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Treball Final de Grau

Characterization of HILIC systems: underivatized silica as stationary phase.

Caracterització de sistemes HILIC: sílice sense derivatitzar com a fase estacionària.

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B:KC Barcelona Knowledge Campus
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It was like a new world opened to me, the world of science, which I was at last permitted to know in all liberty.

Marie Curie

Moltes gràcies als meus pares i al meu germà per estar sempre recolzant-me i animant-me en tot moment; al Miguel Ángel per alegrar-me els dies i fer que sigui tot més fàcil (sobretot per aguantar-me) i als meus tutors, al Xavier i al Martí, per la gran ajuda i suport que m'han donat durant tot el treball.

REPORT

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1. SUMMARY

Hydrophilic interaction liquid chromatography (HILIC) is a type of chromatography especially used for the analysis of polar substances, using mobile phases with a high content of organic solvent and polar stationary phases. Nowadays, its retention mechanism still under study due to its complexity.

In this work, the Abraham's model based on linear free energy relationships (LFER) has been used in order to characterize the study column (Kinetex® with silica support), obtaining information about the mobile phase volume inside the column and the retention mode under certain working conditions. As a way of characterizing this column, the behavior of different homologous series (*n*-alkyl benzenes, *n*-alkyl phenones and *n*-alkyl ketones) has been analyzed with different proportions of mobile phase (acetonitrile and water mixture). According to the mobile phase composition, significative variations on the retention mode and on the mobile phase volume inside the column can be observed.

In fact, this study could be useful for the determination of possible hold-up volume markers, depending on the prevailing retention mode.

Keywords: Chromatography, HILIC, RPLC, Abraham's model, homologous series, mobile phase, stationary phase.

2. RESUM

La cromatografia líquida d'interacció hidrofílica (HILIC) és un tipus de cromatografia utilitzada especialment per l'anàlisi de substàncies polars, emprant fases mòbils amb un alt contingut de solvent orgànic i fases estacionàries polars. Actualment, el seu mecanisme de retenció és objecte d'estudi degut a la seva complexitat.

En aquest treball s'ha utilitzat el model d'Abraham basat en les relacions lineals d'energia lliure (LFER) per tal de caracteritzar la columna d'estudi (Kinetex® amb suport de sílice), obtenint informació sobre el volum de fase mòbil a l'interior de la columna i el mode de retenció en unes determinades condicions de treball. Per dur a terme la caracterització d'aquesta columna, s'ha analitzat el comportament de diferents sèries homòlogues (*n*-alquil benzens, *n*-alquil fenones i *n*-alquil cetones) amb diferents proporcions de fase mòbil (mescla d'acetonitril i aigua). Segons la composició de fase mòbil, s'observen variacions significatives en el mode de retenció i en el volum de fase mòbil dins la columna.

A més a més, aquest estudi també pot ser útil per la determinació de possibles marcadors de volum mort, depenent del mode de retenció predominant.

Paraules clau: Cromatografia, HILIC, RPLC, model d'Abraham, sèries homòlogues, fase mòbil, fase estacionària.

3. INTRODUCTION

3.1. CHROMATOGRAPHY

Chromatography is a physic separation technique based on the distribution of a compound in two phases. On the one hand, an active and immobile phase called stationary phase that could be totally stationary or could be a stationary liquid in a solid surface. On the other hand, an active or inert mobile phase that goes through the stationary phase.

In the two phases, the compounds experience common intermolecular interactions, which can be characterized by an equilibrium constant. [1]

The aim of chromatography is obtaining a good effective (tight chromatographic peaks) and high resolution (separated chromatographic peaks) in the separation of compounds in the chromatogram (the visual output of the signals). This chromatographic separation takes place in a column or in a plane (flat surface).

3.1.1. Chromatographic techniques classification

A specific classification of the chromatographic techniques can be done by the physical states of the phases [1], [2]:

1) Gas mobile phase: the separation of these two chromatographic techniques take place in a column.

- Gas-liquid chromatography (GLC) or Gas chromatography (GC): the stationary phase is a liquid. A partition (absorption) process is the cause of the separation.

- Gas-solid chromatography (GSC): the stationary phase is a solid and an adsorption process carrier out the separation.

2) Supercritical fluid mobile phase: the chromatographic separation happens in a column.

- Supercritical fluid chromatography (SFC): the stationary phase is either a solid or an immobilized liquid. The separation mechanisms are partitioning between bulk phases and interfacial adsorption.

3) Liquid mobile phase: the chromatographic separation takes place in a column or in a flat surface.

- Liquid-liquid chromatography (LLC): the stationary phase is a liquid and involve a separation mechanism of partition. This chromatographic separation occurs in a column.

- Thin-layer chromatography (TLC): the stationary phase is distributed as a thin layer on a flat support. In this type, the mobile phase can ascend through the layer by capillary forces.

- Paper chromatography (PC): the stationary phase is water in a layered support and the mobile phase travels up the stationary phase by capillarity forces.

- Liquid-solid chromatography (LSC) or Normal-phase chromatography (NPC): the stationary phase is a solid and the separation mechanism is adsorption. This type of chromatography uses columns packed with polar stationary phase and a less-polar solvent as the mobile phase.

- Reversed-phase chromatography (RPC): The stationary phase is a nonpolar solid that interact with a polar mobile phase. This type of chromatography uses a column to separate the compounds.

- Size-exclusion chromatography (SEC): the stationary phase is a solid with a controlled pore size distribution and the solutes are separated by size differences (size exclusion separation mechanism).

- Ion exchange chromatography (IEC) or Ion chromatography (IC): the stationary phase is a solid with immobilized ionic groups and the separation mechanism is based on electrostatic interactions between ions in the mobile phase and the stationary phase. All these processes happen in the column.

- Affinity chromatography (AC): the stationary phase is a solid with immobilized molecular recognition sites in which the dominant separation mechanism is the three-dimensional specificity of the interaction between the molecular recognition site and the sample. This chromatographic separation takes place in a column.

3.1.2. Retention parameters

The retention parameters are all the factors that describes a chromatographic process. The "retention time" (t_R) is a parameter indicating the elution time of a compound inside the column. The volume of mobile phase that leaves the column, from the moment the analyte enters the chromatographic phase system to the moment the analyte leaves, is called "retention volume" (V_R) of the analyte [3], [4].

The relation between the retention volume and the retention time is given by the expression,

$$V_R = t_R \cdot F \tag{1}$$

where F is the mobile phase flow (mL min^{-1}).

Besides, the retention time observed on the chromatogram from the injector to the detector is named "gross retention time" ($t_R{}^g$). In other words, it indicates how much time a component spends in the stationary phase from the injection to elution of a retained sample component.

When the analyte is not retained at all by the stationary phase, the volume of mobile phase required to eluate this non-retained analyte is called the "hold-up volume" (V_M) of the chromatographic phase system. Also, we can talk about the "hold-up time" (t_M) as the retention time that a compound take place in the mobile phase during the chromatographic process.

