraction of

the uncharged acid, k_A is the retention of the acid when it is completely charged α_A is the fraction of the charged acid.

The fraction of uncharged acid (α_{HA}) and charged acid (α_A) can be estimated from the Henderson-Hasselbalch equation. The fraction of charged acid is given as: $\alpha_{HA} = 1/1 + 10^{(pH-pK_a)}$ while the fraction of uncharged acid is given as: $\alpha_A = 10^{(pH-pK_a)}/1 + 10^{(pH-pK_a)}$. By inserting the values of α_{HA} and α_A in Eq. (14) yields:

$$k_{\text{acid}} = \frac{k_{HA}}{1+10^{(pH-pK_a)}} + \frac{k_A 10^{(pH-pK_a)}}{1+10^{(pH-pK_a)}} \quad (15)$$

By taking a common denominator, the classical Horváth model for the retention of acids as a function of mobile phase pH is obtained [73].

$$k_{\text{acid}} = \frac{k_{HA} + k_A 10^{(pH-pK_a)}}{1+10^{(pH-pK_a)}} \quad (16)$$

In a similar fashion, the retention of bases can be formulated as:

$$k_{\text{base}} = \frac{k_B + k_{BH} 10^{(pK_a-pH)}}{1+10^{(pK_a-pH)}} \quad (17)$$

Where k_{base} is the retention of the base in a given pH , k_B is the retention of the base when it is completely uncharged and k_{BH} is the retention of the base when it is completely charged. In this case, the base will be completely uncharged when the pH of the mobile phase is at least two pH units above the pK_a of the base and the completely charged when the pH is at least two units below the pK_a of the base.

In **Paper II**, the retention of two weak bases, one charged and one uncharged solute across a range of pH in MMC and RPLC was investigated. The retention of the two bases was modeled in RPLC using Horváth model for bases Eq. (17). In **Paper III**, the retention of three weak acids, benzoic acid (BA), salicylic acid (SA), and PA, was investigated in RPLC. The retention behavior was well described by the Horváth model for acids (Eq. (16)), although the corresponding model fits were not explicitly shown in the paper. A typical representation of the retention of an acid, base, permanently charged and uncharged solutes with mobile phase pH in RPLC is given in Figure 3.

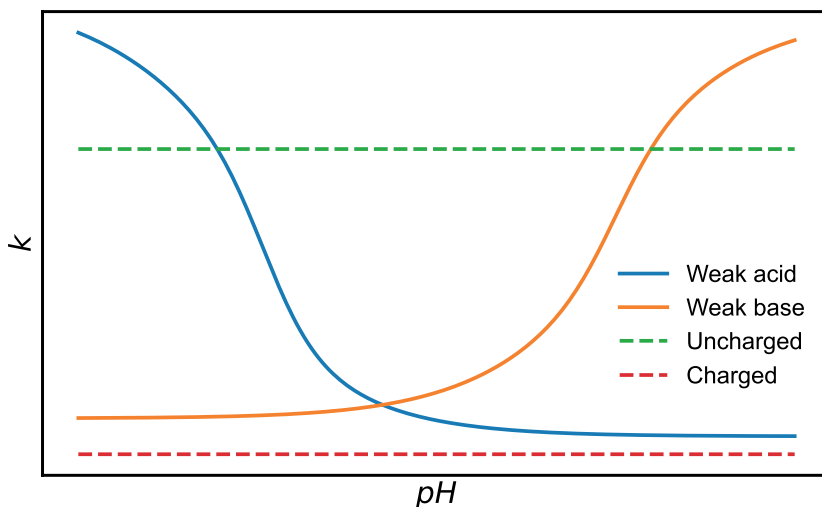


Figure 3. The variation in the retention of an acid, base, permanently charged and uncharged solutes with mobile phase pH in RPLC. The solid lines are the model fit according to Eq. (16) and Eq. (17) for acid and base respectively while dashed lines represent linear regression fits.

Figure 3 shows that permanently charged and uncharged solutes have retention that is essentially not affected by mobile phase pH . By contrast, the retention of weak acid and base show S-shaped curves (sigmoidal) with pH , with the biggest change around their pK_a values. Thus, small pH adjustments near the pK_a can cause large changes in retention, which helps separate solutes that are very similar but have slightly different pK_a values [72]. The same idea can be used in method development to increase retention of solutes that elute too early or to decrease retention of solutes that are held too long, simply by tuning the mobile phase pH .

3.2 Mixed mode chromatography

RPLC is the standard separation technique for a wide range of solutes; however, highly polar and charged compounds often exhibit insufficient retention and limited selectivity [8,74]. Alternative chromatographic modes such as NPLC, HILIC, and ion-exchange chromatography can improve retention for specific classes of solutes, but each has inherent limitations [8,75]. A particularly challenging situation arises when samples contain solutes with widely different polarities or

ionization characteristics, for which none of the above-mentioned chromatographic modes are suitable on their own.

MMC, also referred to as multimodal chromatography, addresses this issue by combining two or more dominant retention mechanisms within a single stationary phase. In MMC, solutes can interact with the stationary phase through multiple mechanisms simultaneously. Unlike RPLC, NPLC, or ion-exchange chromatography, where hydrophobic, hydrophilic, or ionic interactions respectively dominate, mixed-mode systems integrate two or more of these dominant interactions to achieve improved selectivity for complex samples.

In this thesis, **Paper II** investigates and models the retention of charged and ionizable solutes using an Acclaim Mixed-Mode WCX-1 column, a reversed-phase/weak cation-exchange material in which carboxylic acid groups are attached to the termini of C18 ligands (Figure 4).

The ionization state of these carboxyl groups depends on the mobile phase pH . At pH values at least two units above their pK_a , the groups are fully deprotonated and negatively charged; at pH values at least two units below the pK_a , they are fully protonated and uncharged. Between these limits, partial ionization occurs. Consequently, adjustment of the mobile phase pH provides direct control over the surface charge and thus the retention and selectivity of charged solutes.

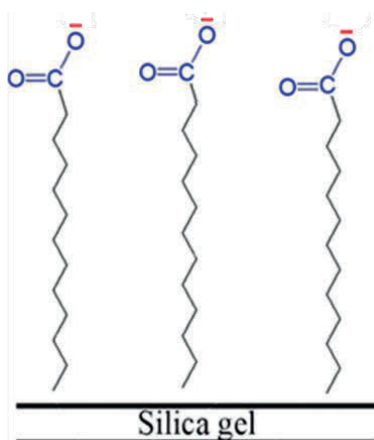


Figure 4. Acclaim Mixed-Mode WCX-1 column (reversed-phase/weak cation exchanger).

As the mobile phase pH increases, progressive deprotonation of the carboxyl groups on the mixed-mode stationary phase generates a negative surface potential that enhances the retention of positively charged solutes through electrostatic attraction, while hydrophobic interactions with the C18 ligands remain operative for both charged and uncharged

species. Accordingly, retention in MMC reflects the combined contribution of hydrophobic and electrostatic interactions, the relative importance of which is governed by solute ionization and the charge state of the stationary phase. In **Paper II**, this behavior is described using an electrostatic free-energy framework in which retention is expressed in terms of a reference adsorption free energy and a *pH*-dependent surface potential arising from ionization of the weak cation-exchange groups on the stationary phase. The full derivation and mathematical formulation of the retention model are presented in **Paper II**.

3.3 Ion-pair RPLC (IP-RPLC)

IP-RPLC is introduced here as a strategy to overcome the limited retention and selectivity observed for charged and highly ionizable solutes in conventional RPLC systems. By incorporating ion-pairing reagents into the mobile phase, IP-RPLC enables the effective separation of solutes that otherwise exhibit low retention or poor selectivity in RPLC [26]. The technique is particularly valuable for large, highly charged molecules such as oligonucleotides and peptides, as well as their impurities and metabolites, for which conventional RPLC often provides insufficient retention and selectivity [30–32].

Compared to conventional RPLC, IP-RPLC offers a broader range of adjustable mobile phase parameters for controlling retention and selectivity. These include the type and concentration of the IPR, mobile phase *pH*, organic-modifier, and ionic strength. This additional flexibility enables the separation of complex mixtures containing ionic, ionizable, and nonpolar compounds within a single chromatographic system [65].

