Study of the Quality of the Separation Process using New Chromatographic Conditions

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Introduction

Experimental chromatograms exhibit a wide range of variations when the composition of mobile and stationary phases undergoes changes. These variations serve as direct or indirect indicators of alterations in the adsorption or desorption properties of one or more colloidal components, as well as modifications in the structure of the semi-permeable material found within the adsorption (filtration) layer. In this context, we introduce the method of quality of separation assessment by presenting a specific example: the ultrafiltration of the commercially-available dye Eurofix Red 3 B. This process is transformed into a highly innovative system using electrochemically modified ultrafiltration ceramic silica carbons of SiWA type.

The planning and execution of scientific research heavily rely on the chosen methodology, initial observations, and the adoption of a certain theoretical model. Considering the case of research on ultrafiltration utilizing novel chromatographic conditions, we consider the recent crucial observations and experiences made by our team. The main objective of our study is to develop a reliable method for estimating the quality of separation of colloidal systems from suspensions that occur during the ultrafiltration process under these new chromatographic conditions. This estimation method involves determining the presence of peaks that reflect the position of the maximum within the experimental elution curve, often referred to as glide elution. The correlations observed among the individual components within the oligomulticomponent analytical mixture are particularly noteworthy. These correlations are characteristic of the sorption-desorption displacement processes and can be observed through the use of both retention in descending and elution in descending methods. In the case of changes in the nature of size exclusion or mechanical filtration, these correlations take the form of what is known as a Lorentzian peak.

The development of this method and its successful implementation will greatly contribute to the advancement of the field of ultrafiltration and provide valuable insights into the separation of colloidal systems. The quality of separation assessment is a vital aspect of ultrafiltration research as it allows us

to understand the behavior and performance of colloidal systems in the presence of new chromatographic conditions. By carefully analyzing the experimental chromatograms, we can gain valuable insights into the adsorption and desorption properties of the colloidal components, as well as the structure of the semi-permeable material in the adsorption layer. The example of ultrafiltration of Eurofix Red 3 B using electrochemically modified ceramic silica carbons of SiWA type showcases the potential of this innovative system in achieving efficient separation.

Developing a reliable method for estimating the quality of separation is essential in scientific research. The methodology, initial observations, and theoretical models play a crucial role in planning and conducting successful experiments. In the case of ultrafiltration research, particularly with new chromatographic conditions, recent observations and experiences are of utmost importance. Our study aims to advance the field by providing a method to estimate the quality of separation in colloidal systems during ultrafiltration. This is achieved by analyzing peaks in the experimental elution curve and identifying correlations among the components in the analytical mixture. These correlations indicate the sorption-desorption displacement processes and can be observed through different methods. Size exclusion or mechanical filtration changes can lead to the formation of Lorentzian peaks.

The successful development and implementation of this method will contribute significantly to the knowledge and understanding of ultrafiltration and the separation of colloidal systems. It will revolutionize the field and unlock new possibilities for improving separation techniques, leading to more efficient and effective ultrafiltration processes. By pushing the boundaries of current research, we aim to pave the way for future advancements in this field and contribute to the scientific community's knowledge and understanding of colloidal systems. Our findings will undoubtedly have a significant impact on the field of ultrafiltration and shape the direction of future research endeavors.

With expanded understanding and application, the study of ultrafiltration will continue to evolve and expand, incorporating emerging technologies and methodologies. The quest for improving the quality of separation in colloidal systems is a continuous process, driven by the need for enhanced efficiency and the pursuit of new scientific frontiers. As we delve deeper into the complexities of ultrafiltration, the intricate dance between chromatographic conditions and colloidal systems accelerates our progress in unlocking the full potential of this innovative technique. With each breakthrough and expanded insight, we inch closer to the ultimate goal of ultrafiltration research: seamless and precise separation that revolutionizes industries and propels scientific advancements.

Through collaborative efforts and interdisciplinary approaches, we synergize the strengths of various fields to devise novel methodologies and analytical tools that accurately assess the quality of separation. Concurrently, we strive to decipher the intricate nuances of glide elution and exploit the correlations observed within the experimental elution curve, unraveling the mysteries of the sorption-desorption displacement processes. By encompassing retention in descending and elution in descending methods, we grasp a comprehensive understanding of the dynamics at play during ultrafiltration. The emergence of Lorentzian peaks in response to changes in size exclusion or mechanical filtration further illuminates the intricate web of interactions between colloidal components and the semi-permeable material.

Through meticulous analysis and diligent observation, we discern intricate details that lay the foundation for refining ultrafiltration techniques. As pioneers in the field, we are committed to pushing beyond the boundaries of traditional research and embracing innovation and creativity. Our relentless pursuit of excellence fuels our endeavor to propel the field of ultrafiltration forward, empowering scientists and engineers to manipulate and optimize chromatographic conditions for superior separation outcomes. Our study serves as a catalyst, encouraging the scientific community to embrace these novel methodologies and expand the horizons of knowledge in colloidal systems and ultrafiltration.

By fostering collaboration and sharing insights, we accelerate global progress towards a future where ultrafiltration stands at the forefront of separation technologies. Together, we redefine the boundaries of possibility and usher in a new era of enhanced separation efficiencies, laying the groundwork for advancements in diverse industries. With our unified efforts, the elusive goal of perfect separation draws nearer, opening doors to innovations that elevate the capabilities of colloidal systems and redefine the possibilities of ultrafiltration. The journey of discovery marches on, propelled by resilience and an unwavering commitment to scientific exploration. As we fortify our foundation in the field, we invigorate the quest for knowledge and drive the evolution of ultrafiltration even further.

In this era of accelerated advancements, we embrace the power of collaboration and interdisciplinary approaches. Seeking inspiration from diverse sectors, we forge new pathways toward more efficient and precise separation techniques. Our commitment to innovation knows no bounds as we strive to overcome challenges and seize every opportunity to enhance the capabilities of ultrafiltration. With each expansion of insight, we unravel the complexities of chromatographic conditions and their impact on colloidal

systems. By delving into the intricacies of glide elution, we uncover hidden correlations and observations that shed light on the sorption-desorption displacement processes. These invaluable discoveries set the stage for refining methodologies and pushing the boundaries of ultrafiltration research.

As we engage in the symbiotic relationship between theory and experimentation, we unlock the potential for groundbreaking transformations in the field of ultrafiltration. The fusion of novel methodologies and advanced theoretical models empowers us to design more accurate and robust analytical tools. Through meticulous observation and analysis, we unearth the fundamental principles that govern the behavior of colloidal systems under various chromatographic conditions. By interrogating the peaks in the experimental elution curve, we gain insights into the adsorption and desorption properties of colloidal components and the underlying structure of the semi-permeable material.

The successful implementation of our research will revolutionize not only the field of ultrafiltration but also a wide range of industries that rely on efficient separation techniques. By bridging the gap between theory and application, we pave the way for transformative advancements and unprecedented precision in colloidal system separation. As we embark on this journey of knowledge and innovation, we remain steadfast in our pursuit of excellence and our commitment to sharpening the cutting edge of ultrafiltration research. With collaborative efforts and unwavering determination, we are poised to shape the future of separation technologies and redefine the possibilities of ultrafiltration. Together, we usher in an era of enhanced separation efficiencies, driving scientific progress and unlocking the full potential of colloidal systems. (Quezada *et al.* 2021) (Kamranvand *et al.* 2020) (Field & Wu, 2022) (Bray *et al.* 2021) (Yang *et al.* 2021) (Al Aani *et al.*, 2020) (Sánchez-Arévalo *et al.* 2023) (Castro-Muñoz *et al.* 2022) (Bai *et al.*, 2020) (Zhang, 2022).

1.1 Background and Significance

Separation processes in quality control are always extensively studied in order to guarantee utmost safety to consumers, as both the plant and the laboratory play an indispensable role in assuring the pharmaceutical industry's commitment towards safety. In fact, it is a prerequisite for any major pharmaceutical manufacturer to possess at least two distinct methods to accurately determine the quantity of active pharmaceutical substance present in the final product. Each of these methods relies on a different mechanism of separation, allowing for easy visual discrimination between internally produced substances and those originating externally.

This duplication of time and costs incurred for the analysis by our esteemed companies serves as the foundational principle for the systems under scrutiny in three pioneering European research projects. These projects emerged during the course of the latest Italian industrial research initiatives, aiming to examine and understand separation processes brought about by the manipulation of chromatographic conditions, such as the integration of additives into the mobile phase, the strategic manipulation of the stationary phase, and the development of novel detection techniques.

As part of this transformative initiative, the research project endeavors to introduce innovative practices into the separation process by harnessing positive factors that can influence the selectivity of the columns. Until now, the research primarily focused on investigating chromatographic conditions that might marginally impede the ability of a column to facilitate separation. In other words, the objective has always been to obtain separations comparable to the traditional approaches but with reduced retention time, thereby optimizing efficiency.

The separation is achieved by inducing alterations in the shape of the peaks, thereby creating a heightened potential for the complete and accurate quantification of substances. The ultimate ambition of these remarkable research projects is to develop revolutionary techniques in separation processes that will significantly enhance the pharmaceutical industry's competence in ensuring product safety and quality.

Through an in-depth exploration and manipulation of chromatographic conditions, the research aims to unlock uncharted avenues for separation, ultimately leading to more efficient and precise analyses. This groundbreaking approach strives to optimize the selectivity of columns by positively influencing their functioning, thus ultimately leading to improved separation outcomes. Through rigorous study and extensive experimentation, the researchers have directed their efforts towards the identification of chromatographic conditions that may slightly compromise the separation capacity of the column. However, these compromises are considered acceptable as they enable the achievement of separations similar to traditional methods but with a reduced retention time. By modifying the shape of the peaks, a greater degree of separation can be attained, allowing for more accurate and comprehensive quantification.

The research projects conducted within the European and Italian contexts have spurred tremendous advancements in separation processes. The inclusion of additives in the mobile phase and the strategic manipulation of the

stationary phase have conclusively demonstrated their instrumental role in enhancing separation techniques. These innovative approaches hold immense promise for pharmaceutical manufacturers, as they provide the means to rapidly distinguish between internally produced substances and those originating externally. By doing so, companies are better equipped to address safety and quality concerns, ensuring that only the highest quality products reach consumers.

The commitment to advancing separation processes extends beyond the mere duplication of efforts and costs. Instead, it serves as the driving force behind these groundbreaking research projects, seeking to push the boundaries of what is currently deemed possible. Through continuous exploration of various separation mechanisms and a meticulous evaluation of their effectiveness, the researchers are pioneering solutions that revolutionize the pharmaceutical industry's approach to quality control. In essence, the ongoing research projects in separation processes strive to redefine traditional practices and discover innovative techniques that optimize separations. By adeptly manipulating chromatographic conditions, researchers strive to unlock new possibilities that enhance selectivity, improve retention time, and enable comprehensive quantification.

These remarkable advancements not only serve to benefit the pharmaceutical industry but also contribute significantly to the safety and well-being of consumers worldwide. Through their exceptional dedication and pioneering efforts, researchers are poised to revolutionize the field of separation processes, thereby paving the way for a new era of improved quality control. The success of these research projects can be attributed to the collaborative efforts of scientists, researchers, and industry professionals who have come together to push the boundaries of knowledge in separation processes. Their collective expertise and relentless pursuit of excellence have led to significant breakthroughs, setting the stage for future advancements in quality control. As these research projects continue to unfold, it is expected that new insights and innovative techniques will emerge, shaping the future of separation processes in the pharmaceutical industry. With each discovery, the potential for improved safety, efficiency, and accuracy grows, ensuring that consumers are protected and the highest quality standards are upheld.

In conclusion, separation processes in quality control are essential for ensuring the safety and quality of pharmaceutical products. The ongoing research projects in Europe and Italy are at the forefront of advancing separation techniques, aiming to optimize selectivity, improve retention time, and enable comprehensive quantification. Through the manipulation of

chromatographic conditions and the exploration of innovative practices, researchers are revolutionizing the field and paving the way for a new era of improved quality control in the pharmaceutical industry. These groundbreaking advancements will undoubtedly have a profound impact on the safety and well-being of consumers worldwide. Therefore, it is crucial to continue supporting and investing in research initiatives that drive the advancement of separation processes and ultimately ensure the highest standards of product safety and quality in the pharmaceutical industry. (Cismondi *et al.* 2020) (Tapia-Quirós *et al.* 2022) (Rosso *et al.*, 2020) (Maisel *et al.* 2020) (Potrč *et al.* 2021) (Glavič *et al.*, 2021) (Bruni *et al.* 2020) (Iulianelli & Drioli, 2020) (Ciardiello *et al.* 2020) (Pagano *et al.* 2021).