Thus, it allows to define the "adjusted retention time (volume)" (t'_R or V'_R), time of a compound retained by the stationary phase. It is expressed by the next expression:

$$V'_R = V_R - V_M \tag{2}$$

In the chromatographic system, there are some sort of connection (capillary tubes and end pieces) that connect the column with the injector and the detector. Therefore, it must be considered the volume of mobile phase that leaves the chromatographic system in time between sample injection to the column entrance and the column exit to the detector. This volume is named "extra-column volume" (V_{excol}).

As a result, if all the parameters are compiled, next parameter relation is acquired (it can also be express by retention times):

$$V_R = V_R^g - V_{excol} = V'_R + V_M \tag{3}$$

So, some of these parameters allow to calculate the "retention factor" (k), time that the analyte spends in the stationary phase in relation to the time that it spends in the mobile phase.

This factor expresses the delay suffered by the analyte due to the stationary phase, with respect to what it would takes to extend across the column at the speed of the mobile phase and without being retained:

$$k = \frac{t_R}{t_M} = \frac{V_R}{V_M} = \frac{V_R - V_M}{V_M} \quad (4)$$

It is important enough to be notice that times can easily be converted into volumes by means of the mobile phase flow rate, allowing measuring V_R and V_M from the recorded retention times of the analytes.

3.2. LIQUID CHROMATOGRAPHY

Liquid chromatography (LC) is a separation technique that uses a liquid mobile phase. This type of chromatography allows the analysis of polar and nonpolar compounds without molecular weight restriction. The instrumentation of liquid chromatography consists in a solvent delivery system involving reservoirs, mixers, valves, pumps, dampers and flow controllers that deliver the desired mobile phase composition to the column; an injector (injection valve or autosampler) for loading the sample solution (usually under ambient conditions) inserting a known volume of it into the pressurized mobile phase flow to be carried to the column and an on-line detector that record continuously on the low pressure side of the system [5]. The detector sends an electric signal to a software system that creates a chromatogram with all the information about the chromatographic separation.

Nowadays, liquid chromatography capable of analyzing very small particles (5 μm approximately) and needs high pressures is called High-performance liquid chromatography (HPLC). If the sample is smaller than 2 μm, the separation technique is named Ultra-high-performance liquid chromatography (UHPLC) and it requires a higher pressure than HPLC. [6]

3.3. HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY

Hydrophilic interaction liquid chromatography (HILIC) is an alternative HPLC mode for separating polar compounds of biological interest and it was first suggested by Alpert in 1990 [7]. Like normal-phase liquid chromatography (NPLC), HILIC uses traditional polar stationary phases such as silica, amino, cyano or zwitterionic groups, even though the mobile phase employed is similar to those used in the reversed-phase liquid chromatography (RPLC) mode: polar solutions of organic solvents that also contains an amount of water or a buffer solution.

HILIC also allows the analysis of charged substances, as in ion chromatography (IC). Figure 1 shows how HILIC complements other chromatographic techniques and extends the range of separation options as mention before. [8].

HILIC has a great amount of advantages in relation to NPLC, RPLC and IC. For instances, polar samples always show good solubility in the aqueous mobile phase used in HILIC, which overcomes the drawbacks of the poor solubility often manifested in NPLC. Moreover, HILIC has established itself as the separation mode of choice for uncharged highly hydrophilic and amphiphilic compounds that are too polar to be well retained in RPLC and may have insufficient charge to allow effective electrostatic retention in IC.

The increase in HILIC applications is shown by the increase of publications on this topic, illustrating that this separation mode is increasingly being adopted by researchers, specially from the end of the 2000s [9].

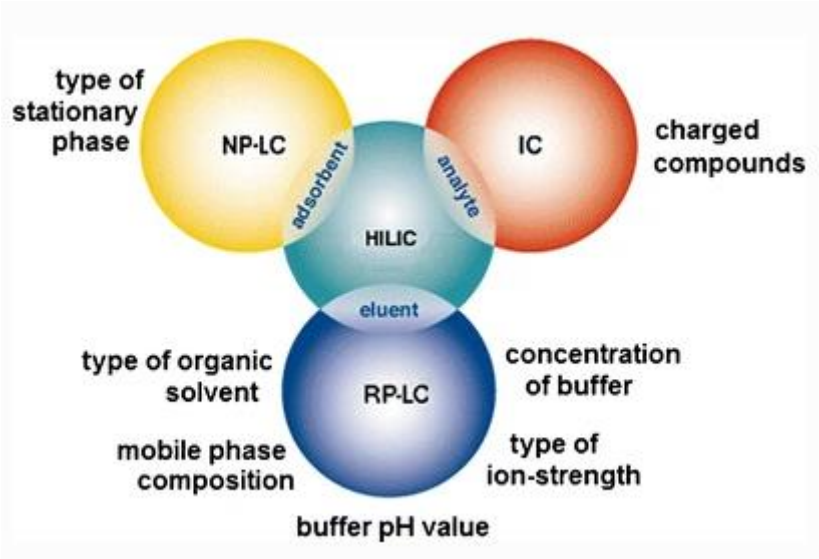


Figure 1. HILIC combines the characteristics of the three methods in liquid chromatography [8].

3.4. RETENTION MECHANISM IN HILIC

Retention mechanisms in HILIC are complex and are currently under investigation.

Polar materials used as stationary phases (SP) in HILIC like silica present a high affinity for polar solvents, specialy water from the hydroorganic mobile phase (MP). As a matter of fact, molecular dynamics studies disclosed the existence of three solvent regions of different composition inside the column, using silica as SP and acetonitrile-water mixtures like a MP[10].

Figure 2 shows these three solvent regions in different colors:

I) A rigid quasi-immobilized water layer (blue). This adsorbed aqueous layer is formed above the silica when mobile phases contain 2-40% water.

II) A diffuse hydroorganic interface region (green), enriched in water, of reduced translational mobility.

III) The nominal acetonitrile-water mixed solvent as the MP (yellow).

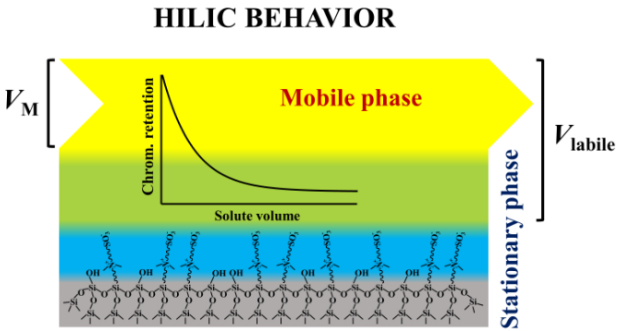


Figure 2. Schematic representation of the different regions inside a chromatographic column (ZILI-HILIC) in HILIC mode [10].