The retention mechanism in IP-RPLC has been a matter of debate for decades. A short description of the different models has already been presented in section 1.2. In **Paper III**, retention in IP-RPLC is described within a mechanistic framework that combines electrostatic interactions with chemical equilibria. Using TBuA as the IPR, the model considers adsorption of the amphiphilic IPR onto the hydrophobic stationary phase, which leads to the formation of a positively charged surface layer. This adsorbed IPR layer generates a surface potential that

promotes retention of oppositely charged solutes through electrostatic attraction.

In addition to this electrostatic contribution, the model accounts for chemical equilibria involving (i) direct adsorption of the solute to the stationary phase and (ii) formation of surface-associated ion pairs between solute ions and adsorbed IPR molecules. Retention in IP-RPLC therefore reflects the combined influence of hydrophobic interactions, electrostatic effects, and ion-pair formation at the stationary phase surface.

As discussed in Sections 2.3.1 and 2.3.2, the retention behavior differs depending on whether the IPR is charged or uncharged. When the IPR is fully charged (low pH), the surface potential is dominated by adsorbed IPR ions, and retention of permanently charged solutes (e.g., sulfonates) is governed primarily by electrostatic interactions. At high pH , where the IPR is uncharged, the electrostatic contribution vanishes and retention is controlled by competition for adsorption sites and solute ionization. For weak acids, the ionization state of the solute must also be considered, resulting in pH -dependent retention behavior.

In contrast to MMC, where the surface potential arises from ionization of functional groups covalently bound to the stationary phase, in IP-RPLC the surface potential is generated by adsorption of the IPR from the mobile phase. This distinction leads to fundamentally different retention behavior and provides additional flexibility in tuning selectivity through mobile phase composition. The full derivation and mathematical formulation of the IP-RPLC retention models are presented in **Paper III**.

Together, pH manipulation, MMC, and IPC provide complementary strategies for controlling the retention and selectivity of charged and ionizable solutes in RPLC, through modulation of solute ionization and the introduction of electrostatic interactions.

4. Adsorption Energy Distribution (AED)

AED analysis is employed to explicitly describe and quantify energetic heterogeneity in chromatographic adsorption processes. Rather than representing adsorption by a single effective interaction energy, AED characterizes adsorption as a distribution of interaction energies, thereby capturing the presence of multiple adsorption sites or interaction modes experienced by a solute on a given stationary phase [76]. The primary aim of using AED analysis in this work is to provide a more physically realistic description of adsorption phenomena and to elucidate how energetic heterogeneity influences retention behavior and peak shape.

In Sections 2 and 3, adsorption and retention were analyzed using mechanistic models in which adsorption is described by an average or effective interaction energy for a given chromatographic phase system. While these models successfully describe equilibrium adsorption and concentration-dependent retention, they do not capture differences in adsorption strength caused by surface heterogeneity. As discussed in Section 1.4, this average description may be insufficient for chromatographic systems in which adsorption is energetically heterogeneous.

This limitation motivates the introduction of AED analysis in the present section. In LC, a single solute may interact with the stationary phase through multiple adsorption sites or interaction modes, each characterized by a distinct adsorption energy. AED analysis captures this behavior by representing adsorption as continuous or discrete distributions of interaction energies rather than a single effective value. In doing so, AED enables direct visualization and quantitative characterization of adsorption heterogeneity and its impact on retention behavior and peak asymmetry.

4.1 Theoretical framework

Traditional adsorption isotherms, such as the Langmuir model, assume uniform adsorption energy across all sites. In reality, as discussed above, adsorption often involves sites with different interaction strengths, leading to adsorption heterogeneity [77]. A straightforward way to account for this complexity is to represent the overall process as

a combination of multiple, independent Langmuir isotherms, each with its own saturation capacity and equilibrium constant (adsorption energy). This approach, referred to as the n -Langmuir model, is described as follows:

$$q(C) = \sum_{i=1}^n q_{s,i} \frac{K_i C}{1+K_i C} \quad (18)$$

Where n is the number of independent adsorption sites, $q_{s,i}$ is mono-layer saturation capacity of the i^{th} site, K_i is the association equilibrium constant for the i^{th} site. If $n=1$, we obtain the Langmuir model; $n=2$, the bi-Langmuir model; and for $n=3$, the tri-Langmuir model, and so on.

While n -Langmuir models allow adsorption heterogeneity to be represented using a small number of discrete site classes, they still impose a priori assumption about the number and nature of adsorption site. A more general and less restrictive approach is provided by AED analysis, which assumes that adsorption energies are continuously distributed rather than confined to a finite number of discrete values.

The AED formulation relies on several key assumptions. Adsorption is treated as the superposition of independent local adsorption processes occurring on a large number of energetically distinct sites. The adsorption energies are assumed to be continuously distributed over a physically relevant range, reflecting gradual variations in interaction strength. For a given energy, local adsorption behavior is described by a selected kernel function. Finally, the AED is constrained to be non-negative and sufficiently smooth, consistent with physically realistic surface heterogeneity. In the present work, smoothness is enforced implicitly through the Expectation–Maximization inversion procedure [45,48,78].

Within this framework, the experimentally measured adsorption isotherm $q(C)$ is expressed as:

$$q(C) = \int_D f(\ln K) \theta \, d \ln K \quad (19)$$

where $f(\ln k)$ is the AED (the unknown to be determined), and θ is the kernel function. The kernel function represents the local adsorption behavior at a specific adsorption energy. The domain D spans the

physically relevant range of adsorption energies, ensuring that all site contributions are included.

For Type-I isotherms, the Langmuir kernel is commonly used:

$$\theta(C, K) = \frac{KC}{1+KC} \quad (20)$$

With this kernel, AED becomes conceptually similar to bi- or tri-Langmuir models, but instead of representing adsorption using a small number of discrete site classes, AED describes a continuous distribution of adsorption energies.

AED provides a powerful visualization tool for adsorption heterogeneity by generating an energetic fingerprint of the chromatographic phase system. Figure 5a shows the Langmuir, bi-Langmuir, and Tóth isotherms, while Figure 5b presents their corresponding AEDs calculated using the Langmuir kernel function.

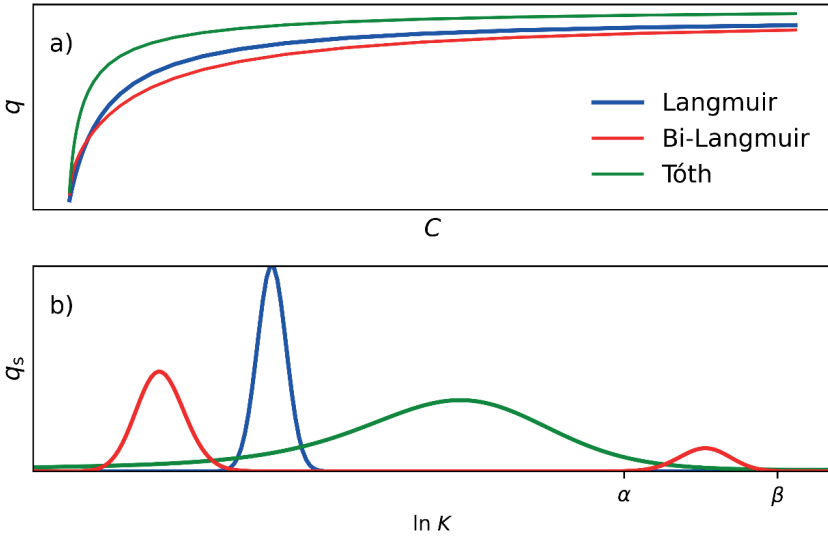


Figure 5. a) Schematic representation of adsorption isotherms for Langmuir (blue line), bi-Langmuir (red line), and Tóth (green line) models, b) Corresponding AEDs calculated using Langmuir kernel function.

Figure 5a shows the three isotherm models, which differ only slightly in curvature and therefore provide limited insight into the nature of adsorption. In contrast, the corresponding AEDs in Figure 5b clearly

reveal the underlying heterogeneity. The Langmuir isotherm produces a unimodal AED, indicating a single, uniform population of adsorption sites with similar energies indicating homogeneous adsorption. Conversely, the bi-Langmuir model yields a bimodal AED, showing two distinct types of sites with different adsorption energies, and therefore energetically heterogeneous adsorption. In contrast, the Tóth model yields a broad, asymmetric energy distribution, representing a surface with graded energetic heterogeneity in which adsorption-site strengths vary continuously rather than occurring as a few distinct site types.