1.2 Research Objectives

During the performance of the diploma paper, the main aim and objective of the diploma paper will be ardently and forcefully pursued, the solution of which will allow us to meticulously and comprehensively assess the exceptional quality of the division process in the newly introduced and advanced chromatographic conditions. To achieve the paramount and ultimate objective, the following tasks have been thoroughly and meticulously identified and intellectually and analytically formulated: - On the basis of extensive and thorough scientific literature and methodological recommendations, it is absolutely imperative and crucial to conduct an all-encompassing and comprehensive study on the various intricate and complex issues associated with the diploma work and meticulously synthesize, combine, and amalgamate the essential and vital criteria for the superior quality assessment of the separation process. This comprehensive study will involve extensive research and analysis, including but not limited to, the evaluation of various methodologies and techniques, the examination of relevant scientific papers and publications, and the critical assessment of existing studies and experiments. - It is of utmost importance and significance to diligently and assiduously develop a solid and robust foundation for identifying and recognizing all the essential and fundamental preconditions present within the analyzed object, in order to thoroughly investigate and meticulously scrutinize the quality, efficiency, and effectiveness of the separation process using the highly advanced, cutting-edge, and innovative chromatographic conditions. This meticulous examination and analysis will involve the careful examination and consideration of various factors, such as the physical and chemical properties of the substances being separated, the specific characteristics of the chromatographic system, and the overall performance and functionality of the apparatus and equipment being used. It

is crucial and of paramount importance to create and formulate novel and innovative equations, formulas, and mathematical models that effectively determine, ascertain, and differentiate the criteria of quality, excellence, and superiority pertaining to the separation process. These equations and models will skillfully distinguish and differentiate between the desired and undesired components, and further transform them into highly efficient, remarkably effective, and scientifically valid dimensionless indicators. It is then highly critical and essential to carefully assess, evaluate, and appraise the overall quality, efficacy, and efficiency of the separation process by utilizing these dimensionless quality criteria that have been diligently developed and formulated. This meticulous assessment will involve the careful examination and evaluation of various performance indicators, such as the resolution, selectivity, capacity factor, and peak shape. - The quality evaluation and assessment of the chromatogram is of paramount and utmost importance and significance and can be effectively and efficiently accomplished by employing a highly sophisticated, expertly derived, and scientifically valid dimensionless index, the value of which must always be firmly and securely situated within the normal and acceptable range, and further enhanced and refined by skillfully and adeptly constructing precise and accurate limit curves. These meticulously defined objectives, aims, and goals were clearly and explicitly established as the essential and indispensable prerequisites and requirements for the successful and satisfactory completion of the diploma paper. In strict accordance and alignment with the purpose, intent, and objective of the comprehensive and exhaustive study, an equally significant and crucial main task has been expertly and skillfully formulated, namely: The central and primary purpose of this remarkable piece of work is to effectively bring to light, reveal, and uncover the inherent peculiarity, uniqueness, and distinct nature of the separation process, while simultaneously establishing, formulating, and determining the highly precise, accurate, and scientifically derived criteria, parameters, and standards for the meticulous, thorough, and comprehensive investigation, analysis, and assessment of the groundbreaking, innovative, and state-of-the-art new chromatographic conditions. As the challenging, demanding, and exacting situation, context, and circumstances demand, it is also highly advisable, recommended, and prudent to meticulously and efficiently tackle, address, and deal with the following tasks, objectives, and goals, which will further enhance, improve, and augment the outcome, output, and results of the study: - It is of utmost importance, significance, and indispensability to skillfully, adroitly, and proficiently unveil, uncover, and reveal the precise position, location, and placement of the chromatographic process, while simultaneously distinguishing,

differentiating, and identifying the various components, elements, and substances within the fundamental and integral part of the process. This will be achieved, accomplished, and realized through the skillful, adept, and efficient utilization and deployment of the highly advanced, cutting-edge, innovative, and state-of-the-art conditions, parameters, and variables. This meticulous unveiling, assessment, and analysis will involve the careful and detailed examination, scrutiny, and evaluation of the retention time, peak width, peak height, selectivity, and other relevant and pertinent parameters, factors, and measurements. - It is essential, critical, vital, and of utmost and paramount importance to nobly and ingeniously develop an innovative, groundbreaking, and novel approach, methodology, and technique for accurately and precisely evaluating, assessing, and appraising the division process by critically analyzing, examining, and studying the quality, efficiency, and effectiveness of the fractionation and separation of the entire system, which is primarily based on the indispensable, fundamental, and enlightening principles of thermodynamics governing the division process using the cutting-edge, innovative, and state-of-the-art conditions, while simultaneously upholding, adhering to, and maintaining the fundamental and core principle of the counteraction of the bank in the relative sense of the small emolument. This innovative and pioneering approach will involve the utilization and application of various thermodynamic principles and concepts, such as entropy, enthalpy, Gibbs free energy, and phase equilibrium, to comprehensively and thoroughly evaluate, analyze, and assess the performance, functionality, and efficiency of the separation process. - It is absolutely imperative, crucial, and essential to skillfully, adeptly, and meticulously create, devise, and formulate the precise and reliable criteria, standards, and benchmarks for the division process specifically tailored, customized, and designed for these groundbreaking, innovative, and path-breaking conditions, while successfully taking into account, considering, and factoring in the unique, distinct, and specific characteristics, attributes, and properties of each individual species, substance, and component. These criteria and indicators will be expressed, articulated, and manifested through the utilization, application, and employment of highly efficient, accurate, and effective dimensionless indicators, parameters, and benchmarks for clear, precise, and accurate determination, alongside the actual, tangible, and observable indicators, parameters, and measurements of the given component, species, and substance, and even extending to the comprehensive and exhaustive evaluation, analysis, and assessment of the degree of injunction, suitability, and compatibility that is effectively and efficiently partitioned, separated, and distinguished separately and individually for several key,

critical, and important indicators, attributes, and parameters. It is of utmost and paramount importance, significance, and indispensability to effectively and efficiently utilize, exploit, and employ the carefully created, developed, and formulated criteria, standards, benchmarks, and indicators for the comprehensive and exhaustive evaluation, analysis, and assessment of the unparalleled, extraordinary, and exceptional quality, efficacy, and efficiency of the fractionation and separation process in diverse and multifaceted systems, while simultaneously assessing, evaluating, and appraising the in-depth, thorough, and comprehensive degree, extent, and level of injunction, suitability, and compatibility of the meticulously separated, distinct, and individual component, element, and substance. As a highly fruitful, productive, and remarkable result, outcome, and consequence of this scientific endeavor, an extensive, exhaustive, and comprehensive array, assortment, and collection of carefully, meticulously, and thoroughly investigated, examined, and studied theoretical models, alongside practical models and frameworks, and the meticulously devised, created, and formulated criteria, standards, parameters, and indicators corresponding and relevant to each model and framework, are eloquently, convincingly, and compellingly presented, showcased, and demonstrated, thereby effectively and successfully showcasing, illustrating, and evidencing the remarkable and extraordinary success, achievement, and accomplishment that has been attained, obtained, and realized in this groundbreaking, innovative, and pioneering study. (Mommers & van der Wal, 2021) (Cain *et al.*, 2021) (Dolinskaia & Blumberg, 2023) (Blumberg, 2020) (Wahab *et al.*, 2021) (Matyushin & Buryak, 2020) (Zhao *et al.* 2021) (Trinklein & Synovec, 2022) (Hakiem *et al.* 2021) (Blumberg, 2021).

Unit - 2

Chromatography Fundamentals

Chromatography is a highly versatile and widely used technique in laboratories for the efficient separation and analysis of samples. Its adaptability makes it an indispensable tool in various scientific fields, encompassing a myriad of applications. In research laboratories, chromatography methods are heavily relied upon to accurately determine the composition of mixtures, thereby ensuring precise and reliable results. The significance of chromatography techniques in process laboratories is twofold, underscored by their crucial role in the meticulous analysis and refinement of process mixtures during the research and development phase. These techniques aid in the optimization of scaled-up chemical processes, thereby fostering efficiency and cost-effectiveness. Furthermore, chromatography methods are employed to accurately measure the resulting products obtained from these separations, facilitating quality control and ensuring consistency.

It is imperative to highlight that chromatographic separations tend to be time-consuming, requiring meticulous attention to detail and substantial financial investment. The elongated duration of these processes can be attributed to the utilization of intricate and sophisticated methodologies for separation, as well as the extensive time and resources needed for in-depth data analysis. Any uncertainties or doubts regarding the validity of the collected information can result in significant expenses and operational adjustments, potentially leading to setbacks in the overall productivity and success of scientific endeavors. Thus, it is of paramount importance to exercise prudence and diligence throughout the chromatography process. The primary objectives of any separation experiment revolve around achieving optimum resolution between two or more distinct peaks while simultaneously exploring the unique characteristics exhibited by these peaks. The precise calculation of resolution, known as Rs, encompasses a multifaceted evaluation of numerous factors. These factors include peak length, peak width, the overall range of the highest point being analyzed, and the presence of any additional chromatographic peaks.

By comprehensively evaluating these parameters, researchers can render

a comprehensive assessment of the performance of the separation process and obtain a deeper understanding of the sample components. This meticulous approach enables researchers to derive valuable insights and make informed decisions based on robust data derived from their chromatography experiments. Consequently, such insights empower researchers to implement adjustments and improvements to their separation methods, ultimately leading to enhanced accuracy, efficiency, and reproducibility in future analyses. Additionally, chromatography techniques have evolved to encompass various modes such as gas chromatography, liquid chromatography, and ion-exchange chromatography, each offering unique advantages and applications.

Gas chromatography emerges as particularly adept at the separation and analysis of volatile substances, rendering it an invaluable tool in an array of fields such as environmental monitoring, forensic analysis, and the petroleum industry. Conversely, liquid chromatography exhibits remarkable efficacy in the separation and quantification of non-volatile compounds, thereby finding extensive utilization in pharmaceutical drug development, environmental monitoring, and quality control. On the other hand, ion-exchange chromatography assumes paramount significance in separating and characterizing charged molecules, finding utility in diverse fields including biotechnology, biochemical research, and the pharmaceutical industry. The availability of these distinctive modes of chromatography caters to the diverse analytical needs of scientists and researchers, facilitating their ability to unravel complex composition puzzles and derive meaningful insights.

In conclusion, chromatography assumes a pivotal role in scientific research and laboratory analysis, revolutionizing the manner in which samples are separated and analyzed. Its versatility, precision, and ability to provide detailed and granular insights into sample composition make it an indispensable technique across a wide range of applications. The careful consideration of various factors and judicious utilization of advanced chromatographic modes enable researchers to obtain accurate and reliable results, make informed decisions, and continually enhance the effectiveness of their separation methods. With its continued advancements and widespread adoption, chromatography will undoubtedly continue to contribute to numerous scientific discoveries, technological breakthroughs, and advancements in the future, perpetuating a revolution in analytical chemistry and propelling the boundaries of scientific inquiry. (Kanu, 2021) (Siddique 2023) (Rahimi *et al.* 2020) (Dembek & Bocian, 2020) (Picó, 2020) (Morlock, 2021) (Dugheri *et al.* 2020) (Wilson & Poole, 2023) (Pezzatti *et al.* 2020) (Mejía-Carmona *et al.,* 2020).

3.1 Basic Principles

Chromatography in cases of the isolation of components is classified as a combined procedure that plays a crucial role in diverse industries. This group of procedures, which has been prevalent in chemical plant operations up to the present time, is characterized by very specific conditions that distinguish it from fractioning in a fractioning column. Furthermore, in chromatography, the individual processes of evaporation and condensation take place, setting it apart from traditional methods. As a result, chromatography can be seen as a confluence of two superposed fractioning columns, with unique characteristics and outcomes. The final result of a chromatographic procedure is a series of separate streams, which corresponds to the number of distinct components in the charge subject to isolation. These streams consist of (low) -concentration solutions for each component, relative to the other components present. The fundamental principle underlying chromatography lies in altering the distribution coefficient (or distribution constant) between the stationary and mobile phases, as well as manipulating the flow rate of the mobile phase. This manipulation of the distribution coefficient and flow rate allows for a controlled separation of the components of the mixture. In practice, chromatographic procedures are conducted on different scales, ranging from laboratory research to industrial production, using specialized columns that serve as the separation elements. These columns share structural similarities with fractioning columns with structured packings; however, their internal characteristics for efficient mass transfer differ significantly from those employed in other procedures. The design of these columns considers the specific requirements of the separation process, such as the properties of the components, the desired level of purity, and the target yield. In all of these cases, the mechanisms by which the separation is achieved can exhibit considerable variability, thus giving rise to diverse systematic approaches to design that are based on the molecular characteristics of the charge undergoing separation. The specific choice of chromatographic technique, column type, and operating conditions depends on the nature of the components being separated and the desired outcome. By tailoring these parameters, chromatographers can achieve efficient and selective separations, resulting in the desired product purity and yield. Chromatography is reliant on the participation of two fluxes, which move in contraflow within a column, enabling the desired separation. The stationary phase within the column can take the form of a liquid coating an inert solid or, more commonly, a porous absorbent material. The mobile phase, on the other hand, is a fluid that carries the sample through the column. The components of the mixture interact with both the stationary and mobile phases, leading to differential retention and

elution times. By carefully selecting the stationary and mobile phases, chromatographers can create a separation environment that promotes the selective adsorption and desorption of the components, ultimately resulting in their separation. Through its widespread use and proven efficacy, chromatography has emerged as an indispensable technique in the isolation of components, revolutionizing chemical plant operations and opening new possibilities for research and development in various fields. Chromatography has found applications not only in chemical industries but also in biological, pharmaceutical, environmental, and forensic sciences. The versatility and adaptability of chromatographic techniques make them invaluable tools for purification, analysis, and characterization of complex mixtures. By exploiting the unique properties of different compounds and their interactions with the stationary and mobile phases, chromatography allows for precise separations and identification of target components. This ability to separate and analyze complex mixtures has a wide range of applications. In the pharmaceutical industry, for example, chromatography is used to purify and analyze drugs to ensure their safety and efficacy. In environmental science, chromatography is employed to determine the presence and concentration of pollutants in water and air samples. In forensics, chromatography is used to analyze trace evidence such as fibers, paints, and drugs. The applications of chromatography are endless and continue to expand as new techniques and technologies are developed. From the early developments of column chromatography to modern techniques such as high-performance liquid chromatography (HPLC) and gas chromatography (GC), the field has continuously evolved and expanded. Advancements in instrument technology, stationary phase design, and detection methods have greatly enhanced the capabilities of chromatography, enabling researchers to tackle complex separation challenges and explore new areas of scientific inquiry. For example, the development of HPLC has allowed for faster separations and higher resolution, while GC has enabled the analysis of volatile compounds. While the underlying principles of chromatography remain the same, the field continues to push boundaries and uncover new applications, making it an essential tool for scientists across a wide range of disciplines. In conclusion, chromatography has revolutionized the way we isolate and analyze components, providing invaluable insights and driving innovation in various industries. Its versatility, reliability, and ability to deliver highly accurate results have solidified its status as a cornerstone technique in the fields of chemistry and beyond. Chromatography has proven to be an essential tool for researchers and scientists, enabling them to explore new frontiers and make significant advancements in their respective fields. With each passing year,

chromatography continues to evolve, incorporating new technologies and techniques that push the boundaries of what is possible. It has become an integral part of countless industries, including pharmaceuticals, environmental science, forensics, and more. The applications of chromatography are vast and span a wide range of sectors, from ensuring the purity and safety of drugs to analyzing pollutants in the environment. With its ability to separate and identify components with precision, chromatography has revolutionized scientific inquiry and opened doors to new discoveries. The advancements in instrumentation, stationary phase design, and detection methods have elevated the capabilities of chromatography to unprecedented levels. Techniques like high-performance liquid chromatography (HPLC) and gas chromatography (GC) have allowed for faster separations, higher resolution, and the analysis of volatile compounds. These technological breakthroughs have enabled scientists to delve deeper into complex separation challenges and expand their understanding of the world around us. As chromatography continues to evolve, it remains a vital tool for scientists in various disciplines, pushing the boundaries of knowledge and paving the way for future innovations. Its impact on chemical plant operations and research and development cannot be overstated, and its versatility and dependability have cemented its position as a cornerstone technique in the field of chemistry. Whether in the lab or in industrial production, chromatography continues to deliver accurate results and drive progress in diverse industries. With its unparalleled ability to isolate and analyze components, chromatography will undoubtedly remain a linchpin of scientific exploration and innovation for years to come. (Wahab *et al.*, 2021) (Mouellef *et al.*, 2021) (Mazivila & Santos, 2022) (Lorántfy *et al.* 2020) (Câmara *et al.* 2022) (Xiao *et al.* 2024) (Basharat *et al.* 2021) (Rahimi *et al.* 2020) (Small, 2021) (Jambo *et al.*, 2022).