The properties of the complex interface region differ from those of the bulk eluent but depends on it. [11]

Alpert suggested in his seminal paper that the analyte retention was based on the hydrophilic partitioning of solutes between bulk eluent and a water-rich layer quasi-immobilized on the surface of the stationary phase in HILIC.

There are essentially three possible retention mechanisms: the analyte partitioning between the MP and the SP, the adsorption mechanism of the analyte onto the silica surface and the preferential adsorption of the organic solvent from the MP on the surface of the SP followed by the partitioning of the analyte into the adsorbed water-rich layer.

The main mechanism depends on the type of SP used and the buffer conditions, including the organic solvent, the type and concentration of salt and the pH. In fact, the retention also depends on different interactions between the solute with the MP, the solute with the SP and the MP with the SP: hydrogen bonding, dipole–dipole interactions and electrostatic interactions [12].

3.5. CONDITIONS IN HILIC

As in all the chromatographic systems, the retention of the analytes depends on the stationary phase, the mobile phase, the temperature, the flow of the mobile phase and others. As far as temperature and the flow are concerned, the optimal values should be chosen according to the dimensions of the column, the effective and resolution of the chromatographic peaks, among others.

However, the stationary phase and the mobile phase are more relevant in the analyte retention.

3.5.1. Mobile phase

There are many polar organic solvents that can be used in HILIC, but the favorable characteristics of acetonitrile (ACN) make it the most used solvent in HILIC. Its characteristics are low viscosity and efficient separations at low pressures. Moreover, ACN has a lower elution strength (e_0 = 0.65), compared to methanol (e_0 = 0.95), ethanol (e_0 = 0.89) and isopropanol (e_0 = 0.82), knowing that the use of stronger eluting components can lead to elution of some analytes in the column hold-up volume. Although the strength of elution of acetone (e_0 = 0.58) is lower than that of ACN, its high absorbance cut-off value (k = 330 nm) makes it impractical for applications with UV detections [9].

Pure ACN and methanol (MeOH) are solvents of similar polarity but there are some differences. On the one hand, ACN is a polar aprotic solvent that do not show proton-donor interactions. Even though, it provides larger differences with respect to the water-rich liquid stationary phase formed on the surface of polar stationary phases. On the other hand, MeOH is a polar protic solvent (like water), which competes to solvate the stationary phase surface and

provides strong hydrogen bonding interactions. Also, it presents an alcohol group, a better hydrogen-bond basicity and a significantly higher hydrogen-bond donor acidity. Regarding these characteristics, HILIC-MeOH and reversed-phase show a similar behavior. [13]

3.5.2. Stationary phase

Polar stationary phases used in the contemporary practice of HILIC separations show large variability in the amount of adsorbed water in aqueous–organic mobile phases, depending on the column type. Typical HILIC stationary phases consist of classical bare silica or modified silica gels with many polar functional groups. Another useful type is the polymer-based stationary phases [14], [15].

There is a great deal of chromatographic columns for HILIC systems. Some examples are:

a) Unmodified bare silica: normally acidic because of the activate silanol groups having a poor stability at basic pH. If the surface is populated with silicon hybrid (Si-H) instead of silanol group, the acidity is slightly reduced even though the polarity decrease.
b) Neutral polar chemically bonded phases: some examples could be diols (hydrogen-bonding properties), amide (basic properties), amino (basic behaviour), cyano (hydrogen-bonding capabilities), polyethylene glycol, sulfalkylbetaine, cyclodextrin, pentafluorophenylpropyl, polypeptide, among others.
c) Zwitterionic columns: it has a sulfobetaine bonded stationary phase, an acidic sulfonic acid group and basic quaternary ammonium groups separated by short alkyl spacers.
d) Ion exchange: styrene-divinylbenzene resins with sulfonated surfaces.

In this work, the chromatographic separation takes place in a Kinetex® HILIC column using a silica stationary phase.

3.6. HOLD-UP TIME IN HILIC. METHODS OF MEASUREMENT

There are different methods in order to determinate hold-up volumes in liquid chromatography: pycnometry, homologous series, minor disturbance of baseline, unretained neutral marker and inorganic salts. [3],[16]

In the pycnometric method the column is filled with two solvents of sufficiently different densities. Hold-up volumes are calculated from the weight difference.

Homologous series method is based on the linear representation of the logarithm of the adjusted retention times for various compounds of the same family versus their corresponding carbon number. Hold-up times are estimated from regression analysis.

A minor disturbance of baseline is obtained by injecting a solution (water or an organic solvent) with a slightly different composition from the mobile phase (normally deuterated) to detect a reliable disturbance peak.

The simplest method is based on the injection of neutral molecules, non-polar for normal-phase (benzene, toluene…) and polar for reversed-phase (formamide, urea…), and it were proposed as suitable unretained markers. In fact, in reversed-phase they are likely to show partial retention and use inorganic salts like $NaNO_3$, KBr, among others.

Because of the complexity of HILIC retention mechanisms (partition of solutes between the bulk of MP and the water-rich layer in the SP), the selection of a suitable hold-up time marker is challenging. These suitable markers are non-polar hydrophobic substances as they should be unable to partition into the water-rich layer.

In some studies, toluene is used as hold-up marker in HILIC, but recent studies indicate that other compounds are less retained than toluene in mobile phases containing acetonitrile or methanol such as alkyl benzene series or alkyl phenome series [16]. Thus, this chromatographic practice (using toluene like a hold-up marker) should be examined with caution.

In this work, the homologous series method has been proposed with the aim of analyzing three homologous series with acetonitrile/water in a HILIC system: alkyl benzene series, alkyl phenome series and alkyl ketone series.

3.6.1. Homologous series method

The homologous series approach for hold-up volume determination is based on linear free energy relationships (LFER) proposed by Abraham, which are widely used to characterize chemical. The free energy of a process can be obtained by an additive combination of the free energies from different chemical interactions that contribute to the overall process, each one proportional to a product of appropriate descriptors of the different compounds that interact.

A specific type of LFER is the linear solvation energy relationship (LSER), which has been widely applied to different partition processes. The solvation parameter model can be applied to measure the retention factor, log k, according to this equation:

$$\log k = c + eE + sS + Aa + Bb + vV \quad (5)$$

where *E*, *S*, *A*, *B* and *V* are solute descriptors; *e*, *s*, *a*, *b* and *v* are the system constants that provide chemical information to characterization the system, in other words, the chromatographic column. All these parameters are related solute-solvent interactions except *c*, which is a system constant. These descriptors represent different interactions:

E: contributions from n- and π-electron pairs.

S: solute polarity/polarizability because of the dipoles and induce dipoles.

A: solute hydrogen-bond acidity.

B: solute hydrogen-bond basicity.

V: McGowan's volume related to the endoergic work of separating solvent molecules to provide a cavity of suitable size for the solute molecule and the exoergic solute-solvent general dispersion interactions.

