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on

CHARACTERIZATION OF FUNCTIONALIZED SILICA PARTICLES USING LINEAR SOLVATION ENERGY RELATIONSHIPS

by

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Abstract

The development of silica nanoparticles as carriers for drug delivery systems has increased exponentially during the last decade. The present work is focused on the characterization of functionalized silica particles using the linear solvation energy relationship model (LSER).

Following different procedures, the bare silica was functionalized adding 3-glycidoxypropylsilane (Epoxy), an ibuprofen group and (3-aminopropyl)triethoxysilane (APTES). The resultant structures were analysed by FTIR. Thereafter, they were package and used as stationary phases.

The retention of 17 solutes in supercritical fluid chromatography (SFC), with carbon dioxide modified with methanol as mobile phase, were evaluated using LSERs. It was observed that the retention and behaviour of the solutes have a correlation among different solute subgroups in all the selected columns. The results showed that polar compounds were more sensitive to the changes of pressure, temperature and modifier concentration, among which the modifier concentration affected the retention the most.

The LSER coefficients, were obtained performing multilinear regressions with five descriptors. The analysis of significance showed that coefficients a and s, related to H-bond donor ability and dipolarity/polarizability capacity respectively, are the dominating solute descriptors. To obtain the trend with the diverse operational conditions, simplified regressions using a and s were performed. The results showed that the simplified model improved the sensitivity of the analysis.

It was concluded that the LSER correlation provides information for the characterization of the functionalized silica particles, although the LSER model is should be improved to predict retention times accurately.

Content

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List of Symbols

Latin Symbols

Symbol	Unit	Meaning
a	-	System coefficient related to hydrogen bond donating ability (basicity)
A	-	Solute descriptor related to hydrogen bond donating ability (basicity)
b	-	System coefficient related to hydrogen bond accepting ability (acidity)
B	-	Solute descriptor related to hydrogen bond accepting ability (acidity)
c	-	Intercept of the LSER regression
D	Å	Diameter
e	-	System coefficient related to excess molar refraction
E	-	Solute descriptor related to excess molar refraction
k	-	Capacity factor
K_D	-	Distribution coefficient
$\dot{m}$	g min^{-1}	Mass flow
P	bar	Pressure
Pc	bar	Critical pressure
R		Resolution
R^2	-	Coefficient of determination
s	-	System coefficient related to polarity and dipolarity
S	-	Solute descriptor related to polarity and dipolarity
t_{Ri}	min	Retention time of compound i
t_0	min	Hold-up time
T	°C	Temperature
Tc	°C	Critical temperature
v	-	System coefficient related to dispersion interactions and cavity effects
V	-	Solute descriptor related to dispersion interactions and cavity effects
V_0	mL	Hold-up volume
V_{SP}	mL	Volume of the stationary phase
V_{MP}	mL	Volume of the mobile phase

Greek Symbols

Symbol	Unit	Meaning
β	-	Phase ratio
μ	cP	Viscosity
π	-	Related to the π interactions
ρ	g cm^{-3}	Density
$\mathcal{D}$	cm^2 s^{-1}	Diffusivity
ϑ	mL/min	Volumetric flow

List of Abbreviations

Abbreviation	Meaning
ABPR	Automated Back Pressure Regulator
APTES	(3-aminopropyl)triethoxysilane
FTIR	Fourier Transform Infrared spectroscopy
GC	Gas Chromatography
GIT	Gastrointestinal tract
GLYMO	(3-Glycidyloxypropyl)trimethoxysilane
HPLC	High Pressure Liquid Chromatography
K	Kromasil®
LSER	Linear Solvation Energy Relationship
MeOH	Methanol
MP	Mobile Phase
MW	Molecular Weight
PDA	Photo Diodo Array
QSPR	Quantitative Structure Property Relationships
SC-CO2	Supercritical CO2
SF	Supercritical Fluid
SFC	Supercritical Fluid Chromatography
Sil	Silica
SP	Stationary Phase
TUHH	Technische Universität Hamburg-Harburg (Hamburg University of Technology)
UV	Ultraviolet

1. INTRODUCTION

1.1. MOTIVATION

Drug delivery systems are engineered technologies for the targeted delivery and/or controlled release of therapeutic agents for humans and animals.

Nanoparticles, with their large surface area and pore volume, are presented as a good choice for their application in drug delivery and biomedicine. Because of these reasons, numerous studies have investigated the silica that include a biocompatibility that made it an excellent candidate for drug delivery and other medical applications.

Even more, the modification of silica with selected functional groups (such as -NH_2, -CN, etc.) affects the drug release and increases the drug diffusion resistance, which opens a new avenue for modifying silica for biomedical applications. To gain more information of the effects of functionalization is the motivation of this work.

1.2. OBJECTIVES.

The aim of this work is to obtain a better understanding of the functionalized silica and its properties. For this aim, a method to characterize the modified silica particles shall be developed, to determine how each modification affects the physicochemical properties of silica and the interaction between silica and pharmaceutical molecules. In order to achieve the application of supercritical fluid chromatography (SFC) applied which allows us to perform the essential studies to characterize the stationary phases.

Moreover, to extract thermodynamic information from the experiments, the use of "Linear Solvation Energy Relationship" model, LSER, was proposed. The main objective of this research is to extend the application of this model to the self-package columns with functionalized silica particles in SFC systems.

1.3. DISERTATION ORGANIZATION

This work consists in 5 chapters and several appendixes that illustrates the information required to its understanding, the results and the conclusions obtained.

Chapter 1, as has been read, introduces the reader in the context of the thesis and add a summary of the objectives and content of the work.

Chapter 2 stablishes the theoretical bases related with the supercritical fluids, the supercritical fluid chromatography and its features and a brief introduction of the LSER model that can help to understand the next pages. In addition of establishing the theoretical bases, this chapter is presented as a summary of the actual state of all these topics and the investigation related to understand them.

Chapter 3 deals with the methodology and technology applied to perform the experimental phase, focusing in the SFC system description. As well as, making a detailed description of the materials used, introducing the solutes, stationary phases and mobile phase.

Chapter 4 begins with the study of the modified silica and their properties. After describing the future stationary phases, the data obtained during the SFC are in-depth studied. The first step summarizes the hold-up time and volume determination analysing the effect of the different conditions in them. Then, the study of retention times and the influence of the temperature, pressure and concentration of modifier in the retention was performed. In the third part of the analysis, the LSER model is applied to characterize the stationary phases, estimate the coefficients and study the influence of temperature, pressure and concentration modifier.

Finally, the Chapter 5 reveals the conclusions developed during the research and summarize some ideas for further projects.

Chapter 6 collects the APPENDIXES , a set of tables, charts and extra information required for the comprehensive understanding of the thesis.

2. GENERAL PRINCIPLES AND STATE OF ART

2.1. SUPERCRITICAL FLUIDS.

2.1.1. HISTORICAL BACKGROUND

Supercritical fluid (SF) dates back almost 200 years, when *Baron Cagniard de la Tour*, Paris 1822, discovered the critical phenomena. Using a Papin's Digester he placed a flint ball in a partially filled digester. Rolling the device, he noticed that a splash sound was generated as the ball penetrated the liquid-vapor interface; however, after heating the system above certain temperature the splashing sound ceased. This was the time that marks the discovery of supercritical fluids. In following publications like *Annales de Chimie et Physuque (C. Cagniard de la Tour,* 1822) or *Nouvelle note sur les effets qu'on obtient par l'application simultanée de la chaleur et de la compression a certains liquids (C. Cagniard de la Tour,* 1823), he studied certain substances, after which consider that his results were not general phenomena but a particularity of the substances that he studied; though he didn't term this new state.

In 1869, *Thomas Andrews* defined the term of "supercritical point" in his *Bakerian Lecture*, describing the behaviour of CO_2 and clarifying that the gas only condense to a liquid or liquid evaporates to a gas below certain temperature and pressure.

In next years, other authors like van der Waals, *Heike Kamerlingh Onnes, Hannay* and *Hogarth*, etc., embarked on different research activities related to supercritical fluids. Some of the discovers and improvements that took place during the early years of the supercritical technology are enlisted in Table 2.1.

Table 2.1 Historical research of Supercritical Fluids.

Year	Author	Summary
1822	Baron Cagniard de la Tour	He noted visually that the gas-liquid boundary disappeared when the temperature of certain materials was increased by heating each of them in a closed glass container. From these experiments the critical point of a substance was discovered.
1869	Andrews	Discover of the critical conditions of CO_2
1879	Hanay and Hogarth	They demonstrated the solvent properties of a fluid above its critical point by dissolving solid inorganic salts in supercritical ethanol.
1928	Eucken and Bressler	They measured and discussed the solubility of solids and liquids in supercritical carbon dioxide and other gases
1936	Wilson, Keith and Haylett	Solexol process was developed for the purification and separation of vegetable and fish oils
1962	Klesper, Corwin and Turner	They first demonstrated the usage of Supercritical Fluid Chromatography (SFC)
1966-1967	Sie, Beersum and Rijnders	They published a series of articles on "High-Pressure Gas Chromatography with Supercritical Fluids" in Separation Science, in which they extensively used carbon dioxide as mobile phase
1970	Zosel K.	He patented the decaffeination of green coffee with supercritical CO_2.

From 80's there were a rapid development of supercritical fluid technologies. In spite this fact, it wouldn't be till 90's, when supercritical fluids took a new role and regained importance. From then on, supercritical fluids have been presented as main candidates for the Green Chemistry due to their compatibility with the environment and wide range of application.

In the next section, some of the fundamental principles which govern the supercritical fluids will be discussed.

2.1.2. DEFINITION

Considering more clarity is needed before proceeding, hereafter a succinct description of supercritical fluids is carried out.

Supercritical fluids are substances at a temperature and pressure above their critical point. Due to this special condition, they share some common features with both gases and liquids. Thereby, it has the density and solvation power similar to a liquid and viscosity and diffusivity close to a gas.

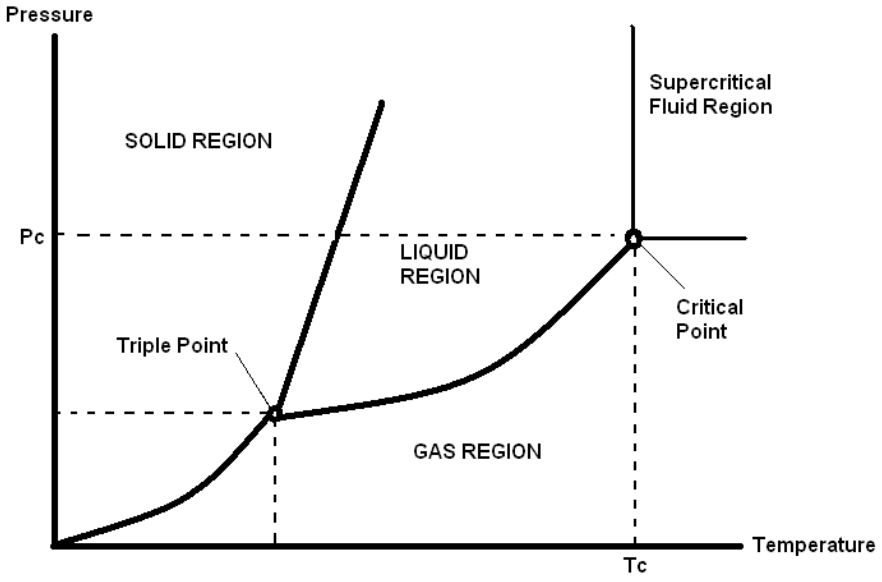


Figure 2.1. Schematic representation of an idealized phase diagram.

So, as it has been settled, main properties that is necessary to consider when it is spoken about SF are: Density, diffusivity and viscosity.

> **Density** of a supercritical fluid is between that of a gas and a liquid, but closer to that of a liquid. In the supercritical region, density of a supercritical fluid increases with increased pressure at constant temperature. When pressure is constant, density of the material decreases with increasing temperature. The dissolving effect of a supercritical fluid is dependent on its density and, for this reason, density should be considered as an essential parameter for analytical techniques using supercritical fluids as solvents.

Diffusivity of a supercritical fluid can be 100 times more than a liquid and 1,000 to 10,000 times less than a gas. Because the supercritical fluids have more diffusivity than a liquid, a solute shows better diffusivity in a supercritical fluid. The diffusivity variation rises when temperature increases, and it downs when pressure decreases. So, higher pressure makes supercritical fluid molecules become closer to each other and reduces diffusivity in the material. Hence, supercritical fluids play an important role for chromatography and extraction processes.

Viscosity for a supercritical fluid is almost the same as a gas, being approximately 1/10 of that of a liquid. Thus, supercritical fluids are less resistant than liquids towards components flowing through. Temperature has little effect on viscosity of liquids, but it can intensely influence that of supercritical fluids.

Table 2.2. Comparison of gases, supercritical fluids and liquids properties. (*Waters Corporation,* 2012)

	Density [g/cm³]	***Viscosity [cP]***	***Diffusivity [cm²/s]***
GASES	10^{-3}	10^{-4}	10^{-4}
SUPERCRITICAL FLUID	10^{-1} - 1	10^{-4} - 10^{-3}	10^{-4} - 10^{-3}
LIQUID	1	10^{-2}	< 10^{-5}

These properties of viscosity, diffusivity, and density are related to each other and the change in temperature and pressure can affect all of them in different degrees.

2.1.3. APPLICATIONS

SFs are regarded as an eco-friendly and sustainable solvents. Nowadays the applications of supercritical fluids involve a wide range of activities in chemical, cosmetics, pharmaceutical or food industry among other fields. Some processes developed in these fields are: extraction, fractionation, impregnation, chemical reactions, drying, cleaning, chromatography and refrigeration.

a) Extraction: From 70's supercritical CO_2 (SC-CO_2) provides a solvation variation that allows, at supercritical temperature and pressure, a high extraction power for nonpolar or small polar molecules. The most popular applications include coffee or

tea processing among others. On the other hand, water extraction has also been carried out to obtain polyphenols and several plant materials.

b) Fractionation: The fractionation of biomass is the current challenge of biorefinery due to the importance and possibilities of their components. The conventional ways to obtain these components require long reaction times and certain pollutant or aggressive agents. So, water in supercritical conditions has been presented as an alternative for the hydrolysis of biomass. (*M.J. Cocero et al*., 2017)

c) Impregnation: The principle of this technic consists in the deposition of a supercritical fluid in which an active substance to impregnate is previously dissolved over a porous solid material (polymers, wood, textiles...). Impregnation is widely used in leather, textile and food conservation industry. (*C. Cejudo Bastante et al*.,2017)

d) Chemical reaction: At supercritical conditions, the opportunities to manipulate the reaction environment (solvent properties) or integrating reaction and separation unit operations, can be reached. Fuels processing, biomass conversion, biocatalysis, polymerization, materials synthesis and chemical synthesis are some of the fields where SFs have taken advantage of their properties.

e) Drying: Using SC-CO_2 this process extracts the water and other solvents from the surfaces without affecting its properties. This method is used to make Lithium batteries, aerogels, or lyophilization of biological matrices and for dry-cleaning of clothes. (*D. Alonso-Domínguez et al.,* 2017)

f) Cleaning: For environmental reasons, avoiding certain solvents, SC-CO_2 can appear as an alternative taking part of membrane cleaning (*J. Krzysztoforski et al.*, 2018), metal cleaning (*Wei-wei Liu et al.*, 2015) or processes of dry-cleaning (*S. Sutano,* 2014).

g) Chromatography: This method is used for the analysis and purification of low to moderate molecular weight molecules and the separation of chiral compounds. Although its principles are similar to HPLC, supercritical fluid chromatography classically uses SC-CO_2 as the mobile phase.

