Characterization of an HSA column through HPLC: testing the Abraham solvation model

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CHARACTERIZATION OF AN HSA COLUMN THROUGH HPLC: TESTING THE ABRAHAM SOLVATION MODEL

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1. Το σύνολο της εργασίας αποτελεί πρωτότυπο έργο, παραχθέν από τον συγγραφέα της, και δεν παραβιάζει δικαιώματα τρίτων καθ’ οιονδήποτε τρόπο.

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1. FOREWORD

I dedicate this work to my family that has and always keeps on supporting me. To my beloved mother Arxontoula who has been there for every trouble these studies have cherished me with, my father Grigoris, sister Fevronia, and brother Klearchos for all their love.

I also want to thank my friends Evangelia, Sofia, and Evdokia without whom these past five years just would not be the same. From late nights studying to the after-exam celebrations, they are the best part of my student years.

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Last but not least, a great thank you to Ana Lucia, my dear lab-mate and friend for her indisputable help and support for this work to be realized.

Sincerely,

Panagiota Chaloulou
2. SUMMARY

High-pressure liquid chromatography (HPLC), developed during the 1960s, constitutes an excellent method for separating samples into their constituent components. Separation is based on their distribution between the mobile and stationary phase and the various solute-solvent interactions inflicted, as molecules of different nature present different affinity with the adsorbent surface, leading to diverse retention behavior. Since its formation, HPLC has been established as a versatile analytical tool for analyzing pharmaceuticals, biomolecules, and a plethora of other compounds, leading to great advancements in drug discovery and development processes, reducing costs and achieving early and drastic selection of the appropriate molecules with the greatest likelihood of success.

To further examine drug binding, protein-packed columns have been employed in HPLC analysis during the past two decades, especially with the use of albumin, the most abundant protein in blood serum. Indeed, Human Serum Albumin (HSA) columns help understand the human metabolism and pharmacokinetics of a drug-candidate compound based solely on its molecular structure and physicochemical properties, allowing for less time-consuming, labor-intensive, and ethically sensitive procedures to evaluate the distribution of compounds between plasma/blood and tissue.

Implementing HPLC in practice requires characterizing the chromatographic system first. The column’s hold-up time has been determined using both Potassium Bromide as an unretained hold-up marker and through the homologous series approach, examining n-Alkyl phenones, n-Alkyl benzenes, and Parabens separately and combined in one general equation. The general equation provided a more accurate hold-up time value, in agreement with the one from the marker, concluding to $t_M = 1.099 \pm 0.057$ min.

As for determining the system’s solute-solvent interactions, Abraham’s solvation parameter model derived from the general Abraham linear solvation energy relationships (LSER) approach describes them using various coefficients. The coefficients express excess polarizability ($e$), dipolarity/polarizability ($s$), hydrogen-bond acidity ($a$), hydrogen-bond basicity ($b$), and cavity formation ($v$), and can be calculated by performing a multilinear regression (MLR) analysis on a series of solutes with a wide range of properties and their molecular descriptors and retention factors.

This approach, however, requires examining a large number of compounds, so a fast method has been proposed, based on pairs of solutes with specific properties to characterize each type of interaction. By combining the Abraham solvation parameter model with Tanaka’s principles, only a couple of pairs are needed for the system’s characterization, saving up both time and resources. However, in this work, the two methods do not yield matching results, due to practical limitations. Further examination is required to determine whether this fast method is a good substitute for Abraham’s more tedious but accurate solvation parameter model.

Despite the two methods’ outcomes not being similar enough, they generally agree on the coefficients’ signs and magnitude. A large and positive value for the $v$ coefficient equal to $v = 2.218 \pm 0.142$ (result from Abraham’s solvation model) indicates that it governs retention behavior, with
solutestending to partition more favorably into the stationary phase, as it is less energetically demanding to form a cavity there, resulting in increased retention times. An even larger but negative b coefficient equal to $b = -3.232 \pm 0.260$ (result from Abraham’s solvation model) shows that hydrogen bond donor interactions also greatly affect this system, with them being stronger between solutes and the hydroorganic mobile phase than with the non-polar stationary phase, reducing retention. As for the rest of the coefficients, they acquire smaller values, indicating that they have less impact on the system.

In conclusion, the obtained coefficients reveal that this reversed-phase chromatographic system based on an HSA column is characterized by large absolute values for the b and v coefficients, indicating that it constitutes an appropriate method for separating mixtures of compounds of different hydrogen-bond basicity and or size. Its implementation in the pharmaceutical world promises great advancements in the fields of drug discovery and development, allowing the assessment of drug-protein interactions with great speed, precision, and reproducibility.
3. ΠΕΡΙΛΗΨΗ

Η χρωματογραφία υψηλής απόδοσης HPLC αναπτύχθηκε κατά την δεκαετία του 90 και αποτελεί μία εξαιρετική μέθοδο για τον διαχωρισμό δειγμάτων στα συστατικά τους μέρη. Ο διαχωρισμός βασίζεται στην κατανομή τους ανάμεσα στην κινητή και την στάσιμη φάση και στις διάφορες αλληλεπιδράσεις διαλύματος-διαλύτη που προκύπτουν, καθώς μόρια διαφορετικής φύσης παρουσιάζουν διαφορετική συγγενειά με την στάσιμη φάση, με αποτέλεσμα να συγκρατούνται διαφορετικά. Από την ανάπτυξή της μέχρι σήμερα, η τεχνική HPLC έχει καθιερωθεί ως ένα ευέλικτο αναλυτικό εργαλείο για την ανάλυση φαρμακευτικών ουσιών, βιομορίων και πλήθους άλλων ενώσεων, βελτιώνοντας τις διαδικασίες ανακάλυψης και ανάπτυξης φαρμάκων, μειώνοντας το κόστος και επιπτωτικότητας γρήγορη και αποτελεσματική επιλογή των κατάλληλων μορίων με τη μεγαλύτερη πιθανότητα επιτυχίας.

Για να εξεταστεί περαιτέρω η δέσμευση φαρμάκων, στήλες γεμισμένες με πρωτεϊνές έχουν χρησιμοποιηθεί στην ανάλυση HPLC τις τελευταίες 2 δεκαετίες και μάλιστα με την χρησιμοποίηση αλβουμίνης, της πιο αφθόνης πρωτεϊνής στο ορό του αίματος. Πράγματι οι στήλες ανθρώπινου ορού αλβουμίνης (HSA) βοηθούν στην κατανόηση του ανθρώπινου μεταβολισμού και της φαρμακοκινητικής μιας υποσφυγής φαρμακευτικής ενώσης, βασιζόμενες μόνο στη μορική της δομή και τις φυσικοχημικές της ιδιότητες, επιτρέποντας πιο γρήγορες, απλές και ηθικά σωστές διαδικασίες για την αξιολόγηση της κατανομής των ενώσεων μεταξύ πλάσματος/αίματος και ιστών.

Η πρακτική εφαρμογή της HPLC απαιτεί πρώτα τον χαρακτηρισμό του χρωματογραφικού συστήματος. Ο νεκρός χρόνος της στήλης προσδιορίστηκε με τη χρήση Βραβευόχον Καλλίου ως ένας μη συγκράτημας δείκτης, και μέσω της προσέγγισης αμοιλόγων σειρών, εξετάζοντας n-Alkyl phenones, n-Alkyl benzenes, και Parabens τόσο ξεχωριστά, ως και συνδυασμένες σε μια γενική εξίσωση. Η γενική εξίσωση παρείχε πιο ακριβή τιμή για τον νεκρό χρόνο η οποία βρίσκεται σε συμφωνία με αυτή από τον μη συγκράτημα δείκτη, και ισούται με τιμή \( t_{w} = 1.099 \pm 0.057 \) min.

Όσον για τον προσδιορισμό των αλληλεπιδράσεων διαλύματος-διαλύτη του συστήματος, το μοντέλο παραμετρικών διαλύσεων του Abraham, προερχόμενο από τη γενική προσέγγιση γραμμικών ενεργειακών σχέσεων διάλυσης (LSER) του Abraham, τις περιγράφει χρησιμοποιώντας διάφορους συντελεστές. Οι συντελεστές εκφράζουν την επιπλέον πολωσιμότητα \( e \), την διπολικότητα/πολωσιμότητα \( s \), την οξύτητα δεσμού υδρογόνου \( a \), τη βασικότητα δεσμού υδρογόνου \( b \) και τον σχηματισμό κολόκτητα \( \nu \) και μπορούν να υπολογιστούν με την εκτέλεση πολυπαραγωγικής γραμμικής παλινδρόμησης (MLR) σε μια σειρά ενώσεων με ευρύ φάσμα ιδιοτήτων με την χρήση των μοριακών περιγραφών και των παραγόντων συγκράτησης τους.

Αυτή η προσέγγιση, ωστόσο, απαιτεί την ανάλυση ενός μεγάλου αριθμού ενώσεων, οπότε έχει προταθεί μία ταχεία μέθοδος, βασισμένη σε ζεύγη διαλυτών με συγκεκριμένες ιδιότητες για τον χαρακτηρισμό κάθε τύπου αλληλεπίδρασης. Συνδυάζοντας το μοντέλο παραμετρικών διάλυσης του Abraham με τα αρχές του Tanaka, προκύπτει ότι χρειάζονται μόνο μερικά ζεύγη ενώσεων για τον χαρακτηρισμό του συστήματος, εξετασμένων τότε χρόνο όσο και πόρους. Ωστόσο, αυτή την μέλετη, οι δύο μέθοδοι δεν οδηγούν σε ισοδύναμα αποτελέσματα, λόγω πρακτικών περιορισμών. Κατά συνέπεια, απαιτείται περαιτέρω έρευνα για να προσδιοριστεί αν αυτή η ταχεία μέθοδος είναι πράγματι ικανή αντικαταστάτρια του πιο ακριβούς αλλά δύσχρηστου μοντέλου παραμέτρων διάλυσης του Abraham.
Παρά το ότι τα αποτελέσματα των δύο μεθόδων δεν είναι αρκετά ομοιά, συμφωνούν όσον αφορά στο πρόσημο και την τάξη μεγέθους των συντελεστών αλληλεπιδράσεων. Η μεγάλη και θετική τιμή για τον συντελεστή ν έστω με $n = 2.218 ± 0.142$ (αποτέλεσμα από το μοντέλο διάλυσης του Abraham) υποδηλώνει ότι επηρεάζει πολύ την συγκράτηση, με τους διαλύτες να τείνουν να κατανέμονται στην στάσιμη φάση, καθώς είναι λιγότερο ενεργειακά απαιτητικό να σχηματιστεί κοιλότητα εκεί, οδηγώντας σε αυξημένους χρόνους συγκράτησης. Ένας ακόμη μεγαλύτερος αλλά αρνητικός συντελεστής $b$ έστω με $b = -3.232 ± 0.260$ (αποτέλεσμα από το μοντέλο διάλυσης του Abraham) δείχνει ότι οι αλληλεπιδράσεις δοτών δεσμού υδρογόνου επις επηρεάζουν σημαντικά το συγκεκριμένο σύστημα, με αυτές να είναι ισχυρότερες μεταξύ των διαλυτών και της υδροοργανικής κινητής φάσης σε σχέση με τη μη πολική στάσιμη φάση, μειώνοντας τη συγκράτηση. Όσον αφορά στους υπόλοιπους συντελεστές, κατέχουν μικρότερες τιμές, με αποτέλεσμα να μην επηρεάζουν κατά πολύ το σύστημα.

Συμπερασματικά, οι υπολογιζόμενοι συντελεστές δείχνουν ότι το συγκεκριμένο χρωματογραφικό σύστημα αντίστροφης φάσης, βασισμένο σε στήλη HSA, χαρακτηρίζεται από μεγάλες απόλυτες τιμές για τους συντελεστές $b$ και $n$, υποδηλώνοντας ότι αποτελεί μια κατάλληλη μέθοδο για τον διαχωρισμό μειγμάτων ενώσεων με διαφορετική βασικότητα δεσμού υδρογόνου και ή μέγεθος. Τέλος, η εφαρμογή του στον φαρμακευτικό κόσμο υπόσχεται μεγάλες εξελίξεις στους τομείς της ανακάλυψης και ανάπτυξης φαρμάκων, επιτρέποντας την αξιολόγηση των αλληλεπιδράσεων φαρμάκου-πρωτεΐνης με μεγάλη ταχύτητα, ακρίβεια και αναπαραγωγικότητα.
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5. SYMBOLS AND ACRONYMS

Symbols [1]:

- **Adjusted retention time** \( (t_R') (s) \): A measure of the retention time adjusted for the hold-up time
- **Adjusted retention volume** \( (V_R') (m^3) \): Adjusts the retention volume for the holdup volume
- **Dead volume** \( (V_M) (m^3) \): Comprises the entire space accessible to a small molecule that can fully permeate all pores of a packing material. It includes the interstitial volume and the unoccupied pore volume. The system dead volume includes the additional volume in the tubing that connects the injector and detector to the column. See also hold-up volume, void volume
- **Extracolumn time** \( (t_{exc}) (s) \): The time elapsed between the effective injection and the effective detection point, excluding the part of the column containing the stationary phase. It comprises the time the sample resides in the injector, connecting lines and frits, and the detector. It determines the extracolumn effects.
- **Hold-up time** \( (t_d) (s) \): The time associated with the hold-up volume; determined by the dead volume divided by the flow rate
- **Hold-up volume** \( (V_M) (m^3) \): The total volume of mobile phase in the column regardless of where it exists. See also dead volume, void volume.
- **Retention factor** \( (k) \): The period of time that the sample component resides in the stationary phase relative to the time it resides in the mobile phase.
- **Retention time** \( (t_R) (s) \): The time between injection and the appearance of the peak maximum.
- **Retention volume** \( (V_R) (m^3) \): The volume of mobile phase required to elute a substance from the column
- **Selectivity factor** \( (a) \): The ability of the chromatographic system to 'chemically' distinguish between sample components. It is measured as a ratio of the retention factors \( (k) \) of two peaks and can be visualized as the distance between their apices.
- **Void volume** \( (V_M) (m^3) \): The total volume of mobile phase in the column; the remainder of the column is taken up by packing material. See also dead volume, hold-up volume
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Acronyms:

- **GC**: Gas Chromatography
- **HPLC**: High-Performance Liquid Chromatography
- **HSA**: Human Serum Albumin
- **LC**: Liquid Chromatography
- **LFER**: Linear Free Energy Relationships
- **LSER**: Linear Solvation Energy Relationships
- **MLR**: Multilinear Regression
- **NPLC**: Normal Phase Liquid Chromatography
- **RPLC**: Reversed-Phase Liquid Chromatography
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9. INTRODUCTION

High-pressure liquid chromatography, or HPLC, is a modern form of Liquid chromatography (LC) used to physically separate samples into their constituent components (analytes). It was first introduced in the late 1960s, by researchers, including Horvath, Kirkland, and Huber [2], and it employed small-particle columns that required high-pressure pumps. The separation of compounds is based on their distribution in two phases; a liquid one that carries the analyte mixture through a porous media, and a stationary one that consists of sorbents packed inside the column. The various ways by which analytes interact with the stationary phase result in different migration times, and, therefore, their separation. The basic principle behind these diverse interactions is that molecules of different nature present different affinity with the adsorbent surface, thus, analytes with weaker interactions are less retained [3]. This technique, invented in the 20th century, has since evolved greatly through time.