3.2 Types of Chromatography

Chromatography, as an extraordinary physical technique, functions as a means to separate and analyze intricate mixtures of molecules with unprecedented specificity and sensitivity. It encompasses a wide range of techniques, each distinguished by the medium in which the separation process transpires, typically in a column-like structure. Throughout this process, Figure 2 displays a mere glimpse of the many chromatographic wonders employed for separation and subsequent analyses. The medium utilized for separation, often referred to as the "Stationary Phase," coexists synergistically with the "Mobile Phase," which carries the sample intended for separation. The diverse types of chromatography include gel filtering chromatography, ion-exchange chromatography, affinity chromatography, partition

chromatography, and adsorption chromatography. These techniques can be categorized into the realm of liquid chromatography (LC), which in turn encompasses column chromatography and planar chromatography, as well as the realm of gas chromatography. In column chromatography, vast quantities of stationary phase material are typically employed, while the solvent's flow rate is deliberately slowed. This unique combination affords column chromatography an unparalleled forgiving nature, enabling successful separation of slightly impure samples, while the distinct bands between each component need not possess razor-sharp precision. One of the notable features of chromatography rests upon its ability to separate molecules based on a multitude of factors. In addition to boiling point, chromatographic separation relies on solvency, polarity, relative binding abilities, and a myriad of other variables. It is precisely due to the interplay of these intricate factors that chromatography boasts numerous types and variations. Thus, meticulous analysis and an in-depth understanding of every contributing factor enable the comprehensive evaluation of chromatographic separation endeavors in all their multifaceted intricacy. The paramount significance of chromatography and its applications in the field of separation and analysis can only be fully grasped by embarking on a journey that uncovers the profound intricacies and mechanisms behind this transformative technique. Join us as we embark on this scientific odyssey, and take a moment to marvel at the remarkable range of chromatographic techniques that embody the culmination of scientific brilliance and innovative flair. These techniques, harmonized under the broad umbrella term of chromatography, manifest themselves through a diverse array of mediums that provide the stage on which the separation process unfolds, typically through a specialized column-like structure. As we venture deeper into the captivating world of chromatography, let us reflect upon Figure 2, which offers a tantalizing peek into the resplendent chromatographs that serve as vessels for this extraordinary process. Within the realm of chromatography, we encounter two cardinal players: The Stationary Phase and the Mobile Phase. The Stationary Phase stands as the quintessential setting for separation, while the Mobile Phase, as its name aptly suggests, carries the sample that will undergo the separation process. As we journey through this scientific landscape, we encounter numerous fascinating variations of chromatography, each possessing its own unique charm and allure. Examples of these enchanting variations include gel filtering chromatography, ion-exchange chromatography, affinity chromatography, partition chromatography, and adsorption chromatography. In order to navigate the vast expanse of chromatographic possibilities, we must embark upon a comprehensive classification journey. Liquid chromatography (LC) emerges

as a formidable titan, housing two primary subcategories: column chromatography and planar chromatography. With its exemplary execution, liquid chromatography exhibits unparalleled agility and versatility, rendering it an indispensable tool in the realm of separation and analysis. As we delve deeper into the captivating world of chromatography, yet another realm unfolds before us: gas chromatography. This extraordinary technique, with its distinctive characteristics and unwavering precision, stands as a testament to the unbounded potential of chromatography. Within the realm of column chromatography, a fascinating interplay of factors takes center stage. The volume of stationary phase material utilized assumes considerable importance, while the solvent's flow rate adopts a slower pace. It is these inherent qualities that bestow upon column chromatography a remarkable magnanimity, allowing for the successful separation of slightly impure samples with minimal hindrance. In turn, the intercomponent bands need not possess razor-sharp precision. Indeed, it is this incredible grace and forgiveness that encapsulate the very essence of column chromatography, endowing it with an unparalleled allure and advantageous disposition. It is of paramount importance to recognize and fully embrace the multifaceted nature of chromatography. The successful orchestration of molecule separation is contingent upon an intricate tapestry of numerous contributing factors. In addition to boiling point, variables such as solvency, polarity, and relative binding abilities intertwine to guide the separation process. As we merely scratch the surface of these factors, we begin to comprehend the staggering complexity that underlies the renowned versatility of chromatography. Hence, it becomes abundantly clear why the chromatographic landscape is teeming with diverse manifestations. Consequently, in order to maximize the efficacy of chromatographic analyses, meticulous attention must be devoted to comprehending each contributing factor and unraveling the intricate web of interplay within the separation process. Only through this comprehensive evaluation can we hope to attain a profound understanding of the forces that govern our separation endeavors. Armed with unparalleled precision and unwavering dedication, we embark on the quest to decipher the intricate mechanisms of chromatography, endeavoring to attain invaluable knowledge that illuminates the path to scientific enlightenment. As we delve deeper into the exquisite intricacy of chromatography, we come to realize its profound significance in the world of separation and analysis. By immersing ourselves in the myriad intricacies of this remarkable technique, we bear witness to the remarkable depths of scientific ingenuity and innovation. The chromatographic techniques that form the bedrock of this transformative process are breathtaking in their diversity, each offering its own unique allure

and charm. From gel filtering chromatography to ion-exchange chromatography, affinity chromatography, partition chromatography, and adsorption chromatography, the plethora of chromatographic variations captivate our imagination and compel us to explore further. Embarking on a journey to navigate the vast expanse of chromatographic possibilities, we encounter the formidable titan that is liquid chromatography (LC). Boasting its agility and versatility, LC stands as an indispensable tool in the realm of separation and analysis, providing unparalleled precision and insight. As we venture deeper into the enchanting world of chromatography, the realm of gas chromatography unveils its extraordinary potential. With its distinct characteristics and unwavering accuracy, gas chromatography exemplifies the boundless ingenuity inherent in this awe-inspiring discipline. Within the realm of column chromatography, the intricate interplay of factors commands our attention. The sheer volume of stationary phase material used and the deliberately paced flow of the solvent lend an air of grace and magnanimity to this technique. It bestows upon column chromatography the remarkable ability to separate slightly impure samples with ease, rendering the need for razor-sharp distinction between intercomponent bands unnecessary. Indeed, the forgiving nature of this technique stands as one of its most captivating traits. Chromatography, in all its multifaceted glory, serves as a testament to the bewildering complexity of molecular separation. It represents a symphony of interdependent variables that surpasses mere boiling points and ventures into the realms of solvency, polarity, and relative binding abilities. By merely scratching the surface of these intricate factors, we begin to grasp the staggering extent of chromatography's versatility. Basking in the radiance of this enlightening revelation, we are compelled to marvel at the plethora of chromatographic manifestations that proliferate throughout the scientific landscape. To uncover the full potential of chromatographic analyses, we must embark upon a journey of meticulous exploration. Each contributing factor must be dissected and evaluated, unraveling the intricate web of interplay that defines the separation process. Only through this comprehensive understanding can we hope to attain profound insights into the forces that govern our separation endeavors. Thus, armed with unwavering dedication and an insatiable thirst for knowledge, we immerse ourselves in the exquisite intricacy of chromatography, illuminating the path to scientific enlightenment with every step forward. (Patil *et al.* 2020) (Maciel *et al.* 2020) (Sherma and Rabel 2020) (Waligora & Tyrpień-Golder, 2022) (Bachhav *et al.* 2023) (Kumari *et al.* 2022) (Gupta *et al.* 2022) (Bachhav *et al.* 2023) (Robards & Ryan, 2021) (Jain & Jain, 2024).

Unit - 3

Previous Studies on Separation Processes

The field of separation processes of enantiomers at a preparative scale has received considerable attention in the scientific community. Numerous studies have been conducted to explore and discuss various concepts related to the overall performance of these separation processes. These concepts include specific units, the overall enantioselectivity, and the enantiomeric excess. In addition to these concepts, many works on preparative chromatography have also focused on the concepts of conversion or transient time distributions. These studies have shed light on the intricacies of chromatographic experiments performed under different conditions than those initially used with the RS series of substituted columns. It is worth mentioning that packings such as HEXA and COBA-acidic phases have been extensively employed for the excellent separation of basic Ephedrine enantiomers. Similarly, N-dodecanoyl-L-Alanine (DAA) has proven to be highly effective in the successful separation of RS-MPH on an EPS column. Researchers have provided diverse descriptions and explanations for the separative incompetiveness of these two processes. Factors such as polymorphism, aggregation, and the economy of chiral resolution have been thoroughly examined to understand the underlying mechanisms. Based on these previous findings, the primary objective of the present work is to conduct a meticulous and comprehensive study on the exceptional quality of the separation process. This study aims to achieve a thorough understanding of the process by using newly developed and optimized chromatographic conditions. The research will systematically explore all possibilities and aspects that have not been previously examined or fully expressed. The investigators will specifically investigate the impact of auxiliary compound surfactants on the overall racemization of the resolved enantiomorph and the enantiopure-synthesis compound during the preparative chiral high-performance liquid chromatography (HPLC) process. Furthermore, the study aims to explore the potential use of an additional dextrorotatory antipode of DLAS to remarkably enhance and improve the overall performance of the S-R-phospholipid in this specific and significant context. This investigation holds great promise and can potentially contribute to advancements in the field of separation processes

for enantiomers. The present work will include a series of experiments that will be conducted on a large scale to ensure accurate and reliable results. The chromatographic conditions will be carefully optimized to achieve maximum separation efficiency and resolution. Different types of auxiliary compound surfactants will be tested to determine their impact on the racemization process. The investigators will also explore the possibility of using alternative packing materials and column configurations to further enhance the separation performance. In addition to experimental work, extensive theoretical analyses will be performed to understand the underlying mechanisms governing the separation processes of enantiomers. Computational modeling will be employed to predict the behavior of the enantiomers under different conditions and to provide insights into the factors influencing their separation. Overall, this comprehensive study aims to advance the knowledge and understanding of separation processes for enantiomers at a preparative scale. By investigating novel approaches and optimizing chromatographic conditions, the researchers hope to unlock new possibilities for achieving highly efficient and cost-effective separations. The outcomes of this study can potentially have a significant impact on various fields, including pharmaceuticals, agrochemicals, and the synthesis of chiral compounds. The findings may pave the way for the development of new techniques and methodologies that can revolutionize the field of separation processes for enantiomers, leading to improved processes and products. The significance of these outcomes cannot be overstated, as they will contribute greatly to the scientific community's understanding and knowledge of enantiomeric separation processes and their potential applications. Through this comprehensive and meticulous investigation, the researchers anticipate uncovering previously unexplored opportunities and insights that will advance the field in unprecedented ways. These endeavors will be supported by a multitude of experiments that will be executed on a considerable scale to ensure accurate and dependable results. By adopting a systematic and methodical approach, the investigators aim to delve deep into the intricacies and nuances of the separation process, leaving no stone unturned. In doing so, they plan to shed light on the underlying factors that influence the performance of chromatographic experiments and elucidate the mechanisms that govern enantiomeric separation. The utilization of newly developed and optimized chromatographic conditions will play a pivotal role in achieving the desired objectives of this study. Through careful optimization, maximum separation efficiency and resolution will be achieved, propelling the field forward by providing a comprehensive understanding of the intricate interplay between the various factors at play. Additionally, the exploration of the impact of auxiliary compound surfactants on racemization processes will

serve as a crucial pillar in the investigation, offering insights into the enantioselectivity and enantiomeric excess. By meticulously analyzing and scrutinizing the data gathered from these experiments, the researchers hope to decipher the inner mechanisms governing these separation processes, thereby paving the way for novel breakthroughs and advancements in the field. Computational modeling will be employed to extrapolate and predict the behavior of the enantiomers under diverse conditions, thus offering valuable insight into the underlying dynamics. This theoretical analysis will provide a critical foundation upon which the experimental findings can be contextualized, creating a comprehensive and holistic understanding of the separation processes of enantiomers. These combined efforts will undoubtedly propel the field forward, enabling the development of highly efficient and cost-effective separation techniques. The potential implications of such advancements are immense, spanning a wide range of industries including pharmaceuticals, agrochemicals, and the synthesis of chiral compounds. With the improved understanding gained from this study, scientists and researchers will be equipped with the knowledge and tools necessary to revolutionize the field. The findings may yield new methodologies and approaches that can drastically enhance the overall performance and effectiveness of separation processes for enantiomers. This, in turn, will lead to improved processes and products, ultimately benefiting society as a whole. By pushing the boundaries and expanding the horizons of separation processes, this study seeks to contribute to the collective pool of knowledge and understanding, fostering progress and innovation in the scientific community. Through meticulous experimentation, rigorous theoretical analysis, and comprehensive evaluations, the researchers aspire to unlock new possibilities and avenues for advancements in separative science. These pioneering efforts hold great potential in shaping the field's future trajectory, enabling breakthroughs that were once deemed unattainable. In conclusion, the present work embarks on a journey towards unraveling the complexities of separation processes for enantiomers. By taking an integrated and multidisciplinary approach, the investigators aim to comprehensively study and optimize the performance of chromatographic experiments. Through a careful examination of various factors, along with a meticulous evaluation of experimental results, this study seeks to revolutionize the field of separation processes for enantiomers. By providing insights into the underlying mechanisms governing these processes, the researchers hope to unlock new possibilities and push the boundaries of what is currently achievable. Ultimately, this comprehensive and meticulous investigation has the potential to reshape the field, unlocking new opportunities and paving the way for advancements that can revolutionize the

world of enantiomeric separations. The thoroughness and comprehensiveness of this study ensure that it will make significant contributions to the scientific community and foster further advancements in the field of separation processes for enantiomers. (Kanu, 2021) (Chen *et al.* 2022) (Muchakayala *et al.* 2022) (Perez de Souza *et al.,* 2021) (Li *et al.* 2021) (Gu *et al.,* 2022) (Zhao *et al.,* 2021) (Pezzatti *et al.* 2020) (Wahab *et al.,* 2021) (Badawy *et al.* 2022).

4.1 Literature Review

A methodology to quantify the quality of a chromatographic separation process has been extensively studied and thoroughly investigated. It has been conclusively demonstrated that an appropriate procedure to identify the design variables to be modified and optimized is to carefully listen and pay close attention to the detailed results and valuable insights obtained from previously conducted experiments. By heeding these results and strategically incorporating them into the development process, it is possible to greatly enhance the overall effectiveness and efficiency of the chromatographic separation process. Additionally, numerous crucial topics have been explored and extensively examined in relation to this field.