Of the aforementioned elements, on following pages of this thesis, the attention focusses on supercritical fluid chromatography,

2.2. SUPERCRITICAL FLUID CHROMATOGRAPHY

Supercritical Fluid Chromatography (SFC) is a specific chromatographic technique which utilizes a supercritical fluid as main solvent.

Although supercritical fluids were defined at the end of 19th century, was not until the end of 20th century when the SFC was re-discovered and hold the investigation attention to reach a rapid development of the technic. Chromatography using supercritical fluids was first described in 1962 by *Klesper et al*. In that work, the authors used mono- and dichlorodifluoromethane as mobile phase to separate porphyrins. Nowadays, the typical implementation of SFC uses carbon dioxide.

It was only in 1980's that the commercialization of SFC instrument started. Those initial chromatograms took as example the gas chromatography, GC, using the open tubular capillary columns, *Novotny et al*. 1981. Despite its high efficiencies and sensitivity, the many improvements in HPLC instrumentation in those days, the independent control of the flow and pressure and facing the possibility of using modifiers and additives; one year later *Gere et al*. modified a regular HPLC adding a backpressure regulator.

In spite of the similarities of both technics, there are contrasting distinctions between HPLC and SFC. Hereafter the differences between both instrumentations are listed:

a) The mobile phase in SFC has a much lower viscosity than it does in HPLC and therefore can be easily operated at flow rates of up to 5 mL/min on a 25cm x 4.6 mm columns.
b) Since CO_2 intended for SFC is a liquid, the pump head for the CO_2 must be chilled to -10 °C, requiring a chiller and a specialized pump head.
c) To maintain a stable supercritical fluid, the back pressure, i.e., after the detector, must be regulated above the critical pressure, usually at ~100 atm, requiring a back-pressure regulator.
d) A high-pressure UV cell is required for SFC since the high pressure is maintained across the detector cell as well.
e) A thermostated column chamber that can function over a 20 – 80 °C range is mandatory for SFC to control temperature effectively whereas in HPLC it is optional.

In Figure 2.2, a schematic model of a SFC instrumentation can be seen. The components of a supercritical fluid chromatograph generally include a CO_2 reservoir, a high-pressure CO_2 pump, a modifier reservoir, a high-pressure modifier pump, a packed column, an oven to regulate column temperature, a detector for determining concentration of the eluted substances, a back-pressure regulator, a sample collector and a data processing and controlling device.

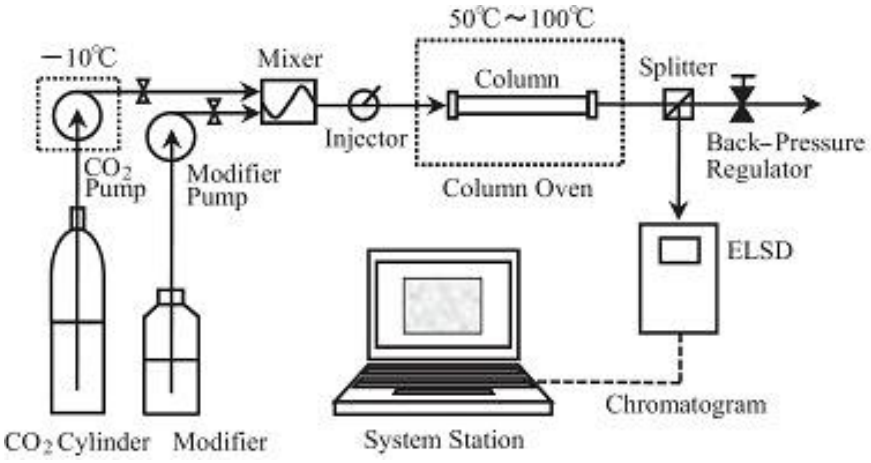


Figure 2.2. Schematic representation of the SFC instrumentation. (*K. Takahashi et al.,* 2013)

In next chapters the equipment will be analysed and described in-depth, analysing the apparatus operated during the experiments.

2.2.1. MOBILE PHASE

The mobile phase is the fluid that flows through a chromatography system, moving the materials to be separated at different rates over the stationary phase

Historically, nitrous oxide, ammonia and carbon dioxide have been used mainly as mobile phase, MP; but actually, the noble gases argon and xenon as well as other hydrocarbons can be used as solvents (*J. Pawliszyn,* 1996). However due to their risks or difficulty to handle them, carbon dioxide is the most popular mobile phase for SFC.

2.2.1.1. Carbon dioxide as mobile phase

The critical point of pure CO_2 is readily achievable, (Tc=31°C and Pc=73 bar), in so doing, CO_2 is presented as the preferred fluid to be used as MP. Besides of the low critical point, other factors that determine this predilection are:

a) Availability: Although massive amounts of CO_2 are used every year (80Mtpa), this gas can be obtained from natural geological reservoirs or as raw material from other industries. The prospective use of CO_2 stands at 140 Mtpa, considering that in the atmosphere, the capacity of CO_2 is estimated to be at least 7.5×10^4 Mtpa, which does not make a significant difference between supply and demand balance.
b) Cost: CO_2 is fairly inexpensive due to it is taken as a by-product from large-scale processes and its recuperation is only a very small fraction of the cost of operation.
c) Safety: The CO_2 is essentially a waste product of animals and humans, and it exists naturally in the atmosphere. Because of this special condition, it is not toxic at low concentrations. Besides, it has been demonstrated that the presence of CO_2 under 0.5% has not any detectable limitations or adverse effect, so it is usually stored in big tanks with a control system to avoid CO_2 leakages.
d) Green chemistry: The elimination of hazardous organic solvents and the search for useful non-hazardous solvents is a prime goal of green chemistry. CO_2 as a liquid or supercritical solvent meets many of the characteristics of an ideal green solvent.
e) Miscibility with polar modifiers: Sometimes the solubility of CO_2 is deficient for high molecular weight and high polar species; in those cases, the introduction of a modifier can lead to a substantial increase in the density of the mobile phase, resulting in a significant change in the retention times and the peaks.

<u>Modifiers</u>

At the beginning of SFC works, the use of capillary columns and the flame ionization detector, force the use of pure CO_2 as mobile phase.

As has been mentioned before, other mobile phases had been employed in SFC. Its use is warranted to increase the range of solute polarity that can be handled by this technique; but more practical way of reaching an extended range of separable compounds is using a mixture of mobile phases.

The aims of adding a modifier to supercritical CO_2 are:

a) To increase the mobile phase polarity.
b) To increase the mobile phase density.
c) Deactivation of active site on the surface of the stationary phase.

Table 2.3. Frequently used modifiers in SFC. (*John A. Adamovicsc*, 1997)

Modifier	*Temp. [°C]*	*Pressure [atm]*	*Molecular mass*	*Dielectric constant at 20 °C*	*Polarity index*
Methanol	239.4	79.9	32.04	32.70	5.1
Ethanol	243.0	63.0	46.07	24.30	4.3
1-Propanol	263.5	51.0	60.10	20.33	4.0
2-Propanol	235.1	47.0	60.10	18.30	3.9
1-Hexanol	336.8	40.0	102.18	13.3	3.5
2-Methoxy ethanol	302	52.2	76.10	16.93	5.5
Tetrahydrurofuran	267.0	51.2	72.11	7.58	4.0
1,4-Dioxane	314	51.4	88.11	2.25	4.8
Accetonitrile	275	47.7	41.05	37.50	5.8
Dichloromethane	237	60.0	84.93	8.93	3.1
Chloroform	263.2	54.2	119.38	4.81	4.1
Propylene carbonate	352.0	-	102.09	69.0	6.1
N,N-dimethylacetamide	384	-	87.12	37.78	6.5
Dimethyl sulfoxide	465.0	-	78.13	46.68	7.2
Formic acid	307	-	46.02	58.5	-
Water	374.1	217.6	18.01	80.1	10.2
Carbon disulfide	279	78.0	76.13	2.64	-

Initially, only small portion of modifier, less than 5%, were employed, due to the increase of pressure and temperature, (*M. Saito,* 2013*)*; however, in SFC those parameters are not a problem and its performance is acceptable even with higher amounts of modifier. So, typically the modifiers are used in a portion between 0 and 50%.

In view of *Encyclopedia of Chromatography,* for at least two-thirds of the works published about modifiers in SFC, methanol is implicated. Although other alcohols are used as modifiers, such as ethanol or isopropanol, other popular co-solvents for HPLC are almost not used in SFC. The wide use of methanol as modifier, may be due to, not only its totally

miscibility with CO_2, but also its high availability, low UV cut-off (205nm) and relatively low toxicity.

Additives

Besides modifiers, sometimes other highly polar compounds, like acids (for example acetic acid, formic acid trifluoroacetic acid), bases (e.g. isoprpylamine, diethylamine) or salts (e.g. ammonium acetate or ammonium hydroxide) can be added to CO_2. These additives dissolved in small amounts, between 0.1-2%, in the modifier usually improves peak shapes.

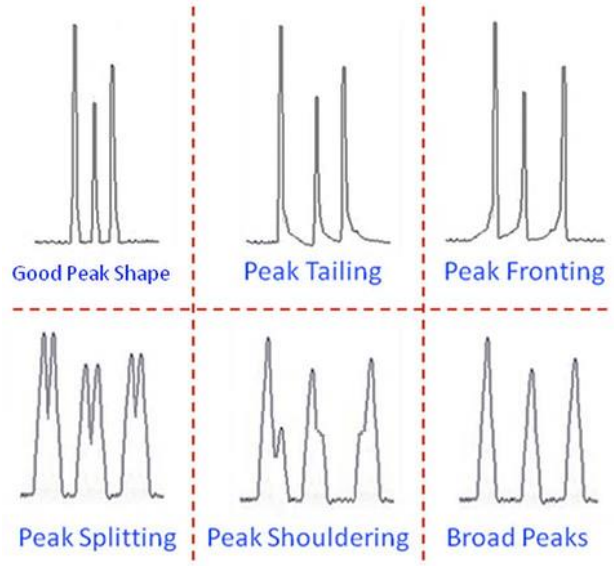


Figure 2.3. Common troubleshooting in chromatography.

Adding them in small portions, an improvement in the chromatogram should be achieved. However, the use of strong acidic additive can affect the pH environment. In the CO_2 case this decrease in the pH may be the origin of the formation of methoxylcarbonic acid, which confers acidity character to the mobile phase. On the other side, basic additives do not affect the yield but can provide a more stable apparent pH.

2.2.2. STATIONARY PHASE

The other phase that takes part in the chromatography system is the stationary phase. As is well known, the stationary phase is a solid, a liquid or a gel on which, the materials to be separated are going to be selectively adsorbed.

The properties that are required for a proper stationary phase are:

a) Porous system: Small pore with diameter between 60 and 120 Å and surface areas greater than 350 m^2/g.
b) High purity
c) Small particles: Spherical particles with diameters between 1.7 and 5 μm, higher ones are in decreasing use.

As noted earlier, the stationary phases, SP, that are commonly utilised in SFC are generally HPLC stationary phases. Therefore, based on bare silica a string of modifications has been explored, bonding several chemistries to introduce a variety of functional groups (alkyl, phenyl, amino, propane-diol, etc.) that provide the stationary phase with unique properties.

Performing a further study, *Lesellier & West* carried out in 2006 an analysis of 100 compounds on 24 stationary phases was carried out at the same conditions and the experimental results were evaluated using a solvation parameter model, which will be introduced in detail in "Linear Solvation Energy Relationship" on following pages. After that, they constructed a spider diagram for the stationary phases that represent certain characteristics of the stationary phases. And shortly after, *Lesellier* states, that the choice of a stationary phase is one of the most important decisions in SFC, considering that the nature of the stationary phase can contribute critically to the retention behaviour, selectivity and separation of the solutes. Figure 2.4 illustrates a reproduction from West and Lesellier original graph that helps the users to select the phases according different properties and identify a suitable stationary phase.

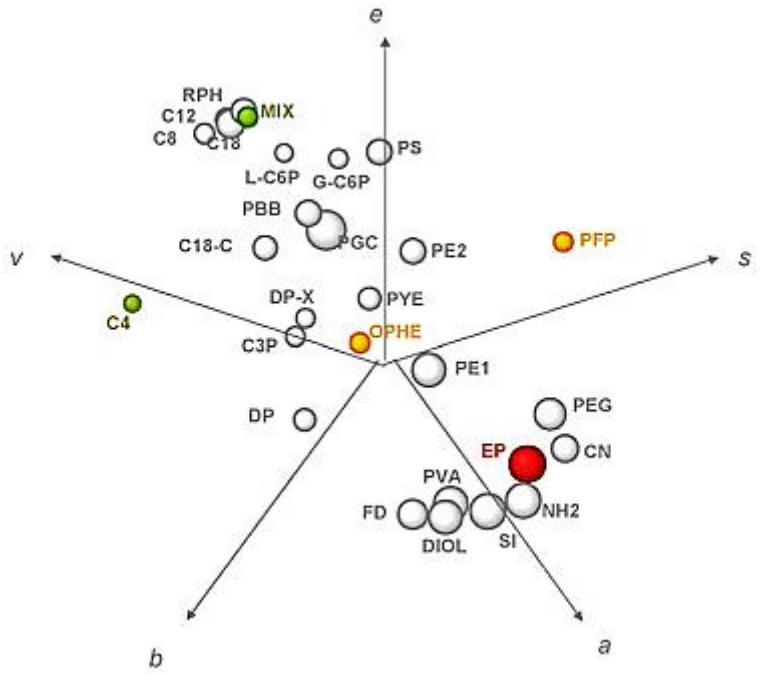


Figure 2.4. Spider diagram for a five-dimensional representation of stationary phase selectivity, evaluated by the solvation parameter model. The coefficients e, s, a, b and v represent specific interactions between the stationary phase and the solute: excess molar refraction, dipolarity/polarizability, acidity, basicity, and volume. (*R. McClain*, 2017)

Nowadays, there are a wide range of good stationary phases with good selectivity, even several stationary phases can be chosen for any specific separation and provide a viable separation. Some alternatives to silica are polysaccharide, zirconia, polystyrene, divinylbenzene or porous graphitic carbon.