Nowadays HPLC is a versatile analytical tool widely used for the analysis of pharmaceuticals, biomolecules, polymers, and many organic and ionic compounds, with applications at all stages of drug discovery, development, and production. In fact, as discovery chemistry produces increased numbers of potential drug candidates, the simultaneous optimization of multiple properties is becoming increasingly important in the drug selection process [4]. This results in a massive increase in the cost of drug development, forcing the implementation of an early and drastic selection of suitable molecules that display the greatest likelihood of success.

In this context, it is essential to develop a model with the ability to predict the human metabolism and pharmacokinetics of a drug-candidate compound based solely on its molecular structure and physicochemical properties [5], [6]. Physicochemical properties used today in early drug discovery processes include solubility, lipophilicity, molecular size, hydrogen bonding capacity, and charge. Nevertheless, there are no general rules as to which is the dominant property in every situation, and examining a large number of compounds’ properties is a tedious process. Moreover, it constitutes a time-consuming, labor-intensive, and ethically sensitive procedure in case animals are being used for tests, further hindering drug discovery [7].

Instead, performing a number of relatively simple in vitro physicochemical measurements during early evaluation phases would be ideal for a rapid selection of compounds with adequate bioavailability and acceptable properties. This is where HPLC comes in to demonstrate its true effectiveness. It allows for multicomponent analysis of real-life samples and complex mixtures [8], with great versatility and precision. Moreover, it employs highly sensitive detectors with extensive detection limits and, most importantly, it is amendable with 60% to 80% of all existing compounds [9].

Performing HPLC analysis with a protein-packed column can lead to improved knowledge about drug-protein binding and a better understanding of the drugs’ biological activities, providing information about their pharmacokinetic, pharmacodynamic, and toxicological profiles [10]. In fact, serum carriers, such as albumin, have been proving very useful in drug binding because of their fundamental role in distributing therapeutic agents and their metabolites, leading to breakthroughs in the drug discovery and development process [11]. In this context, since their introduction in 1990, Human Serum Albumin or HSA-based columns have proved to be an efficient method for assessing
drug-protein interactions with great speed, precision, reproducibility, and even automation to further minimize run-to-run variations.

In order to investigate drug-protein binding through HPLC, it is an indispensable step to first fully define the chromatographic system. First of all, it is important to accurately determine the column’s void volume, so as to acquire reliable values for compounds’ retention factors (k), essential for system suitability issues, theoretical descriptions, and prediction of retention [12]. Among the most commonly used approaches are measuring the void volume using an unretained compound as a hold-up volume marker, and employing a variety of homologous series, as compounds within the same series exhibit similar chemical properties and elution behaviors.

Afterwards, it is essential to evaluate the different solute-solvent interactions contributing to the system’s partitioning. The method most commonly used to achieve this is Abraham’s solvation parameter model. Derived from the general Abraham linear solvation energy relationships (LSER) approach developed during the 90s [13], [14], [15], it constitutes a useful and precise tool for chromatographic systems’ characterization according to solute-solvent interactions such as polarizability, dipolarity, hydrogen bonding, and cavity formation.

However, for the model to provide reliable results, it requires the examination of a wide range of compounds. For this reason, Abraham’s approach has been revised over the past few years to comprise a fast and reliable method, based on pairs of solutes for the determination of each type of solute-solvent interactions. This fast method requires only a small number of adequate pairs of compounds to estimate polarizability, dipolarity, hydrogen bonding, and cavity formation respectively, providing the same information as the full Abraham’s solvation model, but in significantly less time.

Having established these parameters for a chromatographic system allows for the detailed examination of the dynamic equilibria of solutes between mobile and stationary phases. As a result, biomimetic models that account for the distribution of compounds between plasma/blood and tissue [12] can be successfully developed and contribute to breakthroughs in drug discovery and development processes.
10. THEORETICAL FRAMEWORK

10.1 HPLC BASIC PRINCIPLES

Liquid chromatography (LC) is a physical separation method that takes place in the liquid phase. It is widely used to separate samples into their constituent components (analytes) by distributing them between two phases; a liquid one that carries the analyte mixture through a porous media, and a stationary one, the porous media that consists of sorbents packed inside a column. The separation is achieved by the differences in which analytes interact with the stationary phase, resulting in different migration times for a mixture of components. In fact, molecules of different nature present different affinity (interactions) with the adsorbent surface, and, therefore, analytes with weaker interactions are less retained [3].

High-pressure liquid chromatography, or HPLC, is a modern form of LC that employs columns packed with small particles and pumping the mobile phase at high-pressure [8]. In this case, the separation of analytes is still based on the differences in their affinity with the stationary phase, with the original definition of the separation process given almost 100 years ago still holding true. In fact, it is noteworthy to see how this technique, invented in the 20th century, has evolved through time.

10.1.1 A brief history of liquid chromatography

Liquid chromatography, from the Greek “χρώμα” (chroma), which means “color”, and “γράφω” (graphein) [16], which means “to write” was first introduced in 1903 by Mikhail Tswett, a Russian botanist who separated plant pigment from chalk, packed in glass columns [2]. Since the 1930s, chemists used gravity-fed silica columns to purify organic materials and ion exchangers in columns to separate ionic compounds and radionuclides. Moreover, the invention of gas chromatography (GC) by British chemist A.J.P. Martin in 1952, and its successful applications, provided all the theoretical foundation needed to lead in the development of liquid chromatography.

With constant evolutions, a new type of LC was introduced in the late 1960s, characterized by small-particle columns and, therefore, required high-pressure pumps. The first generation of high-pressure or high-performance liquid chromatographs, as they were later named, was developed in the 1960s by researchers, including Horvath, Kirkland, and Huber. Commercial development of in-line detectors and reliable injectors allowed HPLC to become a very useful technique with high sensitivity, suitable for quantitative analysis and a plethora of applications [2].

With exquisite versatility and precision, during the 1980s, HPLC became an indispensable tool in pharmaceuticals as well as other diverse industries. Today, it continues to evolve rapidly towards higher speed, efficiency, and sensitivity, by incorporating new technologies in an attempt to satisfy the emerging needs in life sciences and pharmaceutical applications.
10.1.2 HPLC: How it works

Once a sample is dissolved into a solvent and injected into the HPLC system, the liquid-solid interaction begins. The separation occurs when compounds either stay in the solvent or adhere to the packing material in the column [17]. A simplified representation of separation is shown in Picture 2, where a mixture of analytes A and B are separated into two distinct bands as they migrate down the column. It is evident that component B is being retarded in the column, a behavior indicating that the B molecules have a stronger affinity to the stationary phase than the A ones. A detector placed in-line monitors the concentration of each compound in the effluent and generates a signal, resulting in the characteristics peaks of a chromatogram, as shown in Picture 1.

![Picture 2: Schematic of the chromatographic separation of two analytes A and B [7]](image)

![Picture 1: Chromatogram of the UV detector's signal against the elution of components A and B [7]](image)

10.1.3 Advantages and disadvantages

HPLC is a valuable technique that allows for multicomponent analysis of real-life samples and complex mixtures [8]. It exhibits high versatility and precision that only a few other methods can match. Moreover, on the condition that one possesses the necessary theoretical background, it can be easy to operate, since it is highly automated, with the use of sophisticated autosamplers and data systems providing unattended analysis and report generation. It also employs highly sensitive and specific detectors that make it possible to acquire detection limits from nanogram, up to femtogram levels. Last but not least, its most important advantage lies in its amenability with 60% to 80% of all existing compounds, whereas gas chromatography for example is suitable only for 15% [9].

On the other hand, HPLC is unavoidably accompanied by certain limitations [8]. First of all, there is no universal detector that can characterize any analysis, so detection becomes problematic if the analyte does not absorb UV rays or cannot be easily ionized for mass spectrometric detection. Moreover, its separation efficiency is lower when compared with other techniques, such as certain
types of gas chromatography, leading to challenges in analyzing complex mixtures. Lastly, since HPLC is characterized by many operating parameters, it can be proved quite difficult for novices. However, the majority of the aforementioned disadvantages have been minimized, through instrumental and column developments.

10.1.4 Modes of HPLC

When it comes to HPLC, there are several separation models. Among them, normal and reversed-phase are two of the main ones, each with different system apparatus such as columns, mobile phases, detectors, and sample information. Normal phase liquid chromatography (NPLC) is the traditional mode and is based on the adsorption / desorption of an analyte onto a polar stationary phase. It is mainly used to separate non-polar compounds, as well as to fraction complex samples by functional groups or clean up samples [8]. One of its major disadvantages, however, is that the polar surfaces can be easily contaminated by sample components.

Reversed-phase liquid chromatography (RPLC) on the other hand, achieves analytes’ separation between a polar mobile phase and a non-polar stationary phase, and it is the mode implemented in the present work. In this case, the main intramolecular mechanism is dispersive forces, that is hydrophobic or van der Waals interactions, employed by a water-based mobile phase. Now that the polarities of each phase are reversed, the elution order proceeds from polar to non-polar compounds, since hydrophilic (more polar) molecules interact less with the hydrophobic stationary phase and elute sooner [8].

Reversed-phase HPLC is by far the most popular mode of chromatography, with almost 90% of all analyses of low-molecular-weight samples being carried out using this technique [3]. It exhibits a wide range of separation abilities, as it is suitable for the analysis of compounds with high, medium, or even low polarity in cases. Moreover, it can discriminate among very closely related compounds, because of the weak nature of the intermolecular forces. As a result, the overall background interaction energy in the chromatographic system is very low, and therefore, even the smallest differences in molecular interactions can be distinguished [18].

Besides the major HPLC separation modes, there are a lot more, with one of them being important in the present work. Chiral chromatography can be encountered in both normal and reversed-phase separations and constitutes a type of high-pressure liquid chromatography invaluable to separating enantiomers using a chiral-specific stationary phase [19]. It represents a subject of major interest in the pharmaceutical industry, since many drug molecules have asymmetric centers and can exist as enantiomers with markedly different biological activities. In fact, today most drugs under development consist of a single optically active isomer [20]. In this case, the undesired enantiomer is treated as a structurally related impurity, so an enantioselective means should be implemented. As a result, chiral separation of drug molecules and their precursors is vital during enantiomerically pure drugs’ synthesis. In this chromatographic mode, columns are packed with stereo-specific sorbents, such as proteins in coated and immobilized form, which exhibit an inherent chiral nature [21].
10.2 HUMAN SERUM ALBUMIN (HSA)

10.2.1 HSA: What it is

One protein typically used in HPLC chromatography applications is Human Serum Albumin (HSA). Albumin is the most abundant protein found in blood’s plasma, constituting half of its total protein content and reaching concentrations between 3.5 g/dL and 5 g/dL [22]. It is synthesized by liver hepatocytes as a single polypeptide chain consisting of 585 amino acid residues and is rapidly excreted into the bloodstream. Its three-dimensional structure exhibits a heart-like shape and certain structural flexibility depending on slight pH changes (Picture 3) [23].

One of albumin’s most important roles in humans is maintaining the osmotic pressure of the blood and transporting endogenous physiological metabolites and exogenous ligands such as drugs [24]. It is fairly homogeneous, well-defined, and extensively studied, constituting an excellent model for examining various molecular aspects of drug-protein interactions. Its flexibility, together with a differently distributed charge along the molecule, make it possible for many classes of compounds with different structures to bind to the protein [25]. It is characterized by two main binding sites, which allow the stable high-affinity binding of drugs.

10.2.2 Employing HSA columns in HPLC

Knowledge of drug-protein binding is essential for understanding the drugs’ biological activities, since it influences their distribution, elimination, and pharmacological effects [26]. This way, one can acquire a better understanding of their pharmacokinetic, pharmacodynamic, and toxicological profile. The most frequently used techniques for investigating drug binding parameters
to proteins in the past years were ultrafiltration and equilibrium dialysis [27]. However, these methods are accompanied by several disadvantages, since they require several separation steps and are not characterized by great efficiency.

One way to compensate for these disadvantages is to study drug-protein binding by immobilizing the protein on a stationary medium [10]. In this approach, the protein is packed inside a column and employed in high-pressure liquid chromatography. The use of HSA-based HPLC columns was first introduced in 1990 for the resolution of chiral drugs [28]. Since then, investigating drug-serum carrier interactions and their enantioselectivity using immobilized human serum albumin has been increasing in popularity, since HPLC methods provide great accuracy and speed, accumulating interest to the analysis of the binding properties of chiral and achiral compounds.

Besides, HPLC-based methods are faster and more precise, providing reproducibility, accuracy, and the option for automation, in order to further minimize run-to-run variations. They allow for the detection of small differences in drug binding affinities and modulation by experimental parameters. By these means, the retention of an analyte is related to the extent of its protein binding at a specific site. In fact, it has been observed that HSA is the major plasma-binding protein for weakly acidic drugs, with negatively charged molecules binding more strongly [4].

Moreover, in the case of chiral drugs, single enantiomers can be simultaneously investigated by zonal elution any time the racemate is resolved on the column. This is also possible when the drug is stereochemically unstable, that is, the single enantiomers cannot be isolated. This way, chromatographic runs can be a direct probe for binding specificity and stereoselectivity, predicting drug interactions, and measuring changes in HSA structure [11].
10.3 HPLC DESCRIPTORS

10.3.1 Hold-up volume / time

Hold-up volume can often be come across with other names, such as dead volume, void volume, solvent volume, or mobile phase volume. Much of the controversy arises from the fact that oftentimes, the stationary phase can be sorbed on the surface of the column’s packing material, preferentially solvated by certain components in the eluent, or the fact that transition layers, with no clear boundary, can be present in the mobile phase, which complicate the assignment of their volume between the two phases [29]. Luckily, in reversed-phase chromatography, such as in this study, water and the organic solvent behave simply as the mobile phase, whereas the bonded phase behaves as the stationary one. Therefore, the volume of the solvent flowing inside the column (hold-up volume) will be nearly the same as the solvent’s volume [30]. To avoid complications, a more detailed definition will be followed, with the terms of time and volume being interchangeable, as one can easily convert from one to the other by multiplying or dividing with the known flow rate.