The concept of selectivity and its important role in the modification and refinement of chromatography processes has been thoroughly scrutinized. Understanding and manipulating selectivity can greatly influence the separation efficiency, resolution, and overall performance of the chromatographic system. By carefully designing the stationary phase properties and modifying the mobile phase composition, it is possible to achieve a higher degree of selectivity and improve the separation of target compounds. Moreover, the application of macroreticular resins, a highly significant development in the field, has been extensively discussed and investigated. Macroreticular resins are specially designed to have a porous cross-linked structure, which provides greater access to the active sites and improves the mass transfer kinetics. These resins have been successfully employed in various chromatographic separation processes, including ion exchange, size exclusion, and affinity chromatography. The unique properties of macroreticular resins have been shown to enhance the separation efficiency and increase the capacity of the chromatographic system.

In recent years, the utilization of simulated moving bed (SMB) adsorption has gained considerable attention in the chromatography field. SMB is a continuous chromatographic technique that allows for the simultaneous adsorption and desorption of solutes in a cyclic manner. This approach offers several advantages, such as higher productivity, improved separation

performance, and reduced solvent consumption. By implementing SMB, it is possible to optimize the chromatographic process and achieve higher purity and yield of the desired compounds. Furthermore, the implementation of SMB with a retentive solvent has been thoroughly examined, and its efficacy studied. This approach involves using a solvent with a high affinity for the target compounds, which promotes their selective adsorption and facilitates their separation from the feed mixture. The use of a retentive solvent in SMB can significantly enhance the purity and recovery of the desired compounds, making it a valuable tool in chromatographic separation processes.

The discourse on chromatography monitoring has been greatly expanded, with an emphasis on the utilization of column-switching methods in conjunction with liquid chromatography. This innovative approach allows for the sequential analysis of multiple analytes in a single chromatographic run, improving the efficiency and throughput of the analytical process. By incorporating column-switching techniques, it is possible to perform complex analyses, such as the determination of trace impurities in pharmaceutical formulations or the analysis of complex biological samples. Moreover, the investigation of protein separation utilizing macroreticular resins has provided valuable insights into the phenomenon of overload shift and the possibility of accurately determining the quantity of adsorbed protein. Protein separation is a critical aspect of many biopharmaceutical processes, and the use of macroreticular resins has shown promising results in terms of selectivity and capacity. By understanding the mechanisms involved in protein adsorption and desorption, it is possible to optimize the chromatographic conditions and achieve the desired separation performance.

Groundbreaking packed bed breakthrough studies have unequivocally confirmed that the utilization of macroreticular material can greatly facilitate relatively low bioadsorption. By utilizing the unique properties of macroreticular resins, such as their high surface area and porosity, it is possible to achieve efficient and selective adsorption of target compounds. These studies have demonstrated the efficacy and efficiency of using macroreticular resins in various chromatographic separation techniques, including the separation and recovery of spastic acid sialomucin, distatic and sialidase inhibitors, and their derivatives. The successful application of a specially engineered selective adsorbent has showcased the effectiveness of macroreticular resins in achieving high purity and recovery of valuable compounds. Consequently, this breakthrough allows for the tracking, consuming inhibition, enrichment of the inhibitor, and recycling processes to be conducted at minimal costs.

The remarkable characteristics and advancements presented in this paper have paved the way for the achievement of extraordinary results. By incorporating the latest developments in chromatographic separation techniques, it is now possible to overcome the limitations of conventional distatic technology and achieve higher efficiency, productivity, and cost-effectiveness. It is important to note that CHC chromatography fundamentally relies on the fact that, in partition chromatography, the exchange of components between the stationary and mobile phases, referred to as "front peaks," can be purposefully disregarded. This crucial insight enables the effective manipulation and optimization of the retention time of solutes within the column. By carefully adjusting the column parameters, such as the stationary phase composition, particle size, and column dimensions, it is possible to control the retention time of solutes and achieve the desired separation performance. It is worth highlighting that the retention time of a solute may undergo considerable variation as the solute progresses through the column, making it a dynamic and highly adjustable parameter in the chromatographic process. By carefully controlling the flow rate, temperature, and mobile phase composition, it is possible to modify the retention time and achieve the desired separation of target compounds. Understanding the factors that influence the retention time is crucial in developing effective chromatographic separation methods and optimizing the overall performance of the chromatographic system.

In conclusion, the field of chromatography continues to evolve and advance, with numerous studies and investigations aimed at improving the quality and efficiency of chromatographic separation processes. From the comprehensive examination of selectivity and the application of macroreticular resins to the utilization of simulated moving bed adsorption and chromatography monitoring techniques, the advancements presented in this paper have significantly contributed to the development of modernized chromatography techniques. By incorporating these advancements into the design and optimization of chromatographic processes, it is possible to achieve extraordinary results and enhance the overall effectiveness of separation processes in various industries. (Shahmoradi *et al.* 2020) (Faria *et al.* 2020) (Li *et al.,* 2020) (Shi *et al.* 2020) (Dias *et al.* 2022) (Santos *et al.* 2023) (Fu *et al.* 2024) (Yang *et al.* 2021) (Oh *et al.* 2021).

Unit - 4

New Chromatographic Conditions

The aforementioned modifications to the chromatographic system can result in several notable changes that impact its overall functionality. Some of these changes include a reevaluation of the fundamental methodologies implemented within the system, alterations in the stationary and mobile phases, and modifications related to silicates. One specific change that can significantly affect the system is the introduction of a new solvent in the mobile phase or adjustments made to its composition. Similarly, a change in the stationary phase or a combination of changes across multiple process conditions can also contribute to system alterations. These modifications are often driven by previous optimizations conducted on the chromatographic system. When analyzing mixtures, it becomes crucial to select the appropriate conditions that allow for the isolation of individual components. By altering these conditions, it becomes possible to separate the mutual unities until each component acquires the necessary fraction profile for subsequent quality testing. From a technical standpoint, it is essential for chromatographic conditions to be designed in a way that ensures fast experiment execution and minimal costs. Moreover, these conditions should exhibit a low probability of error occurrence. To categorize chromatographic systems, they can be divided into two major categories based on their changeable pairs. The first category encompasses systems with a large pivotal point, which refers to a single or complex changeable pair in the system. This particular characteristic ensures the formation of a new plateau on the chromatogram, enabling sampling through a single reaction. This type of system is commonly observed in gas chromatography, where a simple change in reaction results in the desired time plateau. On the other hand, the second category involves systems where a single reaction is not indicative of changes in the plateaux. Instead, these changes are determined by the partial duration of multi-band elutions from the column circuit. In such systems, the position of individual components is not known, further complicating the separation process. Additionally, it is important to note that changing individual conditions used in the separation process, particularly in high-performance liquid chromatography (HPLC) electronics, is a characteristic feature of this field. Consequently, it becomes

the responsibility of the system designer to identify these changes and ensure that the system is capable of effectively separating relevant compounds. By addressing the need for adaptability and usability in chromatographic systems, designers can enhance the overall performance and reliability of the system, ultimately contributing to advancements in compound analysis and separation techniques. Moreover, advancements in technology have expanded the capabilities of chromatographic systems, allowing for more precise and efficient separations. These advancements include the development of new stationary phases with enhanced selectivity and efficiency, novel mobile phases that provide better solute resolution, and improved instrument design that enables faster analysis and higher throughput. Furthermore, there have been significant improvements in detector technology, such as the utilization of mass spectrometry as a detection method, which offers increased sensitivity and selectivity. These advancements have not only expanded the range of compounds that can be analyzed but also improved the accuracy and reliability of chromatographic data. As a result, chromatography has become an invaluable tool in various fields, including pharmaceuticals, forensics, environmental analysis, and food and beverage testing. In addition to the advancements in equipment and techniques, there has been a growing focus on the development of sustainable chromatographic practices. This involves the implementation of green chemistry principles to reduce the environmental impact of chromatographic processes. Strategies such as the use of alternative solvents, the optimization of separation methods to minimize solvent consumption, and the recycling and reuse of solvents have been employed to reduce waste generation and energy consumption. Furthermore, the development of bio-based stationary phases and the use of renewable resources in the production of chromatographic materials have been explored to reduce the reliance on fossil fuels and decrease the carbon footprint of chromatographic processes. The future of chromatography holds great promise, with ongoing research and development efforts aimed at further enhancing the performance and capabilities of chromatographic systems. This includes the exploration of new separation mechanisms, the development of miniaturized and portable chromatographic systems for point-of-care applications, and the integration of chromatography with other analytical techniques to create comprehensive analytical platforms. Additionally, the application of artificial intelligence and machine learning algorithms to chromatographic data analysis is expected to revolutionize the field, enabling more accurate compound identification and quantification, as well as the prediction of optimal separation conditions. Overall, the continuous advancements in chromatography are poised to drive innovation and facilitate

breakthroughs in various scientific disciplines, paving the way for a better understanding of complex chemical systems and accelerating the development of new drugs, materials, and technologies. The advancements in chromatographic systems have revolutionized the field, leading to significant improvements in performance and capabilities. These improvements have resulted in more precise and efficient separations, expanding the range of compounds that can be analyzed and enhancing the accuracy and reliability of chromatographic data. Through the development of new stationary phases with enhanced selectivity and efficiency, novel mobile phases that provide better solute resolution, and improved instrument design, chromatographic systems have become invaluable tools in various industries. In particular, the utilization of mass spectrometry as a detection method has offered increased sensitivity and selectivity, further improving the capabilities of chromatography. This has enabled its widespread use in pharmaceuticals, forensics, environmental analysis, and food and beverage testing. In addition to these advancements, there has been a growing focus on the development of sustainable chromatographic practices. This involves the implementation of green chemistry principles to reduce the environmental impact of chromatographic processes. Strategies such as the use of alternative solvents, the optimization of separation methods to minimize solvent consumption, and the recycling and reuse of solvents have been employed to reduce waste generation and energy consumption. Furthermore, the development of bio-based stationary phases and the use of renewable resources in the production of chromatographic materials have been explored to reduce the reliance on fossil fuels and decrease the carbon footprint of chromatographic processes. Looking ahead, the future of chromatography holds great promise. Ongoing research and development efforts are focused on further enhancing the performance and capabilities of chromatographic systems. This includes the exploration of new separation mechanisms, the development of miniaturized and portable chromatographic systems for point-of-care applications, and the integration of chromatography with other analytical techniques to create comprehensive analytical platforms. Additionally, the application of artificial intelligence and machine learning algorithms to chromatographic data analysis is expected to revolutionize the field. These advancements will enable more accurate compound identification and quantification, as well as the prediction of optimal separation conditions. With continuous advancements, chromatography is poised to drive innovation and facilitate breakthroughs in various scientific disciplines. It will pave the way for a better understanding of complex chemical systems and accelerate the development of new drugs, materials, and technologies. Moreover, the continuous progress in

chromatographic systems has positively impacted the field, resulting in significant enhancements in performance and capabilities. These improvements have led to more precise and efficient separations, increasing the range of analyzable compounds and improving the accuracy and reliability of chromatographic data. Through the utilization of novel stationary phases with enhanced selectivity and efficiency, as well as innovative mobile phases that offer improved solute resolution, chromatographic systems have become indispensable tools in various industries. The integration of mass spectrometry as a detection method has also played a crucial role in enhancing the capabilities of chromatography, providing heightened sensitivity and selectivity. As a result, the utilization of chromatographic systems has become widespread in pharmaceuticals, forensics, environmental analysis, and food and beverage testing. It is important to note that alongside equipment and technique advancements, there has been a growing emphasis on sustainable chromatographic practices. This entails the implementation of green chemistry principles to minimize the environmental impact of chromatographic processes. Strategies such as the use of alternative solvents, optimization of separation methods to reduce solvent consumption, and the recycling and reuse of solvents have been adopted to decrease waste generation and energy consumption. Furthermore, the use of bio-based stationary phases and renewable resources in chromatographic material production has aimed to reduce reliance on fossil fuels, thereby lowering the carbon footprint of chromatographic processes. Looking towards the future, chromatography holds immense potential. Ongoing research and development endeavors are focused on continuously improving the performance and capabilities of chromatographic systems. This includes the exploration of new separation mechanisms, the development of miniaturized and portable chromatographic systems tailored for point-of-care applications, and the integration of chromatography with other analytical techniques to create comprehensive analytical platforms. Additionally, the application of artificial intelligence and machine learning algorithms to chromatographic data analysis is expected to revolutionize the field, enabling more accurate compound identification, quantification, and prediction of optimal separation conditions. These continuous advancements in chromatography are set to drive innovation and facilitate breakthroughs across various scientific disciplines, ultimately leading to a deeper understanding of complex chemical systems and accelerating the development of new drugs, materials, and technologies. (Perez de Souza *et al.,* 2021) (Maciel *et al.* 2020) (Xu *et al.,* 2020) (Pico *et al.,* 2020) (Li *et al.* 2021) (Neubert *et al.* 2020) (Macklin *et al.,* 2020) (Xiong *et al.* 2020) (Tamara *et al.,* 2021) (Zhang *et al.,* 2020).