In AED plot, the apex of each peak corresponds to the equilibrium constant of the adsorption site, while the area under the peak represents its saturation capacity. The saturation capacity is obtained by summing the q_s values across the relevant grid points after the AED calculation. In the bi-Langmuir case, the saturation capacity of the second site is determined by taking the sum from α to β as shown in Figure 5b.

In **Papers IV** and **V**, AED analysis was applied to Langmuir, bi-Langmuir, Tóth, and Jovanović isotherm models to visualize and interpret adsorption heterogeneity.

4.2 Numerical estimation

The AED cannot be measured directly; it must be recovered from the experimental isotherm using numerical inversion methods [78–80]. A commonly used technique in chromatographic applications is the Expectation–Maximization algorithm [78]. The procedure starts with an initial guess of the energy distribution (e.g., a uniform profile). A predicted isotherm is calculated from this guess and compared with the experimental data. The distribution is then iteratively updated to improve agreement until convergence is achieved.

In practice, the integral in Eq. (19) is converted into a discrete sum:

$$q_{\text{calc}}(C_j) = \sum_{i=1}^p f(\ln K_i) \cdot \theta(C_j, K_i) \Delta \ln K \quad (21)$$

where q_{cal} is the calculated stationary phase concentration for a given estimate of the distribution function. The energy space is divided into a uniform grid (p is the number of grid points) where the difference between two consecutive grid points is $\Delta \ln K$.

The AED in Eq. (21) is now iteratively updated:

$$f^m(\ln K_i) = f^{m-1}(\ln K_i) \sum_{j=1}^n \frac{q_{\text{exp}}(C_j)}{q_{\text{calc}}(C_j)} \theta(C_j, K_i) \Delta \ln K \quad (22)$$

f^m is the new energy distribution and, f^{m-1} is the previous AED estimate, m is the iteration step, q_{exp} is the experimental adsorption isotherm, q_{calc} is the calculated adsorption isotherm data and is updated in each iteration step using Eq. (21). The iteration is performed until the sum of the root mean square error between q_{calc} and q_{exp} is within a specified tolerance.

In **Papers IV** and **V**, this iterative approach was used to compute the 1D-AED for various adsorption systems using different kernel functions.

4.3 Competitive adsorption heterogeneity

Chromatographic separations are inherently multicomponent processes, where solutes compete for the same adsorption sites on the stationary phase. To describe this process, competitive adsorption isotherm models are used. However, competitive isotherms alone do not reveal how adsorption sites are shared or how different solutes distribute across the energy landscape of the surface.

The two-component AED (2D-AED) approach extends the 1D-AED framework to competitive systems by estimating energy distribution for each component simultaneously. It enables simultaneous characterization of the energetic heterogeneity experienced by two solutes competing for the surface. In this way, 2D-AED provides direct visualization of competitive adsorption phenomena.

The commonly used model for competitive adsorption is the competitive Langmuir model as presented in Eq. (7). Because each component is characterized by its own equilibrium constant, adsorption must be described as a joint function of the adsorption energies of both components. Consequently, the 1D-AED integral formulation (Eq. (21)) is extended to a two-dimensional energy space:

$$q_i(C_1, C_2) = \int_D f_i(\ln K_1, \ln K_2) \vartheta_i d\ln(K_1) d\ln(K_2) \quad (23)$$

D denotes a bounded region in the plane over which the integral is calculated and is related to the appropriate minimum and maximum possible energy values. In this case a competitive Langmuir model is used as a kernel function:

$$\vartheta_i(C_1, C_2, K_1, K_2) = \frac{K_i C_i}{1 + K_1 C_1 + K_2 C_2}, \quad i = 1, 2 \quad (24)$$

Just as the EM algorithm was used to recover the 1D-AED, the same maximum-likelihood framework was extended here to the two-component case. In the 2D-AED calculation, the energy domain is discretized into a grid in both $\ln K_1$ and $\ln K_2$, and the EM algorithm iteratively adjusts the estimated distributions f_1 and f_2 so that the calculated competitive isotherms match the experimental data.

In **Paper IV**, this approach was applied to competitive Langmuir, bi-Langmuir, and tri-Langmuir systems. The resulting 2D-AEDs, shown in Figure 6.

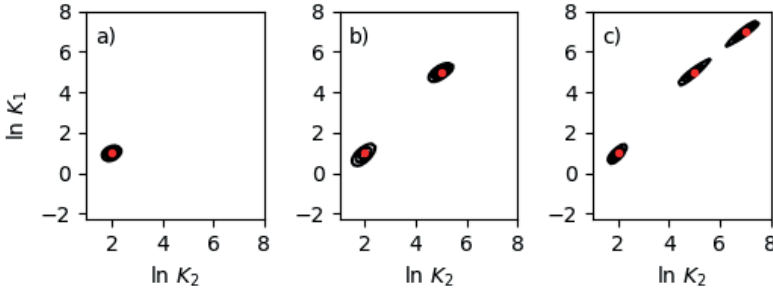


Figure 6. 2D-AED for one component of binary systems using competitive Langmuir model as kernel function. a) 2D-AED for one component of competitive Langmuir isotherm. b) 2D-AED for one component of competitive bi-Langmuir isotherm. c) 2D-AED for one component of competitive tri-Langmuir isotherm.

As shown in Figure 6, 2D-AEDs clearly demonstrate the presence of different adsorption sites, making the heterogeneity of the competitive adsorption process apparent. This visualization of different adsorption sites is not possible directly from isotherm plots. In this thesis, two-component AEDs for different competitive adsorption systems were calculated and presented in **Paper IV**.

5. Discussion of Papers

The studies included in this thesis are closely linked through a common focus on adsorption-based descriptions of chromatographic retention. **Paper I** establishes the experimental foundation by developing reliable methods for quantifying adsorption isotherms of IPRs. These measurements reveal complex, non-ideal adsorption behavior and provide experimentally determined adsorption parameters that serve as direct inputs for the mechanistic retention modeling in **Paper III**. **Papers II** and **III** further introduce competitive adsorption models to describe multicomponent systems. Building on this mechanistic framework, **Papers IV** and **V** extend the analysis to AEDs, providing tools to interpret adsorption heterogeneity and to validate increasingly complex adsorption process. Together, the papers form a coherent progression from experimental adsorption characterization to mechanistic modeling and energetic interpretation.

5.1 Paper I

IP-RPLC has become a gold standard method for the analysis of oligonucleotides [28,81–83]. Commonly used IPRs consist of alkylamines paired with acetate counter-ions; TEtA and TBuA are among the most frequently employed IPRs for oligonucleotide separations [82–85]. Since the adsorption of IPRs to the stationary phase plays a central role in governing retention and selectivity in IP-RPLC, an accurate characterization of this adsorption is essential for mechanistic understanding and quantitative modelling.

A major challenge in this context is the determination of adsorption isotherms for IPRs using conventional chromatographic methods. Trialkylamines such as TEtA and TBuA lack suitable chromophores, are highly polar, predominantly exist in charged form, and often exhibit severe peak tailing, which hinders reliable isotherm determination by standard LC techniques [86–88]. Although adsorption isotherms of tetraalkylammonium ions have been acquired using batch methods [24,89–91]. However, quantification of alkylamines in collected fractions typically requires time-consuming evaporation steps and/or expensive derivatization procedures, with detection based on picrate complex formation or GC using packed columns [90–92].

In **Paper I**, these limitations were addressed by developing and validating an analytical method for quantifying alkylamines in aqueous–organic mixtures. Alkylamines were first converted to their uncharged form using a strong base, followed by extraction into an organic phase and subsequently quantified by gas GC with flame ionization detection.

Due to the highly polar and reactive nature of primary, secondary and lower aliphatic tertiary amines, their performance on routine capillary GC columns is unsatisfactory. They interact with the free silanol group of the fused silica columns resulting in peak tailing, peak splitting or tend to adsorb and decompose on GC liners and columns, resulting in ghost peaks [93–95]. To overcome these issues, a wide-bore capillary column with a thick stationary-phase film (3 μm) and a base-deactivated inlet liner was used. Tripropylamine (TPrA) was used as an internal standard to account for any losses during extraction or injection. A representative chromatogram which shows optimum separation and no peak tailing for TEtA, TPrA and TBuA is shown in Figure 7.