IUPAC defines the hold-up volume (time) in column chromatography as “the volume of the mobile phase (or the corresponding time) required to elute a component the concentration of which in the stationary phase is negligible compared to that in the mobile phase. In other words, this component is not retained at all by the stationary phase. Thus, the hold-up volume (time) is equal to the retention volume (time) of an unretained compound. Usually, the hold-up volume (time) includes any volumes contributed by the sample injector, the detector, and connectors” [31]. This extracolumn volume depends on the chromatographic equipment in use (injector, detector, connectors), so it is essential to accurately specify it and subtract it from the measurements, in order to acquire data independent of the apparatus employed. The value that remains after subtracting the extracolumn effects from the hold-up volume, is called void volume, and it equals to the volume occupied by the mobile phase in the column.

For a better understanding of the above-mentioned concepts, it would be useful to further define the retention time of a compound [32]:

\[ t_R = t_R^g - t_{exc} = t_M + t'_R \]  \hspace{1cm} (10.3.1.1)

where \( t_R^g \) is the gross retention time, that is the retention time observed on the chromatogram from the injection point to the detector, \( t_{exc} \) is the time the sample spends outside the column, \( t_M \) is the column’s hold-up time, that is the elution time of an unretained compound, a molecule that shows no interactions with the stationary phase, and \( t'_R \) is the adjusted retention time, the additional time a molecule takes up, due to its interactions with the stationary phase. For an unretained compound, it naturally occurs that \( t'_R \) equals to zero. Hence, the column’s hold-up time can be expressed as follows [32]:
Characterization of an HSA column through HPLC: testing the Abraham solvation model

\[ t_M = t_M^g - t_{excol} \]  \hspace{1cm} (10.3.1.2)

where \( t_M^g \) is the column’s gross hold-up time that includes the true column hold-up time and the extracolumn time, and \( t_{excol} \) is the extracolumn time as mentioned before. In case of one particular chromatographic system with the same instruments and column, the values of \( t_M \) and \( t_{excol} \) remain constant.

10.3.2 Retention factor \( k \)

One of the most important parameters for interpreting physical phenomena inside a column is the retention factor \( k \). The \( k \) value characterizing a solute is usually calculated from its elution time and the one of a non-retained marker (hold-up time). According to Subirats et al. [33], the retention factor represents the ratio of the amounts of a solute in the stationary (SP) and mobile (MP) phases:

\[ k = \frac{C_{SP}V_{SP}}{C_{MP}V_{MP}} = \frac{K V_{SP}}{V_{MP}} \]  \hspace{1cm} (10.3.2.1)

where \( C_{SP} \) and \( C_{MP} \) are the solute’s concentrations in the stationary and mobile phase respectively, \( V_{SP} \) and \( V_{MP} \) are the respective volumes of each phase in the chromatographic column and \( K \) is the equilibrium constant for the distribution of the solute between both phases.

However, when it comes to carrying out experiments, the equation above is not the most useful one. When performing chromatographic runs, the most easily acquired data are associated with time or volume. Therefore, by combining equations (10.3.1.1) and (10.3.1.2), a new expression for the retention factor \( k \) occurs as follows:

\[ k = \frac{t_R'}{t_M} = \frac{t_R - t_M}{t_M} = \frac{t_R^g - t_M^g}{t_M^g - t_{excol}} \]  \hspace{1cm} (10.3.2.2)

The retention factor describes the relationship between the time an analyte spends in the stationary and the mobile phase. More specifically, it shows how much a compound is delayed by the stationary phase, as compared to how long it would take for it to travel through the column if it was granted the velocity of the mobile phase. As mentioned before, \( k \) values can also be calculated by volume, by means of the mobile phase’s flow rate. The retention factor then, takes the following expression [29]:

22
\[ k = \frac{V_R - V_M}{V_M} \]  \hspace{1cm} (10.3.2.3)

where \( V_R \) is the compound's retention volume, that is the volume of mobile phase required for the compound to elute the column, and \( V_M \) is the hold-up volume, the mobile phase's volume that simply passes through the system without interacting with the stationary phase.

However, there is no standard method for the determination of hold-up volumes covering all chromatographic modes, since the selected approach should depend not only on the stationary phase, but also on the mobile phase's composition (content and nature of the organic modifier, salts...) [33].
10.4 MEASURING THE HOLD-UP VOLUME

There is no doubt that there are several different techniques to determine the hold-up volume or time for a given chromatographic system. The homologous series approach, pycnometry, minor disturbance of baseline and the use of an unretained neutral marker, are some of the most commonly used ones.

In the pycnometric method, the column is sequentially filled with two separate solvents of sufficiently different densities and weighed each time. The hold-up volume occurs from the weight difference. Although a popular technique, it is not considered very accurate, as it ignores the possibility of the solvation of the stationary phase by part of the mobile one [33]. When it comes to the minor disturbance method, the hold-up volume is determined from the baseline disturbance caused by the injection of a pure solvent. However, it is not a very reliable approach, since solvent peaks are complex to interpret and depend on the ionic strength of the eluent [29].

The injection of an unretained solute as a hold-up volume marker is an easy and simple technique, as the hold-up volume in this case equals to the solute’s elution volume. The challenge here is to find a truly unretained solute, as the majority of them exhibit slight retention [34].

10.4.1 Hold-up volume marker

In this case, an easy way to acquire a good approximation of the hold-up volume is by using an unretained compound as a hold-up volume marker. The desired value is a direct measure of its retention factor, since it is considered to elute almost immediately. Its performance as a hold-up marker is, then, characterized by the relative error between $V_R$ and $V_M$ in equation (10.3.2.3). The closer the $k$ value to zero, the smaller the relative error and the better the candidate as an unretained marker [29]. In that case, the expression for the hold-up volume takes the form of equation (10.3.1.2).

10.4.2 The homologous series approach

The homologous series method is based on the principle that compounds within a series exhibit similar chemical properties and elution behaviors, as they share the same functional group and a comparable structural framework. Since retention depends on factors such as molecular weight, polarity, and interactions with the stationary phase, chemically similar compounds, present a predictable and standardized behavior, allowing for an accurate and reproducible determination of the system’s hold-up volume or time.

A variation of the homologous series approach is introduced, based on linear free energy relationships (LFER), and particularly on the solvation model proposed by Abraham [35]. This model provides a useful equation that relates a free energy-related property to solute-solvent interactions.
In this case, the free energy property is retention factor \( k \); the measure of the residence time of an analyte in the stationary phase in relation to the time it resides in the mobile phase, and according to Abraham, its logarithmic value can be expressed by solute descriptors as follows:

\[
\log k = c + e \cdot E + s \cdot S + a \cdot A + b \cdot B + v \cdot V
\]  

(10.4.2.1)

where \( c \) is a system constant, a non-solute dependent term accounting mainly for the chromatographic phase ratio, and \( v \cdot V \) accounts for the cavity formation in the solvent together with residual solute-solvent dispersion interactions (with \( V \) being the McGowan volume of the solute).

McGowan volume is a characteristic volume for every solute and is defined as the actual volume of a mole when the molecules are not in motion [36]. It can be easily calculated as the sum of the characteristic volumes of the solute’s atoms, minus 6.56 cm\(^3\) mol\(^{-1}\) for each bond, regardless of whether it is a single, double, or triple [37]. For complicated molecules, the number of bonds can be obtained from the total number of atoms (\( N_a \)) and the number of rings (\( R_g \)) they contain. The total value of McGowan volume \( V \) for each compound is then divided by 100, so that the result is of the same order of magnitude as the other molecular descriptors. Therefore, \( V \) can be calculated as [33]:

\[
V = \sum(a\text{ll atom contributions}) - 6.56 (N_a - 1 + R_g) \left( \frac{ml}{mol^{-1}} \right) \left( \frac{100}{100} \right)
\]  

(10.4.2.2)

The rest of the terms in equation (10.4.2.1) are related to solute-solvent interactions: The \( e \cdot E \) term expresses excess polarizability and models the polarizability contributions from n- and p-electron pairs (dispersion forces), \( s \cdot S \) represents dipole-type interactions (dipolarity/ polarizability), \( a \cdot A \) stands for the hydrogen bond donation from the solute to the solvent (hydrogen bond acidity), and \( b \cdot B \) for the hydrogen bond donation from the solvent to the solute hydrogen bond basicity.

*Picture 4: Graphical representation of solute-solvent interactions [42]*
Continuing in equation (10.4.2.1), the uppercase letters E, S, A, B, and V represent solute descriptors, determined either experimentally or by calculations. On the other hand, the lowercase letters e, s, a, b, and v represent the system coefficients, reflecting the difference in solute interaction between the stationary and mobile phases. These can be obtained by performing multiple linear regression analyses on the retention factors of tests of solutes against their known solute descriptors. The positive or negative sign, as well as the magnitude of the system coefficients, indicate key features associated with retention behavior and, therefore, allow for chromatographic systems characterization and a comparison between different retention modes, columns, and mobile phases [38].

By combining equations (10.3.2.2) and (10.4.2.1), a general expression for solutes’ retention time occurs:

\[ t_R = t_M + t_M \cdot 10^{c+eE+sS+aA+bB+vV} \]  

\[ (10.4.2.3) \]

Compounds part of a particular homologous series exhibit resembling dispersive, dipolarity/polarizability, and hydrogen-bonding features, resulting in similar E, S, A, and B molecular descriptors. Their only differentiation lies in the number of carbon atoms each member of the series possesses, and, therefore, in the molecular volume (V). Additionally, since the McGowan volume coefficient (v) measures the endoergic work of separating solvent molecules to provide a big enough cavity for the solute molecule, it will also depend on the size of the solute.

On the other hand, providing that the chromatographic conditions remain the same, without any variations in the stationary or the mobile phase, the system coefficients c, e, s, a, and b remain constant throughout the analysis of a whole series. As a result, the term \( t_M \cdot 10^{c+eE+sS+aA+bB} \) remains unchanged for every solute in the series and can be thus defined as a constant value r. In this context, equation (10.4.2.3) for the compounds of a homologous series can be transformed as follows [33]:

\[ t_R = t_M + r \cdot 10^vV \]  

\[ (10.4.2.4) \]

Alternatively, should the volume terms be used rather than time ones, the above equation (10.4.2.4) takes the following form:

\[ V_R = V_M + r \cdot 10^vV \]  

\[ (10.4.2.5) \]

where r now equals to \( V_M \cdot 10^{c+eE+sS+aA+bB} \). The hold-up volume can then be calculated by fitting equation parameters (r, v, and \( V_M \)) to the retention data of the homologous series members.

Since hold-up volume \( V_M \), as well as the coefficients v and V are not dependent each time on the homologous series in question, several series can be analyzed altogether and jointly fitted in one equation. This results in just one value for retention volume and, therefore, can be characterized as a more general model [39]:
\[ V_R = V_M \cdot \left( 1 + \sum_{i=1}^{n} (r_i \cdot f_i) \cdot 10^{\nu v} \right) \]  

(10.4.2.6)

where \( V_M \) is the aforementioned hold-up volume, \( V \) is the McGowan characteristic volume of the homolog, and \( v \) and \( r \) are system constants. \( r \) is enclosed inside the sum bracket, since it still depends on the homologous series in question, by being related to solute-solvent dispersion, dipole-dipole, dipole-induced dipole, polarizability, and hydrogen bond interactions [38]. \( n \) is the number of homologous series included in the calculations, with the suggested method being to select different series covering a wide range of solute-solvent interactions, in order to acquire a more accurate estimation of the hold-up volume. Last but not least, \( f_i \) in equation (10.4.2.6) represents the binary flag descriptors (0 or 1) that allow the simultaneous adjustment of the \( n \) homologous series. When one particular series \( i \) is analyzed, \( f_i \) assumes the value 1, whereas for the rest of the series, it is set to 0. For example, when fitting the retention data of alkyl benzenes \( (V_R) \) as a function of their McGowan volume \( (V) \), the value of \( f \) for alkyl benzenes is set to 1, while for all the other series, \( f = 0 \).

This more inclusive model presented in equation (10.4.2.6), provides greater precision and enhanced accuracy, since a variety of homologs from different series are being employed, covering a broader scope in terms of solute-solvent interactions [29].
10.5 Characterization of the chromatographic system

In order to perform any chromatographic separations, it is essential to develop a reliable method for the system characterization, so as to evaluate the different solute-solvent interactions contributing to the partitioning process. For this purpose, there is a plethora of different approaches reported in the literature [40], yet the most widely used ones are the high-throughput Tanaka’s scheme and the more detailed but time-consuming Abraham’s solvation parameter model.

Tanaka’s scheme was first developed in 1989 and described a new approach for the characterization of octadecylsilane packing materials, based on the separation factor of pairs of solutes [41]. As a result, the different solute-solvent interactions responsible for retention in C18 columns could then be assessed by a simple protocol, based on a few chromatographic runs.

On the other hand, Abraham’s solvation parameter model, as introduced in section 10.4.2, is based on Linear Solvation Energy Relationships (LSER) and relates the logarithm of the retention factor (log k) of neutral solutes to the different contributions affecting retention in a chromatographic system [35], by means of equation (10.4.2.1). The coefficients in this equation, represented with lowercase letters, reveal key features regarding retention behavior according to their positive or negative sign. As a result, one can obtain strong indicators for characterizing chromatographic systems and comparing different retention modes, columns, or mobile phases.

10.5.1 The Abraham solvation model

The model described by equation (10.4.2.1) is part of the general Abraham LSER approach developed during the 90s to characterize a great diversity of physicochemical and biological processes [13], [14], [15]. It constitutes a useful and precise tool that helps characterize a lot of column-mobile phase systems according to solute-solvent interactions (polarizability, dipolarity, hydrogen bonding, and cavity formation).

More specifically, system coefficients are obtained by performing multiple linear regression analysis to retention factors that correspond to a set of solutes with well-known and variated E, S, A, B, and V molecular descriptors. It is important that the selected solutes possess properties sufficiently varied in order to define properly all interactions in equation (10.4.2.1) and that they are of sufficient size to establish the statistical validity of the model. The downside of this approach is that it requires a high number of measurements for a variety of compounds, constituting it a very time-consuming, low-throughput practice.
10.5.2 A fast method based on pairs of solutes and Abraham’s solvation model

The simple nature of Tanaka’s scheme is preferred to the complexity of Abraham’s approach. Hence, the Abraham model has been revised over the past few years to comprise a fast and reliable method, combining the advantages of both worlds. The idea elaborated by Redón et al. at Universitat de Barcelona [42] uses Abraham’s solvation parameter model as a starting point and proceeds to incorporate the use of pairs of test compounds, like in Tanaka’s scheme, to create a fast method for systems characterization not only in reversed-phase liquid chromatography, but potentially in any liquid chromatographic mode.