Retention volume of a solute can be expressed combining equation (4) and (5):

$$V_R = V_M + 10^{c+eE+Ss+aA+bB+vV} \quad (6)$$

If throughout the analysis the chromatographic conditions remain constant and the homologous series is a group of very similar compounds only differing in the number of carbon atoms, *c*+*eE*+*Ss*+*aA*+*bB* is considered like a constant value for all solutes. Considering this approach:

$$V_R = V_M + r \cdot 10^{vV} \quad (7)$$

Where V_M, *r* and *v* are constant values. *V* is the McGowan's molecular volume (mL·mol^{-1}/100). All the descriptors are tabulated and can be calculated experimentally.

In fact, the equation 7 allows know the system constants to characterize the column by only one homologous series. For this reason, the three homologous series, *n*-alkyl benzenes, *n*-alkyl phenones and *n*-alkyl ketones, could be express at the same time by the following equation:

$$V_R = V_M + (r_b + r_p + r_k) \cdot 10^{vV} \quad (8)$$

When making measurements of retention volumes, two behaviors are observed from the LSER homologous series approach, depending on the amount and the composition of the mobile phase, as shown in the Figure 3. On the one hand, the higher the molecular volume and the water content in the mobile phase, the lower the retention in HILIC behavior. On the other hand, the higher the molecular volume and the water content in the mobile phase, the higher the retention in reversed-phase behavior. As a matter of fact, in HILIC mode the hold-up volumes increase with the organic solvent content, whereas in RP mode the hold-up volumes are practically the same.

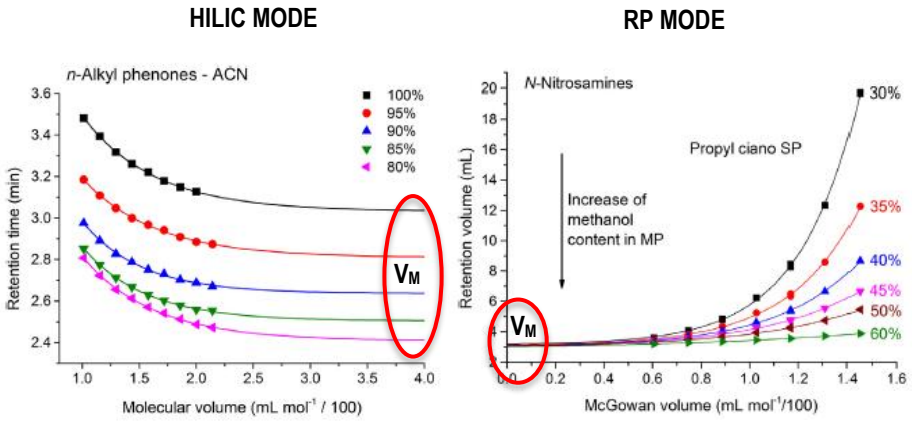


Figure 3. Variation of retention volumes with the McGowan's Volume and fittings according to equation 7 in HILIC and reversed-phase modes [3].

4. OBJECTIVES

The main objective of the present work is the characterization of a HILIC column, a Kinetex® with a silica stationary phase, using acetonitrile-water as the mobile phase by studying the retention modes and the solvent volumes inside this column, from the LSER homologous series approach. The column characterization allows to know information about the retention mechanism by the contribution of the different interactions of the solute with the stationary and mobile phases.

To achieve this aim, the following specific objectives have been summarized:

- Measurement of retention volumes of three homologous series (*n*-alkyl benzenes, *n*-alkyl phenones and *n*-alkyl ketones) and calculation of the fitting parameters of the column with the LFER approach.
- Study of possible hold-up markers that could be used in HILIC or in RPLC.

5. EXPERIMENTAL SECTION

5.1. MATERIAL

The organic solvents, acetonitrile and methanol (Fisher, Loughborough, UK), used as the mobile phase and to dissolve the analytes respectively, were HPLC gradient grade. Water was deionized by a Mili-Q® plus system from Millipore (Billerica, MA, USA). The homologous series studied in this work are compiled in the Table 1, which were bought from Alfa Aesar, Acros organics, Merck, Flucka, Sigma Aldrich and J.T.Baker, all of them with high purity grade (≥99). The column used in this research is described in the Table 2.

Table 1. Molecular descriptors of the homologous series compiled of ChemSpider data base.

Homologous series	Analyte	*E*	*S*	*A*	*B*	*V*
***n*-alkyl benzenes**	**Benzene**	0.61	0.52	0.00	0.14	0.72
	Toluene	0.60	0.52	0.00	0.14	0.86
	Ethylbenzene	0.61	0.51	0.00	0.15	1.00
	Propylbenzene	0.60	0.50	0.00	0.15	1.14
	Butylbenzene	0.60	0.51	0.00	0.15	1.28
	Pentylbenzene	0.59	0.51	0.00	0.15	1.42
	Hexylbenzene	0.59	0.50	0.00	0.15	1.56
	Octylbenzene	0.58	0.48	0.00	0.15	1.84
	Dodecylbenzene	0.57	0.47	0.00	0.15	2.41
***n*-alkyl phenones**	**Acetophenone**	0.82	1.01	0.00	0.48	1.01
	Propiophenone	0.80	0.95	0.00	0.51	1.15
	Butyrophenone	0.80	0.95	0.00	0.51	1.30
	Valerophenone	0.80	0.95	0.00	0.50	1.44
	Hexanophenone	0.78	0.95	0.00	0.51	1.58
	Heptanophenone	0.77	0.95	0.00	0.50	1.72
	Octanophenone	0.77	0.95	0.00	0.50	1.86
	Nonanophenone	0.76	0.95	0.00	0.50	2.00

	Decanophenone	0.75	0.95	0.00	0.50	2.14
***n*-alkyl ketones**	**Propanone**	0.18	0.70	0.04	0.49	0.55
	Butanone	0.17	0.70	0.00	0.51	0.69
	Pentan-2-one	0.14	0.68	0.00	0.51	0.83
	Hexan-2-one	0.14	0.68	0.00	0.51	0.97
	Heptan-2-one	0.12	0.68	0.00	0.51	1.11
	Octan-2-one	0.11	0.68	0.00	0.51	1.25
	Nonan-2-one	0.12	0.68	0.00	0.51	1.39
	Decan-2-one	0.11	0.68	0.00	0.51	1.53
	Undecan-2-one	0.10	0.68	0.00	0.51	1.67
	Dodecan-2-one	0.10	0.68	0.00	0.51	1.82
	Tridecan-2-one	0.10	0.68	0.00	0.51	1.96
	Pentadecan-2-one	0.10	0.68	0.00	0.51	2.24
	Nonadecan-2-one	0.09	0.68	0.00	0.51	2.80

Table 2. Characteristics of the column used in HILIC separation.