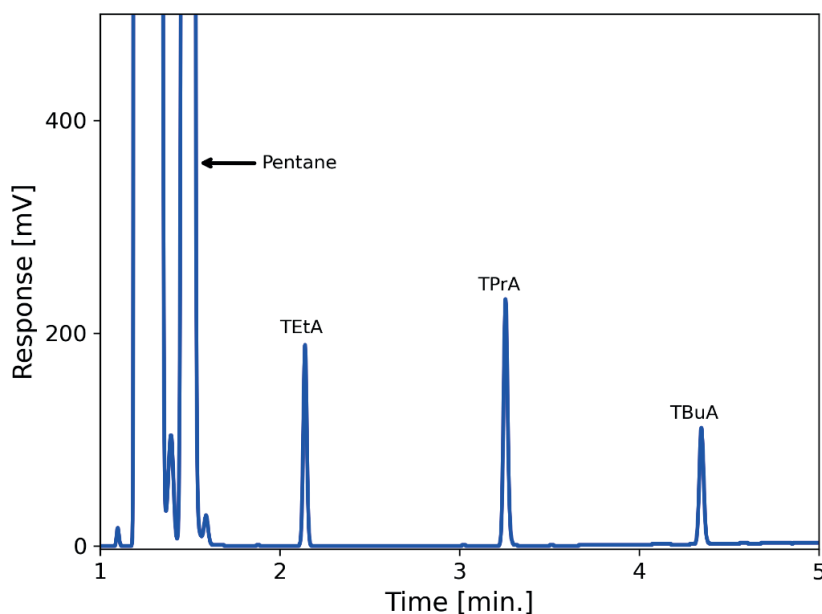


Figure 7. Chromatogram from the GC analysis of standard solution containing 0.400 mM TEtA, 0.300 mM TPrA and 0.100 mM TBuA.

This analytical method forms the basis of acquiring the adsorption isotherm of alkylamines using batch method, in which a C18 column is first equilibrated with a given concentration of IPR and subsequently stripped using an IPR-free eluent. The amount of adsorbed IPR is determined via mass balance from the stripped fractions using the above the developed GC method. A key methodological contribution of this work is the demonstration that the efficiency of the stripping step can be substantially improved by increasing ionic strength through the addition of sodium chloride. This finding highlights the importance of electrostatic interactions in IPR adsorption and allows the adsorption isotherms to be acquired using significantly reduced solvent volumes, improving both accuracy and practicality compared to earlier batch methods.

Using this approach, adsorption isotherms were determined for TBuA and TEtA under conditions relevant to oligonucleotide separations. Notably, the adsorption behavior of the two IPRs was found to differ qualitatively. TEtA exhibited a Type I (Langmuir-like) adsorption isotherm, consistent with monolayer adsorption and with previous reports for quaternary ammonium ions. In contrast, TBuA displayed a Type III (anti-Langmuir) adsorption isotherm, characterized by weak adsorption at low concentrations followed by a sharp increase at higher concentrations, suggesting cooperative or multilayer adsorption phenomena.

This observation is particularly significant, as most existing retention models in IP-RPLC implicitly assume Langmuir-type adsorption of the IPR [96]. The Type III behavior observed for TBuA indicates that such assumptions may not always be valid, especially under condition when the IPR may be present both in charged and uncharged form at the same time and at relatively higher content of organic modifier (70% v/v).

The experimental methods developed in this paper for acquiring adsorption isotherms were subsequently applied in **Paper III** to determine adsorption isotherms of TBuA under different mobile phase conditions. These isotherms provided the quantitative adsorption parameters required for the mechanistic IP-RPLC retention models, in which adsorption of the IPR constitutes a central model input rather than an

adjustable assumption. In addition, the methodology established in **Paper I** provides an essential foundation for the AED analyses presented in **Papers IV** and **V**. AED analysis relies on accurate adsorption isotherms acquired over sufficiently wide concentration ranges, and Paper I establishes practical strategies for obtaining such data for IPRs that are otherwise difficult to quantify. The observed non-ideal and heterogeneous adsorption behavior in **Paper I**, further motivates the use of energy-distribution based interpretations, which are formally developed and applied in the subsequent AED studies.

5.2 Paper II

This paper is divided into two parts. The first part focuses on developing a retention model based on electrostatic theory to predict the pH -dependent retention of charged and ionizable solutes in MMC and to compare this behavior with that observed in classical C18 RPLC. The second part presents the development of a competitive adsorption isotherm model for charged solutes, which is subsequently used to simulate overloaded elution profiles.

In the C18 RPLC system, the retention of uncharged solute (PP) is independent of mobile phase pH across the entire investigated range (pH 2–11.5), indicating unchanged hydrophobic interactions and constant mobile phase composition. Similarly, the retention of permanently charged solute (BTEAC) remains constant up to pH 10, demonstrating negligible electrostatic interactions with the stationary phase. At higher pH values, retention of negatively charged solutes decreases due to electrostatic repulsion from ionized residual silanol groups, indicating the onset of surface charge effects.

For weak bases such as ME and PROP, retention is low at acidic to neutral pH where the solutes are predominantly protonated. As the pH approaches their pK_a values, deprotonation increases hydrophobic interactions with the nonpolar stationary phase, resulting in a sharp increase in retention. Once fully neutralized, further increases in pH do not affect retention. Figure 8 summarizes these retention trends. The retention of weak bases PROP and ME were modeled using the classical Horvath model for bases (Eq. (17)).

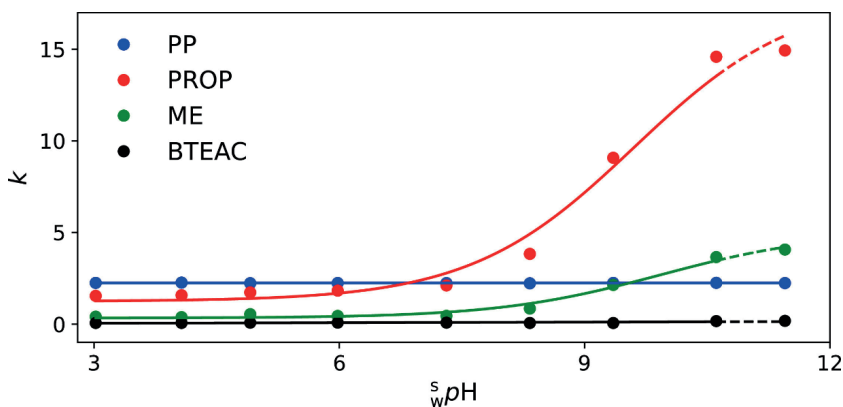


Figure 8: Retention factor for PP (uncharged), ME (weak base), PROP (weak base), and BTEAC (permanently charged) as a function of pH on the C18 column. Solid lines are model fit and symbols are experimental data. PP and BTEAC are modeled using linear regression and PROP and ME are modeled using Eq. (17).

The mixed-mode column used in this study is an RP/weak cation-exchange system containing carboxyl groups attached to the terminal of C18 ligands (see Figure 3 in section 3.2). In this system, pH affects both solute ionization and stationary phase surface charge. As the mobile phase pH increases, ionization of the carboxyl groups generates a negative surface potential, leading to strong electrostatic attraction of cationic solutes. Consequently, the retention of basic solutes on the mixed-mode column increases at substantially lower pH values compared to RPLC. The developed model, which combines solute ionization equilibria with a pH-dependent surface potential, successfully predicts this behavior across the investigated pH range. For the complete derivation of the electrostatic retention model see **Paper II**. The retention of these solutes and the developed surface potential with mobile phase pH is given in Figure 9.