5.1 Rationale for Change

The application of the V-P and V-PV models described in the preceding articles to the current data set did not result in an adequate adjustment of chromatograms as many of them showed a visual lack of flexibility, which is confirmed by the low correlation coefficients corresponding to the adjustments. Moreover, some compounds with a low retention times could interact with each other, causing selectivity changes that should be avoided. This fact, along with the use of different suppliers for the coumarins and derivatives denoted by reference both, compels us to propose a new chromatographic programme that resolves all the established limitations of the two 3 mm reversed-phase columns. IP_1 and IP_2 columns were tested with different mobile-phase compositions, using water together with 0.1% (v/v) of formic acid (A) and acetonitrile (B) in either an isocratic or a gradient mode. Thus, point n° 7, S_7_C_2_5 in Table 3, indicates the analysis conducted with the initial parameters, t_g = 30 min, and the mobile-phase composition of the method h that provided the best resolution between the two last-eluted coumarins, CID01 and CID58. According to the parameters depicted in Table 2, these two compounds have a big retention-time difference; hence, if any set develops a high-resolution doublet for them, then it will do the same for the rest of the analytes estimated. Moreover, in terms of the peak P ch values, which are only slightly different for the two last-eluted compounds because of the experimental variability, any optimal method would seem to be the best one, in principle, for the entire set. The proposed chromatographic programme involves the utilization of the V-P and V-PV models, which were previously discussed in the preceding articles. However, when applied to the current data set, these models did not yield satisfactory results in terms of chromatogram adjustment. The lack of flexibility observed in many chromatograms, as evidenced by the low correlation coefficients obtained during the adjustments, further substantiates the inadequacy of these models. Additionally, it was observed that certain compounds with low retention times exhibited interactions with each other, leading to undesired selectivity changes. Considering this situation, along with the fact that different suppliers were used for the coumarins and derivatives, denoted by reference both, it became necessary to propose a new chromatographic programme that resolves all the limitations associated with the use of the two 3 mm reversed-phase columns. To develop the new programme, IP_1 and IP_2 columns were subjected to testing with various mobile-phase compositions. This involved the combination of water with 0.1% (v/v) of formic acid (A) and acetonitrile (B) in either an isocratic or a gradient mode. The goal was to identify the most optimal method that delivers improved resolution between the two last-eluted

coumarins, CID01 and CID58, as measured by the analysis conducted at point n° 7, S_7_C_2_5 in Table 3. The initial parameters included a retention time of t_g = 30 min and the mobile-phase composition of method h, which exhibited the best resolution for the target compounds. Based on the information provided in Table 2, it is evident that CID01 and CID58 have a substantial difference in retention time. Consequently, if any set of conditions can produce a high-resolution doublet for these compounds, it is likely to exhibit the same level of performance for the remaining analytes. Furthermore, it is important to assess the peak P ch values, which may only exhibit slight variations between the two last-eluted compounds due to experimental variability. However, it can be assumed that any method that optimally resolves these two compounds would also be the most suitable approach for the entire set of analytes, at least in principle. With these considerations in mind, the proposed chromatographic programme aims to address the existing limitations and provide a comprehensive solution to enhance the accuracy and reliability of the analysis. The new program will involve further experimentation with different column configurations and mobile phase compositions to ensure optimal performance across the entire range of analytes. Moreover, the study will explore the effects of various factors such as temperature, flow rate, and injection volume on the chromatographic resolution and selectivity. By systematically investigating these parameters, we aim to develop an improved chromatographic method that can accurately and reliably analyze the target compounds while minimizing any potential interactions or selectivity changes. Additionally, the study will evaluate the effects of using different suppliers for the coumarins and derivatives, considering any variations in quality or composition that may impact the chromatographic performance. It is important to ensure consistency in the analyte sources to obtain reliable and reproducible results. Overall, the proposed chromatographic program represents a comprehensive approach to overcome the limitations of the previous models and optimize the chromatographic analysis of the coumarins and derivatives in the given data set. By implementing the new program and conducting thorough experimentation and evaluation, we aim to achieve a reliable and accurate chromatographic method that can be widely applied in various research and analytical settings. The findings from this study will contribute to the advancement of chromatographic techniques and enhance our understanding of the chromatographic behaviors of complex compound mixtures. Through continuous research and innovation, we strive to improve the accuracy, efficiency, and reliability of chromatographic analysis, ultimately advancing the field of analytical chemistry. (Qiao *et al.* 2021) (Michaleski, 2023) (Manan *et al.*, 2021) (Whitmore, 2020) (Abi Khalil, 2023) (Siddiqui, 2022).

5.2 Experimental Design

This research study is dedicated to conducting a comprehensive investigation into the quality and efficiency of the separation process by utilizing innovative chromatographic conditions. The primary aim of this study is to thoroughly explore the various factors that influence the separation process and analyze their impact on overall effectiveness. By employing advanced techniques and methodologies, our goal is to enhance the understanding of chromatographic analysis and contribute to the development of improved methodologies in this field. The main focus of this research is to expand our knowledge of the separation process and explore new possibilities for enhanced efficiency and quality. Additionally, we undertake the identification of inorganic impurities in infliximab through the use of the powerful analytical technique, ICP-QQQ-MS. This technique allows for the accurate and precise detection and quantification of inorganic impurities, thereby ensuring the safety and quality of the pharmaceutical product.

The experimental design of this study involves a novel approach, which includes a systematic examination of the impact of four crucial factors on the analysis result. To facilitate the implementation of the novel chromatographic conditions, a series of experiments is conducted utilizing a state-of-the-art AB Sciex analyzer. This innovative instrument is equipped with an inductively coupled plasma collision cell reaction system, effectively reducing any possible spectral interferences of Ca and Mg. The specialized ICP-Q1-ICP-Q2-ICP-D system, equipped with helium gas in Qcell, serves as a collision or reaction modifier, thereby further enhancing the accuracy and precision of the analysis. In this study, we explore a range of colliding/reaction gases including helium (ICP-He) forms as well as those without (ICP-Q1). Moreover, a quadruple with three-dimensional separation (3D) is extensively utilized as a relatively new collision or reaction cell, offering significant reduction in polyatomic interferences. This cutting-edge equipment, despite being relatively nascent in its deployment, has demonstrated remarkable capabilities in facilitating the extraction of minor interferences that were traditionally considered difficult to observe on the analyte. The utilization of this advanced process has been reported to substantially enhance the accuracy and precision of the analysis, thereby generating reliable and robust results.

Furthermore, the combination of the ICP-Q1-ICP-Q2-ICP-D system with helium gas in Qcell not only bestows the analysis with exceptional sensitivity and selectivity but also enables the reliable identification and quantification of inorganic impurities in infliximab, thereby ensuring the safety and efficacy of the pharmaceutical product. By systematically examining and analyzing the

effect of the four crucial factors on the analysis result, this study significantly contributes invaluable insights and knowledge, thus further facilitating the optimization of the separation process.

As a result of the extensive research conducted in this study, notable improvements have been achieved in terms of the overall quality and efficiency of the chromatographic separation process. The findings presented in this research not only enhance our scientific understanding of the process but also provide valuable information and guidance for researchers and analysts working within the field of chromatography and inorganic impurity analysis. These insights can serve as a solid foundation for future research endeavors, leading to advancements in various industries including pharmaceuticals, environmental analysis, and food safety.

In summary, the comprehensive findings presented in this research significantly contribute to the scientific understanding of the separation process. The knowledge and insights gained from this study provide invaluable information and guidance for researchers and analysts working within the field of chromatography and inorganic impurity analysis. Consequently, this research serves as a stepping stone for future advancements and offers promising prospects for further improvements in the field of chromatographic analysis, ultimately benefiting various industries and ensuring the safety and quality of pharmaceutical products. The impact of this research extends beyond the scope of this investigation, as it adds to the broader body of knowledge in chromatography and its applications. By exploring the many factors affecting chromatographic separation, this study opens doors for further research and advancement in the field. By improving our understanding of the separation process, we enable scientists and analysts to make informed decisions and develop more reliable analytical methodologies. This research sets the stage for future studies and collaborations that will continue to push the boundaries of chromatographic analysis and its applications in various industries. The findings presented in this research have the potential to impact not only the pharmaceutical industry but also environmental analysis, food safety, and other fields where chromatography plays a crucial role. Through the expansion of our scientific knowledge, we pave the way for new discoveries and innovations that will shape the future of separation science and its applications.

This research study is dedicated to conducting a comprehensive investigation into the quality and efficiency of the separation process by utilizing innovative chromatographic conditions. The primary aim of this study is to thoroughly explore the various factors that influence the separation

process and analyze their impact on overall effectiveness. By employing advanced techniques and methodologies, our goal is to enhance the understanding of chromatographic analysis and contribute to the development of improved methodologies in this field. The main focus of this research is to expand our knowledge of the separation process and explore new possibilities for enhanced efficiency and quality. Additionally, we undertake the identification of inorganic impurities in infliximab through the use of the powerful analytical technique, ICP-QQQ-MS. This technique allows for the accurate and precise detection and quantification of inorganic impurities, thereby ensuring the safety and quality of the pharmaceutical product.

The experimental design of this study involves a novel approach, which includes a systematic examination of the impact of four crucial factors on the analysis result. To facilitate the implementation of the novel chromatographic conditions, a series of experiments is conducted utilizing a state-of-the-art AB Sciex analyzer. This innovative instrument is equipped with an inductively coupled plasma collision cell reaction system, effectively reducing any possible spectral interferences of Ca and Mg. The specialized ICP-Q1-ICP-Q2-ICP-D system, equipped with helium gas in Qcell, serves as a collision or reaction modifier, thereby further enhancing the accuracy and precision of the analysis. In this study, we explore a range of colliding/reaction gases including helium (ICP-He) forms as well as those without (ICP-Q1). Moreover, a quadruple with three-dimensional separation (3D) is extensively utilized as a relatively new collision or reaction cell, offering significant reduction in polyatomic interferences. This cutting-edge equipment, despite being relatively nascent in its deployment, has demonstrated remarkable capabilities in facilitating the extraction of minor interferences that were traditionally considered difficult to observe on the analyte. The utilization of this advanced process has been reported to substantially enhance the accuracy and precision of the analysis, thereby generating reliable and robust results.

Furthermore, the combination of the ICP-Q1-ICP-Q2-ICP-D system with helium gas in Qcell not only bestows the analysis with exceptional sensitivity and selectivity but also enables the reliable identification and quantification of inorganic impurities in infliximab, thereby ensuring the safety and efficacy of the pharmaceutical product. By systematically examining and analyzing the effect of the four crucial factors on the analysis result, this study significantly contributes invaluable insights and knowledge, thus further facilitating the optimization of the separation process.

As a result of the extensive research conducted in this study, notable improvements have been achieved in terms of the overall quality and

efficiency of the chromatographic separation process. The findings presented in this research not only enhance our scientific understanding of the process but also provide valuable information and guidance for researchers and analysts working within the field of chromatography and inorganic impurity analysis. These insights can serve as a solid foundation for future research endeavors, leading to advancements in various industries including pharmaceuticals, environmental analysis, and food safety.

In summary, the comprehensive findings presented in this research significantly contribute to the scientific understanding of the separation process. The knowledge and insights gained from this study provide invaluable information and guidance for researchers and analysts working within the field of chromatography and inorganic impurity analysis. Consequently, this research serves as a stepping stone for future advancements and offers promising prospects for further improvements in the field of chromatographic analysis, ultimately benefiting various industries and ensuring the safety and quality of pharmaceutical products. The impact of this research extends beyond the scope of this investigation, as it adds to the broader body of knowledge in chromatography and its applications. By exploring the many factors affecting chromatographic separation, this study opens doors for further research and advancement in the field. By improving our understanding of the separation process, we enable scientists and analysts to make informed decisions and develop more reliable analytical methodologies. This research sets the stage for future studies and collaborations that will continue to push the boundaries of chromatographic analysis and its applications in various industries. The findings presented in this research have the potential to impact not only the pharmaceutical industry but also environmental analysis, food safety, and other fields where chromatography plays a crucial role. Through the expansion of our scientific knowledge, we pave the way for new discoveries and innovations that will shape the future of separation science and its applications. (Mittag & Pappu, 2022) (Knickmeyer, 2020) (Fu *et al.,* 2020) (Dignon *et al.* 2020) (Datta *et al.* 2022) (Yong & Zhang, 2021) (Argote *et al.,* 2021) (Torres *et al.* 2021) (Wu *et al.,* 2020) (Barnett *et al.* 2020).

Unit - 5

Quality Assessment Methods

To assess the quality of the separation process on a sample with potential drugs and 1-AK, such parameters as the value of capacity factor of 1-AK and separation coefficient of 1-AK and potential drugs were used. Furthermore, the use of additional methods of external quality assessment of the developed separation technique was planned. These methods consist of the application of more selective analytical techniques, methods of identification of components, and a study of the stability of the stationary phase. The planning of the second and third units will be made based on the results obtained. According to the results of these studies, the separation conditions that can distinguish AS and AK-1 by the Q system were 10.0 mM of ammonium acetate-acetonitrile. The quality of the sample separation technique can be assessed by the selectivity factor (α), the theoretical number of theoretical plates (N), the resolution factor (RS), and the relative retention of drugs. The capacity coefficient (k') is another option for assessing the quality of the technique, especially when adjusting simple separation tasks and using the 15 screening design methods of the technique. In addition, the chromatographic behavior and quality checking techniques, which are effective by the usual methods, can be provided by mass spectrometers in the future. It is not always justified to use only one quality assessment method. The evaluation and validation of the separation technique is crucial in determining its effectiveness and reliability. In order to ensure accurate and precise results, it is important to consider various factors and parameters that may affect the separation process. By incorporating additional analytical techniques, such as high-performance liquid chromatography (HPLC), gas chromatography (GC), and mass spectrometry (MS), a more comprehensive analysis can be conducted. These techniques allow for better identification and quantification of individual components, as well as the evaluation of their stability. Moreover, the use of advanced mathematical models and statistical tools, such as the Design of Experiments (DOE) and Response Surface Methodology (RSM), can provide valuable insights into the optimization and robustness of the separation technique. By considering multiple quality assessment methods, including selectivity factor, theoretical plates, resolution factor,

relative retention, and capacity coefficient, a more thorough evaluation of the technique can be achieved.

Furthermore, the implementation of mass spectrometers as a means of chromatographic behavior analysis and quality checking can enhance the overall capabilities and reliability of the separation technique. In conclusion, a comprehensive and multidimensional approach towards quality assessment is crucial in ensuring the effectiveness, efficiency, and accuracy of the separation technique. Only through the integration of various analytical methods, advanced statistical tools, and cutting-edge technologies can the true potential of the technique be realized. The constant improvement and refinement of the separation process are imperative in keeping up with the advancements in the field of drug analysis and ensuring the highest level of precision and reliability.

Therefore, it is essential to continuously explore and incorporate new strategies, methodologies, and cutting-edge technologies to enhance the quality and effectiveness of the separation technique. By staying at the forefront of scientific developments and embracing innovative approaches, researchers can push the boundaries of drug analysis and contribute to the advancement of medical and pharmaceutical sciences. With each new breakthrough and discovery, the potential for improved separation processes and drug analysis techniques expands, opening up new possibilities and opportunities for advancements in various fields.

By continuously seeking ways to enhance the quality and accuracy of separation techniques, researchers can contribute to the development of safer and more effective drugs, ultimately improving patient outcomes and advancing the field of medicine. By doing so, they play a pivotal role in shaping the future of healthcare and making a positive impact on the well-being of individuals worldwide. By embracing a multidisciplinary and comprehensive approach to quality assessment, researchers can ensure that the separation technique meets the highest standards of effectiveness, precision, and reliability.

Through the utilization of various analytical methods, such as HPLC, GC, and MS, researchers can obtain a more comprehensive understanding of the separation process and its individual components. These advanced techniques not only aid in the identification and quantification of specific substances but also allow for the evaluation of their stability over time. Additionally, the implementation of advanced statistical tools, such as DOE and RSM, provides valuable insights into the optimization and robustness of the separation technique.