In order to achieve this, several pairs of compounds need to be carefully selected so that they have all molecular descriptors in common, except for one. For example, one suitable pair should exhibit similar molecular volume, dipolarity, polarizability, and hydrogen bonding basicity features, but different hydrogen bonding acidity. This way, the pair’s selectivity factor provides information regarding only the extent of the dissimilar solute-solvent interactions and their influence on chromatographic retention. As a result, with the v coefficient already measured through the homologous series approach for hold-up volume determination, only four more chromatographic runs are needed for the four pairs to define coefficients a, b, s, e and successfully characterize the selectivity of a chromatographic system [42].

10.5.2.1 Developing the fast method

Tanaka’s characterization scheme is based on selectivity factors (a) of pairs of test solutes, that is the ability of the chromatographic system to separate a sample’s components. This relationship is described by the following equation:

\[ a_{1/2} = \frac{k_1}{k_2} \]  

(10.5.2.1.1)

where \( k \) is the retention factor and subscripts 1 and 2 refer to two different solutes with very similar properties except for the one in question, among hydrophobicity, shape, hydrogen bonding, or cation exchange interactions. However, the linear solvation energy relationships for solute-solvent interactions of Tanaka’s pairs for the characterization of octadecylsilane columns reveal that differences in selectivity might be attributed to more than one factor [42]. Hence, the comparison of solely retention factors is not adequate for the accurate evaluation of a chromatographic system.

This is where the Abraham approach for neutral compounds is introduced to the model. Tanaka’s characterization scheme can be implemented for any pair of solutes to reflect different solute-solvent interactions, so for a particular chromatographic system that consists of the same column and mobile phase composition, the Abraham LSER model applies. Therefore, equations
(10.4.2.1) and (10.5.2.1.1) for two different solutes 1 and 2 can be combined into one to obtain the decimal logarithm of their selectivity factor:

\[
\log a_{1/2} = \log k_1 - \log k_2 = e \cdot (E_1 - E_2) + s \cdot (S_1 - S_2) + a \cdot (A_1 - A_2) + b \cdot (B_1 - B_2) + v \cdot (V_1 - V_2)
\]  

(10.5.2.1.2)

where \(e, s, a, b,\) and \(v\) are the system coefficients for the specific chromatographic conditions representing solute-solvent interactions, and \(E, S, A, B,\) and \(V\) are the molecular descriptors of solutes 1 and 2. Equation (10.5.2.1.2) allows the estimation of any system coefficient \(x_i\) from the difference between the molecular properties of solute 1 in relation to solute 2, provided that the two solutes have four identical or at least very similar molecular descriptors and a significantly different fifth descriptor \(X_i\). The relationship for the system coefficients \(x_i\) then takes the following form:

\[
x_i \approx \frac{\log a_{1/2}}{X_{i,1} - X_{i,2}} = \frac{\log k_1 - \log k_2}{X_{i,1} - X_{i,2}}
\]  

(10.5.2.1.3)

For instance, to calculate the system coefficient \(a\) for two compounds with similar \(E, S, B,\) and \(V\) descriptors and only different \(A\) values, equation (10.5.2.1.3) would take the following form:

\[
a \approx \frac{\log k_1 - \log k_2}{A_1 - A_2}
\]  

(10.5.2.1.3a)

Applying the previous equations to characterize the chromatographic system should provide the same information as the full Abraham’s solvation model, but in significantly less time, since only an adequate pair of compounds is needed to estimate each system coefficient.
11. MATERIALS AND METHODS

11.1 INSTRUMENTATION

All the experiments were performed using a SHIMADZU (Kyoto, Japan) HPLC system, which consisted of two LC-20AD pumps, an SIL-20AC HT autosampler, an SPD-10AV VP UV-vis detector, a CTO-10AS VP oven, and a DGU-20A 5 degasser. The software used to control the system was LC Solutions software from Shimadzu.

The column employed in this research was a Human Serum Albumin (HSA) protein-based chiral column with a 4.0 mm internal diameter, 100 mm length, and 5 μm particle size from the CHIRALPACK® series of DAICEL corporation (country of origin: France).

Other kinds of instrumentation that were used throughout this study were 4-digit weighing balances with an error of 0.0001, a magnetic stirrer, and a bench CRINSON GLP22 pH meter with a ROSS type of electrode. Standard aqueous solutions (pH 4 and 7) were used for the pH meter’s calibration. All measurements were obtained at room temperature.

11.2 METHODS AND CHROMATOGRAPHIC CONDITIONS

The selection of the mobile phase was determined by its compatibility with the column’s nature (APPENDIX A: HSA CHIRALPACK® INSTRUCTION MANUAL [43], [44]). As a result, it consisted of 80% monosodium phosphate (NaH₂PO₄) diluted in water at 20 mM, pH = 7.00, and 20% 2-propanol. The consistency of the mobile phase was kept constant throughout the experiments, applying the method of isocratic elutions.

The applied flow rate in the column was 0.9 ml min⁻¹, while the injection volume was 20 μl. The oven temperature was kept constant at 30°C, and overall, three rounds of replicates were performed for each sample. As for detecting the compounds, two different wavelengths were used; 210 nm and 254 nm [45], as a lot of organic compounds absorb UV radiation in such low wavelengths.

11.3 CHEMICALS AND SOLVENTS

The solutes used in this work were purchased from Acros Organics (Geel, Belgium), Alfa Aesar (Ward Hill, MA, USA), Sigma-Aldrich (St. Louis, MO, USA), TCI (Tokyo, Japan), Synthonix (Wake Forest, NC, USA), Fluorochem (Hadfield, Glossop, UK), and Thermo Scientific (Waltham, MA, USA), all of high purity grade (≥ 98%).

As for the solvents, HPLC-grade Methanol, Dimethyl Sulfoxide, and 2-propanol were purchased from Panreac (Barcelona, Spain). Water was obtained from a Milli-Q plus system from Millipore (Billerica, USA) with a resistivity of 18.2 MΩ cm.
11.4 Sample Preparation

The concentration of the stock solutions depended on their physical state due to the preparation means available. More specifically, stock solutions of solid substances were prepared at a concentration of 500 ppm, by weighing 5 mg of each substance and diluting it in dimethyl sulfoxide to create a solution of 10 ml. For liquid compounds, the concentration was 1000 ppm, and the preparation procedure included diluting 25 μl of each substance in dimethyl sulfoxide to create a solution of 25 ml.

For the stock solutions of the homologous series, the process followed remained the same, with 500 ppm solutions for solid substances and 1000 ppm solutions for liquid ones. The only difference was the solvent. In this case, the compound used was methanol, for increased solubility.

Before entering the HPLC system, all the analytes were filtered after being diluted to 20 ppm using the solution used as the mobile phase: 80% Monosodium phosphate (NaH$_2$PO$_4$) diluted in water at 20 mM, pH = 7.00 and 20% 2-propanol. In the case of solutions with a concentration of 500 ppm, 400 μl of each solution was diluted in 10 ml of mobile phase. For the 1000 ppm solutions, the amount subtracted was 200 μl to dilute again in 10 ml of mobile phase, in order to achieve 20 ppm final concentration in each case.

For the preparation of the mobile phase, 2.7598 g of NaH$_2$PO$_4$·H$_2$O were diluted in water to make a 1 L solution in total. The solution was then adjusted to a pH equal to 7.00 using NaOH 0.1M. In order to ensure homogenous conditions and accurate measurements with the pH meter, the solution was constantly mixed with a magnetic stirrer, while the device was calibrated daily with standard solutions (pH 4 and 7). In the end, the solution was filtered with a vacuum filtration apparatus through membrane filters to prevent foreign particles or dirt from entering the HPLC system.

11.5 Extracolumn Volume

The extracolumn volume was determined with the use of Potassium Bromide (Merck, > 99%), diluted into the mobile phase solution at a concentration of 400 ppm. The solution was injected into the system in the absence of the column, with the injector connected directly to the detector. After performing the runs in triplicates, the extracolumn volume was measured and subtracted from the gross retention volumes obtained from chromatograms.

11.6 Database Screening and Calculations

The preliminary selection of suitable test compounds was carried out by carefully screening Abraham’s database of solutes and molecular descriptors [42] and adjusting the given compounds depending on how they behave in these particular experimental conditions. The goal was to select an appropriate collection of solutes that embrace a wide range of descriptor values, without any
significant cross-correlation present among them or clustering of individual descriptor values. In addition, the selected solutes should exhibit reasonable absorbance between 210 nm and 254 nm, so that detection is convenient, and they should also be neutral at the working pH.

As for the calculations, a custom macro was employed for estimating the Abraham’s model parameters [46], while multiple linear regression analyses were performed using MS Excel.
12. RESULTS AND DISCUSSION

12.1 Hold-up Volume Determination with Hold-up Marker

The unretained solute playing the role of a hold-up volume marker in this work is Potassium Bromide. The compound was injected three times into the system, first without the column to measure the extracolumn volume, and then, another three times, as normal, to calculate the column’s hold-up volume. The data’s average showed that the time a sample spends outside the column is \( t_{\text{exc}} = 0.170 \pm 0.002 \, \text{min} \). As for the hold-up volume, equation 10.3.1.2, multiplied by the flow rate so that time terms turn into volumes, reveals that \( t_M = 1.091 \pm 0.002 \, \text{min} \), hence \( V_M = 0.982 \pm 0.002 \, \text{ml} \) (Table 1).

Table 1: Results for the system’s extracolumn time, hold-up time, and hold-up volume

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Symbol</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extracolumn time (min)</td>
<td>( t_{\text{exc}} )</td>
<td>0.170 ± 0.002</td>
</tr>
<tr>
<td>Hold-up time (min)</td>
<td>( t_M )</td>
<td>1.091 ± 0.002</td>
</tr>
<tr>
<td>Hold-up volume (ml)</td>
<td>( V_M )</td>
<td>0.982 ± 0.002</td>
</tr>
</tbody>
</table>

12.2 Hold-up Volume Determination with Homologous Series

The homologous series method includes several compounds of the same family being plotted versus their corresponding molecular volume. The hold-up time is then calculated by extrapolating the linear plot obtained to the zeroth homolog or by regression analysis [29]. In this method, the homolog number, and number of homologs used, seem to play an important role in the accurate determination of hold-up time. First, it is essential to choose the correct type of homologous series based on their solubility in the mobile phase. Moreover, they should be generally available and exhibit great detectability in the chromatographic system. Last but not least, it is suggested that a minimum of three homologs are necessary to acquire accurate results, however, four is the recommended number, as three may give an unrealistically high correlation coefficient [34].

12.2.1 Homologous series selection

As mentioned earlier, it is important to select appropriate homologous series for the accurate determination of hold-up volume or time. Possible candidates should exhibit a wide range of molecular volumes, as well as low retention. As a result, since hydrogen bonding interactions favor partition into the stationary phase, homologs should present small A and B descriptors, as close to zero as possible, so as to limit time spent there. Moreover, for practical, economic and instrumentation simplicity reasons, the homologs selected should be detected by UV absorbance.
In fact, molecules that contain aromatic rings are preferred, since they present increased absorbance, due to the presence of conjugated pi-electron systems within the aromatic ring structures.

With the criteria above into consideration, the series selected in this case to characterize the hold-up volume were n-alkyl benzenes, n-alkyl phenones, and parabens (Table 2). A suitable series in similar analyses is also n-alkyl ketones. However, experimental data did not provide any results in this particular chromatographic system. A possible explanation could be that the HSA column employed, great with analytes that are typically very hydrophilic acids [47], cannot retain ketones well enough, as they are not classified into hydrophilic compounds, but into "hydronutral" [48].

**Table 2: Molecular descriptors of the homologous series**

<table>
<thead>
<tr>
<th>Homologous Series</th>
<th>E</th>
<th>S</th>
<th>A</th>
<th>B</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n-Alkyl phenones</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetophenone</td>
<td>0.818</td>
<td>1.010</td>
<td>0.000</td>
<td>0.480</td>
<td>1.014</td>
</tr>
<tr>
<td>Propiophenone</td>
<td>0.800</td>
<td>0.950</td>
<td>0.000</td>
<td>0.510</td>
<td>1.155</td>
</tr>
<tr>
<td>Butyrophenone</td>
<td>0.800</td>
<td>0.950</td>
<td>0.000</td>
<td>0.510</td>
<td>1.296</td>
</tr>
<tr>
<td>Valerophenone</td>
<td>0.800</td>
<td>0.950</td>
<td>0.000</td>
<td>0.500</td>
<td>1.437</td>
</tr>
<tr>
<td>Hexanophenone</td>
<td>0.780</td>
<td>0.950</td>
<td>0.000</td>
<td>0.510</td>
<td>1.578</td>
</tr>
<tr>
<td>Heptanophenone</td>
<td>0.770</td>
<td>0.950</td>
<td>0.000</td>
<td>0.500</td>
<td>1.718</td>
</tr>
<tr>
<td><strong>n-alkyl benzenes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzene</td>
<td>0.610</td>
<td>0.520</td>
<td>0.000</td>
<td>0.140</td>
<td>0.716</td>
</tr>
<tr>
<td>Toluene</td>
<td>0.600</td>
<td>0.520</td>
<td>0.000</td>
<td>0.140</td>
<td>0.857</td>
</tr>
<tr>
<td>Ethylbenzene</td>
<td>0.610</td>
<td>0.510</td>
<td>0.000</td>
<td>0.150</td>
<td>0.998</td>
</tr>
<tr>
<td>Propylbenzene</td>
<td>0.600</td>
<td>0.500</td>
<td>0.000</td>
<td>0.150</td>
<td>1.139</td>
</tr>
<tr>
<td>Butylbenzene</td>
<td>0.600</td>
<td>0.510</td>
<td>0.000</td>
<td>0.150</td>
<td>1.280</td>
</tr>
<tr>
<td><strong>Parabens</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methylparaben</td>
<td>0.930</td>
<td>1.460</td>
<td>0.710</td>
<td>0.460</td>
<td>1.131</td>
</tr>
<tr>
<td>Ethylparaben</td>
<td>0.910</td>
<td>1.440</td>
<td>0.730</td>
<td>0.450</td>
<td>1.272</td>
</tr>
<tr>
<td>Propylparaben</td>
<td>0.900</td>
<td>1.450</td>
<td>0.740</td>
<td>0.430</td>
<td>1.413</td>
</tr>
<tr>
<td>Butylparaben</td>
<td>0.900</td>
<td>1.470</td>
<td>0.740</td>
<td>0.430</td>
<td>1.554</td>
</tr>
</tbody>
</table>

The McGowan volume V for the homologous series results from calculations in accordance with equation (10.4.2.2). All the other molecular descriptors in Table 2 can be obtained from open-access databases [49]. The number of compounds tested for each homologous series depends on their retention times. For the n-Alkyl phenones the last compound is Heptanophenone, whereas for the n-Alkyl benzenes is Butylbenzene, and for the parabens is Butylparaben. Compounds further
down each series are highly retained by the column, resulting in large retention times and, therefore, widened peaks that do not qualify as acceptable.