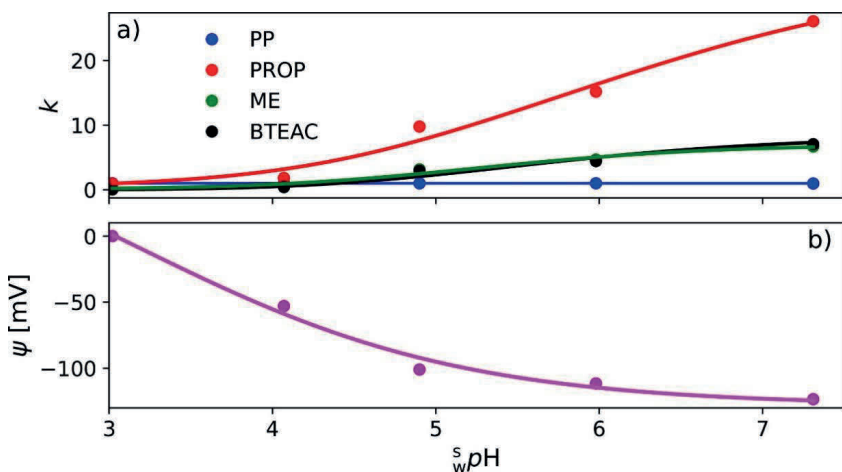


Figure 9. (a) Retention factor of PP, ME, PROP, and BTEAC as a function of mobile-phase pH on the mixed-mode column. Symbols represent experimental data, and solid lines represent predictions of the mechanistic retention model described in Paper II (b) Surface potential as a function of pH derived from the retention behavior of BTEAC shown in (a). Symbols represent experimental values, and the solid lines represent the corresponding model prediction in Paper II.

To gain deeper insight into the underlying adsorption mechanisms, single-component adsorption isotherms were acquired for all solutes using the elution by characteristic point method [46,97,98]. The results showed that uncharged solute followed classical Langmuir behavior, independent of pH and column type, whereas charged solutes required an electrostatically modified Langmuir model (Eq. (4)) to adequately describe their adsorption. The monolayer saturation capacity of the C18-WCX column was generally higher than that of the C18 column for both the charged and uncharged solutes leading to higher retention on this column. This could be attributed to the larger surface area of the C18-WCX column compared to that of the C18 column. In case of cationic solutes, the higher saturation capacity can also be attributed to the negative surface charges on the mixed mode column.

A major contribution of this paper is the derivation of a competitive adsorption isotherm model based on electrostatic theory, which explicitly accounts for electrostatic interactions of charged solutes. The model incorporates both competition for adsorption sites and electrostatic attraction or repulsion arising from already adsorbed charged species (Eq. (8)). Its validity was demonstrated by accurately

simulating overloaded elution profiles for several binary competitive systems. In all cases, strong agreement between simulated and experimental elution profiles confirms that the model reliably describes the underlying adsorption processes in competitive systems.

In most cases, for charged, uncharged, and ionizable solutes, the elution profiles exhibited normal shapes consistent with Type I adsorption isotherms on both C18 and mixed-mode columns. However, a markedly different behavior was observed for charged solutes on mixed-mode columns when the mobile phase pH ($\bar{s}pH \approx 4$) was close to the pK_a of the ionizable functional groups attached to the terminal ends of the C18 ligands. Under these conditions, the overloaded elution profiles were severely deformed, indicating a more complex adsorption process. An example of such behavior is shown in Figure 10a, where the overloaded elution profile of propranolol on a mixed-mode column deviates strongly from a conventional peak shape.

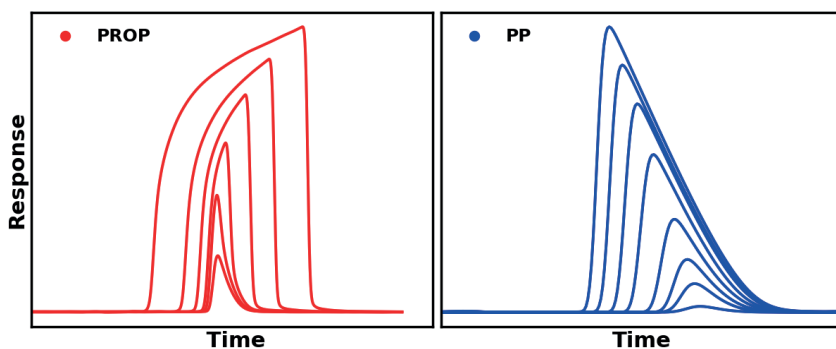


Figure 10. Overloaded elution profiles for PROP and PP on the mixed mode column at pH 4 ($\bar{s}pH$). a) Elution profiles of PROP at progressively increasing concentrations. b) Elution profiles of PP at progressively increasing concentrations.

Deformed overloaded elution profiles have previously been reported in RPLC for ionizable solutes at mobile phase pH values close to the pK_a of the solute [99] or due to difference in the pH of the diluent and eluent [100,101]. In such cases, the coexistence of charged and uncharged solute species or diluent-eluent pH mis-match lead to nonlinear and complex adsorption behavior, resulting in distorted peak shapes. In the present system, however, both ionizable and permanently charged solutes were present predominantly in a single charged form and the pH

of the eluent and diluent was the same. Instead, the complexity arises from the partial ionization of the surface functional groups on the mixed-mode stationary phase when operating near their pK_a . Under these conditions, the surface contains a heterogeneous population of charged and uncharged sites, which can lead to complex adsorption process and consequently, to deformed overloaded elution profiles.

This interpretation is further supported by the observation that the elution profiles of the uncharged solute PP remained symmetric and well behaved at the same pH (Figure 10b). Because the adsorption of uncharged solutes is not directly affected by the ionization state of the surface functional groups, no peak deformation was observed in this case. Together, these results suggest that overloaded peak deformation in MMC can arise not only from solute ionization, as commonly reported in RPLC, but also from partial ionization of the stationary phase surface. This finding highlights an additional mechanism by which pH -dependent surface chemistry can influence adsorption behavior and peak shape of charged solutes, and it warrants further systematic investigation.

5. 3 Paper III.

Paper III extends the scope of this thesis to IP-RPLC by developing a mechanistic retention model that captures the combined effects of mobile phase pH and IPR concentration on the retention of charged and ionizable solutes. The model integrates electrostatic surface potential effects arising from IPR adsorption, surface ion-pair formation between solutes and adsorbed IPR, and solvophobic interactions due to direct solute–stationary phase binding. Together, these contributions enable predictive retention modeling across a broad pH range (pH 2–12) and IPR concentrations (0–10 mM).

In this study, a conventional C18 column (XBridge) was used with TBuA as IPR and selected sulfonates and carboxylic acids as charged and ionizable solutes, respectively. This is relatively a complex system, in which pH not only affects the ionization of solute ions (acids) but it also affects the ionization of IPR. Change in IPR ionization alters its adsorption on the stationary phase, which in turn affects solute retention. Since the adsorption of charged and uncharged IPR differs substantially, two limiting IPR states were considered: fully charged and

fully uncharged. Similarly, solutes may be permanently charged (e.g., sulfonates) or exist in partially ionized, fully ionized, or unionized forms (weak acids). A schematic illustration of the resulting phase system and the relevant interactions is shown in Figure 11 for sulfonate ions.

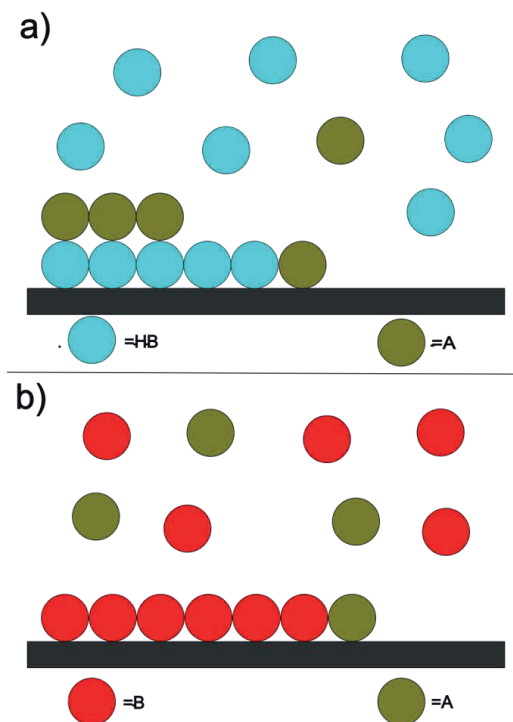


Figure 11. Description of the phase system for sulfonates in IP-RPLC. a) when the IPR is fully charged and b) when the IPR is completely uncharged. Green circles indicate charged sulfonate ions (A), blue circles represent charged IPR (HB), and red circles represent uncharged IPR.

In Figure 11a (low pH), the IPR is fully protonated and adsorbs as a positively charged layer (blue circles). Charged sulfonate ions (green circles) form ion-pair with adsorbed IPR and also directly bind to the stationary phase surface. All these interactions are represented by their own chemical equilibria.