At last, graphitic coal, was tested by *Lesellier* adding different modifiers to mobile phase, for instance, methanol, hexane, acetonitrile, etc. After carrying out several experiences, certain interaction between solutes and stationary phase and between mobile phase and solutes were detected, being greater the stationary phase and solutes interactions, especially in cases were acidic solutes were used.

Independently of their nature, the stationary phases can undergo changes over the chromatography. According to *Colin F. Poole*, there are two possible mechanisms to perform those changes:

a) Leading with polymerical stationary phase, the polymer may swell by adsortion of mobile phase components, changing the stationary phase volume, the phase ratio and some chemical properties and its nature.
b) The mobile phase or the solvents can be adsorbed onto stationary phase surface, changing the appearance of the stationary phase.

Both changes cause changes in the stationary phase that can influence the chromatograph process.

Throughout this project, several silica-based stationary phases are used, so it has been considered correct to focus in this material and expand the information about the silica as stationary phase.

2.2.2.1. Silica as stationary phase

Silica, also known as silicon dioxide or SiO_2, is a colourless, white, chemical compound. It is known as one of the most abundant compounds in the earth's crust (59% of total composition) and it can be used for several purposes, from industrial application to the food and pharmaceutical industry.

The use of silica as mobile phase is inheritance from the beginning of SFC when the models were GC and HPLC. Bare silica was the most straightforward, but a number of other bonded-silica stationary phases can be also use.

So actually, silica is combined with several chemical compounds in order to modify the properties of silica. The ligand is bounded via a siloxane bridge (Si-O-Si) making the chains susceptible to hydrolytic attack if the chromatography is performed with a low pH mobile phase. The nature of the surface modifications will affect the selectivity of the stationary phase and take an important role in the retention

2.2.3. PARAMETERS IN SFC

2.2.3.1. Retention time

The retention in chromatography is determined by the interaction of the samples with the stationary phase. It is given by:

$$t_{Ri} = t_0(1 + K_D\beta) \quad (1)$$

Where t_0 is the hold-up time, K_D is the distribution coefficient and β is the phase ratio.

The retention time is affected strongly by nature and composition of the mobile phase; hence, changing the composition of the mobile phase, the retention and the selectivity vary (Janssen et al., 1991).

2.2.3.2. Hold-up volume and time

Hold-up time, or dead time, is the minimum time in which a sample that is not retain in the stationary phase reaches the detector.

It is tightly related with the hold-up volume, V_0, that is the volume of mobile phase that leaves the column during the passing of an unretained substance along it.

Normally this volume keeps constant for a chromatographic phase at a defined pressure and temperature; however, it is important to mention that the hold-up time can vary affected for these conditions.

2.2.3.3. Capacity factor

This parameter, also known as capacity ratio, k_i, quantify the time that a sample remains in the stationary phase relative to the time that it remains in the mobile phase. So, for a certain k_i the interaction between the solutes and the stationary phase can be measured.

In equation (1) the product $K_D\beta$ represents the capacity factor, k.

It is important to note that the capacity factor is independent on column geometry or mobile phase flow rate, but it can be affected by other parameters that change the retention times of the solutes, such as pressure, presence of modifiers or temperature.

It can be shown as:

$$k_i = \frac{K_i\, V_{SP}}{V_{MP}} \quad (2)$$

Where k_i is the capacity factor of the compound i, K_i is a distribution factor of the compound i,V_{SP} is the volume in the stationary phase and V_{MP} is the volume in the mobile phase

However experimentally it can be determined using the hold-up time and the retention times of a sample:

$$k_i = \frac{t_{Ri} - t_0}{t_0} \quad (3)$$

The values for k_i vary depending on the hold-up time and the retention times for each solute. For high k_i values, long retention and analysis times are required.

2.2.3.4. Other parameters

Other parameters can be interesting to the characterization and analysis of results in chromatography, for instance: efficiency, selectivity and resolution.

The **efficiency** of a column is related with the number of theoretic plates, N, and it measures the dispersion of the analyte band that passes through the column. Experimentally it can be related with the width of the peak. The slimmer the peak is, the higher the efficiency is.

The **selectivity** is represented by a selectivity factor, α. It is the rate between the most retained analyte and the less one. It is related with the distance between peaks.

$$\alpha = \frac{k_j}{k_i} \quad (4)$$

Where k_j is the capacity factor of the most retained compound and k_i is the capacity factor of the less retained compound.

The **resolution** is a measure of the capacity to separate analytes. It is a quantitative measure of how well two elution peaks can be differentiated in a chromatographic separation. It is defined as the difference in retention times between the two peaks, divided by the combined widths of the elution peaks.

$$R = \frac{2\left[t_{Rj} - t_{Ri}\right]}{W_i - W_j} \quad (5)$$

Where t_R are the retention times of compound i and j and W is the elution peak width of each one. If the resolution is greater than one, the peaks can usually be differentiated successfully.

2.2.4. EFFECT OF OPERATION PARAMETERS.

The variation of some conditions, pass on the results of the process. Temperature and pressure influence directly the density of mobile phase, and this change result in a change of diffusivity, surface tension and viscosity change. Furthermore, the variation of these parameters can affect the properties of the solutes and the mobile phase. In Figure 2.5 the variation of density can be merely displayed to obtain a general overview of this variation.

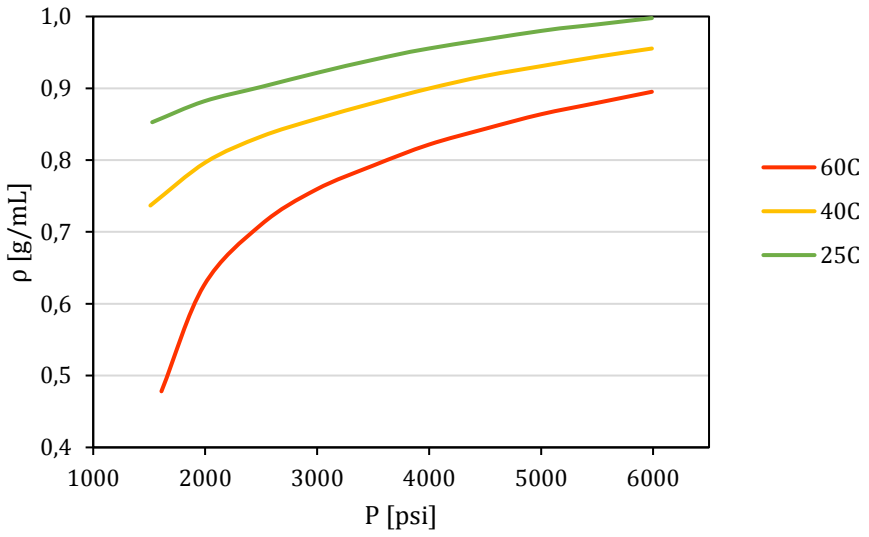


Figure 2.5. Variation of density as function of pressure at three different temperatures (25, 40 and 60°C) for a mixture CO_2:MeOH (95:5 mol/mol)

The influence of those physical parameters has been discussed in several papers *(A.Tarafder et al*. 2015; *A. Hiitz et al*.,1990) due to the higher importance of the dependence of some chromatographic variables. Capacity factor, selectivity or resolution are greatly affected by the variations on pressure, temperature, density, and mobile phase composition comparing with HPLC or GC, where the chromatographic variables are generally monotonous functions of the physical parameters.

2.2.4.1.Temperature

Increasing the temperature of the process at constant pressure the density of the mobile phase decreases. The effect of temperature on analyte retention at constant pressures could be more complex, because, any variation in temperature oppositely affects the density, which in turn leads to a counter-acting effect on retention.

Besides the retention times, temperature affects the selectivity too, although some studies show that the mobile phase modifier composition is a major influence.

2.2.4.2.Pressure

Properties relevant to SFC vary with pressure along the column and do this more dramatically in the region of the critical point. The density of mobile phase increases with pressure, resulting normally in a higher solubility of analytes in SC-CO_2. This in turn, facilitated its elution from the stationary phase, hence an increase in the pressure is accompanied by a small decrease in selectivity, resolution and retention times.

2.2.4.3.Modifier concentration

According to *Martin Enmark*, (*M.Enmark et al.,* 2016) the methanol fraction is the most important factor that governs the retention process. As has been settled before, any increase in pressure or decrease in temperature involves in a reduction in the retentions times; however, they may be considered as playing a minor role comparing with the methanol fraction variation.

2.2.5. APPLICATIONS

Relevance of SFC has increased in the last 40 years, therefore its application has been increased and nowadays a wide range of fields use the SFC for analysis and separation.

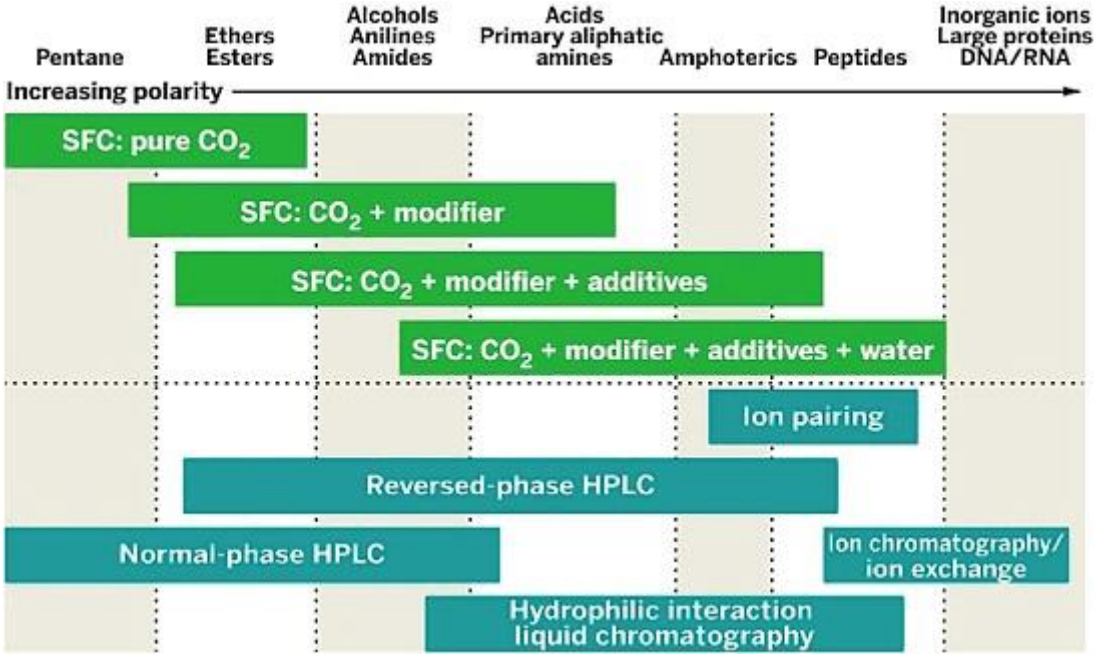


Figure 2.6. An overview of the application range of SFC with co-solvents and additives (*C.H.Armaud, 2014*).

Initially many of the applications of SFC were to obtain organic non-polar compounds. With the inclusion of modifiers, a wide variety of SFC applications was introduced. New examples of the use of SFC for analysis of a variety of complex oligomeric mixtures including polypropylene glycol, polysiloxanes, fluorocarbon oligomers (i.e. -3M's fluoro-chemical surfactant Fluorad 171, and Kel-F) and high molecular weight alcohols are shown. The use of SFC for separation of mono-, di-, and triglycerides at low operating temperatures can be found in the literature too (*D. Giron et al.,* 1992).

With all the modifiers and additives that has been discussed before, the technic provides a wide range of possibilities. Some of the SFC applications are shown in Figure 2.6 generally.

2.3. LINEAR SOLVATION ENERGY RELATIONSHIP (LSER).

A wide range of models can be used to study and quantify the solute-solvents interactions that take place between two phases.

Models of Quantitative structure property relationships (QSPR) are used to relate a chemical property with the response variable. For SFC, retention is the main property of interest, so the model is known as QSRR, Quantitative Structure Retention Relationship. This model is statistically derived relationships between chromatographic parameters and the quantities (descriptors) characterizing the molecular nature of the analytes.

QSRR has found its application to get insight into the molecular mechanism of separation operating in a given chromatographic system, identify the most informative structural descriptors of analytes, evaluate complex physicochemical properties of analytes, evaluate properties of stationary phases and predict retention for a new analyte.

2.3.1. LSER INTRODUCTION

First model, proposed by *Kamlet and Taft et al.*, was used to describe solvation effects on physicochemical process. This model was described using solvent parameters. Those parameters were later used to describe solute characteristics and study the solubility properties in several medium. Abraham presents a technique using solutes descriptor instead of solvent. The model for the description of supercritical chromatographic systems is given by:

$$Ln\ k = c + eE + sS + aA + bB + vV \qquad (6)$$

In the equation, the capital letters represent the solute descriptor related to different interactions, while lower case letters represent the system parameters. The coefficients represented by lower case letters are determined by measuring the selected property for a varied amount of solutes and then carrying out a multilinear-regression.

The model terms eE, sS, aA, bB, and vV describe the influence of dispersion interaction, polarizability and dipolarity interaction, hydrogen bond donating ability (basicity), hydrogen bond accepting ability (acidity), and molecular size, respectively, on the retention of individual solutes in a given system. If experimental conditions remain constant or vary

in the same way during the test, a direct comparison of stationary phases comparing e, s, a, b, and v profiles is possible.

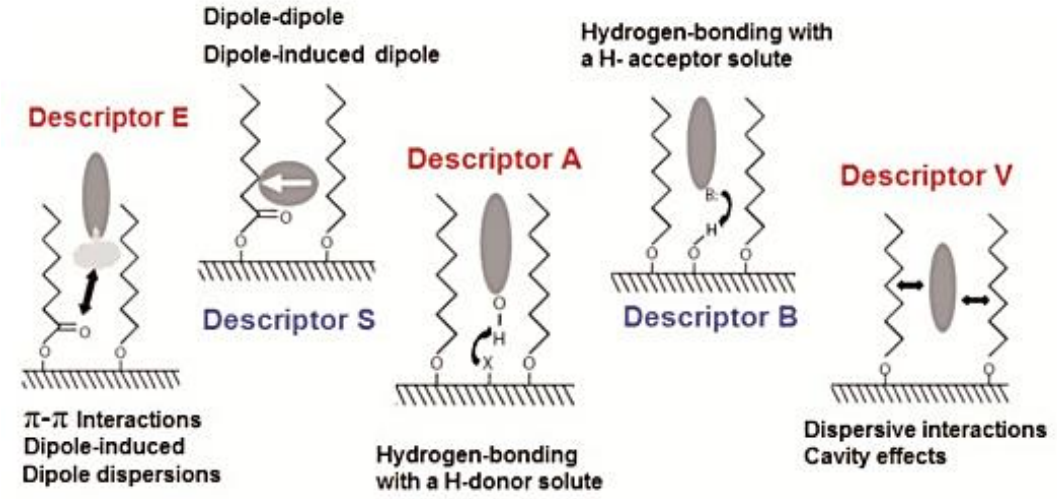


Figure 2.7. Principle of the solvation parameter model and the interactions related to each solute descriptor. (*C.H. Collins et al., 2014*)

The c is a constant that depends exclusively on the columns parameters, such as porosity or volume. The rest of the coefficients can be understood as a difference in the relation between the stationary phase and the mobile phase (*West & Lesellier*, 2005) and it can be represented as following:

$$x = x_{stationary} - x_{mobile} \quad (7)$$

Where x represents the system coefficients. The positive or negative value of the coefficient studied will determine the domain of a phase above the other. If the result is a positive coefficient, it indicates a stronger interaction of the stationary phase with the solutes and the opposite case, a negative coefficient, means the domain of the interaction between the mobile phase and the solutes.