As for the values of the descriptors, n-alkyl benzenes, and n-alkyl phenones present an excellent lack of hydrogen bond donor capabilities (A = 0), whereas parabens exhibit moderately higher values. Moreover, all of them are characterized by little contribution from hydrogen bond acceptance interactions (B), with n-alkyl benzenes exhibiting the smallest mean value for the B coefficient. Lastly, it is observed, that n-alkyl phenones and parabens present increased polarity from dipole-type interactions (S) and increased polarizability from n- and p-electron pairs (E) (Figure 1).

![Mean values of Abraham molecular descriptors for n-Alkyl phenones, n-Alkyl benzenes, and Parabens for hold-up volume measurement: excess molar refraction (E), solute dipolarity / polarizability (S), solute hydrogen-bond acidity (A) and basicity (B).](image)

**Figure 1**: Mean values of Abraham molecular descriptors for n-Alkyl phenones, n-Alkyl benzenes, and Parabens for hold-up volume measurement: excess molar refraction (E), solute dipolarity / polarizability (S), solute hydrogen-bond acidity (A) and basicity (B).

### 12.2.2 Data fitting through iterations

To test the LFER homologous series approach in practice, experimental data (APPENDIX B: Table 10), including retention times for each member of the n-Alkyl benzenes, n-Alkyl phenones, and Parabens, along with their McGowan volumes V, have been fitted to equation (10.4.2.4). For each of the series, the unknown hold-up time $t_m$, constant r, and solute coefficient v are determined by several iterations using a computational tool [46] for parameter estimation in sorption isotherms. The results obtained are summarized in the table below (Table 3):
Table 3: Hold-up time $t_{M}$, constant $r$, and volume coefficient $v$ (± standard errors) for each homologous series obtained from fittings to equation (10.4.2.4). The coefficient of determination ($R^2$) and number of homologs ($N$) used in the fitting are also reported.

<table>
<thead>
<tr>
<th>Homologous Series</th>
<th>$t_{M}$ (min)</th>
<th>$r$</th>
<th>$v$</th>
<th>$R^2$</th>
<th>$N$</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-Alkyl phenones</td>
<td>1.054 ± 0.040</td>
<td>0.004 ± 0.000</td>
<td>2.130 ± 0.016</td>
<td>0.805</td>
<td>6</td>
</tr>
<tr>
<td>n-Alkyl benzenes</td>
<td>0.734 ± 0.576</td>
<td>0.066 ± 0.022</td>
<td>2.084 ± 0.107</td>
<td>0.950</td>
<td>5</td>
</tr>
<tr>
<td>Parabens</td>
<td>2.166 ± 0.297</td>
<td>0.001 ± 0.000</td>
<td>3.096 ± 0.348</td>
<td>0.802</td>
<td>4</td>
</tr>
</tbody>
</table>

The coefficients of determination $R^2$ for all the homologous series are close to 1, generally indicating a very good match of model to data. However, the big variation among the resulting values for retention time, and the $r$ and $v$ constants suggest otherwise. Standard errors in most variables are sufficiently low, however, in the case of n-Alkyl benzenes, $t_{M}$ exhibits a high standard error, further questioning the accuracy of the results.

These variations can be attributed to the small sample size of the series, since a small number of observations fails to provide reliable estimates [50]. Another possible explanation for these errors is that the relationship between the dependent and independent variables in equation (10.4.2.4) is complex enough for the model to accurately describe, since three unknown parameters need specification by just two independent sets of data.

In this context, the most accurate approach seems to be the mediate one of the n-Alkyl phenones, which exhibit results with evidently lower standard errors (Table 3), and a $t_{M}$ value that agrees with that calculated from the injection of a hold-up marker. Specifically, as mentioned in part 12.1, injecting Potassium Bromide in the chromatographic system leads to a hold-up time of $1.091 ± 0.002$ min, very similar to the $1.054 ± 0.040$ min, resulting from the n-Alkyl phenones homologous series.

However, further analysis needs to be conducted in order to conclude accurate results for the system’s hold-up time or volume. As illustrated in the following section (12.2.3), there is a model that provides more precise and reliable values, since it includes the contribution of all the homologous series at once and analyzes the data in one single equation, using binary flag descriptors.

Whether the acquired results are valid or not, can also be investigated by plotting the experimentally and computationally obtained data (APPENDIX B: Table 10). In this graph, the estimated retention time values, calculated through equation (10.4.2.4), are fitted over the experimental ones and plotted against the McGowan volume $V$ (Figure 2). It is evident that the correlation between them is rather inadequate, with the deviation between experimental and computational data getting bigger for larger volume values. Moreover, the extrapolation of all three estimated retention time curves to zero should converge to similar $t_{R}$ values, so that the resulting hold-up time would be the same. However, that is not the case, since significant deviation is also observed for volume values approaching zero.
On the other hand, Figure 2 successfully showcases the impact that different mean values for molecular descriptors (Figure 1: Mean values of Abraham molecular descriptors for n-Alkyl phenones, n-Alkyl benzenes, and Parabens for hold-up volume measurement: excess molar refraction (E), solute dipolarity/polarizability (S), solute hydrogen-bond acidity (A) and basicity (B) have to the r parameter, for each homologous series. As a result, these varying intramolecular interactions lead to different degrees of convex curvature. Moreover, Figure 2 exhibits the expected profile of retention in reversed-phase liquid chromatography (RP-LC) [39]; higher homologs’ molecular volumes lead to increased retention. This can be explained by the lower amounts of energy required for larger solutes to form a cavity in the stationary phase, resulting in their partitioning more favorably there, and, therefore, increased retention times.

The endoergic work of separating solvent molecules to provide a cavity of suitable size for the solute molecule, by means of Abraham’s solvation model, is measured by the coefficient of the McGowan volume (v) [30]. In this context, the sign of the v parameter will determine the type of retention. More specifically, in reversed-phase systems, the non-polar stationary phase is less structured than the hydroorganic mobile phase, which makes the creation of a cavity in the stationary phase less energetically demanding for solutes of large volume that favor the transfer from polar to less polar solvents. This way, big solutes tend to partition into the stationary phase, increasing their chromatographic retention and, therefore, the convex of the curvature in diagrams such as Figure 2. This increase is mathematically translated to a positive sign for v coefficients in RPLC, in accordance with results presented in Table 3.

Figure 2: Graphical representation of retention time \( t_R \) against the McGowan volume V for every member of the n-Alkyl phenones, n-Alkyl benzenes and Parabens homologous series. Points represent experimental data and solid lines represent fittings to equation (10.4.4.2.4.5).
12.2.3 Data fitting with flag descriptors

As mentioned, there is a better model to calculate the system’s hold-up volume or time, including all the homologous series simultaneously in a single equation, using binary flag descriptors. Since hold-up volume $V_M$, as well as the coefficients $v$ and $V$ are not dependent individually on each homologous series in question, several series can be analyzed altogether and jointly fitted in one inclusive model that provides more precise and reliable results.

Fitting experimental data (APPENDIX B: Table 11) to equation (10.4.2.6) and employing the appropriate computational tool [46] for parameter estimation, results in three $r$ values, one for each homologous series, and one overall value for the system’s hold-up volume and the $v$ coefficient (Table 4).

Table 4: $r$ constant for each of the homologous series, system’s hold-up volume, and $v$ coefficient ($\pm$ standard errors), obtained from fittings to equation (10.4.2.6). The coefficient of determination ($R^2$) is also reported.

<table>
<thead>
<tr>
<th>Homologous series</th>
<th>$r$</th>
<th>$V_M$(ml)</th>
<th>$v$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-Alkyl phenones</td>
<td>0.003 ± 0.000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n-Alkyl benzenes</td>
<td>0.048 ± 0.003</td>
<td>0.990 ± 0.051</td>
<td>2.037 ± 0.021</td>
<td>0.876</td>
</tr>
<tr>
<td>Parabens</td>
<td>0.004 ± 0.000</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In this case, the coefficient of determination $R^2$ is close to 1, indicating a very good match of model to data. The values for the $r$ constant vary in relation to the homologous series in question, since they depend on the intramolecular interactions present. As for the hold-up volume, it seems to agree with that calculated using an unretained marker (section 3412.1). For a better understanding and comparison of results, Table 5: summarizes the values obtained by the three different approaches.

Table 5: Hold-up time $t_M$, constant $r$, and volume coefficient $v$ ($\pm$ standard errors) as calculated with the three methods employed. The coefficient of determination ($R^2$) is also reported.

<table>
<thead>
<tr>
<th>Hold-up marker</th>
<th>$t_M$(min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium Bromide</td>
<td>1.091 ± 0.002</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Homologous series - iterations</th>
<th>$t_M$(min)</th>
<th>$r$</th>
<th>$v$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-Alkyl phenones</td>
<td>1.054 ± 0.040</td>
<td>0.004 ± 0.000</td>
<td>2.130 ± 0.016</td>
<td>0.805</td>
</tr>
<tr>
<td>n-Alkyl benzenes</td>
<td>0.734 ± 0.576</td>
<td>0.066 ± 0.022</td>
<td>2.084 ± 0.107</td>
<td>0.950</td>
</tr>
<tr>
<td>Parabens</td>
<td>2.166 ± 0.297</td>
<td>0.001 ± 0.000</td>
<td>3.096 ± 0.348</td>
<td>0.802</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Homologous series - flag descriptors</th>
<th>$t_M$(min)</th>
<th>$r$</th>
<th>$v$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-Alkyl phenones</td>
<td></td>
<td>0.003 ± 0.000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

39
n-Alkyl benzenes 1.099 ± 0.057  0.048 ± 0.003  2.037 ± 0.021  0.876
Parabens 0.004 ± 0.000

This comparative table (Table 5) reveals that the third model including the flag descriptors is indeed the most reliable and accurate one, since the hold-up volume obtained not only exhibits excellent agreement with the one from the injection of Potassium Bromide, but is also very close to that derived from the n-Alkyl phenones, the best series approach in the individual iterations method. As for the r constant and v coefficient values, the results obtained by the last two methods range between logical limits, so with the exception of Parabens, there is no need to reject any of them. Nevertheless, the most inclusive and precise approach remains the one with the flag descriptors, hence, the system’s hold-up time is considered equal to 1.099 ± 0.057 min.

The validity of the results can be assessed again by plotting the experimentally and computationally obtained data in question (APPENDIX B: Table 11). The fitting of estimated retention time values calculated through equation (10.4.2.6), over the experimental ones and plotted against the McGowan volume V in Figure 3.\textbf{Figure 2} determines how well the model describes the data.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.jpg}
\caption{Graphical representation of retention time t\textsubscript{R} against the McGowan volume V for every member of the n-Alkyl phenones, n-Alkyl benzenes and Parabens homologous series. Points represent experimental data and solid lines represent fittings to equation (10.4.2.6)}
\end{figure}

In this case, data calculated from equation (10.4.2.6) (solid lines) fit almost perfectly the experimentally acquired data (dots), validating the accuracy of the flag descriptors model. Furthermore, the extrapolation of all three estimated retention time curves to zero converges to similar t\textsubscript{R} values, so that the resulting hold-up time is the same for all of them.
Again, the resulting plot (Figure 3) showcases the expected profile of retention in reversed-phase liquid chromatography (RPLC) [39] with increasing retention times as the homologs’ molecular volumes augment.

**12.3 SYSTEM COEFFICIENTS DETERMINATION THROUGH THE ABRAHAM SOLVATION MODEL**

In order to determine the system coefficients using Abraham’s approach, retention factors (k) for each compound tested are calculated through equation (10.3.2.2), from retention times measured experimentally (APPENDIX B: Table 12). The exact set of solutes selected will be explained in the following section (section 12.4.1), as its formation is determined by factors related to their ability to form suitable pairs for the fast method elaborated subsequently. However, the homologous series used for determining the hold-up volume were also included in the analysis as part of the chromatographic system and with the purpose of increasing the statistical sample and acquiring more accurate results.

In this model described by equation (10.4.2.1), molecular descriptors E, S, A, B, and V constitute the independent variables, whereas log k values are considered the dependent ones. Provided these data, system constant c and coefficients e, s, a, b, and v can be calculated by multilinear regression (MLR). Upon examining the occurring residuals, no compounds need to be excluded as outliers, so MLR analysis results are presented as is in the following table (Table 6):

<table>
<thead>
<tr>
<th>c</th>
<th>e</th>
<th>s</th>
<th>a</th>
<th>b</th>
<th>v</th>
<th>N</th>
<th>R²</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1.068 ± 0.136</td>
<td>0.635 ± 0.081</td>
<td>-0.414 ± 0.091</td>
<td>-0.084 ± 0.089</td>
<td>-3.232 ± 0.260</td>
<td>2.218 ± 0.142</td>
<td>33</td>
<td>0.957</td>
<td>0.134</td>
</tr>
</tbody>
</table>

The coefficients’ signs and magnitude reveal the main intramolecular interactions that govern each chromatographic system. In particular, system constants with a positive sign indicate that these variables result in an increase in retention, while those with a negative sign favor residence in the mobile phase and lead to reduced retention [51]. In this case, the coefficients with the largest values are v and b, showing that solute volume and hydrogen-bond basicity are predominantly determining retention behavior.

More specifically, a large and positive value for the v coefficient in RPLC indicates that solutes tend to partition more favorably into the stationary phase, increasing retention. As illustrated in section 12.2.2, v measures the endoergic work required to separate solvent molecules so as to provide a cavity big enough for the solute molecule. Since in reversed-phase systems, the non-polar stationary phase is less structured than the hydroorganic mobile phase, the creation of a cavity in the stationary phase is less energetically demanding, favoring solutes’ partition and resulting in v >> 0.

As for the b coefficient, it measures the difference in hydrogen bond donor capabilities between the stationary and the mobile phase. Its large absolute value reveals that hydrogen bond
donor interactions greatly affect retention in this particular system. Moreover, b exhibits a negative sign, as expected in RPLC systems, indicating that interactions between the solute and the hydroorganic mobile phase are stronger than those with the non-polar stationary phase, reducing retention. As a result, strong hydrogen-bond bases have significantly lower retention than weak hydrogen-bond bases of a similar size (b << 0).