In Figure 11b (high pH), the IPR is fully uncharged (red circles). Although uncharged TBuA adsorbs strongly, it does not generate surface charge and therefore cannot promote electrostatic retention. Instead,

it competes with sulfonates for surface sites. As a result, only a small fraction of sulfonate ions adsorbs, leading to markedly reduced retention.

A similar conceptual framework applies to acids, which may exist in charged, partially charged, or uncharged forms depending on pH , while the IPR may also be charged or uncharged. The full set of phase-system descriptions and the corresponding retention models for these limiting cases are presented in **Paper III**.

Increasing IPR concentration enhances sulfonate retention at low pH through surface potential effects and ion-pair formation at the stationary phase. At high pH , where IPR is uncharged, sulfonate retention decreases due to competitive adsorption between the solute and IPR. Since TBuA remains ionized up to approximately pH 7, its adsorption behavior is essentially constant within this range. Because sulfonates are permanently charged, their retention at a given IPR concentration is independent of pH up to 6.3. Accordingly, plotting sulfonate retention versus IPR concentration yields a single retention curve across different pH values (Figure 4a–b, Paper III). These findings indicate that the pK_a of TBuA in the stationary phase is lower than in the bulk mobile phase, likely due to the acetonitrile-rich adsorbed layer, as higher organic content reduces the observed pK_a of weak bases.

In contrast, for ionizable carboxylic acids, mobile phase pH directly affects solute ionization. Although IPR adsorption remains constant up to pH 7, acid retention varies with pH , resulting in distinct retention–IPR concentration curves at different pH values. This behavior is illustrated in Figure 6a–b of **Paper III** for the three acids examined. The adsorption of TBuA at pH 3 and pH 12 was acquired using the batch method described in **Paper I**.

In the developed model, it is assumed that solute ions interact with the IPR already adsorbed on the stationary phase to form ion-pairs on the surface, described by the equilibrium constant K_{HBA} . In this paper, K_{HBA} was found to increase with solute hydrophobicity, indicating that the interaction between solute ions and the adsorbed IPR is not purely electrostatic but includes a significant hydrophobic contribution.

In addition to retention modeling, **Paper III** reports, for the first time, systematic peak deformations under analytical conditions in IP-RPLC. While peak shapes generally improved with increasing IPR concentration, pronounced tailing, fronting, and peak splitting occurred at specific intermediate IPR concentrations and disappeared when the IPR level was either increased or decreased. These distortions were attributed to co-elution of solute peaks with system-perturbation peaks arising from disruption of the established IPR adsorption equilibrium upon sample injection. When solute and perturbation peaks propagated through the column at similar velocities, peak overlap occurred, leading to severe deformation. Representative chromatograms illustrating both peak improvement and deformation are shown in Figure 12.

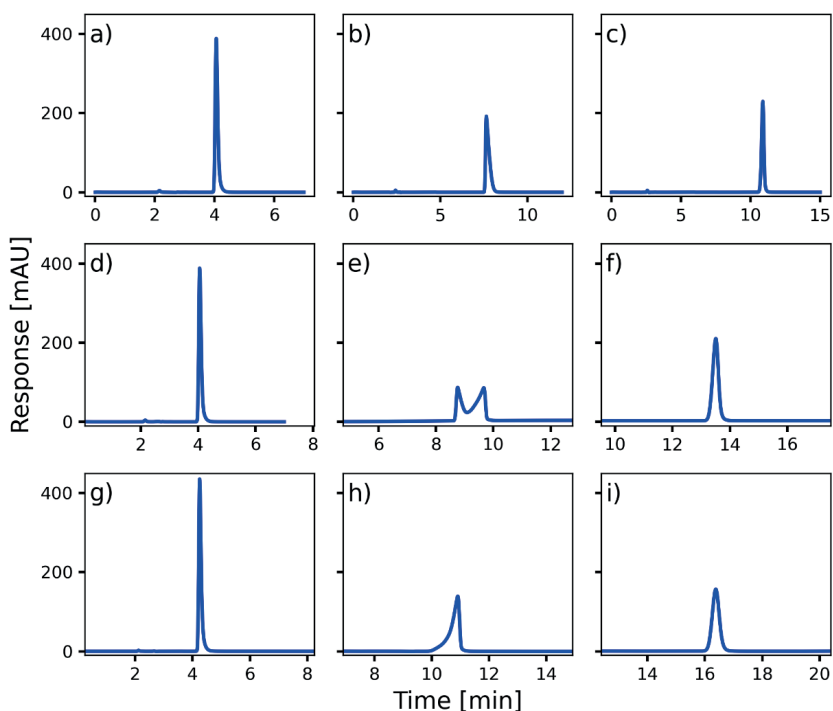


Figure 12. Chromatograms for some selected separations. In a-c) injection of para toluene sulfonate at pH 6 using eluents containing 0, 2, and 4 mM IPR, respectively. d-f) injections of salicylic acid at pH 6 using eluents containing 0, 2, and 4 mM IPR, respectively. g-i) injections of salicylic acid at pH 5 using eluents containing 0, 2, and 4 mM IPR, respectively.

5.4 Paper IV

1D-AEDs are effective for visualizing adsorption heterogeneity and assessing isotherm models but are limited to systems involving a single solute. In chromatographic separations, which are inherently multi-component processes, two or more solutes interact and compete for the stationary phase surface. Under such competitive conditions, 1D-AEDs cannot describe how adsorption sites and adsorption energies are shared between components. This limitation motivates the extension of AED analysis to multi-component systems. **Paper IV** therefore introduces a 2D-AED framework that enables quantitative visualization of adsorption heterogeneity under competitive conditions.

Paper IV first highlights the role of 1D-AEDs as diagnostic tools for visualizing adsorption heterogeneity, guiding isotherm model selection, and assessing whether adsorption isotherms have been acquired over a sufficiently wide concentration range. Building on this foundation, the AED concept is extended to two-component systems using competitive adsorption data.

The numerical framework used for 1D-AED was adapted to the competitive case by formulating the adsorption isotherm as a double integral over two adsorption-energy dimensions corresponding to the two equilibrium constants (Eq. 23). A competitive Langmuir isotherm was employed as the kernel function, enabling simultaneous visualization of heterogeneity for both components.

The 2D-AED approach was first validated using synthetic adsorption data generated from Langmuir, Bi-Langmuir, and Tri-Langmuir models, demonstrating that the method accurately resolves the number of adsorption sites, their energies, and saturation capacities (section 4.3).

The method was subsequently applied to experimental competitive adsorption data. For the methyl mandelate – ethyl mandelate binary system, the 2D-AED yielded single, unimodal distributions for both components, indicating that a simple competitive Langmuir model is sufficient to describe the adsorption process. However, the distributions were not fully resolved, suggesting that the adsorption isotherms were not acquired over a sufficiently wide concentration range. Despite this

limitation, the AED-derived parameters agreed well with experimental values, confirming the validity of the chosen isotherm model (Fig. 4 in **Paper IV**).

For other binary systems involving benzyl alcohol (BAL), 2-phenylethanol (PE), and 2-methylbenzyl alcohol (MBA), the 2D-AED analysis also produced unimodal energy distributions, suggesting that a single-site competitive model may describe the adsorption process. However, the broad and asymmetric nature of these distributions indicates significant adsorption heterogeneity. Although conventional nonlinear regression yielded statistically acceptable fits, the AED revealed that the underlying adsorption process was more complex and cannot be fully described by a simple competitive Langmuir model. This interpretation is consistent with the original authors' observations that their isotherm reduces to a competitive Langmuir form only under specific conditions [102]. This is indicated for the three binary systems in Figure 13.

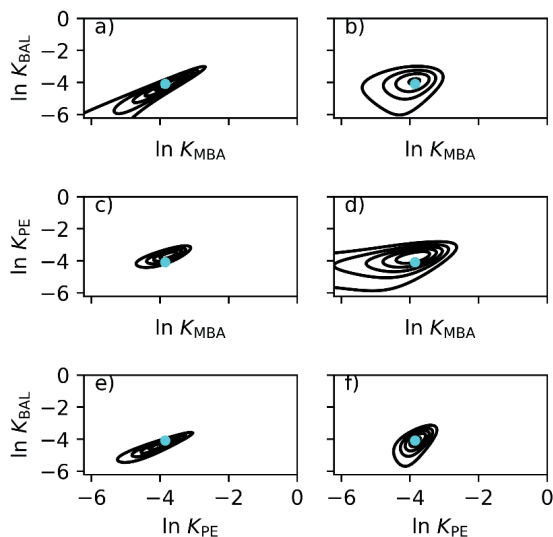


Figure 13. Calculated 2D-AED from adsorption data for system for a) BAL and b) MBA for the BAL/MBA system, c) PE and d) MBA for the PE/MBA system, and e) BAL and f) PE for the BAL/PE system. The cyan dots indicate the estimated K values from the model fit to competitive Langmuir model.