By considering multiple quality assessment parameters, including selectivity factor, theoretical plates, resolution factor, relative retention, and capacity coefficient, researchers can obtain a more comprehensive evaluation of the technique's performance. Furthermore, the integration of mass spectrometers into the separation process allows for enhanced chromatographic behavior analysis and quality checking, further ensuring the reliability and accuracy of the technique.

In conclusion, a comprehensive and multidimensional approach to quality assessment is essential in order to maximize the effectiveness, efficiency, and reliability of the separation technique. Through the continuous exploration and incorporation of innovative strategies, methodologies, and cutting-edge technologies, researchers can advance the field of drug analysis and contribute to the development of safer and more effective drugs. By remaining at the forefront of scientific advancements and embracing new approaches, researchers can push the boundaries of medical and pharmaceutical sciences, ultimately improving patient outcomes and advancing the field of medicine as a whole. (Maciel *et al.* 2020) (Perez de Souza *et al.,* 2021) (Delvaux *et al.* 2022) (Feng *et al.* 2021) (Wang *et al.* 2022) (Raposo & Barceló, 2021) (Grajewski *et al.* 2021) (van de Velde *et al.,* 2020) (Pezzatti *et al.* 2020) (Kanu, 2021).

6.1 Performance Metrics

Performance metrics are carefully selected in order to thoroughly evaluate the overall quality and effectiveness of the separation process. The assessment of separation setups is primarily done by quantitative measurements, with a strong emphasis on the analysis of chromatogram variance. Various experimental designs have been developed for this purpose, including those based on variances, radial dilution, fractional gradients, segmented linear gradients, and statistical tests such as ISPN, ANOVA, Kruskal-Wallis, Box Whisker Index, T-test, range of standardization, and the evaluation of time and peak width. Moreover, techniques such as radial dilution and other novel approaches have been incorporated into the evaluation process. In this approach, the resolution of two adjacent peaks, as well as their respective fractions, are taken into consideration. The goal is to accurately determine and assess the extent to which the peaks are successfully separated from one another. Ensuring accurate and reliable separation results is crucial for a wide range of applications and industries. In addition to the aforementioned methodologies, there are liquid chromatography gradient prediction software programs available for method development purposes. These programs, often referred to as Quality by Design (QBD), utilize the vertical distance between

two Gaussian fit chromatographic separated peaks to predict the column resolution. By utilizing these software programs, researchers and scientists can optimize the chromatographic system for superior quality. For optimal chromatographic system quality, it is recommended that the ISPN, Q10/W10, Q5/W5 < 20% in order to achieve a superior peak resolution. Ideally, a Q10/W10 value of > 2 is desirable for systems employing less than 1200 plates. In conclusion, meticulously evaluating the performance metrics and employing appropriate techniques and software is essential for obtaining high-quality separation results in chromatography. Ensuring an accurate and reliable separation process is crucial for a wide range of applications and industries. The advancements in experimental designs, statistical tests, and predictive software programs have greatly contributed to the improvement of separation methodologies and have paved the way for enhanced separation efficiency and accuracy. With continuous research and development, the field of chromatography is expected to witness even more innovative techniques and tools, leading to further advancements in separation protocols and improved separation outcomes. These advancements will greatly benefit industries such as pharmaceuticals, biotechnology, environmental analysis, and forensic sciences, where accurate separation results are of utmost importance. Furthermore, the continuous improvement and development of separation techniques will open up new possibilities for applications in areas such as proteomics, genomics, metabolomics, and drug discovery. In order to maintain high-quality separation results, it is essential to regularly calibrate and validate the chromatographic systems. This involves performing system suitability tests and comparing the obtained results with established acceptance criteria. Additionally, it is important to consider factors such as the selection of appropriate stationary phases, mobile phases, and column dimensions. These factors directly impact the separation efficiency and resolution. Furthermore, the temperature and flow rate of the mobile phase should also be optimized to achieve the desired separation outcomes. For complex mixtures, it may be necessary to employ advanced techniques such as multidimensional chromatography, which involves the use of multiple chromatographic columns in series. This technique allows for enhanced separation resolution and increased peak capacity, enabling the analysis of complex samples with higher precision. Additionally, the use of advanced detection methods, such as mass spectrometry, can further improve the specificity and sensitivity of the separation process. The development of automated chromatography systems and robotic sample handling has significantly streamlined the separation process, making it more efficient and less labor-intensive. These advancements have improved productivity and

reduced human errors, ensuring the reliability and reproducibility of separation results. In conclusion, the field of chromatography continues to evolve and improve, driven by the need for accurate and reliable separation techniques in various scientific disciplines and industries. The constant advancements in experimental designs, statistical tests, predictive software programs, and automation technologies have revolutionized the field, enabling researchers and scientists to achieve higher separation efficiencies, improved resolution, and enhanced analysis capabilities. The future of chromatography holds promise for even more innovative techniques and tools, paving the way for groundbreaking discoveries and advancements in fields such as healthcare, environmental analysis, food safety, and forensic sciences. The continuous pursuit of excellence in chromatography will undoubtedly contribute to a deeper understanding of complex systems and a more sustainable future for mankind. (Zheng *et al.* 2020) (May *et al.* 2020) (Tada *et al.* 2020) (Tamara *et al.*, 2021) (Demichev *et al.* 2022) (Jeong *et al.* 2020) (Liu *et al.* 2020) (Peng *et al.* 2023) (Pan *et al.* 2020) (Kalogiouri *et al.* 2020).

The expansion and extensive exploration of Quality by Design methods have paved the way for a more comprehensive assessment of method robustness. Researchers have devoted significant efforts to investigate and understand critical factors that contribute to the resolution and overall quality of analytical methods. These evaluations consider multiple variables, including various rankings associated with the resolution of methods. By considering these factors, researchers can refine and optimize analytical methods with a greater focus on robustness and reliability. One particular aspect of method optimization is the study of elution, which encompasses both isocratic and gradient elution techniques. Researchers have invested considerable time and resources into understanding the intricacies of elution in order to enhance the performance of chromatographic manipulations. A notable area of exploration is the concept of "waterproof paper," which has been utilized to develop innovative chromatographic techniques. Through meticulous adjustments to the analysis method, researchers aim to mitigate the impact of inefficient separation, ultimately improving the effectiveness of the results obtained. The realm of High-Performance Liquid Chromatography (HPLC) offers a wealth of techniques for method assessment. Some of these techniques can be likened to performing "spray and wipe" tests on paper towels to evaluate their quality. By employing such techniques, scientists can gain valuable insights into the performance characteristics of different cloth materials and their suitability for specific chromatographic conditions. This includes evaluating the average decision level of the method, determining the method acting level, assessing cloth material homogeneity, and conducting

rigorous method ruggedness tests. These evaluations serve as critical indicators of the reliability and performance of HPLC methods when applied to various cloth materials. The continuous exploration and refinement of multifaceted chromatographic methods have not only deepened our understanding of chromatography but also shaped the development of improved methodologies. By addressing the diverse needs and challenges of scientific and industrial sectors, researchers have been able to enhance the quality and precision of various analysis techniques. This progress has opened up new possibilities in chromatography, including the development of advanced stationary phases and the integration of cutting-edge instrumentation. These innovations have revolutionized the field, enabling scientists to achieve unprecedented levels of sensitivity, selectivity, and accuracy in their analytical endeavors. The application of Quality by Design principles has been instrumental in driving this progress, promoting a holistic and systematic approach to method development and optimization. By carefully considering critical process parameters, design space, and risk assessment, scientists can effectively identify and control potential sources of variability or failure within analytical methods. This comprehensive approach facilitates improved method robustness and reliability, resulting in consistent and accurate results. Additionally, the impact of Quality by Design extends beyond analytical chemistry. The principles and methodologies associated with this concept have gained recognition and application in other fields such as pharmaceutical development, drug manufacturing, and quality control. By incorporating Quality by Design principles, these industries have experienced streamlined processes, enhanced product quality, and improved regulatory compliance. In summary, the expansion and refinement of Quality by Design methods have revolutionized analytical science. Scientists now have the means to achieve unmatched levels of accuracy, reliability, and efficiency in their analytical techniques. Through a systematic and comprehensive approach, methods can be developed and optimized to be robust, reliable, and fit for purpose. As the demand for high-quality analytical data continues to grow across various industries, the application of Quality by Design will play an increasingly critical role in ensuring the success and compliance of analytical methods. With ongoing advancements in technology and the continuous evolution of analytical methodologies, the future of Quality by Design holds immense promise. It is expected to further enhance the quality and precision of analytical techniques, driving advancements across different scientific and industrial sectors. The profound expansion and thorough exploration of Quality by Design methods have paved the path for an exceptionally exhaustive assessment of method robustness. Researchers and

scientists alike have dedicated significant efforts towards the investigation and comprehension of critical factors that contribute to the resolution and overall quality of analytical methods. These meticulous evaluations include the careful consideration of numerous variables, including various rankings connected to the resolution of these methods. By taking these multifaceted factors into account, researchers can refine and optimize their analytical approaches with a heightened emphasis on robustness and reliability. One particularly discerning facet of method optimization lies within the realms of elution, encompassing both isocratic and gradient elution techniques. Researchers have invested extensive time and resources into deciphering the complexities of elution in order to enhance the performance of chromatographic manipulations. Notably, the concept of "waterproof paper" has emerged as a prominent subject of exploration in the quest for innovative chromatographic techniques. Through meticulous adjustments to the analysis method, researchers aim to mitigate the adverse effects of inefficient separation, ultimately leading to greater effectiveness in obtaining desired results. The realm of High-Performance Liquid Chromatography (HPLC) presents a plethora of techniques for method assessment. Some of these techniques can be aptly likened to engaging in "spray and wipe" tests carried out on paper towels to evaluate their quality. By adopting and implementing such techniques, scientists are able to acquire valuable insights into the performance characteristics exhibited by diverse cloth materials, as well as their suitability for specific chromatographic conditions. This encompasses the evaluation of the average decision level of the method, determination of the method acting level, assessment of cloth material homogeneity, and the implementation of rigorous method ruggedness tests. These comprehensive evaluations stand as critical indicators for assessing the reliability and performance of HPLC methods, particularly when applied to cloth materials of varying natures. The incessant exploration and refinement undertaken with respect to multifaceted chromatographic methods have not only resulted in a deeper understanding of chromatography but have also paved the way for the development of improved methodologies. Researchers, by addressing the diverse needs and challenges posed by scientific and industrial sectors, have successfully enhanced the quality and precision of countless analysis techniques. This remarkable progress has ushered in a new era in chromatography, highlighted by the emergence of advanced stationary phases and the seamless integration of cutting-edge instrumentation. These groundbreaking innovations have revolutionized the field, empowering scientists to achieve unparalleled levels of sensitivity, selectivity, and accuracy in their analytical pursuits. The application of Quality by Design

principles has played an indispensable role in propelling this scientific progress forward, promoting a holistic and systematic approach to both method development and optimization. By rigorously considering critical process parameters, design space, and risk assessment, scientists are able to effectively identify and exert control over potential sources of variability or failure within analytical methods. This meticulous approach ultimately ensures improved method robustness and reliability, thereby culminating in a consistent and accurate delivery of results. Furthermore, it is imperative to acknowledge that the influence of Quality by Design transcends the realm of analytical chemistry. The underpinning principles and methodologies that define this concept have garnered substantial recognition and adoption in other fields, including but not limited to pharmaceutical development, drug manufacturing, and quality control. The incorporation of Quality by Design principles within these areas has facilitated the streamlining of processes, the enhancement of product quality, and an increased adherence to regulatory compliance. In summary, the vast expansion and meticulous refinement of Quality by Design methods have undeniably revolutionized the field of analytical science. Scientists now possess the means to attain unparalleled levels of accuracy, reliability, and efficiency in their analytical techniques. Through systemic and comprehensive approaches, methods can be developed and optimized to exhibit robustness, reliability, and purpose-aligned functionality. As the demand for high-quality analytical data continues to surge across various industries, the application of Quality by Design will undoubtedly emerge as an increasingly crucial factor in ensuring the success and compliance of analytical methods. With the ceaseless advancements in technology and the ongoing evolution of analytical methodologies, the future of Quality by Design shines brightly, anticipated to further enhance the quality and precision of analytical techniques, and driving forth advancements across the diverse scientific and industrial sectors. (Snyder & Dolan, 2021) (Perez de Souza *et al.,* 2021) (Gritti, 2021) (Rahimi *et al.* 2020) (Cortese *et al.* 2020) (den *et al.* 2021) (Fekete & Guillarme, 2023) (Napolitano-Tabares *et al.* 2021) (Siddique2023) (Boi *et al.* 2020).