The coefficients and their chemical and structural meaning will be thoroughly studied in Chapter 4 at "Analysis of the parameters according the stationary phase".

Although the model has not many changes in last years, obtaining a deeper understanding of the process can clarify concepts. The original model principles state that: the transfer of a solute into a solvent can be explained by a three-step process: starting with a cavity

formation, following with the solute insertion in the cavity and ending with the intermolecular interaction of the solute.

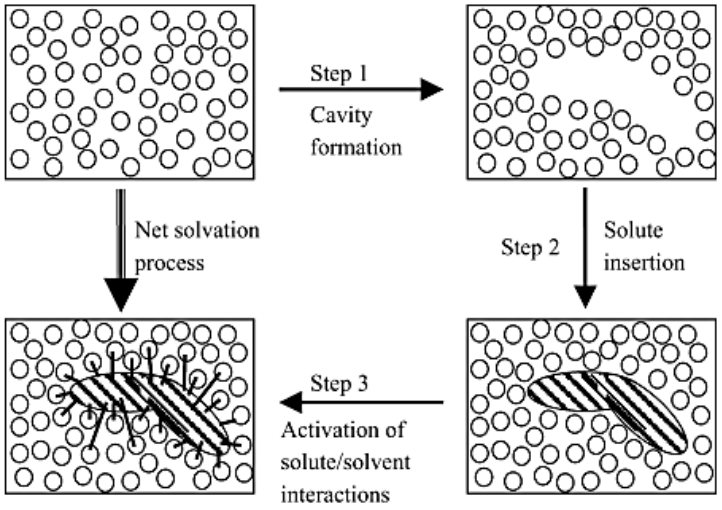


Figure 2.8. Model of the solvation process: Step 1, solvent cavity formation. Step 2, solute insertion. Step 3, activating solute/solvent interactions (*M. Vitha et al.*, 2006).

In this way, the cavity formation occurs when the intermolecular interaction between the solvent molecules is lower transferred. After that, the hole given is replenish by the solute and when the cavity is full of it, the solute-solvent interaction "start turning on". the solute is transferred from one phase to another. This process results in a change in the free energy that is characterized by an equilibrium constant.

2.3.2. PROCEDURE

To obtain a properly model, it is important to carry out a good selection of representative solutes. Since the equation is represented by six parameters is important to have at least x+1 compounds to analyse. The amount of solutes involved in the study should be enough to consider the model representative. The much varied and more representative the solutes are, the better will the model be due to the importance of covering a wide range of properties. According to *West and Lesellier*, a larger set of solutes are required to obtain more accurate results.

The solutes selected to this purpose, will be presented in Chapter 3 "Methodology and materials".

2.3.3. STRENGTHS AND DRAWBACKS

Following this introduction, it is important to know the strengths and drawbacks of the model. Although, the model is very plenty there are some limitations that should be known when it is applied:

a) LSERs does not take differences between enthalpic and entropic contributions to the retention.
b) This model contains a lot of chemical information, but due to its complexity it is hardly ever analysed or interpreted.
c) The equation that describes the model is a linear relationship, nowadays there is not a state that ensure the true of this assumption; but it works empirically.
d) Although there are different parameters in the equation, some interactions are not covered by them and they do not have a term to describe it
e) The optimization of retention time predictions with LSER is not accurate enough due to the beforementioned reason and the covariance between some parameters.

On the other hand, after settled the deficiencies of the model, is important to highlight the advantages that it can offer:

a) LSER model offers a relatively thorough understanding of various chemical interactions that govern the process studied.
b) Comparing it with another models and parameter sets, LSER and its parameters can be used to study a wide range of phenomena.
c) LSER use general, instead of local, parameters, so it can reflect the real chemical effects more accurately.
d) Its applicatopn does not need an extra-work due to the measurements needed to get the parameters are the same as the routine process. It is important to note that in this case is useful to make prior considerations to arrange a proper system.
e) LSER is widely studied in the science community and the amount of papers has increase fast.

3. MATHERIALS AND METHODS

3.1. MATERIALS

In this section, all materials and chemicals that were used in this work, as well as the preparation procedure, are described. First the phases are described for this work. Then the selected solutes and their properties are shown. The last point of this sections depicts the chemical and method selected to determine the hold-up time.

3.1.1. MOBILE PHASE

Supercritical CO_2 was selected as mobile phase. The CO_2 with a purity of 99.99995% was supplied by Westfalen and stored in a cylinder of 50 L. During the experiment a heating blanket was used in order to sustain a sufficient inlet pressure.

With a view to increase the polarity of the mobile phase, methanol was added as organic modifier. Methanol is supplied by ROTH with a purity of (99.9%). The modifier concentration in the mobile phase was varied depending on the experimental condition.

3.1.2. STATIONARY PHASE

As it was described before, silica columns are the most common stationary phases for SFC. Consequently, in this work silica gel with modifications were selected as stationary phases.

All the HPLC columns used for this work were carefully selected. The stationary phase was packed into a stainless-steel column with an inner diameter of 4.6 mm and a length of 50 mm, as well as an identical inner roughness.

3.1.2.1.Silica

In this work, commercial silica from Kromasil®, manufactured by AkzoNobel, was used as basis of all experimentations. Three kinds of silica were selected to perform the experiments. Their properties are shown in the table below:

Table 3.1. Properties of silica (Kromasil).

Commercial product	*Kromasil 60-5-SIL*	*Kromasil 100-5-SIL*	*Kromasil 300-5-SIL*
Abbreviation	k60	k100	k300
Batch	0000012677	0000017909	0000144519
Particle size, µm	5	5	5
Phase	silica	silica	silica
Pore size, Å	60	100	300

To study a way of obtaining different drug loading capacities, several modifications of the silica structure were done. Hereafter a detailed description of the production process is included.

Preparation of ibuprofen anchored silica

Silica, which was used for this purpose, was dried overnight before starting with the functionalisation.

This process can be subdivided into two main processes: Silica functionalisation and preparation of ibuprofen-anchored silica itself.

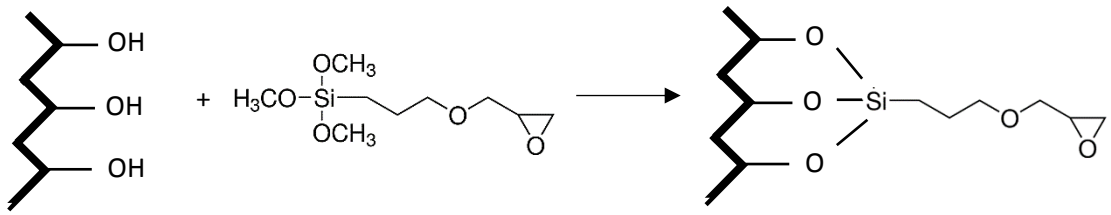


Figure 3.1. Reaction of functionalisation of silica with GLYMO.

First process started with silica samples (6 g) being added to 100 mL of toluene and 8mL of (3-Glycidyloxypropyl)trimethoxysilane, GLYMO, and maintained in an oil bath to keep temperature constant at 130 °C for 4 hours. The reaction took place with reflux. After reaction, the product was washed with toluene and diethylether.

Lastly, a continuous extraction was run overnight in a soxhlet apparatus using a mixture of diethylether-dichloremethane (1:1 v/v). Drying the sample was a required step after extraction, so using a vacuum oven (T= 80°C) the sample was settled overnight.

The material of this set was named as Sil-kXX-EPOXY serie. The 'XX' refers to the pore size of each material given in Table 3.1.

In second process, functionalised silica (2.5 g) was added to ibuprofen (2.5 g), toluene (50 mL) and trimethylamine (1.72 mL) solution. The suspension was stirred overnight at reflux. After that, a washing process using a sequence of solvents (toluene, methanol, distilled water, methanol and diethylether) to remove the spare ibuprofen took place.

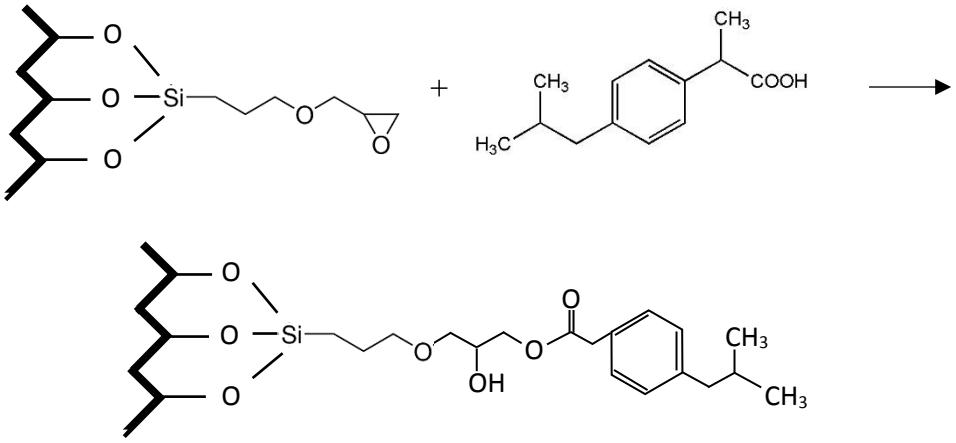


Figure 3.2. Reaction of functionalisation of silica with Ibuprofen.

Ending the washing process, a soxhlet extraction was carried out using a mixture of diethylether-dichloremethane (1:1 v/v) at 90°C overnight. The extraction product was settled in a vacuum oven (T= 90 °C) in an overnight process.

Finally, samples were duly stored and named as Sil-kXX-ibuprofen series. The 'XX' refers to the pore size of each material given in Table 3.1.

Preparation of APTES grafted silica

Silica that was used for these experiments was dried overnight before starting.

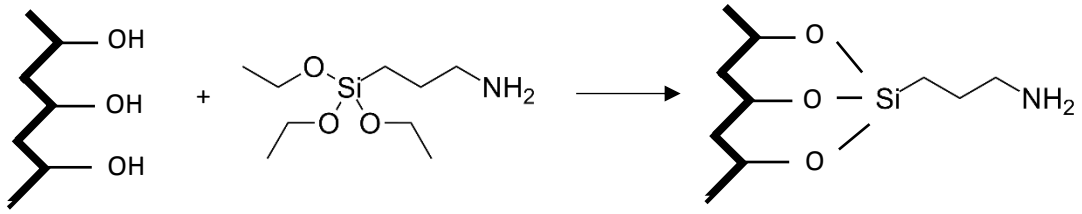


Figure 3.3. Functionalisation of silica with APTEs.

After drying, silica (2.5 g) was added in a flask which contains 125 mL of toluene. Subsequently *x* g of (3-aminopropyl)triethoxysilane, APTES, were added. "*x*" represents the amount of APTES that should be added depending on the kind of silica that were used. It was calculated considering the amount of -OH groups each type of silica has. Results and amounts of APTES used for each silica are displayed in the table below:

Table 3.2. Calculus and quantities of APTES.

	OH **[g/kg]**	***OH*** **[mol/kg]**	***Specific surface area*** **[m^2/g]**	***OH*** **[mol/m^2]**	***APTES*** **[g/g_{silica}]**
K60	91.99	10.22	574	22.21	2.262
K100	53.94	5.99	270	244.54	1.326
K300	205.92	22.88	94	17.82	5.065

To maintain the temperature constant (T= 130 °C) during the refluxed reaction, the flask was immersed in an oil bath overnight.

The product was filtered and washed three times with toluene and, after that, dried at 80 °C overnight in a vacuum oven.

Finally, the products were duly stored and named as Sil-kXX-NH_2 series. The 'XX' refers to the pore size of each material given in Table 3.1.

3.1.3. SOLUTES

The solutes that were used for the experiments were thoroughly selected. A total of 17 solutes were chosen. All the selected solutes are commercially available from several suppliers. Namely: From EMSURE, Pyridine for analysis; from FLUKA, Phenol (>99.5%), Naphthalene (≥98.0%), Benzoic Acid (≥99.5%); from Honeywell; Nitrophenol; from MERCK, pure Caffeine, Benzene; from ALDRICH, p-Nitrotoluene (99%), Butyl benzoate (99%), Ethyl benzoate (>99%), Anthracene (99%); from ROTH, Vanillin, n-Hexane (≥98%); from SIGMA, Nicotineamide (≥99.5%); from SIGMA-ALDRICH, Anisole (≥99%), Nitrobenzene (≥99.0%), P-Cresol (≥99%) and Toluene from ROTH.

The 17 solutes selected were named formerly. In Figure 3.4, their structures are shown.

The wavelength of the UV signal, in which the solutes were detected, depends on the nature of the solutes analysed. The range of wavelength is between 200-260 nm. The choice of the wavelength was decided considering the absorption spectrum and the clarity of the peak shape.