Together, these findings demonstrate the value of the 2D-AED as a mechanistic tool for visualizing adsorption heterogeneity and identifying when more advanced adsorption models are required.

5. 5 Paper V

Paper V provides a comprehensive discussion of the theoretical basis, mathematical formulation, and physicochemical interpretation of AEDs, and explains their role in describing adsorption heterogeneity, retention mechanisms, and peak asymmetry in LC. In addition to summarizing previous studies where AEDs were used to explain chromatographic separations, the paper introduces new perspectives and practical approaches that improve the application and interpretation of AED analysis in LC systems.

First, the importance of AED in revealing adsorption heterogeneity is highlighted by calculating AEDs for Langmuir, bi-Langmuir, and Tóth isotherm models, as discussed in detail in Section 4.1 and shown in Figure 5. These examples illustrate how different isotherm models correspond to distinct energetic landscapes, even when their macroscopic adsorption behavior appears similar.

The paper then presents key limitations and critical factors for reliable AED calculations. It shows that the concentration range of the adsorption isotherm data, together with the number of grid points and iterations, strongly influences AED resolution. In particular, insufficient concentration ranges can result in incomplete AEDs and may falsely suggest homogeneous adsorption.

This effect is illustrated in **Figure 14a** using a bi-Langmuir adsorption system. When the adsorption isotherm is acquired over a sufficiently wide concentration range (black curve), both high-energy sites, which dominate adsorption at low concentrations, and low-energy sites, which become relevant only at higher concentrations, are properly probed. The resulting AED is bimodal, with two well-resolved distributions corresponding to the high- and low-energy adsorption sites.

In contrast, when the isotherm is measured over a reduced concentration range (red curve), only the high-energy sites are adequately probed. The available data contains insufficient information to resolve the low-energy adsorption sites, causing the AED to remain underconstrained at low energy. This leads to an incomplete AED in which the low-energy site is not resolved, despite the underlying system being heterogeneous.

Figures **14b** and **14c** further demonstrate the influence of iteration number and grid resolution on AED shape and stability, emphasizing the importance of appropriate numerical settings for reliable AED interpretation.

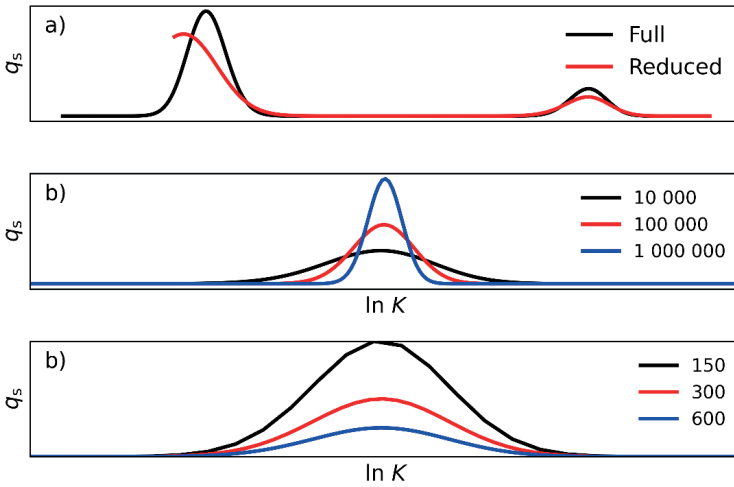


Figure 14. a) Effect of concentration range on AED derived from a bi-Langmuir adsorption isotherm. Using a reduced concentration range (red), the lower-energy adsorption site is not resolved compared to the full dataset (black). b) Effect of iteration number on AED calculation for a Langmuir model. c) Effect of the number of grid points on AEDs for the Langmuir model.

A major contribution of this work is demonstrating how the choice of kernel function in AED calculations affects both the shape and the parameters of the resulting AED. For a system described by a bi-Jovanović model, AEDs were calculated using both Langmuir and Jovanović kernel functions. Although both kernels produced bimodal AEDs, the Jovanović kernel yielded parameters that closely matched the true adsorption isotherm values, as indicated by the arrows in Figure 15a. In a second system described by the Tóth model, AEDs calculated using the Tóth and Langmuir kernels differed significantly. When the Tóth kernel was used, adsorption heterogeneity was incorporated directly into the kernel, resulting in a unimodal and symmetric AED (Figure 15c). In contrast, use of the Langmuir kernel caused the heterogeneity to be reflected in the shape of the AED itself (Figure 15b). In

both cases, the true equilibrium constant, indicated by arrows, aligns well with the Tóth model. These examples clearly highlight the importance of selecting an appropriate kernel function for reliable AED analysis.

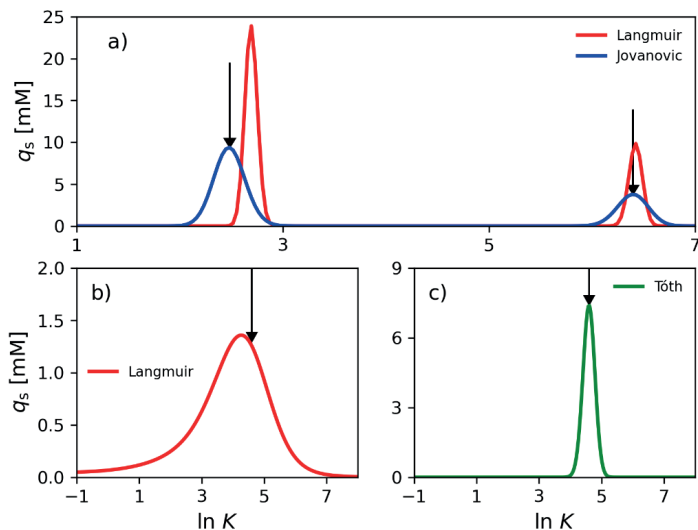


Figure 15. AED results for a) the bi-Jovanović and b-c) the Tóth adsorption isotherm. In a) the AEDs were calculated using both Langmuir and Jovanović kernel functions. In b), the AEDs were calculated using Langmuir and c) Tóth kernel functions. Arrows indicate the true K -values.

Beyond methodological aspects, a second and equally important contribution of **Paper V** is the quantitative and physically grounded explanation of chromatographic peak tailing. Using the AED framework, peak asymmetry was directly linked to adsorption heterogeneity rather than treated as an empirical peak-shape phenomenon. Contrary to the common view that residual silanols are inherently detrimental, the results show that high-energy adsorption sites associated with silanols can be beneficial when properly controlled. For systems described by a bi-Langmuir model, increasing the saturation capacity of the high-energy site reduces peak asymmetry (Figure 16a,c), while decreasing the energy difference between low and high-energy sites also improves peak shape (Figure 16b,d). This behavior is closely analogous to MMC, where charged functional groups are deliberately introduced to improve retention and separation of ionic solutes. Together, these results

establish peak tailing as a predictable consequence of adsorption heterogeneity and demonstrate that it can be quantitatively analyzed and systematically controlled using AED framework.