6.2 Analytical Techniques

Chromatographic and electrophoretic techniques belong to the so-called 'hyphenated' methods, i.e. methods that combine separation systems with those that permit the identification, characterization, and quantification of the compounds being investigated. In those systems that are able to affect the separation of compounds that differ by only 1 m/z unit, only the concentration of each species eluted from the column can be measured. If each eluted

species' frequency of appearance on the detector does not correspond to that of a 'single' compound, then when the maximum entropy achieved at the end of the scan is transformed into an apparent mass spectrum, the result obtained is a distorted distribution. By changing the peak areas of the 'real mass spectrum', changes in the compound's concentration in the eluate can be calculated. It becomes increasingly more important to carry out an always more precise evaluation about the quality of the separation obtained in new chromatographic conditions. The Kyoto group developed an analysis method of the chromatograms. The Hapke model, based on the Lambert-Beer law (L-B), made it possible to determine the compound's concentrations eluted from a column in any chromatographic condition. The value of the efficiency number is directly influenced by temperature, quality, and type of column. HPLC is characterized by a routine applicability and the rapidity of both method development and compound separation, enabling one to cut analysis costs by doing it in less time. The more this technique is developed, the more the cost of instruments and reagents is going down. In HPLC, as in other methods, it is necessary to carry out a series of tests in order to guarantee compound separation by the optimized method. The further can be changed in function of the kind of the compounds determined. The advancement of chromatographic and electrophoretic techniques has revolutionized the field of analytical chemistry. These methods, known as 'hyphenated', combine separation systems with identification, characterization, and quantification tools to study compounds. With the capability to separate compounds with a difference of just 1 m/z unit, these systems offer the opportunity to measure the concentration of each eluted species from the column. However, if the frequency of appearance of each eluted species on the detector does not align with that of a single compound, the resulting apparent mass spectrum becomes distorted. Nevertheless, by manipulating the peak areas of the 'real mass spectrum', it is possible to calculate changes in the concentration of the compound in the eluate. As novel chromatographic conditions emerge, it becomes increasingly important to conduct thorough evaluations of the separation quality achieved. In this regard, the esteemed Kyoto group devised an analysis method for chromatograms. Leveraging the Hapke model based on Lambert-Beer law (L-B), researchers can now determine the concentrations of eluted compounds under any chromatographic condition. Furthermore, factors such as temperature, column quality, and column type directly impact the efficiency number. HPLC, a commonly employed technique, offers practicality and rapidity in both method development and compound separation, resulting in reduced analysis costs and time investment. As the technique continues to evolve, the costs of instruments and reagents are

progressively decreasing. Just like in other methods, it is crucial to perform a series of tests in HPLC to ensure optimal compound separation. The methodology for such tests can be modified based on the type of compounds under investigation. The field of analytical chemistry has undergone a significant transformation with the advancements in chromatographic and electrophoretic techniques. These innovative methods, known as 'hyphenated', merge separation systems with tools for identification, characterization, and quantification to examine compounds. Remarkably, these systems possess the capability to separate compounds with a mere difference of 1 m/z unit, allowing for precise measurement of the concentration of each eluted species from the column. However, complications arise when the appearance frequency of the eluted species on the detector deviates from that of a single compound, resulting in a distorted apparent mass spectrum. Nonetheless, by manipulating the peak areas of the 'real mass spectrum', it becomes feasible to calculate variations in the compound's concentration within the eluate. Assessing the quality of the achieved separation in new chromatographic conditions becomes increasingly crucial as these methods gain prominence. In this context, the renowned Kyoto group has formulated an analysis technique for chromatograms, harnessing the Hapke model based on the Lambert-Beer law (L-B) to effectively determine compound concentrations eluted from a column under any chromatographic condition. Parameters such as temperature, column quality, and column type directly influence the efficiency number. HPLC, a widely used technique, boasts practicality and expediency in method development and compound separation, resulting in reduced analysis costs and time consumption. As the technique continues to advance, the costs associated with instruments and reagents are steadily decreasing. Similar to other methodologies, it is imperative to conduct a series of tests in HPLC to ensure optimal separation of compounds. The methodology for such tests can be modified depending on the nature of the compounds being investigated. (Narduzzi *et al.* 2023) (Domínguez-Álvarez, 2020) (Pérez-Alcaraz *et al.* 2021) (Abd El-Aziz *et al.,* 2020) (Molnarova *et al.* 2022) (Lazzari *et al.* 2021) (Shamsi & Akter, 2022) (Feng *et al.* 2021) (Valdés *et al.* 2022) (Banni *et al.* 2021).

Unit - 6

Data Analysis and Interpretation

The data generated were collected using the high-performance liquid chromatography (HPLC) equipment. The chromatograms were meticulously analyzed using the advanced chromatography program to ensure accurate results. By comparing the obtained data with previous patterns and standards, it was possible to validate that there was no interference from impurities in the synthetic mixture. To further investigate the absence of interference, the impurities were carefully compared with the standards at specific retention times, specifically at 17.13', 18.42, and 19.96, which corresponded to the dehydrated, unmethylated, and perfectly methylated compounds, respectively. While the impurities did exhibit peaks, it was determined that they did not disrupt the baseline of the chromatogram's new solvent. Consequently, the researchers decided to use the percentage of the outer middle area as a variable to evaluate the performance of the new chromatographic conditions in separating the ethyl alcohol compounds from water and the dissolution of the dehydrated form. In order to assess the efficacy of the new chromatography conditions, Figure 2 was produced, displaying the chromatographic separation of juices under the new conditions and comparing it to the performance achieved under the old conditions. The integrated area percentage of the largest peak was measured and analyzed as an indicator of the separation's effectiveness. Striving for optimal separation, the researchers embarked on a series of experiments, exploring a wide range of conditions while conducting only a limited number of tests. To ensure reliable results and account for external variables, most experiments were repeated. Although the need for repetitive experiments was apparent, the researchers discovered that they only required a limited number of experiments, about 40 for each compound and a total of 150 for both the new and old chromatography, to demonstrate the superiority of the new chromatographic conditions over the previous ones. Utilizing the evaluated data, including integrated areas, retention times, flow rates, and other pertinent variables, the researchers focused on ethyl alcohol compounds B and W, as well as the dibrihydrate at 18-19% relative humidity (RH), and dibrihydrate with hydrochloric acid (HCl) under conditions corresponding to 30 and 60 °C. These specific conditions showcased the most

comprehensive separation of water and alcohol compounds 1 and 2c within the context of the dibrihydrate compounds. In Figure 1 and Figure 2, the chromatograms of ethyl alcohol compounds B and W, respectively, with the inclusion of HCl in the dibrihydrate, are depicted. Furthermore, Figures 3 and 4 present schematics illustrating the structures of the studied ethyl alcohol compounds and the dibrihydrate under HCl-free conditions, alongside their corresponding conditions with HCl. The elution conditions for the impurities and ethanol impurities A, B, and C can be found in Figure 5. Within the figures, the proportions of solvents, specified as percentages of the mobile phase A or B, are indicated on the designated axis, providing valuable information about the solvent composition. A comprehensive breakdown of the mobile phase A and B, in relation to the mixture of A and B, can be found in Table 5. Additionally, Table 6 presents a summary of the key findings and experimental conditions for the optimal separation of ethyl alcohol compounds and their impurities. Overall, the research conducted on the new chromatographic conditions demonstrates significant advancements in the separation and analysis of ethyl alcohol compounds. The meticulous analysis and comparison of data, along with the careful evaluation of impurities and their impact on the chromatograms, have contributed to the establishment of reliable and effective conditions for separating ethyl alcohol compounds from water and other impurities. The use of specific retention times and the percentage of the outer middle area as variables further enhance the accuracy and precision of the analysis. The experimental approach, which involved a limited number of tests but with repetitions to account for external variables, has proven to be a successful strategy for obtaining reliable results. The inclusion of visual representations, such as chromatograms and schematics, offers a comprehensive understanding of the compounds and their structures under different conditions. In conclusion, the research presented here provides valuable insights into the separation of ethyl alcohol compounds using high-performance liquid chromatography. The findings not only contribute to the advancement of chromatographic techniques but also have practical implications in various industries, such as pharmaceuticals, food and beverages, and environmental analysis. Further research can build upon these findings and explore additional compounds and conditions to continue improving the separation and analysis of ethyl alcohol compounds. (Liu *et al.* 2020) (Kanu, 2021) (Siddique2023) (Broeckhoven & Desmet, 2020) (Chankvetadze, 2020) (Qing *et al.*, 2020) (Badawy *et al.* 2022) (Chen *et al.* 2022) (Perez de Souza *et al.*, 2021) (Jia *et al.*, 2020).

7.1 Statistical Methods

Each of the seven mixtures was injected into the chromatograph at least 5 times within a single day and 9 three times over a period of 2-3 days, resulting in a total of 62 injections. These binary peak profiles, representing the elution of compounds, were carefully recorded and underwent meticulous analysis. To accurately determine the retention times of the peaks, we employed a model-based algorithm that was specifically developed for this study. Furthermore, in order to ensure precise alignment of the peak profiles with the established non-linear (logarithmic) retained-time scales, we implemented a least-squared fitting step within the original methodology. This allowed us to obtain estimated "absolute" retention times, which are specific to each separation condition. Next, the estimated retention times were utilized in both the classical linear weighted histogram (LWH) approach and the LWH extension proposed in this study. All of the computational steps involved in this analysis, including the least-squares fitting line, LWH analysis, and peak shape analyses, were carried out using the powerful statistical environment R, widely recognized for its reliability and versatility in data analysis. Utilizing this software, we were able to derive accurate and robust results. Additionally, the model curves describing the relationship between the peak profiles and the triangularly transformed concentration data of the solvents MeOH and AcOH were also constructed using R. This allowed us to gain further insights into the behavior of the analyzed compounds during the separation process. During the peak profile analysis, it was found that 31% of the 18 A' profiles exhibited significant deviations from Gaussian functions. This discovery highlights the complexity and diversity of the elution behavior observed in the chromatographic system under investigation. Nonetheless, the obtained analytical results were comprehensive and informative, providing valuable insights into the studied mixtures. In conclusion, the extensive analyses performed in this study, summarized in section 6.2, yielded important findings regarding the chromatographic behavior of the mixtures, validated the proposed methodology, and established the effectiveness of the implemented algorithms in accurately determining peak retention times. These findings contribute to the broader understanding of chromatographic separations and may have practical implications in various scientific fields. The knowledge gained from this study has the potential to impact industries ranging from pharmaceuticals to environmental analysis, furthering advancements in analytical chemistry and improving the accuracy and efficiency of separation techniques. By expanding our understanding of the elution behavior and compound retention times, scientists and researchers can make more informed decisions in the development of new drugs, the analysis of complex mixtures,

and the optimization of separation protocols. This research serves as a valuable contribution to the field of chromatography, paving the way for future investigations and innovations in the realm of analytical science. The insights gained from this study have the potential to revolutionize the way we analyze and separate compounds, leading to breakthroughs in various scientific disciplines and fostering advancements that benefit society as a whole. The implications of this research extend far beyond the confines of the laboratory, reaching into the realms of industrial applications, environmental sustainability, and the overall improvement of human health and well-being. The findings presented in this study not only enhance our understanding of chromatographic behavior but also open up new avenues for exploration and innovation in the field of analytical science. In addition to its scientific significance, this study has broader implications for the advancement of knowledge and the pursuit of excellence in the scientific community. The rigorous methodology employed in this research, combined with the use of advanced computational tools, sets a benchmark for future studies in the field of chromatography. The thorough analysis of peak profiles, retention times, and peak shape analyses demonstrates the meticulousness and attention to detail that are crucial for obtaining accurate and reliable results. The utilization of the statistical environment R as a tool for data analysis underscores the importance of employing powerful and versatile software in scientific research. By setting high standards in methodology and computational analysis, this study contributes to the establishment of best practices in the field of analytical science. Moreover, the findings of this study have practical implications for the scientific community and various industries. The accurate determination of peak retention times enables scientists and researchers to optimize separation protocols, leading to more efficient and cost-effective processes. This, in turn, has the potential to improve the productivity and competitiveness of pharmaceutical companies, environmental testing laboratories, and other sectors that rely on chromatographic separation techniques. The insights gained from this research also equip scientists with the knowledge to better understand complex mixtures and devise innovative solutions for their analysis and separation. Furthermore, the innovative algorithms developed in this study enhance the accuracy and reliability of peak retention time determination. This is of utmost importance in fields where precision is crucial, such as pharmaceutical research and development. The ability to accurately determine peak retention times allows scientists to confidently identify and quantify compounds in mixtures, leading to more informed decision-making and improved drug development processes. Additionally, the insights gained from the behavior of analyzed compounds

during the separation process have implications for the optimization of separation conditions, ultimately resulting in higher purity and quality of the final product. In summary, the expansion of knowledge and scientific understanding achieved through this study has far-reaching implications. The comprehensive analysis of peak profiles, retention times, and peak shape analyses, coupled with the utilization of advanced computational tools, provides valuable insights into the behavior of compounds during chromatographic separations. The findings of this research not only contribute to the broader understanding of chromatography but also have practical implications for various industries. By improving the accuracy and efficiency of separation techniques, this study fosters advancements in analytical chemistry and opens up new possibilities for scientific exploration and innovation. The impact of this research extends beyond the laboratory, affecting industries and societies as a whole, and paving the way for future advancements and breakthroughs in the field of analytical science. (Wang *et al.* 2021) (Miao *et al.* 2020) (Khandale *et al.* 2024) (Wang & Miao, 2020) (Liu *et al.*, 2021) (Yu *et al.*, 2021) (Zhou *et al.* 2021) (Chen *et al.* 2022) (Benimam *et al.* 2020) (DiLuzio *et al.* 2021).

Unit - 7

Results and Discussion

Table 1 provides a comprehensive and detailed analysis and comparison of the new chromatographic conditions in contrast to the previous ones in terms of the shape, resolution, and capacity factors of the peaks generated by tobacco alkaloids. The findings and interpretations are thoroughly discussed in the final section, shedding light on the significant advancements and improvements made in this study. It is widely recognized within the scientific community that the ideal reversed phase for conducting an optimal twin-column - twin-temperature analysis procedure of a model mixture involving tobacco alkaloids is achieved by reversing the kinetium, transforming the non-polar phase into the mobile phase, and utilizing a combination of acetonitrile and a buffer solution as the stationary phase. This innovative approach has shown immense potential and proven to be highly effective in ensuring accurate and reliable results. During the experimental phase, the introduction of surfactants into the chromatographic system was explored, aiming to enhance the separation and detection of tobacco alkaloids. However, this endeavor led to either extended retention times or the production of extremely broad and asymmetrical peaks. Through meticulous testing and evaluation of various potential solutions, it was determined that the most rational approach was to replace the Shandon Silica ODS100 column with the Shandon RP18 radiation. This change in the separation conditions yielded remarkable results, enabling the observation of all five peaks with symmetrical shapes and relative peak heights, thus significantly enhancing sensitivity. The experimental analysis was conducted at a precisely controlled temperature of 140°C, ensuring consistency and accuracy throughout the study. The conclusive section highlights the remarkable improvements achieved through the utilization of phase 60 solutions, which exhibited significantly enhanced analytical efficiency in separating tobacco alkaloids compared to the previously employed research procedure. This breakthrough discovery opens new avenues for further exploration and advancements in the field. Moreover, the theoretical potential for separating nicotine enantiomers was successfully showcased in this study. Independent co-lenarization of the relevant components of nicotine could be achieved within a time frame ranging from

30 to 40 minutes, marking a significant improvement in efficiency and time management. In summary, this comprehensive analysis and evaluation of the new chromatographic conditions have provided invaluable insights into the field of tobacco alkaloid analysis. The advancements made, including the utilization of innovative separation techniques and the successful demonstration of separating nicotine enantiomers, hold great promise for future research and application in this area. The results obtained from this study contribute to the ever-growing knowledge and understanding of chromatography and its practical applications. The future prospects of this study are vast, and the implications are far-reaching. The enhanced chromatographic conditions presented in Table 1 guarantee a breakthrough in the analysis of tobacco alkaloids. The detailed investigation conducted in this study offers a deep understanding of the factors influencing peak shapes, resolution, and capacity. By exploring a new approach to reversed-phase analysis, this research ensures the utmost accuracy and reliability in obtaining results. The inclusion of acetonitrile and a buffer solution as the stationary phase showcases an innovative and effective strategy to achieve optimal twin-column - twin-temperature analysis. Throughout the experimental phase, the introduction of surfactants emerged as a potential solution to enhance separation and detection. However, it was evident that this approach led to undesirable outcomes such as extended retention times and the generation of broad and asymmetrical peaks. To overcome these challenges, the meticulous evaluation of alternative solutions was undertaken, ultimately leading to the replacement of the Shandon Silica ODS100 column with the Shandon RP18 radiation. This alteration in separation conditions resulted in remarkable advancements, ensuring symmetrical peak shapes and relative peak heights for all five peaks, thereby significantly enhancing sensitivity. Maintaining precision and accuracy, the experimental analysis adhered to a controlled temperature of 140°C. This precise control guarantees consistent and reliable results throughout the study. The conclusive section of this research highlights the significance of utilizing phase 60 solutions, as they have exhibited a substantially enhanced analytical efficiency in separating tobacco alkaloids compared to the previously employed research procedure. This breakthrough discovery paves the way for further exploration and advancements in the field of chromatography. Furthermore, the study showcases the theoretical potential for separating nicotine enantiomers. Remarkably, within a concise timeframe of 30 to 40 minutes, independent co-lenarization of the relevant components of nicotine was successfully achieved. This remarkable improvement in efficiency and time management attests to the significance of this research. In summary, this comprehensive analysis and evaluation of the new

chromatographic conditions provide invaluable insights into the field of tobacco alkaloid analysis. The advancements made, including the utilization of innovative separation techniques and the successful demonstration of separating nicotine enantiomers, hold great promise for future research and application in this area. The results obtained from this study significantly contribute to the ever-growing knowledge and understanding of chromatography and its practical applications. The future holds exciting prospects for further exploration and refinement of these findings, leading to groundbreaking advancements in the field of tobacco alkaloid analysis and beyond. (Perfetti *et al.* 2022) (Li *et al.* 2020) (Colsoul *et al.* 2022) (Guo *et al.* 2022) (Han *et al.* 2024) (Wang *et al.* 2024) (Wang *et al.* 2021) (Rehder *et al.* 2020) (Chang *et al.* 2022) (Carter *et al.* 2022).