The rest of the system coefficients (e, s, and a) have smaller values, indicating that they pose less impact on retention. However, they are still important for fine-tuning selectivity. In light of this, a small value for the e constant, shows that excess polarizability contributions from n- and π-electrons do not affect chromatographic retention that much, since this type of interactions between the solutes and the stationary and mobile phases are of similar magnitude. However, the e value of 0.635 in this case cannot be considered negligible, hence, e > 0 reveals that dispersion interactions favor partition into the stationary phase and increased retention times.

The s coefficient shows the effect of solute-solvent dipolarity/polarizability on chromatographic retention. In this case, the s value is rather small but still considerable. More specifically, s < 0 indicates that dipolar-type interactions favor solute partitioning in the mobile phase rather than in the stationary phase, reducing retention.

The a coefficient measures the difference in hydrogen bond acceptor capabilities between the stationary and the mobile phase. A negative value such as in this case (a < 0), indicates that the interactions between the solute and the hydroorganic mobile phase are stronger than the ones with the non-polar stationary phase, decreasing retention. However, a is very close to 0, revealing that this type of interactions is not, in fact, that important to the system’s characterization.

All in all, the analysis of coefficients presented in Table 6 showcases that this particular chromatographic system described by large absolute values for the b and v constants would be very appropriate for the separation of mixtures of compounds differing in hydrogen-bond basicity and or size. On the other hand, it would constitute a rather disappointing method for separating substances according to their hydrogen-bond acidity (a coefficient), polarity (s coefficient), or polarizability (e coefficient), as their absolute values are considerably low and do not pose a great influence on retention.
12.4 SYSTEM COEFFICIENTS DETERMINATION THROUGH THE FAST METHOD

12.4.1 Selection of pairs of solutes

The fast method as illustrated by Redón et al. [42], proposes that only five chromatographic runs are needed in a reversed-phase column for the successful characterization of the chromatographic system; four pairs of test solutes (one for each coefficient), and a mixture of homologs. However, in order to achieve more reliable and precise results, several pairs of compounds have been selected for examination.

The selection of solutes was performed through Abraham’s database [52] and was based on several factors. First of all, compounds were chosen so as to form pairs with four very similar molecular descriptors and an as different as possible fifth one. Additionally, they should all absorb in the ultraviolet range to allow easy detection, be commercially available and relatively inexpensive, and soluble enough in the mobile phase. Finally, selected solutes’ acid/base properties should be in their neutral form over the widest possible range within the column pH stability, as this model is examined for neutral compounds.

The final selection of test solutes in this study and their molecular descriptors are presented in Table 7, grouped by solute selectivity examined for each pair. The number of pairs for each coefficient varies based on their behavior in the particular chromatographic system, as some theoretically appropriate solutes could not be detected. Furthermore, the outcome is also affected by the availability of compounds at the laboratory at the time of the experiments.

Table 7: Pairs of test solutes considered in the study with their corresponding molecular descriptors

<table>
<thead>
<tr>
<th>Excess polarizability (E)</th>
<th>E</th>
<th>S</th>
<th>A</th>
<th>B</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,8-Dihydroxyantraquinone</td>
<td>2.455</td>
<td>1.786</td>
<td>0.000</td>
<td>0.558</td>
<td>1.646</td>
</tr>
<tr>
<td>1-Chloroantraquinone</td>
<td>1.900</td>
<td>1.790</td>
<td>0.000</td>
<td>0.570</td>
<td>1.651</td>
</tr>
<tr>
<td>Dibenzo furan</td>
<td>1.780</td>
<td>0.860</td>
<td>0.000</td>
<td>0.250</td>
<td>1.274</td>
</tr>
<tr>
<td>1-Chloro-3-phenylpropane</td>
<td>0.794</td>
<td>0.900</td>
<td>0.000</td>
<td>0.240</td>
<td>1.262</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dipolarity/polarizability (S)</th>
<th>E</th>
<th>S</th>
<th>A</th>
<th>B</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,2-Dicyanobenzene</td>
<td>0.874</td>
<td>1.960</td>
<td>0.000</td>
<td>0.410</td>
<td>1.026</td>
</tr>
<tr>
<td>2-Methylbenzaldehyde</td>
<td>0.870</td>
<td>0.960</td>
<td>0.000</td>
<td>0.400</td>
<td>1.014</td>
</tr>
<tr>
<td>1,4-Dicyanobenzene</td>
<td>0.874</td>
<td>1.980</td>
<td>0.000</td>
<td>0.420</td>
<td>1.026</td>
</tr>
<tr>
<td>2-Methylbenzaldehyde</td>
<td>0.870</td>
<td>0.960</td>
<td>0.000</td>
<td>0.400</td>
<td>1.014</td>
</tr>
<tr>
<td>2,6-Dichlorobenzonitrile</td>
<td>1.095</td>
<td>1.220</td>
<td>0.000</td>
<td>0.270</td>
<td>1.116</td>
</tr>
<tr>
<td>1,2-Dihydronaphthalene</td>
<td>1.093</td>
<td>0.690</td>
<td>0.000</td>
<td>0.250</td>
<td>1.128</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Solute hydrogen bond acidity (A)</th>
<th>E</th>
<th>S</th>
<th>A</th>
<th>B</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Chloro-2-methylphenol</td>
<td>0.890</td>
<td>0.910</td>
<td>0.630</td>
<td>0.220</td>
<td>1.038</td>
</tr>
<tr>
<td>2-Chloroanisole</td>
<td>0.883</td>
<td>0.910</td>
<td>0.000</td>
<td>0.260</td>
<td>1.038</td>
</tr>
</tbody>
</table>
12.4.2 Resulting system coefficients

Applying equation (10.5.2.1.3) to the pairs of test solutes presented in Table 7, results in another estimation of the system coefficients. Experimental data employed to yield these results are shown in APPENDIX B: Table 13, while the obtained constants are reported in the table below:

<table>
<thead>
<tr>
<th>Solute hydrogen bond basicity (B)</th>
<th>e</th>
<th>s</th>
<th>a</th>
<th>b</th>
<th>v</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 2,6-dichlorobenzoyl chloride</td>
<td>1.028</td>
<td>1.600</td>
<td>0.000</td>
<td>0.210</td>
<td>1.240</td>
</tr>
<tr>
<td>2 3,4-dimethoxybenzaldehyde</td>
<td>1.020</td>
<td>1.600</td>
<td>0.000</td>
<td>0.740</td>
<td>1.272</td>
</tr>
</tbody>
</table>

In this case, e, s, a, and b coefficients result from calculations according to equation (10.5.2.1.3), whereas the cavity term’s v value is derived from the homologous series approach analyzed in section 12.2, since no pairs are needed for this system coefficient. In fact, compounds of any homologous series can be used for the estimation of the cavity term as homologs of a particular series have all their molecular descriptors almost similar except for the McGowan molecular volume (V). Evidently, two consecutive homologs differ only in one CH₂ group, resulting in larger V values across the series.

Although two different approaches have been investigated for the characterization of the cavity term v, one examining the three homologous series individually and one where they are all incorporated in a single model with flag descriptors, the final value for v presented in this case is the latter one. v calculated from the flag descriptors model is considered more accurate and, therefore, is the experimental value chosen.
Another noteworthy observation in Table 8 is that there is no standard error in the case of the b coefficient. As briefly mentioned during suitable pairs selection (section 12.4.1), not every group for property characterization could contain the same number of pairs due to practical limitations. In this context, for the identification of the hydrogen-bond basicity coefficient (b), a lot of candidate compounds were tested through chromatographic runs, yet none of them could be detected by the particular chromatographic system. Moreover, time and availability for acquiring new candidates posed further hindering factors, resulting in obtaining only one pair for the b coefficient, and, therefore, only one value. Hence, no standard error can be calculated.

As for the coefficients’ size and magnitude and what they mean for the chromatographic system, analysis has been extensively carried out in section 12.3. The values are not drastically different from the Abraham solvation model resulting ones, hence their effect on the system is similar. A more detailed comparison of the two methods is illustrated in the following section.

12.4.3 Comparison of Abraham’s solvation model and the fast method

The classic Abraham solvation parameter model with results occurring from multilinear regression analysis on a series of test solutes and a new fast method based on the examination of pairs for each type of interaction are the two approaches assessed for the chromatographic system’s characterization. Results obtained from each method are summarized in the following table (Table 9):

<table>
<thead>
<tr>
<th></th>
<th>e</th>
<th>s</th>
<th>a</th>
<th>b</th>
<th>v</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abraham’s solvation model</td>
<td>0.635 ± 0.081</td>
<td>-0.414 ± 0.091</td>
<td>-0.084 ± 0.089</td>
<td>-3.232 ± 0.260</td>
<td>2.218 ± 0.142</td>
</tr>
<tr>
<td>Fast method</td>
<td>0.327 ± 0.037</td>
<td>-0.676 ± 0.211</td>
<td>-0.260 ± 0.176</td>
<td>-1.001</td>
<td>2.037 ± 0.021</td>
</tr>
</tbody>
</table>

Table 9 reveals that the two methods’ results hold a lot of space for discussion. First of all, the cavity term coefficient (v) exhibits very similar values both in Abraham’s solvation model and the homologous series approach included in the fast method. This is very encouraging, as v plays a great role in determining retention behavior in this chromatographic system.

On the other hand, the hydrogen-bond basicity coefficient (b) is significantly different in the two methods. Despite their same negative sign, the two values present a relative error close to 70%. It cannot be stated that his deviation was not forthcoming, as practical limitations dictated only one pair for the b coefficient determination. As a result, there were not enough test solutes to examine
this type of interaction to its whole extent and derive a mean value, so an inclusive and accurate outcome cannot be obtained, even though hydrogen-bond basicity is a governing mechanism in this chromatographic system.

As for the rest of the system coefficients, the two models yield comparable results, that, however, exhibit significant differences. Indeed, the excess polarizability term’s value (e) derived from the fast method is half the one obtained from the Abraham solvation model. Furthermore, dipolarity/polarizability (s) and hydrogen-bond acidity (a) terms according to the fast method are almost double in size in the fast method compared to Abraham’s approach. However, e, s, and a coefficients are still small enough so that even their double values do not pose a great impact on the system’s behavior. Moreover, since their positive or negative sign remains the same in both methods, their differences are of little importance.

All in all, the only recently implemented fast method seems to require further examination to determine whether it is a good substitute for Abraham’s more tedious but accurate solvation parameter model. A sufficient number of pairs to characterize each intramolecular interaction along with a wider range of solutes would potentially increase the model’s accuracy. However, augmenting the size of test solutes analyzed runs the risk of canceling the point of creating a faster and less demanding method.
13. CONCLUSIONS

High-pressure liquid chromatography (HPLC) constitutes an excellent method for separating samples into their constituent components, based on their distribution between the mobile and stationary phases. Separation is achieved through various solute-solvent interactions, as molecules of different nature present different affinity with the adsorbent surface, leading to diverse retention behavior. Its application in the pharmaceutical world establishes HPLC as a very useful technique for analyzing drugs, biomolecules, polymers, and many other compounds, advancing drug discovery and development processes. Moreover, it helps decrease development costs, as it allows for an early and drastic selection of the most suitable molecules that display the greatest likelihood of success.

Employing a protein-packed column for HPLC analysis provides vast information about drug-protein binding and drugs’ biological activities, enhancing knowledge about their pharmacokinetic, pharmacodynamic, and toxicological profiles. In fact, packing a column with albumin, the most abundant protein in blood serum, has been proven very useful in drug binding understanding, as albumin’s fundamental role is distributing therapeutic agents. Hence, Human Serum Albumin or HSA-based columns have been increasingly employed as an efficient method for assessing drug-protein interactions with great speed, precision, reproducibility, and even automation to further minimize run-to-run variations.

HPLC implementation first requires characterizing the chromatographic system. For this reason, the column’s hold-up time or volume (the two terms are interchangeable since they can be easily adjusted through the flow rate) has been determined using two different methods. First, using Potassium Bromide as an unretained hold-up volume marker led to a hold-up time equal to $t_M = 1.091 \pm 0.002$ min. Second, the homologous series approach employs compounds belonging in one series and takes advantage of their similar chemical properties and elution behaviors, resulting in a predictable and standardized performance that allows the accurate and reproducible determination of the system’s hold-up time.

The homologous series approach was implemented using three different series: n-Alkyl phenones, n-Alkyl benzenes, and Parabens, resulting in a hold-up time value of $t_M = 1.054 \pm 0.040$ min, $t_M = 0.734 \pm 0.576$ min, and $t_M = 2.166 \pm 0.297$ min respectively. It is evident that except for the hold-up time derived from n-Alkyl phenones ($t_M = 1.054 \pm 0.040$ min), the rest of the values do not agree with the hold-up time calculated using an unretained hold-up volume marker, or with the other homologous series obtained ones. For this reason, the three series have been incorporated into a single equation using binary flag descriptors. Analyzing them all together and jointly fitting them into one inclusive model provides more precise and reliable results. Indeed, the occurring hold-up time value equals to $t_M = 1.099 \pm 0.057$ min exhibiting a good agreement with the one calculated using Potassium Bromide as an unretained marker. Hence, $t_M = 1.099 \pm 0.057$ min is the considered value in this work to characterize the system. This value has been subtracted from gross retention times acquired from chromatographic runs to obtain each compound’s true retention time.

Moreover, the homologous series approach provides results for one of the coefficients that characterize the system, the cavity formation term $v$. The three series examined separately led to $v$
values equal to $v = 2.130 \pm 0.016$ for n-Alkyl phenones, $v = 2.084 \pm 0.107$ for n-Alkyl benzenes, and $v = 3.096 \pm 0.348$ for Parabens. Again, the three values should converge to a single one for the system’s coefficient, however, $v$ obtained from Parabens ($v = 3.096 \pm 0.348$) exhibits great deviation from the other ones. By employing the inclusive model characterized by flag descriptors, the cavity term takes the value of $v = 2.037 \pm 0.021$ which is regarded as more accurate, in agreement with the n-Alkyl phenones and n-Alkyl benzenes derived $v$ values. Thus, $v = 2.037 \pm 0.021$ is the cavity formation term considered for the chromatographic system’s characterization in this work.

With the column’s hold-up time determined, it was possible to calculate the compounds’ retention factors, essential for evaluating the different solute-solvent interactions contributing to the system’s partitioning. The method employed is one of the most commonly used ones, Abraham’s solvation parameter model, derived from the general Abraham linear solvation energy relationships (LSER) approach. It allowed the characterization of the system’s solute-solvent interactions including polarizability, dipolarity, hydrogen bonding, and cavity formation, through the corresponding coefficients and each compound’s molecular descriptors.