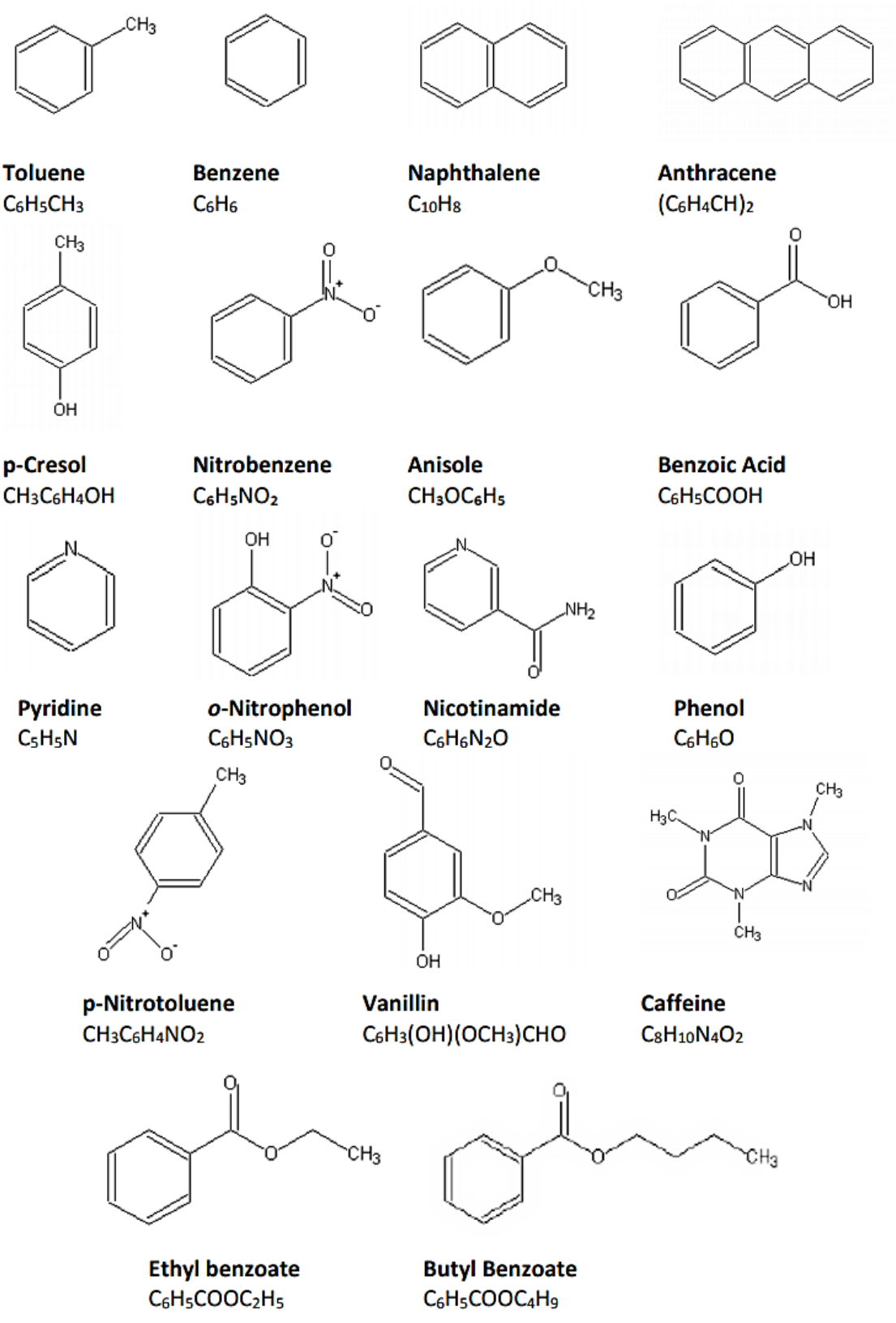


Figure 3.4. Structure of solutes

The solutes selected were presented by *West and Lesellier*, 2008, among one hundred chemicals. According to them, our solute set had been selected to represent the most commonly functional groups. This selection allows us to make a most complete characterization of the packing materials and obtain representative coefficients.

The Abraham descriptors of the solutes can be seen in Table 3.3.

Table 3.3. Solutes selected for evaluation.

SOLUTES	***E***	***S***	***A***	***B***	***V***
Toluene	0.58	0.63	0	0.12	0.8573
Benzene	0,56	0,69	0	0,12	0,7164
Caffeine	1,48	1,9	0	1,27	1,3632
Benzoic Acid	0.75	1.08	0.57	0.44	0.9317
Phenol	0,78	0,9	0,5	0,39	0,7751
Vanillin	1,02	1,46	0,44	0,76	1,1313
Naphthalene	1,27	1,02	0	0,17	1,0854
Anthracene	1,99	1,34	0	0,23	1,4544
p-Nitrotoluene	0,85	1,2	0	0,21	1,0315
Nitrobenzene	0,83	1,26	0	0,21	0,8906
Anisole	0,62	0,79	0	0,33	0,916
p-Cresol	0,81	0,85	0,5	0,39	0,916
o-Nitrophenol	0,96	1,24	0,11	0,35	0,9493
Butylbenzoate	0,64	1,05	0	0,46	1,4953
Ethyl Benzoate	0,64	1,04	0	0,45	1,2135
Pyridine	0,6	0,82	0	0,4	0,6753
Nicotinamide	1,04	1,68	0,49	0,94	0,9317

E, Excess moral refraction; S, Dipolarity/polarizability; A, Hydrogen bond acidity; B, Hydrogen bond basicity; V, McGowans's characteristic volume.

Table 3.4. Properties of solutes.

	MW	*Density*	*Nº of hydrogen bond*		
Solute	**[g/mol]**	**[g/mL]**	**Donors**	**Acceptors**	**Dielectric constant**
Toluene	92,14	0,871	0	0	2.4
Benzene	78,11	0,876	0	0	2.3
Naphthalene*	128,17	1,14	0	0	2.3
Anthracene	178,23	1,25	0	0	2.35
Anisol	108,14	0,995	0	1	4.3
Pyridine	79,10	0,9819	0	1	12.5
Ethyilbenzoate	150,17	0,995	0	2	6
Butylbenzoate	178,23	1,01	0	2	5.9
p-Nitrotoluene*	137,14	1,12	0	3	23.8
Nitrobenzene	123,11	1,196	0	3	35
Caffeine	194,19	1,23	0	6	
p-Cresol	108,14	1,034	1	1	7.5
Phenol	94,11	1,07	1	1	11
Benzoic Acid*	122,12	1,27	1	2	
Vanillin*	152,15	1,056	1	3	17.8
p-Nitrophenol*	139,11	1,48	1	4	9.5
Nicotinamide*	122,13	1,4	2	3	

Dielectric constant had been added to this table. The value of those dielectric constant can be used as a way of estimate the retention times in the column. It is known from *Terry A. Berger (T. A. Berger et al.,* 1991*)* that high values of polarity increase the retention times and polarity is related directly with the dielectric constant. Thus, as higher is the dielectric constant, higher should be the polarity and therefore higher retention times would be detected.

3.2. INSTRUMENTATION AND METHODOLOGY.

3.2.1. SAMPLES PREPARATION

To prepare the solution that were used in the SFC, the solutes were measured and then dissolved in methanol to achieve a 0.1 mg/ mL concentration. To obtain that concentration, diluting solutions were prepared, reaching 1 mg/mL solutions first and then diluting them with methanol until the desired concentration. Final solutions were prepared in 2 mL vials, preparing simultaneously several vials and storing them. Each vial was identified with the name of the containing solute and the date (day/month) of preparation. For instance: "Caffeine 13/12".

This preparation was made four times during the experimental phase to avoid the decay of the solutes stored in the vials.

3.2.2. HOLD-UP TIME DETERMINATION.

Determine the hold-up time, $t_{0,}$ is an essential step in all fields of chromatography. The calculation of retention factors and porosity of the column require this parameter.

The available methods to determine it without affecting the stationary phase structural properties are separated into static methods and dynamic methods.

a) Static methods: The measure of hold-up times takes place after disconnecting the columns from the chromatograph. The most used in this group is the "weight-difference method". The most important drawbacks in this method are, first of all, it does not consider the solvation of the stationary phase and, secondly, it does not consider that the hold-up determination is affected with the pressure drop along the column because it is estimated under atmospheric pressure conditions.
b) Dynamic methods: These methods measure the hold-up volumes of columns while the mobile phase pass through the column at a constant velocity. The most common used are the hold-up volume marker method, the minor disturbance method and the inverse size exclusion chromatography.

As it was mentioned above, the static methods do not consider the solvation of the stationary phase, as well as the last two methods of dynamic ones.

For this reason, the hold-up times are determined using the hold-up volume marker method. Nitrous oxide was dissolved in methanol to use it as hold-up marker.

Nitrous oxide is provided by Westfalen with a purity of 99.995% and stored in a cylinder of 50 L. To prepare the samples N_2O was pumped into a glass tube with pure methanol for 10 minutes.

Nitrous oxide is not stable for a long time in vials. When the samples were stored, it is important to keep it hermetically sealed in order to maintain the gas dissolved in the methanol. However, in the time that they were opened or after two weeks of storage, the N_2O peaks were diffused and it was necessary to prepare a new set of samples.

The detection of nitrous oxide was performed at the wavelength of 195nm

3.2.3. SUPERCRITICAL FLUID CHROMATOGRAPHIC SYSTEM

Once the samples and the vials to determine the hold-up time were prepared, the chromatographic analysis could begin. Following pages describe the column preparation and the equipment necessary to perform the chromatographic analysis.

3.2.3.1. Column preparation

Although a lot of chromatography suppliers offer pre-packed columns, the columns used in this work were prepared in the laboratory.

Mainly two methods of column preparation can be distinguished:

a) Dry package: consisting of tapping the column on a hard surface while the packing is added via a stationary funnel fitted with a distribution head
b) Slurry package: the adsorbent is mixed with the solvent and then this slurry is poured into the prepared column.

Although the dry filling is easy to perform, the slurry method provides more efficient and more reproducible columns, besides with this method the air bubbles from the column package are eliminated. For these reason, the slurry-package method was selected.

The slurry must be prepared with enough particles to fulfil the column.

Figure 3.5. Slurry package system

The stationary phase, one of the silica series, is selected. Approximately 0.7g of silica are measured to fulfil the column. It is important, to fill the column with the stationary phase to avoid dead spaces or channels.

Using approximately 10 mL of hexane, supplied by ROTH, a slurry is prepared. Installing a reservoir in the top end of the column, the other one is sealed following the "sandwich technique" shown in Figure 3.5. The slurry is stored in the reservoir and fresh hexane is pumped into the system with the aid of a pump that supplies the system a pressure of 400 bar.

Without leaking, the solvent should be pumped for approximately 5 minutes. Then the column is disconnected from the reservoir and the spare particles in the inlet are meticulous removed from the top with a plastic object. Finally, the column is closed with the same "sandwich technique".

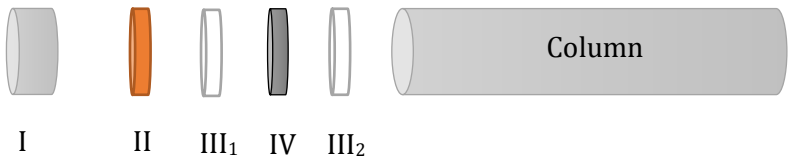


Figure 3.6. "Sandwich technique" to closing the columns. (I) tap, (II) fitting gum, (III_1 and III_2) filter papers and (IV) metal sieve.

It is important to dry the column in an overnight cycle before starting with the chromatograph analysis. This process takes place using a mixture of CO_2 and methanol (90:10) at 40 °C and 200bar.

3.2.3.2.SFC instrumentation

The chromatographic analysis was carried out using the Waters Acquity UPC² system.

The equipment consists in 5 modules, as it is seen in Figure 3.7, namely: Binary solvent manager, Sample manager, Convergence manager, Column manager and PDA detector.

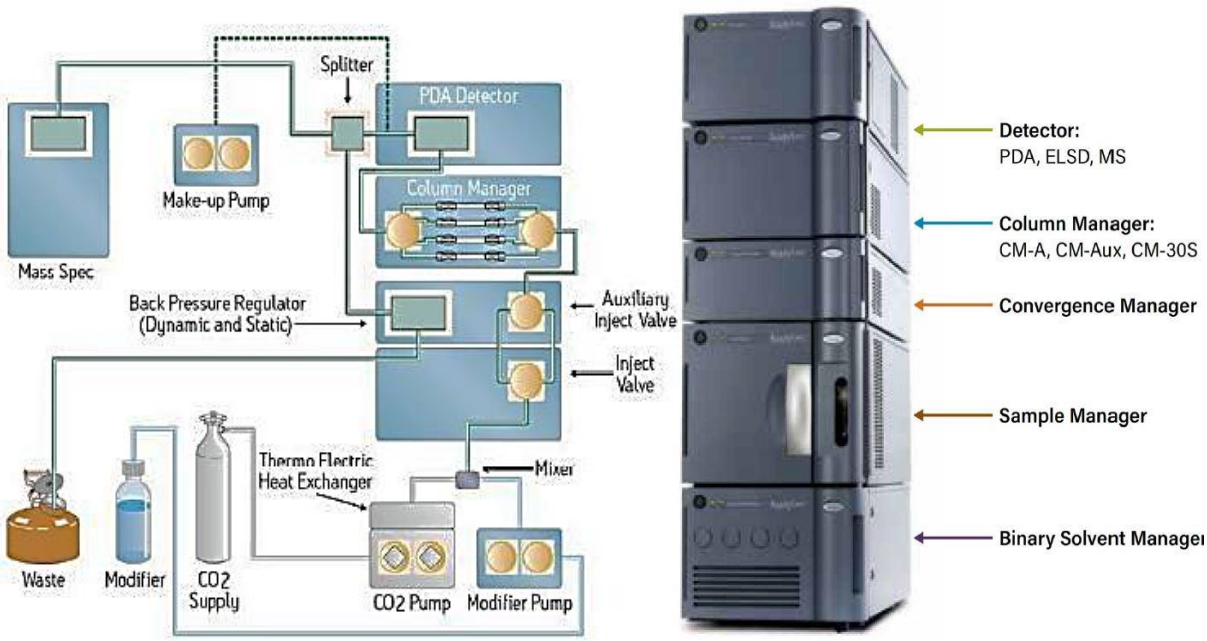


Figure 3.7. The ACQUITY UPC² System (*Waters guide*, 2018).

According with the instrument specifications provided by Waters:

BINARY SOLVENT MANAGER: The system is prepared with two detached pumping systems. The co-solvent pumping system can deal with four solvents (B1, B2, B3 and B4) that can be selected in the program. While the pumping system for $C0_2$ is directly connected with the supplying cylinder. The operating flow rate range is from 0.001 to 4.0 mL/min in 0.001 mL. The maximum operating pressure is 413bar for 3 mL/min flows and up to 293bar up to 4 mL/min.

SAMPLE MANAGER: The system is provided with 2 plates with 48 positions for 2.00 mL vials each one. The injector can achieve until 99 injections per sample with a volume range from 0.1 to 50µL in 0.1 µL increments. The system counts with a two wash programs: strong

and weak wash solvent. Besides of that the temperature of the samples can be controlled in the chamber and it can be configured from 4.0 to 40.0°C.

CONVERGENCE MANAGER: It has an automated back pressure regulator (ABPR). The ABPR allows for controlled depressurization of the compounds of interest and CO_2 for supercritical fluids systems. The control precision of ABPR shall be <±0.5bar.

COLUMN MANAGER: It is designed to support two columns as standards (150 mm maximum length) or four columns (50mm) up to 4.6 mm internal diameter. The columns have two independent heat/cool zones to regulate the temperature of the columns. It can attain temperatures from 4 to 90 °C in 0.1°C increments.