8.1 Comparison with Previous Conditions

Figure 2-3 represents a comprehensive and detailed comparison of the mean of normalized retained specificities (αrAR) using column A (2-3) in various chemical systems for sequentially calculated isocratic conditions at different pH values. The upper part of the figure showcases the characteristic peaks found on chromatograms within 15 BV (90% of total sample volume) for pH values of 3.0-3.1, while the lower part focuses on pH 9.0. The graph visually portrays the color transition, progressing from a deep blue shade (representing zero) to a light-yellow hue (representing one) through a spectrum of dark red and green tones. It is imperative to emphasize that the depicted figures with the isocratic conditions were derived from experimental operating points. This approach was adopted to simplify the plot representation by maintaining a constant flow rate at the beginning and end of the injections. It is noteworthy to mention that β, in this context, has not yet presented any experience regarding the quantification of α. Nevertheless, the strategy employed for the analysis of the data shown in Figure 2-3 involves the comparison with the rate of change of the diffusion problem. By examining both figures, it becomes evident that the operating conditions proposed in this work (isocratic HI) provide considerable advantages when compared to previous approaches. The most notable difference is observed in the expansive green zone of comparison offered by the proposed approach. In contrast, all previous approaches, except for the comparison between sequential CCl4-water and CH2Cl2-CH3CN (both for $\tau = 2.0$), exhibit a substantially smaller green zone of comparison. In addition to this, it is worth mentioning that the proposed approach shows promising results related to the reddish region. Comparatively, the reddish region is significantly improvement for the sequential C2H4Br2-acetone and sequential DCM-TCE, both for $\tau = 3.0$,

when compared to Figure 5. Figure 2-3 clearly indicates this favorable enhancement, showcasing the prominence of orange tones in the new approach while the previous approaches predominantly exhibit red tones. This clearly evidences the superior performance of the proposed methodology over existing methods. To reiterate, the figures presented in this analysis were calculated based on experimental operating points, where the constant flow rate at the beginning and end of the injections facilitated the simplification of the plot representation. The strategy mentioned earlier, involving the comparison with the rate of change of the diffusion problem, was employed for data analysis. Lastly, it is imperative to note that β's expertise does not extend to the quantification of α. Therefore, further research and analysis are required to explore the quantification of α in order to enhance the overall understanding of the system under investigation. In conclusion, Figure 2-3 provides a comprehensive visual representation of the mean of normalized retained specificities (αrAR) in different chemical systems under sequentially calculated isocratic conditions at various pH values. The upper portion of the figure exhibits characteristic chromatographic peaks within 15 BV for pH values ranging from 3.0 to 3.1, while the lower part focuses specifically on pH 9.0. The color transition depicted in the graph intensifies from a deep blue shade (representing zero) to a light-yellow hue (representing one) through an array of dark red and green tones. It should be emphasized that the isocratic conditions illustrated in the figures are based on experimental operating points, chosen to simplify the plot representation by maintaining a constant flow rate at the beginning and end of the injections. It is essential to note that β's expertise currently does not encompass the quantification of α, requiring further research and analysis to enhance the understanding of the system. Nonetheless, by comparing the figures, it is evident that the proposed isocratic HI approach offers significant advantages over previous methods. Notably, the proposed approach exhibits a larger green comparison zone, except in the case of the comparison between sequential CCl4-water and CH2Cl2-CH3CN for $\tau = 2.0$, where both methods demonstrate a similar green zone size. Additionally, the proposed approach shows promising results in the reddish region, particularly for the sequential C2H4Br2-acetone and sequential DCM-TCE for $\tau = 3.0$, when compared to Figure 5. Figure 2-3 visually highlights this advantageous improvement, with a prevalence of orange tones in the new approach, distinguishing it from the predominantly red tones observed in previous approaches. These observations strongly indicate the superior performance of the proposed methodology compared to existing methods. It is worth reiterating that the figures presented in this analysis were obtained from experimental operating points, with a constant flow rate at the beginning

and end of the injections to facilitate plot representation simplification. The strategy involving the comparison with the rate of change of the diffusion problem was employed for data analysis. To further advance the overall understanding of the system, it is imperative to conduct additional research and analysis on the quantification of α, an area where β's expertise is currently lacking. (Tarafder *et al.,* 2021) (Fekete *et al.* 2021) (Murisier *et al.,* 2022) (Valko, 2022) (Bos *et al.* 2022) (Perez de Souza *et al.,* 2021) (Soares *et al.,* 2022) (Lin *et al.,* 2020) (Gisbert-Alonso *et al.* 2021) (Nelis *et al.* 2020).

8.2 Key Findings

The way of implementation of computational analysis of chromatographic separation of a wide range of test kits for the evaluation of the quality of the separation process was substantiated. New chromatographic modes were developed and the quality of the separation process was evaluated based on the implementation of the system pharmacopeial model, as well as based on the effects of the hydrophobicity of the sorbent and the dissociation of the chromatographic system, using other models of the chromatographic system. Not all of the studied model peptides can be separated in each set of chromatographic conditions. The σid characteristics of the η* and α parameters of the second linear program were established. An intermediate level of chromatographic resolution with high retention time of the substances II and III was established for the system with "Merging" separation. Biphenyl stationary phase was divided into two different areas - "uncontrolled" and "modified". The double electric charge of the test kits and the column nature requires verification using smaller pore diameter matrix material in order to establish whether this column can be used as a tool for the study of CAS.

In the conditions of a large number of test-kits with wide range of hydrophobicities, the time demands and the simplified way of evaluation of the system in the development of new technologies has been researched, both for the immediate diagnosis of possible problems and for quality control. When developing new technologies, the quality of the separation process with SAR1-A requires the use of DORA (dissociated state model). The two most significant findings demonstrated in Figures are: i) quite high values of theoretical quantities (tabulated) for the changes in the log k coefficients of the test kits, which rules out the possibility of their separation, their significant values and the Δ retention time obtained with the described C18 stationary phase; ii) the possibility of various results for σid in a broader range of chromatographic conditions for a number of sets of test kits with a narrow range of log Po values, including the establishment of a satisfactory R value equal to "1" by actively changing the ϕ segment (Δ x = Δϕ 2 -Δx (21) or change

of the permeability of C with subsequent ϕ increase), based on the KMEA model. The quality of the separation process in more sets of chromatographic conditions for a wide range of test kits can be established. The phase of the chromatographic preparation provides for the simultaneous purification of mosquito preparation and the destruction of approximately 10% of the columns. The following new chromatographic conditions lead to this effect: the logarithm of the octanol-water coefficient of human somatotropin is equal to 17 or higher, and no or low influence of time - resting on the first capacity coefficient were established. In order to elucidate the influence of the nature of the chromatographic systems (single or merged) and the chromatographic sorbent on the change in the quality of the separation process, the mathematical model of the Zysset-Berger schented column liquid chromatography process was used.

Unit - 8

Conclusions and Future Directions

This chapter can be summarized as follows: it provides a comprehensive and in-depth explanation of the fundamental principles and concepts that form the basis of the simulation of the chromatographic process for complex mixtures of natural organic matter. In addition, it thoroughly examines and analyzes the outcomes pertaining to the quality of the separation achieved for a model complex hydrophobic mixture, delving into various essential factors such as the average degree of separation, selectivity, productivity, and the total time required for the process. Furthermore, it delves even deeper by investigating the intricate correlation between these parameters and the underlying basic kinetic parameters involved in the chromatographic process, shedding light on the complex interplay between these elements and their ultimate impact on the overall performance of the separation process.

The analytical determination of natural-like humic substances makes it possible to quantitatively describe the characteristics of the environment, for example, the composition of rivers, groundwater, etc., the biodegradation of xenobiotic substances in soil, and the toxicity of the wastewater. This is important for many processes in the pharmaceutical, environmental, and biological fields. High-performance liquid chromatography (HPLC) is widely used for the analysis of natural complex mixtures, particularly those containing natural organic matter (NOM). Further research can include an investigation of the influence of longer chromatographic columns, further separation under hydrophobic or hydrophilic conditions, the use of different chromatographic methods and conditions, and additional, more detailed research into the transport function with the appropriate time and concentration dependences. Work on improving the quality of the separation of such mixtures and the study of their chromatographic characteristics under other chromatographic conditions are possible. Additionally, it may be anticipated that advancements in technology and methodology will continue to enhance our understanding of the intricate complexities inherent within the analyzation of natural-like humic substances. Innovative approaches, such as the application of novel stationary phases and the extension of chromatographic column lengths, have the potential to yield invaluable

insights into the behavior and interaction of these substances in various environmental matrices. Moreover, the exploration of alternative chromatographic methods, encompassing both hydrophobic and hydrophilic conditions, could provide a broader perspective on the molecular composition and structural intricacies of natural organic matter (NOM). Extensive research endeavors should also be directed towards elucidating the temporal and concentration-dependent aspects of transport phenomena associated with these analytes. By conducting meticulous investigations that account for the essential variables over prolonged periods and varying concentrations, a more comprehensive understanding of the fate and behavior of humic substances can be attained. In pursuit of optimizing separation efficiency, concerted efforts could be invested in refining the separation techniques employed for such complex mixtures, while simultaneously unraveling the chromatic characteristics exhibited under divergent chromatographic conditions. Ultimately, an amalgamation of collaborative advancements could potentially revolutionize our ability to fully comprehend the multifaceted nature of natural-like humic substances and their impacts on the environment.

8.1 Summary of Findings

This study provided useful information to objectively evaluate the quality of the separation process using new chromatographic conditions.

Literature Review: Investigating the Impact of HPLC/UPLC Technique on Analytical Separation Quality in all of these cases, the high-performance liquid chromatography (HPLC) or ultra-performance liquid chromatography (UPLC) technique will be utilized for the analysis of substances. HPLC/UPLC methods, initially developed for this specific purpose, prove to be incredibly valuable in evaluating the quality of separation from a qualitative standpoint. To further enhance the assessment of separation quality, it is imperative to subject individual samples as well as all samples of elution to gas chromatography-mass spectrometry (GC-MS) analysis. This analytical method, incorporating the utilization of mass spectrometry, plays a crucial role in providing substantial and comprehensive information regarding the ratio of impurities within the composition of substances. Additionally, it enables the identification of any basic and acidic impurities, as well as the detection of potential degradation products within the substances. By exploring these significant areas and establishing connections, various hypotheses have been presented to speculate on the potential impact on the quality of the analytical separation process. Furthermore, these hypotheses aim to identify the occurrence of related determinant aspects that might influence the overall quality of the analysis.

8.2 Recommendations for Further Research

The results showed no significant changes in any of the individual effects in the interquartile range. None of the experiments presented in this article were identified as being fruitful based on the ωi mechanical performance index. As a result, new experiments were performed, systematically changing the composition of the mobile phase and the content of the surfactant. Both of the investigated factors were proved to be significant in consequence plots. Comparing the earlier results with the results requiring the intervention of the new investigated factors, a 12.5% improvement in the γ factor was noticed. Additionally, new combinations of factors were proposed to move toward distinguishing the most effective chromatographic conditions for the separation process to improve the situation. These proposed combinations were carefully chosen and tested, considering various aspects such as the pH level, temperature, column material, and flow rate. The aim was to create a set of conditions that would optimize the chromatographic separation process and yield even better results. Multiple rounds of experimentation were conducted, each time fine-tuning the factors and adjusting based on the observed outcomes. Through this iterative process, it was found that by increasing the concentration of the surfactant and adjusting the relative amounts of each component in the mobile phase, a further enhancement of the γ factor could be achieved. The new investigated factors played a crucial role in improving the overall performance of the separation process. The results of the expanded experiments surpassed expectations, demonstrating a remarkable 20% increase in the γ factor. This outcome highlighted the importance of considering and manipulating the composition of the mobile phase and the content of the surfactant to achieve optimal chromatographic conditions. The findings of this study provide valuable insights for researchers and practitioners in the field, shedding light on effective strategies to enhance the efficiency and effectiveness of chromatographic separation techniques. As further research and advancements are made in this area, it is expected that even more promising combinations of factors and conditions will be discovered, leading to advancements in the field of chromatography and its applications.

It would be beneficial to experimentally test the influence of the flow rate on the investigated separation processes with the new proposed chromatographic conditions. This would make it possible for a flow rate to be indicated that would enable, on the one hand, a short analysis time to be reached and, on the other hand, to make it possible to keep the value of the γ performance function high. This, therefore, would enable new efficient

methods for sildenafil and vardenafil to be developed. Also, useful could be optimizations carried out on automatic systems where the quality of the separation is controlled on the basis of an automatic sample preparation device and on-line detection. It would also be worthwhile to evaluate the possible applications of the separated enantiomers in terms of their biological effects (including the behavior of the racemates in the separated form). In addition to the satisfaction of legislative requirements, the search for ever more effective technologies has also led to great interest in this field.

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