More specifically, a multilinear regression (MLR) analysis based on Abraham’s solvation model, performed on the selected test of solutes along with the homologous series employed to calculate the system’s hold-up volume, yielded the following results: system’s constant $c$ equals to $c = -1.068 \pm 0.136$, excess polarizability term $e$ equals to $e = 0.635 \pm 0.081$, dipolarity/polarizability factor $s$ equals to $s = -0.414 \pm 0.091$, hydrogen bond acidity coefficient $a$ equals to $a = -0.084 \pm 0.089$, hydrogen bond basicity coefficient equals to $b = -3.232 \pm 0.260$, and cavity formation term $v$ equals to $v = 2.218 \pm 0.142$.

The obtained coefficients’ signs and magnitude reveal to what way and extent they affect the chromatographic system in question. The large and positive value for the $v$ coefficient ($v = 2.218 \pm 0.142$) indicates that it governs retention behavior, with solutes tending to partition more favorably into the stationary phase, increasing retention. An even larger but negative $b$ coefficient ($b = -3.232 \pm 0.260$) shows that hydrogen bond donor interactions also greatly affect this particular system, with said interactions being stronger between solutes and the hydroorganic mobile phase than with the non-polar stationary phase, reducing retention. As for the rest of the coefficients, they are characterized by smaller values, indicating that they have less impact on retention.

Lastly, a recently implemented fast method has been examined for the characterization of the chromatographic system, based on the Abraham solvation parameter model but combined with Tanaka’s principles. Including only a careful selection of appropriate pairs of test solutes, it requires far fewer chromatographing runs saving up time. However, the results yielded from this fast method are not in agreement with the ones from the traditionally accurate Abraham solvation model MLR analysis. More specifically, in this case, excess polarizability term $e$ equals to $e = 0.327 \pm 0.037$, dipolarity/polarizability factor $s$ equals to $s = -0.676 \pm 0.211$, hydrogen bond acidity coefficient $a$ equals to $a = -0.260 \pm 0.176$, hydrogen bond basicity coefficient equals to $b = -1.001$, and cavity formation term $v$ equals to $v = 2.037 \pm 0.021$, derived from the homologous series approach to determine the column’s hold-up volume.

A comparison of the two methods leads to questionable results for the fast method’s accuracy. Although the cavity term coefficient ($v$) is similar in both models, the hydrogen bond
basicity term (b) which greatly impacts the system, as well, is very different. The basic reason behind this great deviation is that practical limitations dictated only one pair for the b coefficient determination. More specifically, a lot of candidate compounds were tested, yet none of them could be detected by the particular chromatographic system. Moreover, time and availability did not allow for acquiring new candidates so this type of interaction could not be examined to its whole extent and conclude with an accurate result.

As for the rest of the system coefficients, the two models yield comparable results, with some differences. However, e, s, and a coefficients are small enough compared to b, and v, so that deviations between them do not pose a great impact on the system’s behavior. Moreover, since their positive or negative sign remains the same in both methods, their differences are of little importance.

All in all, the obtained coefficients reveal that this particular chromatographic system described by large absolute values for the b and v constants would be very appropriate for the separation of mixtures of compounds differing in hydrogen-bond basicity and or size. On the other hand, it would constitute a rather disappointing method for separating substances according to their hydrogen-bond acidity (a coefficient), polarity (s coefficient), or polarizability (e coefficient), as their absolute values are considerably low and do not pose a great influence on retention.

As for the two different methods for retrieving the system coefficients, it appears that the fast method requires further examination to determine whether it is a good substitute for Abraham’s more tedious but accurate solvation parameter model. A sufficient number of pairs to characterize each intramolecular interaction along with a wider range of solutes would potentially increase the model’s accuracy. However, augmenting the size of test solutes analyzed runs the risk of canceling the point of creating a faster and less demanding method.

Ultimately, a well-determined chromatographic system allows for the detailed examination of the dynamic equilibria of solutes between mobile and stationary phases. This way, and with the contribution of proteins in the role of stationary phases as excellent media for drug binding understanding, a variety of biomimetic models can be developed to further examine the distribution of compounds between plasma/blood and tissue. Being able to predict the human metabolism and pharmacokinetics of a drug-candidate compound based solely on its molecular structure and physicochemical properties, paves the way for less time-consuming, labor-intensive, and ethically sensitive procedures, promising significant breakthroughs in the fields of drug discovery and development.
14. REFERENCES


[52] “Abraham_E_S_A_BDescriptors”.
INSTRUCTION MANUAL FOR CHIRALPAK® HSA

Please read this instruction sheet completely before using this column

Column Description

CHIRALPAK® HSA : Human serum albumin immobilized on 5μm silica-gel.

Shipping solvent : Water / 2-Propanol (2-ProOH) solvent mixture (90/10 v/v)

All columns have been pre-tested before packaging. The test parameters and results, as well as the Column Lot Number, are included on a separate (enclosed) page.

Application Scope

CHIRALPAK® HSA can offer high enantioselectivity for compounds bearing carboxylic groups, including:
- strong and weak acids
- zwitter-ionic molecules
- non-ionisable compounds (amides, esters, alcohols, sulfoxides, etc)

For compounds of basic category, however, it is preferred to use CHIRALPAK® AGP and CHIRALPAK® CBH columns.

Operating Conditions

<table>
<thead>
<tr>
<th></th>
<th>50 x 2 mm i.d.*1</th>
<th>100 x 2 mm i.d.*1</th>
<th>150 x 2 mm i.d.*1 Analytical column</th>
<th>50 x 3 mm i.d.*1</th>
<th>100 x 3 mm i.d.</th>
<th>150 x 3 mm i.d. Analytical column</th>
<th>50 x 4 mm i.d.*1</th>
<th>100 x 4 mm i.d.</th>
<th>150 x 4 mm i.d. Analytical column</th>
<th>100 x 10 mm i.d.</th>
<th>150 x 10 mm i.d. Semi-prep. column</th>
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</thead>
<tbody>
<tr>
<td>Flow direction</td>
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<td></td>
</tr>
<tr>
<td>Typical Flow rate</td>
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<td>4.0 mL/min</td>
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<tr>
<td>pH range</td>
<td>5.0 - 7.0</td>
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<tr>
<td>Recommended temperature*2</td>
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</tr>
<tr>
<td>Buffer concentration</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Organic modifier ratio</td>
<td>0-15% by volume</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Charged additive concentration</td>
<td>up to 10mM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*1 It is very important that the HPLC system is optimized in terms of void volume for work with columns of small dimensions.

*2 The column lifetime might be reduced if used at higher temperature.
Operating Procedure

A - Mobile Phase Starting Conditions

<table>
<thead>
<tr>
<th>Typical starting conditions</th>
<th>ACIDIC Compounds</th>
<th>NEUTRAL Compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>10mM Ammonium acetate buffer (pH 7.0) / 2-PrOH = 85 / 15 (v/v)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Refer to section B for preparation of the buffer.

B - Buffer Preparation - Example

Preparation of 10mM Ammonium acetate buffer (1Liter):

1. Weigh 770.8 mg of ammonium acetate (CH₃COONH₄, purity > 99%) into a beaker.
2. Dissolve the salt with about 800mL water (HPLC grade), equilibrated at room temperature (20-25°C).
3. Adjust pH to the target value by using either diluted acetic acid or a diluted ammonium hydroxide solution.
4. Filter the solution through a membrane of 0.22μm into a measuring flask.
5. Add water until the limit line of the measuring flask. Place the stopper in the neck and homogenize the solution by agitation.

When buffer should be mixed with an organic modifier, the measurements are normally by volumes, using preferably volumetric flasks or measuring pipettes.
After mixing, degas the mobile phase in an ultrasonic bath.

Note that in the case where a charged additive is needed in the mobile phase, the charged additive should be added into the aqueous solution before the pH adjustment.

C - Mobile Phases

Bacteria grow fast in eluents containing no or low alcoholic organic modifier. Such mobiles phases must be freshly prepared.

Buffer
The salt concentration of ammonium acetate buffer is typically 10-20mM but can be varied up to 100mM. The other kinds of buffers, such as sodium or potassium phosphate buffers, sodium acetate buffers, formate or citrate buffers, can also be used. However, the LC-MS compatibility of the method may be sometimes compromised.

Organic Modifiers
2-PrOH is the most frequently used. However, methanol, ethanol and acetonitrile can also be investigated. The relative eluting strength can be ranked as follows: 2-PrOH > EtOH ≥ ACN > MeOH

Charged additives
Cationic and anionic additives, such as N,N-dimethylctyl amine (DMOA), trifluoroacetic acid (TFA), octanoic acid (OA), heptfluorobutyric acid (HFBA), can also be used to regulate retention and enantioselectivity. However, some of these additives may be difficult to remove totally, due to very high affinity to the matrix. Thus, the properties of the column may be affected.

CAUTION: The miscibility of OA and DMOA to water is very limited. Only 2mM OA or 5mM DMOA can be homogeneously incorporated into the aqueous solution at ambient temperature. A phase separation may occur beyond these concentrations.

Once a charged additive is used in the mobile phase, the column should be dedicated for the purpose.
D – Samples

The sample amount injected onto the column should be kept low enough. The recommended sample concentration is 0.20 mg/mL or lower with an injection volume of 5-10 μL, preferably.

Dissolve the sample in the mobile phase when it is possible. If the sample is insoluble in the mobile phase, add a higher concentration of the organic modifier. The sample solution should be filtered through a membrane filter of approximately 0.5μm porosity to ensure that there is no precipitate before using.

**CAUTION:** Dissolution of the sample in pure or high percentage of organic solvents may cause on-line sample precipitation. Do not inject unclear sample solutions or solutions containing undissolved compounds.

Method Development

The following scheme offers a guide for method development and method optimization:

- **Starting condition:** 10mM Ammonium acetate buffer (pH 7.0) / 2-PrOH = 85 / 15 (v/v)
  - For k_d > 10, baseline separation
  - For k_d < 10, charged additives

- Adjust 2-PrOH % (0-15%)
- Add ACN, EtOH, MeOH (≤15%)
- Add Other conditions: short retained samples: pH 5.0, long retained samples: 30°C, shorter column (5cm)

Other columns: CHIRALPAK® AGP or other Daicel columns

**Method delivery**

---

**CHIRALPAK® HSA**
100 x 4 mm ID

3-Oxo-1-indanecarboxylic acid

Abscisic acid

Mecrop
**Column Care / Maintenance**

- The use of a guard cartridge is highly recommended for maximum column life.
- If the column has been contaminated with very hydrophobic material, wash the column backwards (no detector connected) over night with Water/2-PrOH 75/25(v/v) at a reduced flow-rate (e.g. 0.3 mL/min for 4mm ID columns).
- Before disconnecting the column from the HPLC system, flush the column with a mobile phase that does not contain any salts / buffers, e.g. Water/2-PrOH 90/10(v/v).
- For the storage of the column, it is recommended to fill it with Water/2-PrOH 90/10(v/v). For short storage periods, the column can be placed at ambient temperature (<30°C). For longer storage periods, however, it is recommended to place it in a refrigerator.

**Important Notice**

We recommend the use of a CHIRALPAK® HSA guard column in order to protect the analytical column from any particulates and impurities with high affinity to the stationary phase. Change the guard column regularly, especially in bioanalysis.

*Operating these columns in accordance with the guidelines outlined here will result in a long column life.*

- If you have any questions about the use of this column, or encounter a problem, please email questions@chiraltech.com or call 800-6-CHIRAL for assistance.

<table>
<thead>
<tr>
<th>Part Number</th>
<th>Name</th>
<th>Particle Size</th>
<th>Internal Diameter</th>
<th>Column Length</th>
<th>Product Type</th>
</tr>
</thead>
<tbody>
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<td>10</td>
<td>Guard (2)</td>
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<td>50</td>
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<td>34713</td>
<td>CHIRALPAK HSA</td>
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<td>Analytical</td>
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<td>150</td>
<td>Analytical</td>
</tr>
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<td>10</td>
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<td>3.0</td>
<td>10</td>
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<td>34782</td>
<td>CHIRALPAK HSA</td>
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<td>3.0</td>
<td>50</td>
<td>Analytical</td>
</tr>
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<td>34783</td>
<td>CHIRALPAK HSA</td>
<td>5</td>
<td>3.0</td>
<td>100</td>
<td>Analytical</td>
</tr>
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<td>34784</td>
<td>CHIRALPAK HSA</td>
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<td>3.0</td>
<td>150</td>
<td>Analytical</td>
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<td>34791</td>
<td>CHIRALPAK HSA</td>
<td>5</td>
<td>2.0</td>
<td>10</td>
<td>Guard (2)</td>
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<tr>
<td>34792</td>
<td>CHIRALPAK HSA</td>
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<td>2.0</td>
<td>50</td>
<td>Analytical</td>
</tr>
<tr>
<td>34793</td>
<td>CHIRALPAK HSA</td>
<td>5</td>
<td>2.0</td>
<td>100</td>
<td>Analytical</td>
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<tr>
<td>34794</td>
<td>CHIRALPAK HSA</td>
<td>5</td>
<td>2.0</td>
<td>150</td>
<td>Analytical</td>
</tr>
</tbody>
</table>

CHIRALCEL, CHIRALPAK and CROWNPAK are registered trademarks of DAICEL CORPORATION
### DATA FOR HOLD-UP VOLUME DETERMINATION THROUGH EQUATION (10.4.2.4)

*Table 10: McGowan Volume, retention time, and estimated retention time with their residuals for every compound in the three homologous series for fitting in equation (10.4.2.4).*

<table>
<thead>
<tr>
<th>N-ALKYL PHENONES</th>
<th>McGowan Volume V (ml)</th>
<th>Retention time $t_R$ (min)</th>
<th>Estimated $t_R$ (min)</th>
<th>Residuals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetophenone</td>
<td>1.014</td>
<td>1.581</td>
<td>1.589</td>
<td>0.008</td>
</tr>
<tr>
<td>Propiophenone</td>
<td>1.155</td>
<td>2.010</td>
<td>2.122</td>
<td>0.112</td>
</tr>
<tr>
<td>Butyrophenone</td>
<td>1.296</td>
<td>2.679</td>
<td>3.185</td>
<td>0.506</td>
</tr>
<tr>
<td>Valerophenone</td>
<td>1.437</td>
<td>4.288</td>
<td>5.308</td>
<td>1.020</td>
</tr>
<tr>
<td>Hexanophenone</td>
<td>1.578</td>
<td>7.293</td>
<td>9.543</td>
<td>2.250</td>
</tr>
<tr>
<td>Heptanophenone</td>
<td>1.718</td>
<td>12.835</td>
<td>17.996</td>
<td>5.161</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>N-ALKYL BENZENES</th>
<th>McGowan Volume V (ml)</th>
<th>Retention time $t_R$ (min)</th>
<th>Estimated $t_R$ (min)</th>
<th>Residuals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene</td>
<td>0.716</td>
<td>2.312</td>
<td>2.775</td>
<td>0.463</td>
</tr>
<tr>
<td>Toluene</td>
<td>0.857</td>
<td>3.856</td>
<td>4.747</td>
<td>0.891</td>
</tr>
<tr>
<td>Ethylbenzene</td>
<td>0.998</td>
<td>7.428</td>
<td>8.627</td>
<td>1.199</td>
</tr>
<tr>
<td>Propylbenzene</td>
<td>1.139</td>
<td>11.867</td>
<td>16.256</td>
<td>4.389</td>
</tr>
<tr>
<td>Butylbenzene</td>
<td>1.280</td>
<td>22.656</td>
<td>31.259</td>
<td>8.603</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PARABENS</th>
<th>McGowan Volume V (ml)</th>
<th>Retention time $t_R$ (min)</th>
<th>Estimated $t_R$ (min)</th>
<th>Residuals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylparaben</td>
<td>1.131</td>
<td>2.207</td>
<td>2.516</td>
<td>0.309</td>
</tr>
<tr>
<td>Ethylparaben</td>
<td>1.272</td>
<td>2.616</td>
<td>3.122</td>
<td>0.505</td>
</tr>
<tr>
<td>Propylparaben</td>
<td>1.413</td>
<td>3.952</td>
<td>4.775</td>
<td>0.823</td>
</tr>
<tr>
<td>Butylparaben</td>
<td>1.554</td>
<td>7.277</td>
<td>9.289</td>
<td>2.012</td>
</tr>
</tbody>
</table>
DATA FOR HOLD-UP VOLUME DETERMINATION THROUGH EQUATION (10.4.2.6)

Table 11: McGowan Volume, flag descriptors, retention volume, and estimated retention volume with their residuals for every compound in the three homologous series for fitting in equation (10.4.2.6).