Column	Manufacturer	Support	Particle size [µm]	Pore size [Å]	Dimensions [mm]
Kinetex®	Phenomenex (Torrance, CA, USA)	Silica	5	100	150 x 4.6

5.2. INSTRUMENTATION

All the HILIC measurement were performed on a Shimadzu (Kyoto, Japan) HPLC system, which was formed by two LC-20AD pumps, a SIL-20AC auto-injector, a CTO-10ASvp oven, an SPD-10AVvp UV/Vis detector and a controller.

The injection volume and the flow rate through the Kinetex® column were 1 µL and 1 mL min^{-1} respectively. The temperature of the oven was 25ºC.

5.3. EXPERIMENTAL PROCEDURE

5.3.1. Measurement of extra-column volume

All the retention times obtained by the chromatographic separation (gross retention time) need subtracted the extra column contribution in order to obtain the retention times of the analytes only in the column. This extra-column was determinate in other studies injecting

1 µL of 0.5 mg mL^{-1} aqueous solution of potassium bromide in absence of column, using water as eluent and a flow rate of 0.5 mL min^{-1}, each in triplicate. The extra-column volume was 0.12 mL in this chromatographic system.

5.3.2. Homologous series

Stock solutions of homologous series were prepared in different Eppendorf with methanol at a concentration of 5 mg mL^{-1}, each of them diluted to 0.5 mg mL^{-1} in different vials in order to be injected in duplicate.

6. RESULTS AND DISCUSSION

In this work, the study of three homologous series (*n*-alkyl benzenes, *n*-alkyl phenones and *n*-alkyl ketones) was carried out using a Kinetex® column with silica support. Figure 4 allows to see the measured retention volumes depending on the McGowan's volume for the three homologous series using acetonitrile/water as mobile phase mixture in the range between 10% and 90%. Mobile phases with 100% and 0% ACN were not studied because on the first one, the quasi-immobilized water layer would not form in the absence of water; the second one due to its too high retention. These figures show two different chromatographic modes of retention in both homologous series.

On the one hand, approximately from 90% to 40% of ACN exhibits a HILIC behavior. In these range, the higher number of carbons of each analyte from each homologous series imply a lower retention volume. It means that the most non-polar compounds (the ones with the highest number of carbons) are less retained in comparison with the most polar (the ones with the lowest number of carbons) since the first ones have less affinity for the quasi-immobilized water layer formed previously and adsorbed on the silica support. Moreover, above 70% of ACN, the higher water content means lower retention volume due to the retention being associated with the difficulty in inserting the analyte in the diffuse hidroorganic interface region (water enriched) by the solvent-solvent intermolecular forces. However, from 60% to 40% of organic solvent, the quasi-immobilized water layer remains diffusive with the hydroorganic interface region (two layers mixture). It produces an increase of the retention volume with rising percentages of water.

In fact, at 30% of ACN, we cannot distinguish a single retention mode because of the combination of HILIC and RPLC behaviors.

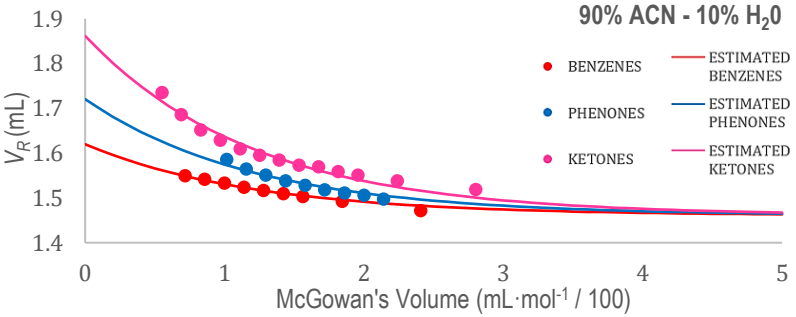
On the other hand, down to 20% of organic solvent, the retention volumes started to increase with the amount of water, prevailing the reversed-phase behavior. In this range, the mobile phase and the stationary phase water layer decrease its polarity differences up to the point where the analytes of the homologous series could interact directly with the surface of the stationary phase, furthering the partition of the hydroorganic interface region.

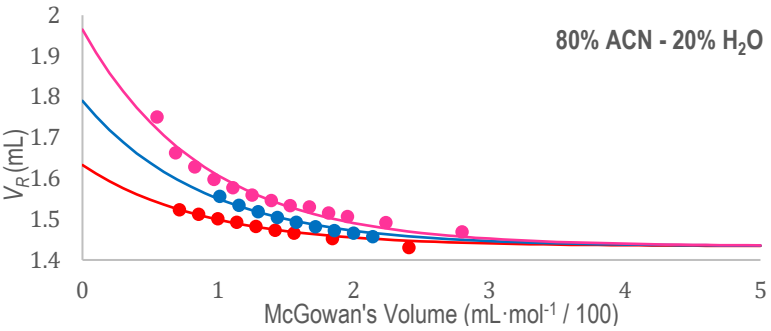
Furthermore, from the retention volumes obtained experimentally, the characterization parameters of the Kinetex® column are calculated by the LSER model with the equation 7 (parameters to each homologous series) and 8 (parameters to the three homologous series at the same time). These parameters (V_M, r, v) and the different errors are compiled on the Tables 3 and 4 for each amount of mobile phase. All the fitting parameters of this column are represented in the Figure 5 for each homologous series and the three at the same time.

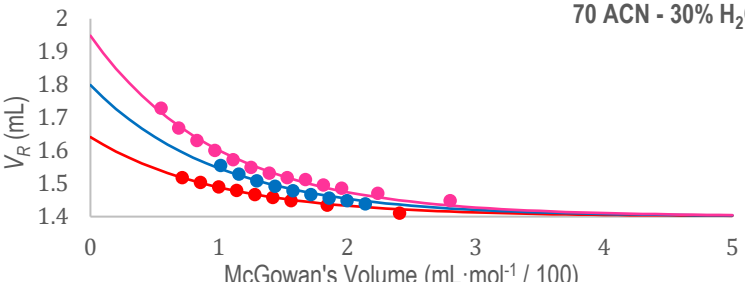
As a matter of fact, the parameters from 30% of ACN in both cases (each homologous series and the triseries), the fitting could not be made as the two retention behaviors predominate at the same time in this range. It means that, as show in the tables, in the transition from HILIC mode to RPLC mode, the parameter v changes the sign (from negative to positive). Therefore, it is to be expected that in 30% of organic solvent, the v is practically 0 ($10^v = 1$), thus, a flat curve is obtained:

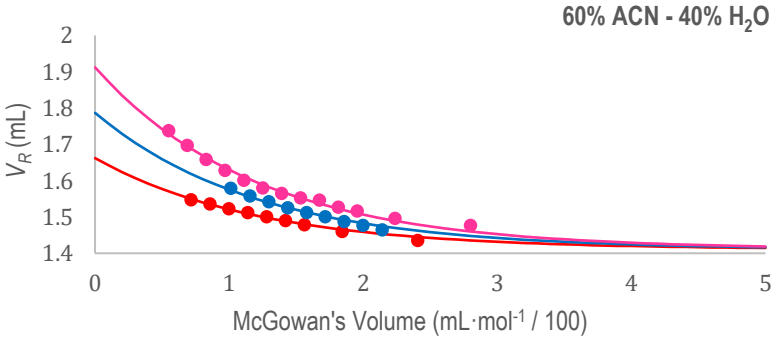
$$V_R = V_M + r \tag{9}$$

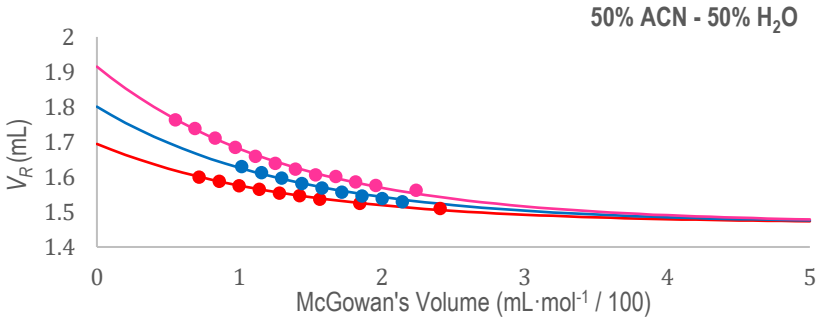
This v coefficient represents the hydrophobicity of the solutes in the system, which one tends to be negative in HILIC mode and positive in RPLC, as shown in the figure and the tables mentioned before. Another fitting parameter is r, which means different interactions between the analytes and the chromatographic system. As represented in the figure, in HILIC behavior the values are positive and in RPLC they tend to 0. Finally, the fitting hold-up volumes were similar for the three homologous series.




80% ACN - 20% H2O
VR (mL)
McGowan's Volume (mL·mol-1 / 100)


70 ACN - 30% H2O
VR (mL)
McGowan's Volume (mL·mol-1 / 100)


60% ACN - 40% H2O
VR (mL)
McGowan's Volume (mL·mol-1 / 100)


50% ACN - 50% H2O
VR (mL)
McGowan's Volume (mL·mol-1 / 100)

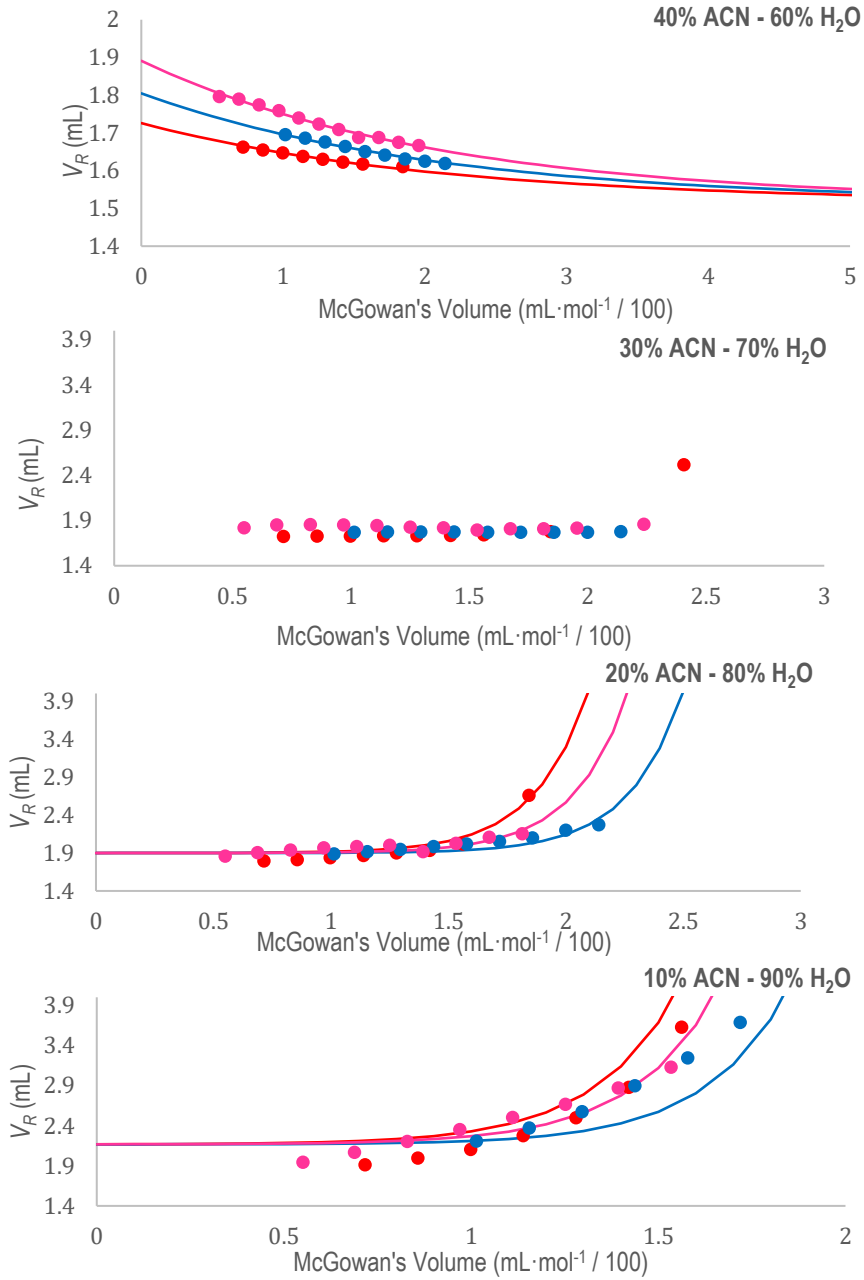


Figure 4. Retention volumes of Kinetex® for both homologous series depending on the amount of the organic solvent added to the mobile phase. Solid lines show fittings to Equation 7.

Table 3. Fittings of Kinetex® column to Equation 7 for each homologous series with acetonitrile mobile phase composition (Standard errors in grey). N is the number of analytes; R_{adj}^2 is the fitting error; RMSE is the Root Mean Square Error.