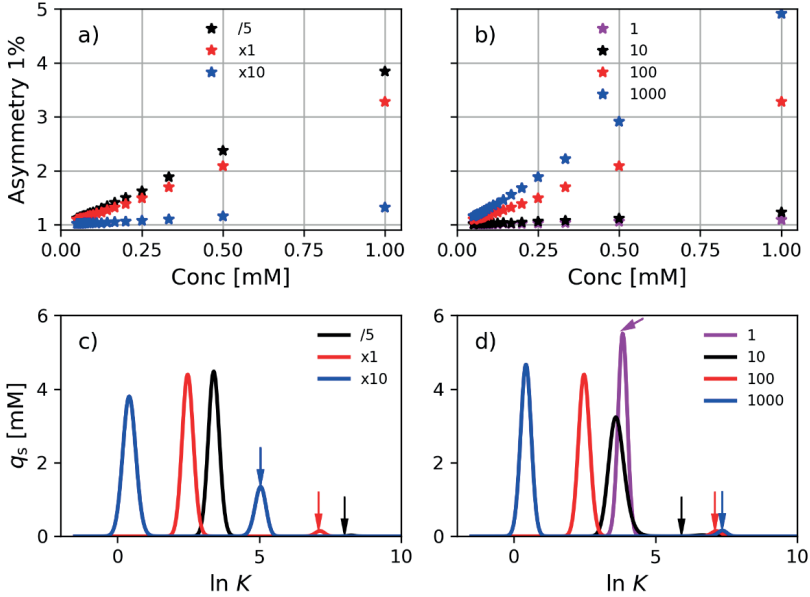


Figure 16.: Asymmetry factors calculated at 1% peak height are shown in (a) for varying saturation capacities of the second (high-energy) adsorption site: five times smaller ($/5$), equal ($\times 1$), and ten times larger ($\times 10$) than the original value; and in (b) for decreasing distances between the two adsorption sites, both plotted against sample concentration. The corresponding AEDs are shown in (c) and (d), respectively. Arrows in (c) and (d) indicate the adsorption energy of the high-energy site. All AEDs were calculated using 100,000 iterations, the Langmuir kernel function, and 600 grid points.

Overall, **Paper V** establishes AED analysis as a fundamental tool for understanding adsorption heterogeneity in LC. By integrating theory, numerical methods, visualization strategies, and mechanistic interpretation, the paper goes beyond a traditional review and provides practical guidance for calculating, interpreting, and validating AEDs. In addition, it highlights key limitations and practical considerations such as concentration range requirements, kernel selection, and numerical convergence, that are essential for the reliable use of AED analysis. These insights directly support the modeling strategies and experimental findings presented in the other papers of this thesis.

6. Concluding remarks and future perspectives

This thesis develops a mechanistic framework for understanding the retention of charged and ionizable solutes in RPLC, MMC, and IP-RPLC. In MMC and IP-RPLC, retention models based on electrostatic theory were established (**Papers II and III**), demonstrating how surface potential arising either from ionization of stationary phase functional groups or from adsorption of IPRs, governs the retention of charged solutes. To enable quantitative modeling in IP-RPLC, a practical analytical method was developed to measure adsorption isotherms of IPRs that lack chromophores and are difficult to quantify using conventional chromatographic techniques (**Paper I**). In addition, AED analysis was further developed and extended to multi-component systems (**Papers IV and V**), providing a quantitative framework for describing adsorption heterogeneity, competitive adsorption, and chromatographic peak asymmetry. Together, these contributions enable a physically grounded description of retention, adsorption and peak shape in LC.

A key experimental finding of this work is that adsorption of typical alkylamines used as IPRs is generally assumed to follow Type I (Langmuir-like) behavior, which was also observed in **Papers I and III** of this thesis. However, deviations from Langmuir behavior can occur under specific mobile phase conditions. In particular, adsorption of TBuA exhibited Type III (anti-Langmuir) behavior when the mobile phase pH was close to its pK_a and when high fractions of organic modifier (70 v% acetonitrile) were used (**Paper I**). This behavior is consistent with reports for ionizable solutes near their pK_a , where partial ionization and changes in solvation can lead to non-ideal adsorption. These results demonstrate that adsorption of alkylamine-based IPRs cannot always be assumed to be Langmuirian and is strongly influenced by mobile-phase pH and composition.

A central conceptual outcome of this thesis is the demonstration that chromatographic peak asymmetry is a direct and quantitative manifestation of adsorption heterogeneity (**Paper V**). Using the AED framework, peak tailing was linked to the distribution and strength of heterogeneous adsorption sites rather than to empirical peak-shape descriptions. The results show that residual silanol sites, traditionally viewed

as the primary cause of peak tailing, can be beneficial when properly controlled. Increasing the saturation capacity of high-energy silanol sites or reducing the energy difference between high and low-energy sites leads to more symmetric peaks. This behavior is closely related to MMC, where charged functional groups are deliberately introduced into C18 ligands to improve retention and selectivity for ionic solutes. These findings demonstrate that peak asymmetry is not an unavoidable defect of stationary phases, but a predictable and tunable consequence of adsorption heterogeneity.

The results further show that ion-pair formation between charged solutes and already adsorbed IPR in IP-RPLC cannot be described as a purely electrostatic process (**Paper III**). Instead, the strength of this interaction increases with solute hydrophobicity, indicating that surface ion-pair formation involves a significant hydrophobic contribution. This insight challenges the common assumption of purely electrostatic ion-pair interactions and has important implications for understanding selectivity and for rational choice of IPRs in IP-RPLC. Additionally, this study demonstrates that the pK_a of tributylamine in the stationary phase is lower than in the bulk mobile phase likely due to the acetonitrile-rich adsorbed layer, as higher organic content reduces the observed pK_a of weak bases.

Another important and previously unreported finding of this work is the systematic deformation of solute peaks observed in IP-RPLC under analytical conditions (**Paper III**). These distortions occurred only at specific IPR concentrations, where solute peaks co-eluted with, or eluted in close proximity to, system-perturbation peaks generated by disruption of the established IPR equilibrium upon injection. When the IPR concentration was increased or decreased from this specific value, the perturbation and solute peaks no longer overlapped, and normal peak shapes were restored. This direct correlation demonstrates that peak deformation arises from overlap with mobile phase perturbations propagating through the column, providing a mechanistic explanation for distorted peak shapes in IP-RPLC and highlighting the importance of accounting for such effects during method development.

In addition, severe peak deformation of charged solutes was observed under overloaded conditions in MMC at mobile phase pH values close

to the pK_a of the ionizable functional groups on the stationary phase (**Paper II**). Similar peak distortions have previously been reported in RPLC when operating near the pK_a of the solute, where coexistence of charged and uncharged species leads to complex adsorption behavior and distorted peak shapes. The present observations suggest that, in MMC, operating near the pK_a of surface functional groups may induce peak deformation due to partial ionization of the stationary phase surface and the resulting complex adsorption behavior. This hypothesis requires further experimental verification and represents an important topic for future studies.

Together, these results provide a coherent physical picture linking adsorption heterogeneity, electrostatic and hydrophobic interactions, and chromatographic peak shape. This framework can be applied to modern pharmaceutical separations, particularly for highly charged solutes such as therapeutic oligonucleotides and complex peptides, where severe peak tailing, distorted peak shapes, and limited selectivity remain major challenges [103]. The AED framework and the adsorption-based interpretation developed in this thesis provide practical tools for diagnosing these problems and guiding rational choices of stationary phase, mobile phase pH , and IPRs.

Future work should extend these concepts to biopharmaceutical systems. In particular, AED analysis should be applied to ion-pair separations of therapeutic oligonucleotides to quantify the energetic origins of peak tailing and selectivity. Integration of the mechanistic retention and competitive adsorption concepts would enable retention prediction and control of peak deformation. Further development of stationary phases with reduced non-specific adsorption sites and hybrid mixed-mode functionality, guided by AED-based characterization, offers a promising route to improving peak shape and resolution for highly charged molecules.

By providing a quantitative and physically grounded description of how adsorption heterogeneity and coupled electrostatic–hydrophobic interactions govern chromatographic behavior, this thesis establishes a foundation for the rational design and optimization of liquid chromatographic separations for current and emerging drug modalities.

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Fundamental Investigations of Retention and Adsorption in LC with Emphasis on Charged Solutes

Reversed-phase liquid chromatography (RPLC) is a widely used separation technique. However, it often provides inadequate retention and separation of charged and very polar solutes. These limitations arise from heterogeneous adsorption involving complex interactions between solutes and stationary phase surfaces.

This thesis focuses on two main areas. The first is the development of mechanistic retention models for such solutes in mixed-mode chromatography (MMC) and ion-pair chromatography (IPC), with RPLC serving as a reference technique (**Paper I–III**). In both separation modes, retention is governed by electrostatic surface potential, arising from charged surface groups in MMC and from adsorption of ion-pair reagents in IPC, together with hydrophobic interactions. Competitive adsorption models are developed and applied in retention modelling and simulations of overloaded elution profiles.

The second focus is adsorption heterogeneity and its impact on chromatographic behavior (**Paper IV–V**). Adsorption energy distribution (AED) analysis is extended to two-component systems to visualize competitive adsorption. For single-component AED, practical limitations and requirements are identified, and its roles in explaining peak tailing and retention mechanisms are established.

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