PDA DETECTOR: The photo diode array detection is the preferred technology used in supercritical fluid chromatography. It obtains spectral profiles from ultraviolet to near the IR region of the samples that are eluting the column. The wavelength range is from 190 to 800 nm. It is equipped with a Deuterium lamp as light source and has a pressure limit of 6000 psi (413 bar).

3.2.4. FTIR ANALYSIS

The modified silicas were prepared in small vials for FTIR analysis.

Before performing the measurements, a background measurement was needed. After cleaning the module, a small amount of the sample was placed and pressed. The measurements were performed twice to analyse small discrepancies.

4. RESULTS AND DISCUSSION

4.1. FUNCTIONALIZATION OF THE COLUMNS.

The initial phase of the experimental research was the preparation of the functionalized columns from bare silica. The three groups selected for this purpose, as well as the procedure to obtain the modifications, were mentioned in the Chapter 3 "Material and Methods" where their structure can be consulted. They were: Epoxy, ibuprofen and amine.

In order to confirm the success of these modifications, an analysis with Fourier-transform infrared spectroscopy, FTIR, was performed before starting with the column package.

In Figure 4.1 the FTIR results for the Sil-k60-series are shown, the Sil-k100 and Sil-k300 series can be consulted in Appendix A. The chart shows the characteristic peaks for silica at 798, 960, and 1060 cm^{-1} owing to Si-O-Si symmetric stretching, Si-OH stretching, and Si-O-Si asymmetric stretching vibrations, respectively. Those values correspond to the expected ones, according with literature, (*P. Larkin,* 2011*).*

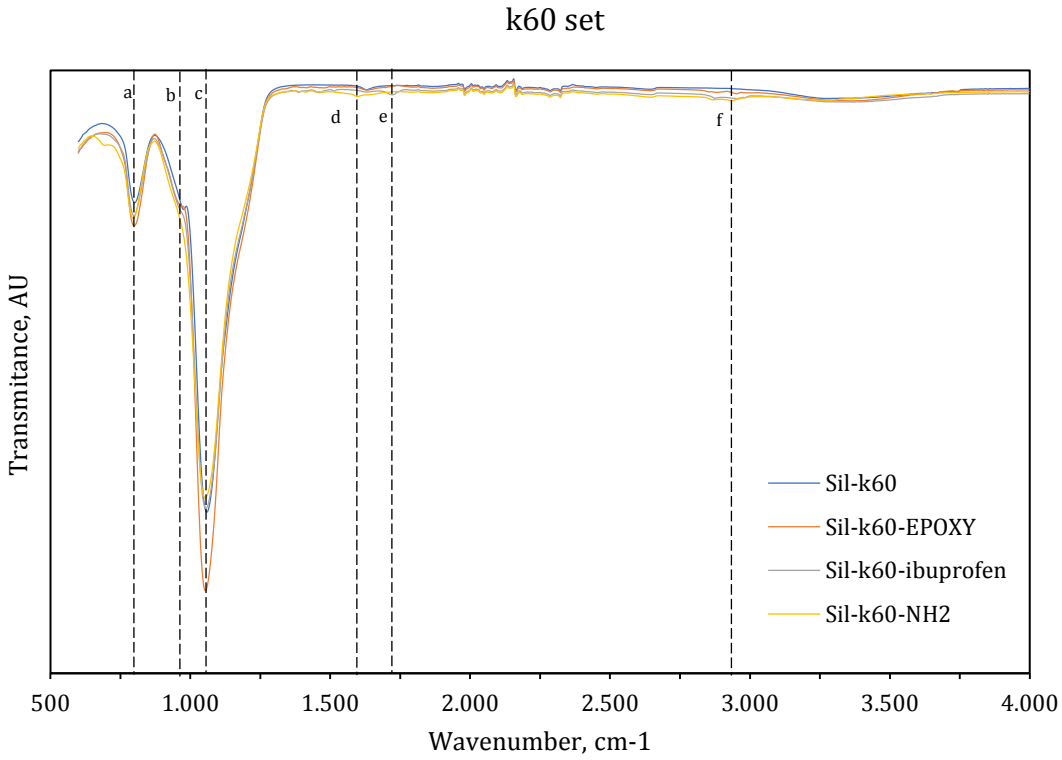


Figure 4.1 Infrared spectra of Sil-k60 and Sil-k60 functionalizations. (a) Si-O-Si symmetrical stretch at 798 cm^{-1} (b) Si-OH stretch at 960 cm^{-1} (c)$Si-O_2$ stretch at 1060 cm^{-1} (d) NH_2 stretch at 1585 cm^{-1}(e) Ester stretch at 1742 cm^{-1} (f) CH_3 stretch at 2980 cm^{-1}.

The functionalization of silica was performed using the abovementioned functional groups. The FTIR analysis reveals the presence of new groups, depending on the stationary phase analysed.

The ibuprofen modification spectrum, showed in grey in the chart, reveals a group peak at 1742 cm^{-1} that represent the presence of the characteristic ester group according with Peter J. Larking who places the stretch between 1720-1750 cm^{-1} (*P. Larkin*, 2011). Another example of the success of the modification is the amine line, plotted in yellow, that shows a peak in the wavenumber near the 1580 and 1630 characteristic of the NH_2 bond.

4.2. HOLD-UP TIME AND VOLUME

As it was said in Chapter 3 "Materials and Methods", the hold-up time was determined injecting nitrous oxide dissolved in methanol in the system as hold-up marker.

This parameter is not related to the retention process and depends on the flow rate and physical characteristics of the column (length, diameter, porosity of stationary phase); nevertheless, the determination of the hold-up times is critical for the subsequent data analysis. So, it should be performed in a properly way in order to ensure a good measure, with precision and accuracy for each condition.

4.2.1. EFFECT OF MODIFIER IN THE HOLD-UP TIME.

The retention times of N_2O for each column were measured, obtaining the hold-up time, t_0, for each condition. These measurements were carried out at the same conditions that the chromatographic analysis of the 17 solutes was done, maintaining the same pressure and temperature that were used for the set.

Before addressing the analysis of the measurements, it is important to highlight that the nitrous oxide spectrum has the highest resolution between 173 to 190 nm (*Selwyn et al.,* 1977). In spite this fact, the system cannot reach wavelengths below 190 nm. Therefore, as it was settled in section "Hold-up time determination", all measurements were performed at 195 nm.

Analysis has been down to decide whether to determine the hold-up time with modifier. As it can be shown in Figure 4.3 (a), the chromatograms obtained for the nitrous oxide with 10% of modifier (MeOH) in the mobile phase were full of noise, so the peak was unrecognizable and became impossible to determine the hold-up time.

To solve this setback, a bibliography research was performed, considering the alternative of another method to determine the hold-up time. However, according to *Vajda and Guichon* (2013), in mixtures of supercritical carbon dioxide and methanol the void volume determined with nitrous oxide has only small variations. These changes can be caused by the formation of a small layer in the surface of the stationary phase produced by the addition of methanol in the mobile phase. Considering this fact, the hold-up volumes for different methanol concentrations (v/v%) were determined and shown in the Figure 4.2.

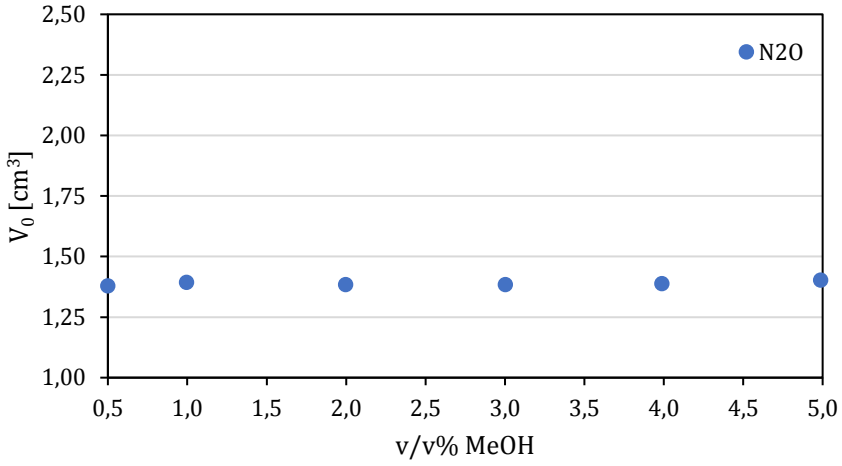


Figure 4.2 Comparison of the hold-up volumes determined from the retention time of N_2O peaks and from the first negative disturbance caused by the injection of solvent molecules. (*Vajda and Guichon,* 2013).

The small variations in this determination, with a standard deviation of 0.008, allow us to change the percentage of methanol in the mobile phase without modifying the retention times of nitrous oxide. The constant value of the void volume, even changing the methanol concentration in the mobile phase, leads us to believe that the retention time of the nitrous oxide is only dependent of the volumetric flow and density.

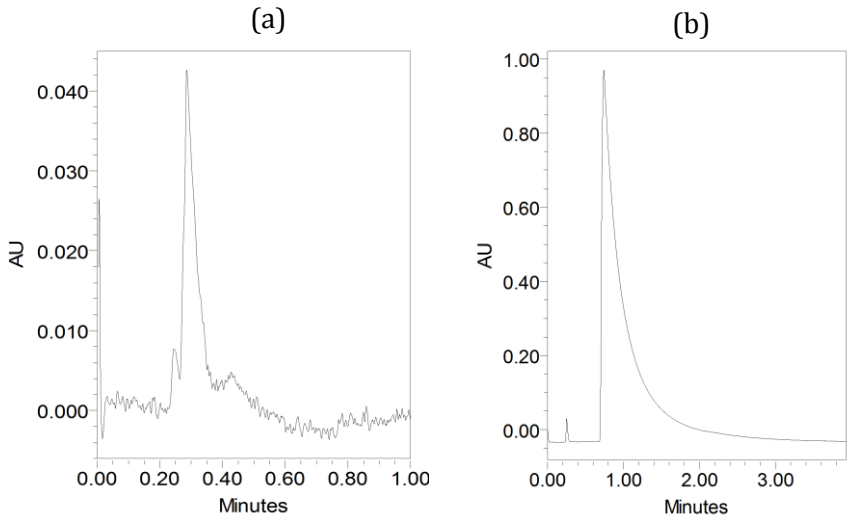


Figure 4.3_ Chromatogram of N_2O for hold-up determination measured for "Sil-k60-NH_2". Conditions: (a) 200bar/40°C/10% MeOH. (b)200bar/40°C/0% MeOH.

The figure 4.3 shows the chromatogram obtained injecting the nitrous oxide dissolved in methanol with pure CO_2 for the same column as chart (b). In this figure, two peaks are observed clearly and now it can be seen legible peaks. The most retained and tailed one corresponds to the methanol and the small and sharp one at the beginning is the nitrous oxide. So, with this new operational condition, the hold-up time can be determined without disturbances.

Because of the reasons discussed above, the hold-up time was determined without modifier in mobile phase

4.2.2. TREND OF HOLD-UP WITH THE PRESSURE.

Due to its inert nature. nitrous oxide was selected as hold-up marker. Because of this reason, regardless of the condition, it is foreseeable that the retention in the column would be constant. In the previous section, *Vajda and Guichon* (2013) have already demonstrated that this theory may be applied for the variation of methanol concentration in the mobile phase.

The variation of the hold-up times was measured using nitrous oxide dissolved in methanol as it was said in the chapter 3 "Material and Methods". The hold-up volume obtained for each condition would be constant because of the inert nature of the compound. In spite this fact, the hold-up time were different due to the change of mobile phase density when de

pressure was changed. The plot of the variation of retention times with the pressure, shown in Figure 4.4, reveals a dependence of the hold-up time with the pressure. An increase in the pressure causes an increase in the hold-up time for every column.

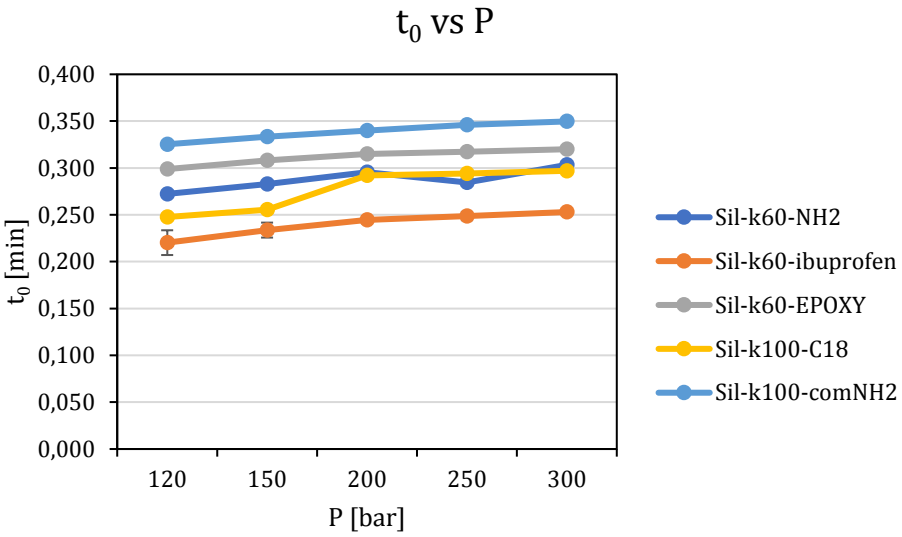


Figure 4.4. Nitrous oxide retention times against the pressure variation for all the columns of the proposed case study.

This result suggests a correlation between the hold-up time and the pressure. So, if it is considered that the evolution of the retention times of nitrous oxide is corresponding to the variation of the density with the pressure, a relationship between them is proposed.

4.2.3. TREND OF HOLD-UP WITH THE TEMPERATURE.

In the same way, it is expected that any variation in temperature conditions do not lead to a variation in the retention time of nitrous oxide. However, as it is shown in the previous paragraph, the nitrous oxide seems to be not completely inert.

The Figure 4.5 represents the variation of the hold-up times determined using nitrous oxide dissolved in methanol against the variation of temperature from 25 to 60. As the chart reveals, an increase in the temperature conditions cause a decrease in the hold-up times to all the selected columns.