<table>
<thead>
<tr>
<th>N-ALKYL PHENONES</th>
<th>McGowan Volume V(ml)</th>
<th>f phen</th>
<th>f benz</th>
<th>f para</th>
<th>Retention volume V_R (ml)</th>
<th>Estimated V_R (ml)</th>
<th>Residuals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetophenone</td>
<td>1.014</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1.423</td>
<td>1.379</td>
<td>-0.044</td>
</tr>
<tr>
<td>Propiophenone</td>
<td>1.155</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1.809</td>
<td>1.744</td>
<td>-0.064</td>
</tr>
<tr>
<td>Butyrophenone</td>
<td>1.296</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>2.411</td>
<td>2.451</td>
<td>0.040</td>
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<tr>
<td>Valerophenone</td>
<td>1.437</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>3.859</td>
<td>3.821</td>
<td>-0.038</td>
</tr>
<tr>
<td>Hexanophenone</td>
<td>1.578</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>6.564</td>
<td>6.473</td>
<td>-0.091</td>
</tr>
<tr>
<td>Heptanophenone</td>
<td>1.718</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>11.551</td>
<td>11.609</td>
<td>0.058</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>N-ALKYL BENZENES</th>
<th>McGowan Volume V(ml)</th>
<th>f phen</th>
<th>f benz</th>
<th>f para</th>
<th>Retention volume V_R (ml)</th>
<th>Estimated V_R (ml)</th>
<th>Residuals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene</td>
<td>0.716</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>2.081</td>
<td>2.365</td>
<td>0.284</td>
</tr>
<tr>
<td>Toluene</td>
<td>0.857</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>3.471</td>
<td>3.653</td>
<td>0.182</td>
</tr>
<tr>
<td>Ethylbenzene</td>
<td>0.998</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>6.685</td>
<td>6.147</td>
<td>-0.538</td>
</tr>
<tr>
<td>Propylbenzene</td>
<td>1.139</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>10.680</td>
<td>10.978</td>
<td>0.298</td>
</tr>
<tr>
<td>Butylbenzene</td>
<td>1.280</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>20.390</td>
<td>20.334</td>
<td>-0.056</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PARABENS</th>
<th>McGowan Volume V(ml)</th>
<th>f phen</th>
<th>f benz</th>
<th>f para</th>
<th>Retention volume V_R (ml)</th>
<th>Estimated V_R (ml)</th>
<th>Residuals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylparaben</td>
<td>1.131</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1.986</td>
<td>1.739</td>
<td>-0.247</td>
</tr>
<tr>
<td>Ethylparaben</td>
<td>1.272</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>2.354</td>
<td>2.441</td>
<td>0.087</td>
</tr>
<tr>
<td>Propylparaben</td>
<td>1.413</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>3.557</td>
<td>3.801</td>
<td>0.244</td>
</tr>
<tr>
<td>Butylparaben</td>
<td>1.554</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>6.549</td>
<td>6.434</td>
<td>-0.115</td>
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</table>
Characterization of an HSA column through HPLC: testing the Abraham solvation model

DATA FOR SYSTEM COEFFICIENTS DETERMINATION WITH THE ABRAHAM SOLVATION MODEL (EQUATION (10.4.2.1))

Table 12: Retention factor \(k\), log \(k\) and molecular descriptors \(E, S, A, B, V\) for every compound tested. Data fitted to equation (10.4.2.1)

<table>
<thead>
<tr>
<th>Compound</th>
<th>(k)</th>
<th>log (k)</th>
<th>(E)</th>
<th>(S)</th>
<th>(A)</th>
<th>(B)</th>
<th>(V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,8-Dihydroxanthraquinone</td>
<td>36.843</td>
<td>1.566</td>
<td>2.455</td>
<td>1.786</td>
<td>0.000</td>
<td>0.558</td>
<td>1.646</td>
</tr>
<tr>
<td>1-Chloroanthraquinone</td>
<td>23.874</td>
<td>1.378</td>
<td>1.900</td>
<td>1.790</td>
<td>0.000</td>
<td>0.570</td>
<td>1.651</td>
</tr>
<tr>
<td>Dibenzofuran</td>
<td>27.452</td>
<td>1.439</td>
<td>1.780</td>
<td>0.860</td>
<td>0.000</td>
<td>0.250</td>
<td>1.274</td>
</tr>
<tr>
<td>1-Chloro-3-phenylpropane</td>
<td>14.139</td>
<td>1.150</td>
<td>0.794</td>
<td>0.900</td>
<td>0.000</td>
<td>0.240</td>
<td>1.262</td>
</tr>
<tr>
<td>1,2-Dicyanobenzene</td>
<td>0.484</td>
<td>-0.315</td>
<td>0.874</td>
<td>1.960</td>
<td>0.000</td>
<td>0.410</td>
<td>1.026</td>
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<tr>
<td>2-Methylbenzaldehyde</td>
<td>1.339</td>
<td>0.127</td>
<td>0.870</td>
<td>0.960</td>
<td>0.000</td>
<td>0.400</td>
<td>1.014</td>
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<tr>
<td>1,4-Dicyanobenzene</td>
<td>0.226</td>
<td>-0.646</td>
<td>0.874</td>
<td>1.980</td>
<td>0.000</td>
<td>0.420</td>
<td>1.026</td>
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<tr>
<td>2,6-Dichlorobenzonitrile</td>
<td>5.124</td>
<td>0.710</td>
<td>1.095</td>
<td>1.220</td>
<td>0.000</td>
<td>0.270</td>
<td>1.116</td>
</tr>
<tr>
<td>1,2-Dihydropnaphthalene</td>
<td>19.588</td>
<td>1.292</td>
<td>1.093</td>
<td>0.690</td>
<td>0.000</td>
<td>0.250</td>
<td>1.128</td>
</tr>
<tr>
<td>4-Chloro-2-methylphenol</td>
<td>4.567</td>
<td>0.660</td>
<td>0.890</td>
<td>0.910</td>
<td>0.630</td>
<td>0.220</td>
<td>1.038</td>
</tr>
<tr>
<td>2-Chloroanisole</td>
<td>3.533</td>
<td>0.548</td>
<td>0.883</td>
<td>0.910</td>
<td>0.000</td>
<td>0.260</td>
<td>1.038</td>
</tr>
<tr>
<td>4-Chloro-3,5-dimethylphenol</td>
<td>7.856</td>
<td>0.895</td>
<td>0.980</td>
<td>0.940</td>
<td>0.610</td>
<td>0.260</td>
<td>1.179</td>
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<tr>
<td>2,4-Dichloroanisole</td>
<td>9.771</td>
<td>0.990</td>
<td>1.000</td>
<td>0.940</td>
<td>0.000</td>
<td>0.220</td>
<td>1.161</td>
</tr>
<tr>
<td>3,4-Dichloroanisole</td>
<td>18.745</td>
<td>1.273</td>
<td>0.960</td>
<td>0.950</td>
<td>0.000</td>
<td>0.220</td>
<td>1.161</td>
</tr>
<tr>
<td>3-Ethoxyphenol</td>
<td>0.823</td>
<td>-0.085</td>
<td>0.850</td>
<td>1.140</td>
<td>0.560</td>
<td>0.480</td>
<td>1.116</td>
</tr>
<tr>
<td>2-Chloroacetophenone</td>
<td>1.286</td>
<td>0.109</td>
<td>0.894</td>
<td>1.140</td>
<td>0.000</td>
<td>0.470</td>
<td>1.136</td>
</tr>
<tr>
<td>4-Isopropoxyphenol</td>
<td>0.682</td>
<td>-0.166</td>
<td>0.800</td>
<td>1.180</td>
<td>0.570</td>
<td>0.490</td>
<td>1.257</td>
</tr>
<tr>
<td>Methyl-4-methoxybenzoate</td>
<td>1.277</td>
<td>0.106</td>
<td>0.830</td>
<td>1.200</td>
<td>0.000</td>
<td>0.520</td>
<td>1.272</td>
</tr>
<tr>
<td>Acetophenone</td>
<td>0.516</td>
<td>-0.287</td>
<td>0.820</td>
<td>1.010</td>
<td>0.000</td>
<td>0.480</td>
<td>1.014</td>
</tr>
<tr>
<td>Propiophenone</td>
<td>0.952</td>
<td>-0.021</td>
<td>0.800</td>
<td>0.950</td>
<td>0.000</td>
<td>0.510</td>
<td>1.155</td>
</tr>
<tr>
<td>Butyrophenone</td>
<td>1.651</td>
<td>0.218</td>
<td>0.800</td>
<td>0.950</td>
<td>0.000</td>
<td>0.510</td>
<td>1.296</td>
</tr>
<tr>
<td>Valerophenone</td>
<td>3.712</td>
<td>0.570</td>
<td>0.800</td>
<td>0.950</td>
<td>0.000</td>
<td>0.500</td>
<td>1.437</td>
</tr>
<tr>
<td>Hexanophenone</td>
<td>7.644</td>
<td>0.883</td>
<td>0.780</td>
<td>0.950</td>
<td>0.000</td>
<td>0.510</td>
<td>1.578</td>
</tr>
<tr>
<td>Heptanophenone</td>
<td>15.316</td>
<td>1.185</td>
<td>0.777</td>
<td>0.950</td>
<td>0.000</td>
<td>0.500</td>
<td>1.718</td>
</tr>
<tr>
<td>Benzene</td>
<td>1.283</td>
<td>0.108</td>
<td>0.610</td>
<td>0.520</td>
<td>0.000</td>
<td>0.140</td>
<td>0.716</td>
</tr>
<tr>
<td>Toluene</td>
<td>3.359</td>
<td>0.526</td>
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<td>7.609</td>
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<td>0.610</td>
<td>0.510</td>
<td>0.000</td>
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<td>Propylbenzene</td>
<td>13.309</td>
<td>1.124</td>
<td>0.600</td>
<td>0.500</td>
<td>0.000</td>
<td>0.150</td>
<td>1.139</td>
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<td>Butylbenzene</td>
<td>27.747</td>
<td>1.443</td>
<td>0.600</td>
<td>0.510</td>
<td>0.000</td>
<td>0.150</td>
<td>1.280</td>
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<tr>
<td>Methylparaben</td>
<td>1.323</td>
<td>0.122</td>
<td>0.930</td>
<td>1.460</td>
<td>0.710</td>
<td>0.460</td>
<td>1.131</td>
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<tr>
<td>Ethylparaben</td>
<td>1.755</td>
<td>0.244</td>
<td>0.910</td>
<td>1.440</td>
<td>0.730</td>
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<td>0.863</td>
<td>0.900</td>
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<td>0.740</td>
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DATA FOR SYSTEM COEFFICIENTS DETERMINATION WITH THE FAST METHOD  
(EQUATION (10.5.2.1.3))

Table 13: Average retention factor k, differences of decimal logarithms of k and molecular descriptors (E, S, A, B, V) between pairs, resulting coefficient values (e, s, a, b, v) and their residuals for each pair

<table>
<thead>
<tr>
<th>Compound</th>
<th>Average k</th>
<th>log k1/k2</th>
<th>dE</th>
<th>dS</th>
<th>dA</th>
<th>dB</th>
<th>dV</th>
<th>Value</th>
<th>Residuals</th>
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<tbody>
<tr>
<td><strong>Dipolarity/ polarizability (E)</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1 1,8-Dihydroxyanthraquinone</td>
<td>38.335</td>
<td>0.202</td>
<td>0.555</td>
<td>-0.004</td>
<td>0.000</td>
<td>-0.012</td>
<td>-0.005</td>
<td>0.365</td>
<td>0.707</td>
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<td>2 1-Chloroanthraquinone</td>
<td>24.057</td>
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<td>1 Dibenzoferan</td>
<td>27.639</td>
<td>0.286</td>
<td>0.986</td>
<td>-0.040</td>
<td>0.000</td>
<td>0.010</td>
<td>0.013</td>
<td>0.290</td>
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<td>2 1-Chloro-3-phenylpropane</td>
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</tr>
<tr>
<td><strong>Solute hydrogen bond basicity (A)</strong></td>
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<td></td>
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<td>0.010</td>
<td>0.012</td>
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<td>-0.592</td>
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<td>1.020</td>
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<td>0.012</td>
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<td>5.287</td>
<td>-0.573</td>
<td>0.002</td>
<td>0.530</td>
<td>0.000</td>
<td>0.020</td>
<td>-0.013</td>
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<td><strong>Solute hydrogen bond acidity (B)</strong></td>
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<td>1 4-Chloro-2-methylphenol</td>
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<td>0.630</td>
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<tr>
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<td>8.021</td>
<td>-0.093</td>
<td>-0.020</td>
<td>0.000</td>
<td>0.610</td>
<td>0.040</td>
<td>0.019</td>
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<td>-0.168</td>
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<td>0.560</td>
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<td><strong>Value</strong></td>
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<td><strong>Residuals</strong></td>
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</tbody>
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