Organic Modifier-Homologous Serie	(v/v)	V_M		*r*		*v*		N	R_{adj}^2	RMSE
ACN - *n*-alkyl benzenes	**90%**	1.409	0.009	0.199	0.006	-0.207	0.018	9	0.999	0.001
	80%	1.372	0.006	0.227	0.003	-0.245	0.015	9	0.999	0.001
	70%	1.365	0.005	0.254	0.002	-0.305	0.016	9	0.999	0.001
	60%	1.347	0.018	0.289	0.012	-0.217	0.029	9	0.998	0.002
	50%	1.482	0.005	0.224	0.005	-0.384	0.029	9	0.998	0.001
	40%	1.576	0.014	0.163	0.005	-0.371	0.089	8	0.994	0.002
	30%									
	20%	1.816	0.014	0.000	0.000	2.052	0.166	8	0.995	0.023
	10%	2.002	0.059	0.001	0.000	2.256	0.094	8	0.999	0.103
ACN - *n*-alkyl phenones	**90%**	1.469	0.006	0.391	0.029	-0.526	0.049	9	0.998	0.001
	80%	1.418	0.006	0.410	0.019	-0.472	0.035	9	0.999	0.001
	70%	1.399	0.006	0.507	0.025	-0.509	0.034	9	0.999	0.001
	60%	1.343	0.019	0.418	0.007	-0.249	0.028	9	0.999	0.001
	50%	1.454	0.011	0.379	0.006	-0.328	0.030	9	0.999	0.001
	40%	1.493	0.072	0.320	0.047	-0.192	0.085	9	0.993	0.002
	30%									
	20%	1.804	0.034	0.021	0.011	0.628	0.095	9	0.992	0.012
	10%	2.343	0.145	0.001	0.001	1.744	0.146	9	0.992	0.219
ACN - *n*-alkyl ketones	**90%**	1.521	0.007	0.458	0.030	-0.629	0.056	13	0.990	0.006
	80%	1.480	0.010	0.646	0.072	-0.738	0.091	13	0.979	0.012
	70%	1.442	0.006	0.586	0.020	-0.581	0.031	13	0.997	0.005
	60%	1.459	0.007	0.516	0.014	-0.492	0.028	13	0.997	0.005
	50%	1.535	0.008	0.445	0.019	-0.501	0.043	13	0.993	0.006
	40%	1.466	0.142	0.416	0.124	-0.165	0.090	11	0.986	0.006
	30%									
	20%	1.649	0.339	0.170	0.288	0.253	0.245	9	0.943	0.024
	10%	1.001	0.187	0.608	0.147	0.353	0.045	8	0.999	0.015

Table 4. Fittings of Kinetex® column to Equation 7 for the three homologous series at the same time with acetonitrile mobile phase composition (Standard errors in grey). N is the number of analytes; R_{adj}^2 is the fitting error; RMSE is the Root Mean Square Error.

Organic Modifier-Homologous Serie	(v/v)	V_M		r_{benz}		r_{phen}		r_{ket}		v		N	R_{adj}^2	RMSE
ACN - Triseries	**90%**	1.461	0.011	0.159	0.018	0.260	0.018	0.401	0.017	-0.358	0.039	31	0.976	0.009
	80%	1.434	0.010	0.199	0.021	0.356	0.031	0.531	0.032	-0.486	0.048	31	0.972	0.011
	70%	1.401	0.008	0.240	0.014	0.398	0.019	0.548	0.019	-0.437	0.030	31	0.989	0.008
	60%	1.411	0.010	0.251	0.013	0.376	0.014	0.501	0.012	-0.359	0.026	31	0.990	0.007
	50%	1.468	0.007	0.226	0.009	0.333	0.008	0.447	0.007	-0.323	0.016	30	0.996	0.004
	40%	1.516	0.017	0.210	0.019	0.289	0.017	0.375	0.012	-0.205	0.020	28	0.994	0.004
	30%													
	20%	1.899	0.024	0.000	0.000	0.000	0.000	0.000	0.000	1.887	0.383	27	0.838	0.072
	10%	2.166	0.102	0.002	0.001	0.001	0.000	0.001	0.001	1.940	0.142	25	0.977	0.309

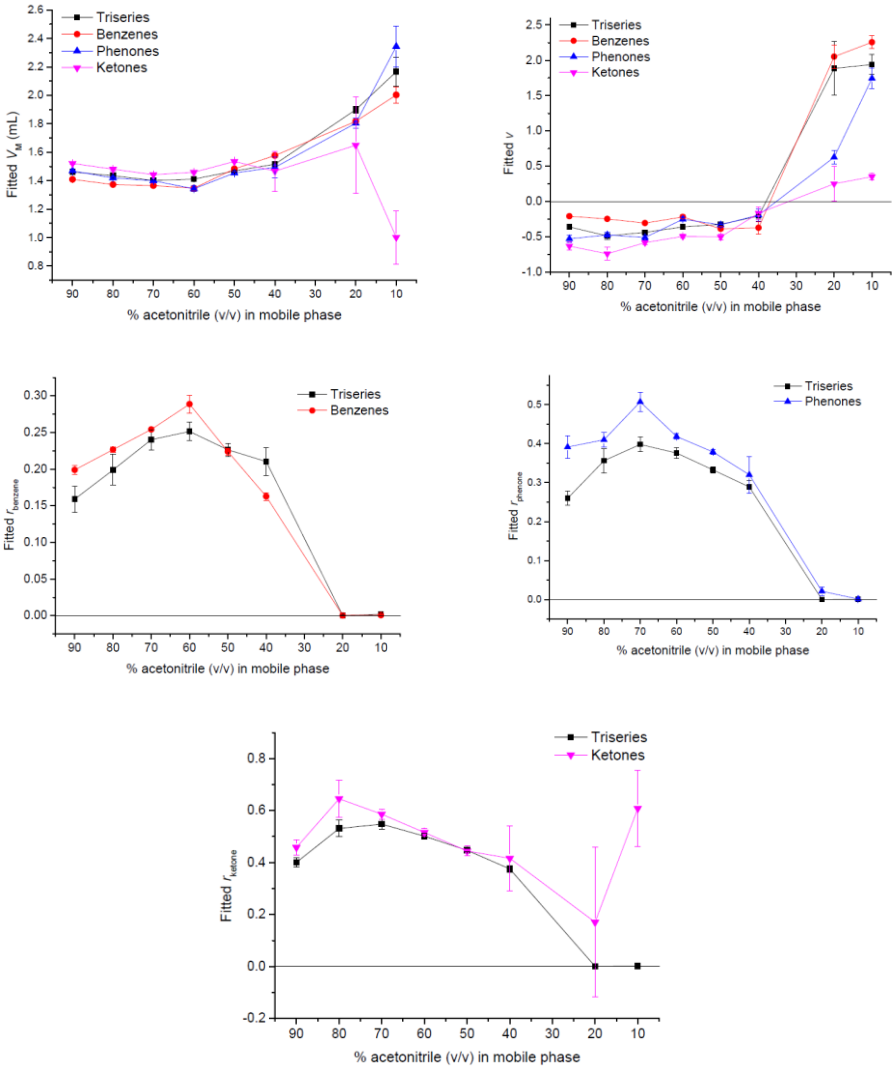


Figure 5. Fitting parameters of Kinetex® for each homologous series and the three at the same time depending on the organic solvent content added to the mobile phase.