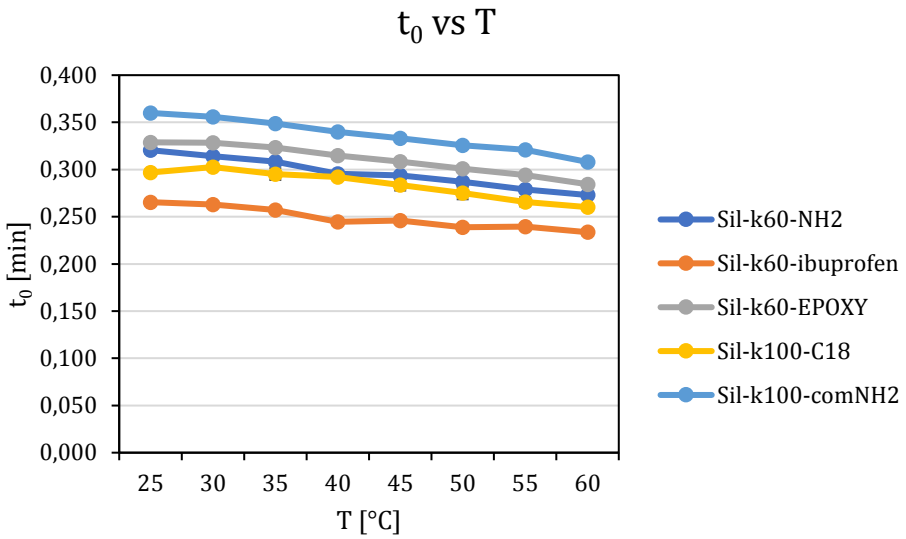


Figure 4.5. Nitrous oxide retention times against the temperature variation for all the columns of the proposed case study.

This result suggests, as in the previous case, a correlation between the hold-up time and the temperature and if it is considered that the evolution of the retention times of nitrous oxide is corresponding to the variation of the density with the temperature, a relationship between them is proposed.

4.2.4. HOLD-UP VOLUME.

The previous analysis reveals a linear relationship with both parameters, temperature and pressure, suggesting that they do not have a real influence in the variation of the retention time of nitrous oxide. Therefore, it is hypothesized that the density is the main cause for this variation, as it was abovementioned.

In spite the fact that the hold-up times calculated with nitrous oxide varied, it is supposed that the void volume of each column is constant regardless the experimental conditions. To verify this hypothesis and check whether the retention times are affected by the density of the mobile phase, a calculus of the hold-up volume was performed.

It is known that the void volume can be calculated using:

$$V_0 = \vartheta \times t_0 \tag{8}$$

Where: V_0 is the hold-up volume [mL]
ϑ is the volumetric flow [mL min^{-1}]
t_0 is the hold-up time [min]

Due to the difference of pressures from the inlet point of the column, the system pressure, and the outlet, given by the back-pressure the volumetric flow in the column is expressed as an average between the volumetric flow in the inlet and the outlet of the column:

$$\vartheta = \frac{\vartheta_{in} + \vartheta_{out}}{2} \tag{9}$$

Then the following relation relates the mass flow with the density and the volumetric flow:

$$\dot{m} = \rho \times \vartheta \tag{10}$$

Combining both expressions, the void volume is given by:

$$V_0 = \frac{\dot{m} \times \left(\frac{1}{\rho_{in}} + \frac{1}{\rho_{out}}\right)}{2} \times t_0 \tag{11}$$

Where ρ_{in} and ρ_{out} are the density of the mobile phase, CO_2 with 0% of methanol in the case of the hold-up time determination. This equation is considering that the mass flow is constant in all the column.

Applying this equation to the determination of nitrous oxide for the three functionalized columns, the three hold-up volumes were obtained.

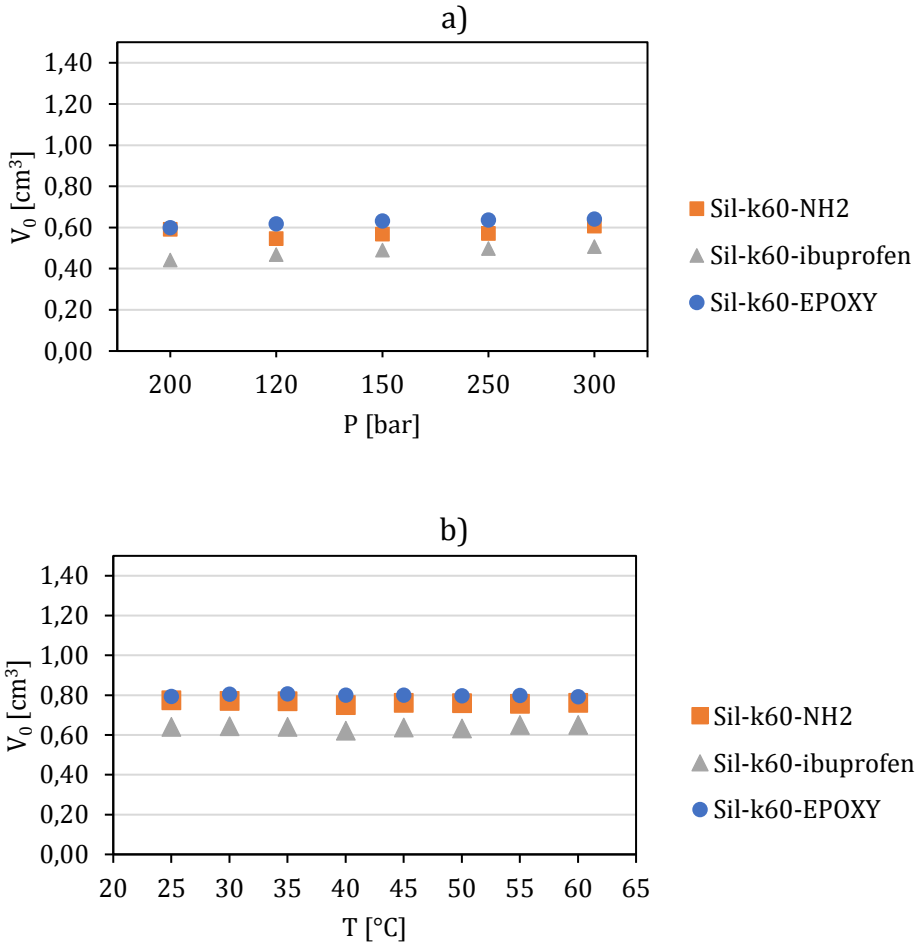


Figure 4.6. Comparison of the hold-up volumes determined from the retention time of N_2O peaks for a pressure variation (a) and a temperature variation (b).

The Figure 4.6 summarizes the change of the hold-up volume due to an increase in the pressure (a) and in the temperature (b). The values of void volume are almost constant, with a maximum standard deviation of 0.01 for the "Sil-k60-ibuprofen"; but the changes are more obvious in the case of pressure, with a maximum standard deviation of 0.03 for the "Sil-k60-NH_2". Therefore, the void volume can be considered constant, so the initial hypothesis can be considered true.

In the view of the results, it can be concluded that the density of the mobile phase is the main reason of the changes in the retention times of the nitrous oxide.

4.3. PROPERTIES OF COLUMNS.

The analysis of three different series of stationary phases, Kromasil 60, 100 and 300, was originally planned. However, several problems with the equipment delayed the original study. Due to this hiccup, the initial experiments were changed and only the analyses of the modified Kromasil 60, listed in Table 4.1, were performed. Two commercial stationary phases were analysed to complete the case study:

a) The first one is an amine-modified silica for direct chromatography. The column was packed in the laboratory with the same technique that was used to pack the Kromasil 60 columns, explained in "Column preparation".
b) The second one was completely different, selecting a well-known C18 stationary phase for reverse chromatography. This column was commercial, so it was not needed to package the column.

Table 4.1. Summary of the stationary phases used to perform the experiments and its properties.

Stationary phase	*Pore size [Å]*	*Loading [µmol/m²]*
Sil-k60- EPOXY	60	1.5
Sil-k60- ibuprofen	60	0.9
Sil-k60-NH_2	60	2.0
Sil-k100-C18	100	
Sil-k100-comNH_2	100	

The selected columns were initially analysed following two different criteria:

a) Polarity: The columns packed in the laboratory are all polar stationary phases with varied polarity depending on the functionalization of the silica group. It is supposed for these columns that more polar the solute is, more retained it will be. However, the C18 column is a non-polar stationary phase, with which the less polar compounds should be first eluted. This grouping criterion allows us to compare the differences between the columns considering the variation of polarity due to the functionalized groups.

b) Pore size: The diffusivity of the system can be strongly affected by the pore size. The amine column presented with two different pore seizes, named 60 Å and 100 Å, allows to study the influence of this parameter. It is supposed that the smaller the pore size is, the larger the surface area will be and, therefore, the material more retentive will be.

4.3.1. POLARITY.

The polarity is caused by the separation of the positive and negative charge densities. Molecules that do not cancel the dipole moments due to their polar nature or geometry can be considered polar.

Looking into the stationary phases that were selected for this study, "Sil-k100-C18" is non-polar because of its long chain with methyl groups where the main elements are H and C. Although they are a bit more polar, columns packaged with "Sil-k60-EPOXY" and "Sil-k60-ibuprofen" can be consider non-polar too due to its lack of polar groups or the presence of groups that significantly reduces the global polarity. Finally, the amine columns "Sil-k60-NH_2" and "Sil-k100-NH_2" have to be consider as polar ones because of the presence of the amine group that charge the chain.

Analysing all the columns together, the greatest differences of retention are detected in the most polar (Benzoic acid, Phenol, o-Nitrophenol and Nicotinamide) and the most non-polar samples (Toluene, Benzene, Naphthalene and Anthracene). Predictably, non-polar compounds have larger retention times for "Sil-k100-C18", making a notable difference to the most polar compounds.

The group of solutes that do not present strongly polarity or non-polarity are going to be named "intermediate solutes". Although the retention time of the so-called "intermediate solutes" are similar in both cases, to have a clearer and simpler point of view, "Sil-k100-C18" and "Sil-k100-comNH_2" were taken to analysis.

These stationary phases are going to be compared together because they are supposed to be the least polar and the most polar one, respectively and they have same pore sizes, so only the influence from the surface modification shall be considered.

Predictably, the retention times on the non-polar stationary phase "Sil-k100-C18" are larger than on the polar stationary phase "Sil-k60-NH_2" for non-polar compounds, such as Anthracene (an obvious non-polar). The "intermediate solutes"; those that cannot be marked as extreme polar or non-polar, have similar times on polar and non-polar phases; deviating to one of the extremes depending on its polarity. So, polar solutes like Benzoic acid or Nicotinamide have a clear polar behaviour while Anisole, p-Nitrotoluene among others, have similar times with both columns. To illustrate it, the capacity factor of each solute was plotted for both columns, as we can see in Figure 4.7.

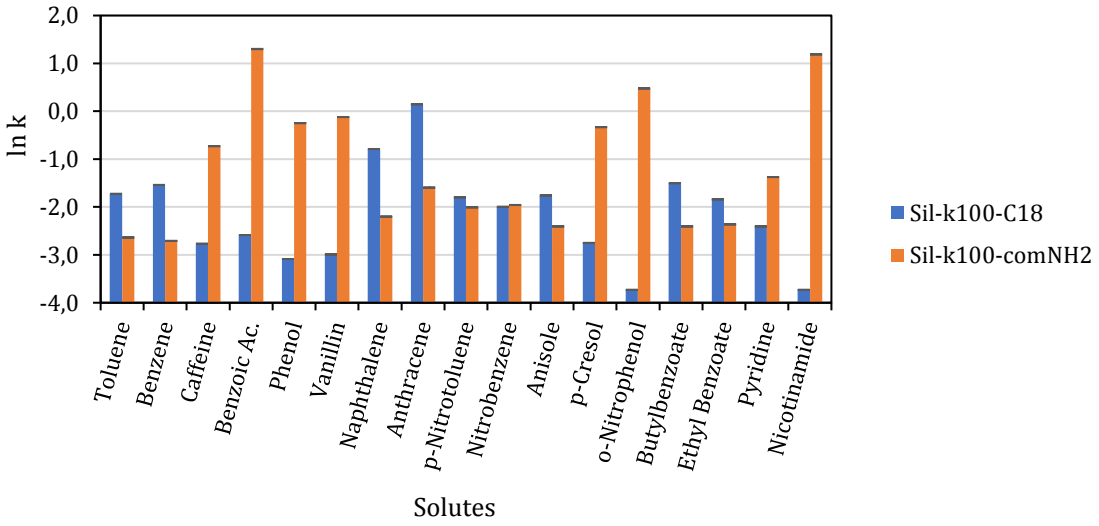


Figure 4.7. Comparison of capacity factors obtained with the commercial columns using 17 solutes. Conditions: 200bar/40°C/10% MeOH. (Maximum error: ±0.005)

After considering both columns, the existence of a dependence between the polarity of the stationary phase, the polarity of the solutes and the capacity factor, and consequently the retention times, can be concluded.

Having analysed, the polarity, its influence predominates over the pore size. In spite the relationship between the diffusion and the pore size, the porosity is not a key issue in selecting the material. Nevertheless, the ibuprofen singularity opens the window of perform a study about the influence of the modified chain volumes. Moreover, the polarity should be highly taken into consideration, due to its relationship with the material interactions and solutes retention times.

4.4. SOLUTES AND RETENTION TIMES

4.4.1. CLASSIFICATION OF SOLUTES.

The classification of solutes was performed after a preliminary analysis of the results. Finding that, due to the number of compounds, the analysis of solutes can be tangled. To ease the study of subsequent paragraphs the classification of the 17 solutes used for the characterization was done.

According to the descriptors of LSER model, initially a classification between solutes with hydrogen bond acceptor capacity and without it was settled. The basic group was still too wide to be analysed, so a second step selection was performed analysing the structure of each compound.

Thus, the solutes were classified in four "families" according the abovementioned criteria. Namely: Non-polar analytes, Basic analytes, Strong basic analytes, Basic/acid analytes. For ease, the chromatogram of each solute is shown in Figure 4.8 and it is evident, according to the colour range selected, the classification of grouping performed.

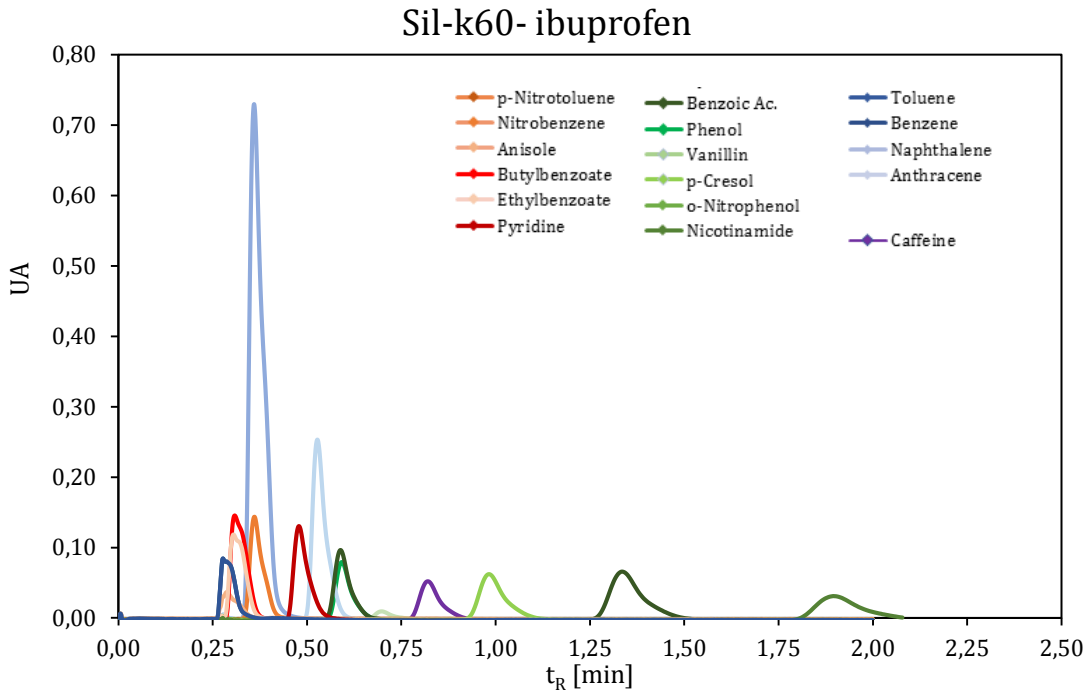


Figure 4.8. Chromatogram of 17 solutes for the column "Sil-k60-ibuprofen". Conditions: 200bar/40°C/10% MeOH. The classification of solutes is shown in range of colours (blue: Non-polar analytes, red: basic analytes, violet: strong basic analytes, green: Acid/basic analytes).