Once the fitting parameters have been calculated, it can be studied if some of the analytes could be used like a hold-up marker in HILIC or in RPLC. If the experimental retention volumes (Table of appendix 1) are compared with the fitting hold-up volumes for each homologous series (Table 3), two types of hold-up markers could be observed. For HILIC retention mode dodecylbenzene, decanophenone and nonadecane-2-one could be used like a marker, for each homologous series since for all of the compounds, the experimental retention volumes are the ones getting closer to the fitting hold-up volumes. However, for RPLC retention mode some compounds that could be used are benzene, acetophenone and propanone for each homologous series.

In other words, the lower retention volume, the better marker in HILIC, whereas the higher retention volume, the better marker in RPLC.

7. CONCLUSIONS

The main goal of this work was the characterization of a Kinetex® HILIC column, studying the retention modes inside the column and its retention parameters. The following points allows to summarize the work conclusions:

- HILIC columns can exhibit two different behaviors (HILIC and reversed-phase), depending on the mobile phase composition and its amount. Bellow 30% of acetonitrile in the mobile phase, the behavior changes from HILIC to reversed-phase.
- The homologous series approach allows to characterize the column by the fitting parameters.
- From the fitting parameters, specifically from the fitting hold-up volume, different hold-up markers could be obtained depending on the experimental retention volume such as dodecylbenzene, decanophenone and nonadecane-2-one for HILIC and benzene, acetophenone and propanone for RPLC.

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9. ACRONYMS

A	Solute hydrogen-bond acidity
AC	Affinity chromatography
ACN	Acetonitrile
B	Solute hydrogen-bond basicity
E	Contributions from n- and π-electron pairs
GLC or GC	Gas-liquid chromatography or Gas chromatography
GSC	Gas-solid chromatography
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High-performance liquid chromatography
IEC or IC	Ion exchange chromatography or Ion chromatography
LC	Liquid chromatography
LFER	Linear free energy relationships
LLC	Liquid-liquid chromatography
LSC or NPC	Liquid-solid chromatography or Normal-phase chromatography
LSER	Linear solvation energy relationship
MeOH	Methanol
MP	Mobile phase
NPLC	Normal-phase liquid chromatography
PC	Paper chromatography
RP	Reversed-phase
RPC	Reversed-phase chromatography
RPLC	Reversed-phase liquid chromatography
S	Solute polarity/polarizability
SEC	Size-exclusion chromatography
SFC	Supercritical fluid chromatography

SP	Stationary phase
TLC	Thin-layer chromatography
UHPLC	Ultra-high-performance liquid chromatography
V	McGowan's volume

APPENDICES

APPENDIX 1: RETENTION VOLUMES OF THE STUDY COMPOUNDS

V_R (mL)	Substance	McGowan V	90%ACN	80%ACN	70%ACN	60%ACN	50%ACN	40%ACN	30%ACN	20%ACN	10%ACN
1.1	Benzene	0.720	1.550	1.523	1.519	1.548	1.600	1.663	1.725	1.795	1.915
1.2	Toluene	0.860	1.542	1.512	1.504	1.536	1.588	1.655	1.727	1.813	1.996
1.3	Ethylbenzene	1.000	1.533	1.502	1.490	1.523	1.576	1.647	1.729	1.837	2.107
1.4	Propylbenzene	1.140	1.524	1.493	1.480	1.512	1.566	1.638	1.729	1.865	2.276
1.5	Butylbenzene	1.280	1.517	1.482	1.467	1.501	1.555	1.630	1.730	1.898	2.501
1.6	Pentylbenzene	1.420	1.509	1.473	1.458	1.490	1.547	1.623	1.734	1.935	2.878
1.7	Hexylbenzene	1.560	1.503	1.466	1.449	1.478	1.537	1.618	1.740	2.016	3.630
1.8	Octylbenzene	1.840	1.492	1.453	1.435	1.460	1.525	1.611	1.775	2.659	9.470
1.9	Dodecylbenzene	2.410	1.472	1.431	1.411	1.435	1.510	1.640	2.516		
	Substance	McGowan V	90%ACN	80%ACN	70%ACN	60%ACN	50%ACN	40%ACN	30%ACN	20%ACN	10%ACN
2.1	Acetophenone	1.010	1.586	1.556	1.555	1.579	1.630	1.696	1.771	1.885	2.209
2.2	Propiophenone	1.150	1.565	1.534	1.529	1.558	1.613	1.687	1.774	1.918	2.372
2.3	Butyrophenone	1.300	1.551	1.519	1.509	1.542	1.598	1.677	1.775	1.947	2.575
2.4	Valerophenone	1.440	1.538	1.504	1.492	1.526	1.582	1.664	1.774	1.985	2.900
2.5	Hexanophenone	1.580	1.528	1.493	1.479	1.512	1.569	1.651	1.771	2.017	3.246
2.6	Heptanophenone	1.720	1.518	1.482	1.468	1.500	1.557	1.642	1.770	2.050	3.688
2.7	Octanophenone	1.860	1.511	1.472	1.456	1.488	1.546	1.632	1.769	2.100	4.541
2.8	Nonanophenone	2.000	1.506	1.466	1.449	1.477	1.539	1.626	1.770	2.198	5.835
2.9	Decanophenone	2.140	1.497	1.458	1.439	1.465	1.530	1.620	1.776	2.271	9.141
	Substance	McGowan V	90%ACN	80%ACN	70%ACN	60%ACN	50%ACN	40%ACN	30%ACN	20%ACN	10%ACN
3.1	Propanone	0.550	1.735	1.751	1.729	1.738	1.763	1.797	1.818	1.859	1.947
3.2	Butanone	0.690	1.686	1.663	1.669	1.697	1.739	1.790	1.850	1.904	2.068
3.3	Pentan-2-one	0.830	1.652	1.628	1.631	1.658	1.712	1.775	1.854	1.939	2.203
3.4	Hexan-2-one	0.970	1.629	1.598	1.601	1.628	1.685	1.759	1.852	1.966	2.352
3.5	Heptan-2-one	1.110	1.610	1.577	1.573	1.601	1.659	1.740	1.844	1.985	2.503
3.6	Octan-2-one	1.250	1.595	1.559	1.550	1.580	1.640	1.724	1.827	2.002	2.669
3.7	Nonan-2-one	1.390	1.584	1.546	1.532	1.565	1.623	1.709	1.818	1.918	2.869
3.8	Decan-2-one	1.530	1.573	1.534	1.518	1.553	1.607	1.688	1.796	2.025	3.129
3.9	Undecan-2-one	1.670	1.570	1.530	1.513	1.546	1.602	1.689	1.807	2.107	3.196
3.1	Dodecan-2-one	1.820	1.559	1.515	1.496	1.527	1.587	1.675	1.809	2.153	
3.11	Tridecan-2-one	1.960	1.551	1.507	1.487	1.516	1.576	1.667	1.814		
3.12	Pentadecan-2-one	2.240	1.538	1.492	1.471	1.496	1.562	1.664	1.860		
3.13	Nonandecan-2-one	2.800	1.519	1.469	1.449	1.477	1.562	1.683			