The following chart shows us the solutes, highlighting the importance of the first criteria selected to do the classification. The second criteria is less evident; however, the cluster has similarities with the "Table 3.4" shown in the Chapter 3 "Material and Methods" that can help the reader to understand the final grouping.

Table 4.2. Solutes classification according their basicity and acidity.

SOLUTES	E	S	A	B	V
			Non-polar analytes		
Toluene	0.58	0.63	0	0.12	0.8573
Benzene	0,56	0,69	0	0,12	0,7164
Naphthalene	1,27	1,02	0	0,17	1,0854
Anthracene	1,99	1,34	0	0,23	1,4544
			Basic analytes		
p-Nitrotoluene	0,85	1,2	0	0,21	1,0315
Nitrobenzene	0,83	1,26	0	0,21	0,8906
Anisole	0,62	0,79	0	0,33	0,916
Butylbenzoate	0,64	1,05	0	0,46	1,4953
Ethyl Benzoate	0,64	1,04	0	0,45	1,2135
Pyridine	0,6	0,82	0	0,40	0,6753
			Strong basic analytes		
Caffeine	1,48	1,9	0	1,27	1,3632
			Basic/Acid analytes		
o-Nitrophenol	0,96	1,24	0,11	0,35	0,9493
Vanillin	1,02	1,46	0,44	0,76	1,1313
Nicotinamide	1,04	1,68	0,49	0,94	0,9317
Phenol	0,78	0,9	0,5	0,39	0,7751
p-Cresol	0,81	0,85	0,5	0,39	0,916
Benzoic Acid	0.75	1.08	0.57	0.44	0.9317

To check the grouping, all the columns and solutes were analysed. The chromatogram showed the grouping, seeing the non-polar and basic analytes with similar retention times. To ensure that the "families" were properly selected, the results obtained with another column are shown in Figure 4.9. Hereafter, the "Sil-k60-NH_2" column was selected to show

how the logarithm of the capacity factor evolves with the pressure, temperature and concentration of modifier.

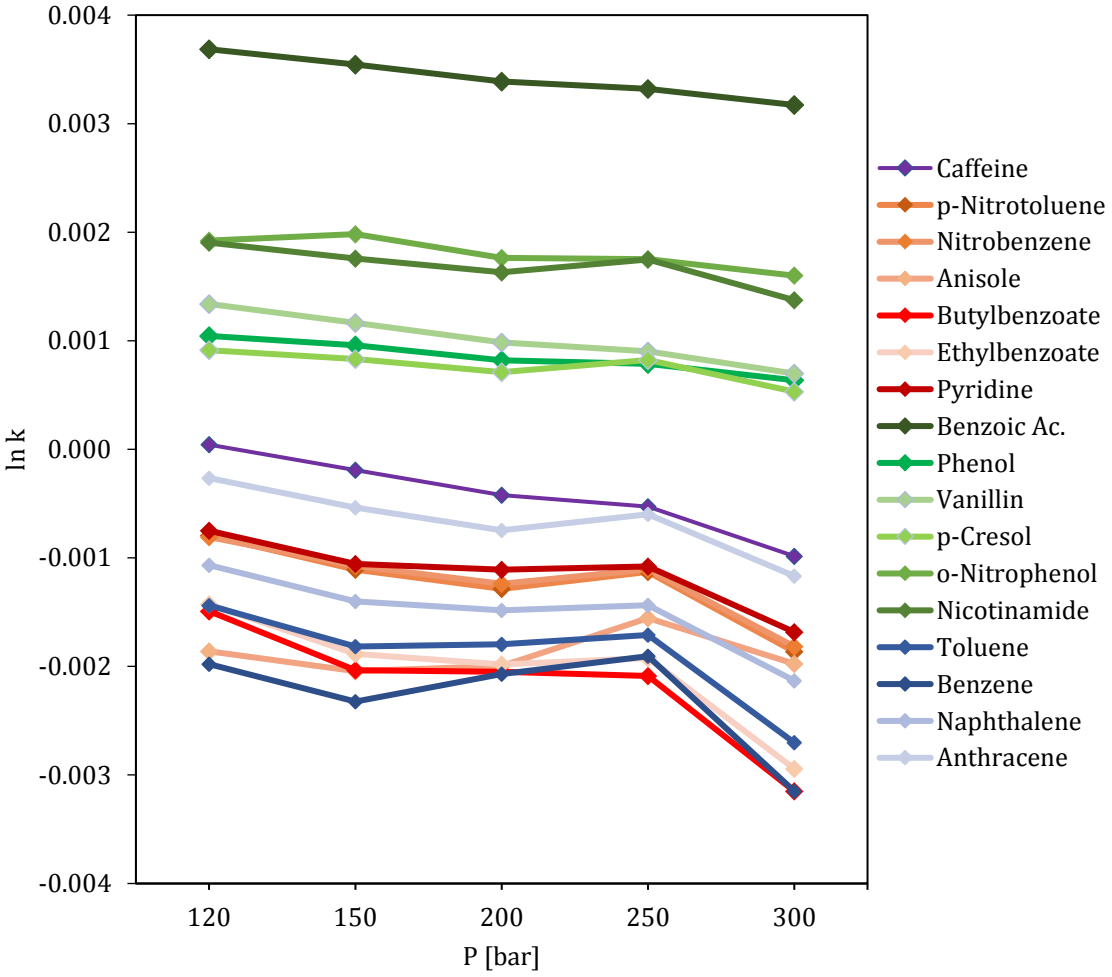


Figure 4.9. Influence of the pressure in the logarism of capacity factor for the column "Sil-k60-NH_2". Conditions: 200bar/40°C/10% MeOH. The classification of solutes is shown in range of colours (blue: Non-polar analytes, red: basic analytes, violet: strong basic analytes, green: Acid/basic analytes).

The basic/acid analytes, that are depicted in green. are clearly distinguished with the higher logarithm of capacity factor; so, the longest retention times. In spite the fact that the evolution of this property will be analysed in following chapters, the almost linear evolution with the increasing pressure follows a trend that, in the case of basic and non-polar grouping, is missed. The lack of trend can be due to the low retention times, that affect strongly the capacity factors leading a bigger standard deviation.

In next sections the influence of pressure, temperature and concentration of modifier for the different grouping are analysed. To this aim a representative solute of each family was selected: Naphthalene, of non-polar analytes; p-Cresol, of basic/acid analytes; Pyridine, of basic analytes and Caffeine, of strong basic analytes.

4.4.2. INFLUENCE OF PRESSURE

The influence of pressure was evaluated for all the columns abovementioned. The analysis of the influence of the pressure in the retention times and the peak shapes is of particular importance.

The retention times decrease with the increasing pressure, as well the capacity factor. At low pressures the mobile phase is more compressible, which makes the volumetric flow rate bigger resulting in shorter retention times and smaller capacity factors. The density of the mobile phase increases with the pressure, enhancing the solubility of the solutes in the mobile phase, so resulting in lower retention times.

In Figure 4.10, not only the influence in retention times but the shape of the peaks can be analysed. It is important to emphasize the presence of secondary peaks in Caffeine, p-Cresol and Pyridine at high pressures.

As it was expected. for all the selected groups the solutes are more retained by the stationary phase at low pressures than at high pressures. The shape of the peaks suffers an improvement while the pressure increases in all the cases, being particularly evident in the case of Caffeine and p-Cresol. The retention times for both compounds present more variation than the retention times from Naphthalene and Pyridine what makes easier determine the improvement in the peak shape.

Caffeine, selected to represent the strong basic analytes, has the larger variation due to its high polarity and its solubility. Naphthalene, as a representant of the non-polar analytes is almost not affected by changes in pressure, maintaining the shape and the proportionality in the peaks. And Pyridine presents a small variation as well. This similarity may be caused because of its similar basic nature.

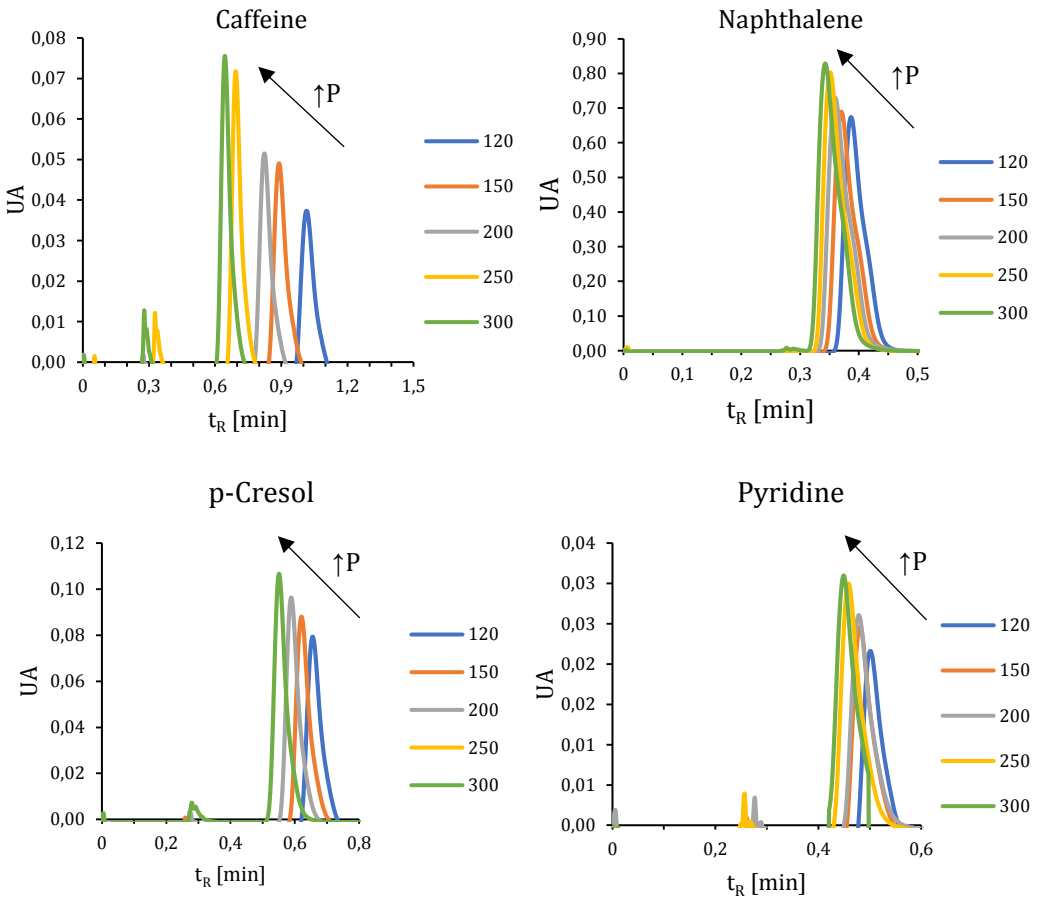


Figure 4.10. Influence of the temperature in peak shape for the column "Sil-k60-ibuprofen". Conditions: 40°C/10% MeOH. (**Caffeine**: strong basic**, Naphthalene**: Non-polar, **p-Cresol**: Acid/basic**, Pyridine**: basic)

The Figure 4.11 shows the variation of the logarithm of the capacity factor with the increase of the pressure for the column "Sil-k60-NH_2" measured at 40°C with 10% of modifier in the mobile phase and a flow rate of 2 mL min^{-1}.

It is clearly shown that Caffeine is more retained by the stationary phase and the variation of the capacity factor is more evident with the increase, the capacity factor (and consequently the retention) decrease when the pressure increases. In the same way, p-Cresol presents a quite big variation in the retention times; however, a certain proportion in the times and shapes of the peaks is kept along the pressures. The highest pressures for naphthalene and pyridine compound present a deviation of the linearity and it can be caused by the high densities. As it was mentionend in previous parragraphs the increase of pressure causes a increase in the density and this dependence is accentuated particularly at high pressures.

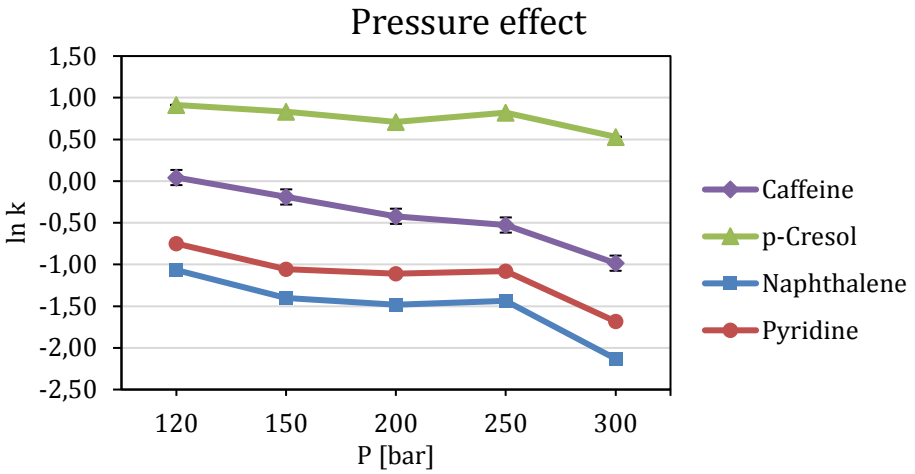


Figure 4.11. Influence of the pressure in retention times for the column "Sil-k60-NH_2". Conditions: 40°C/10% MeOH. (blue: Non-polar, red: basic, violet: strong basic, green: Acid/basic). Standard error: 0.001-0.092

4.4.3. INFLUENCE OF TEMPERATURE

The influence of temperature was evaluated for the five columns selected for this research. Although some authors (*M. Enmark et al*. 2014) have pointed out that it has a minor effect in the retention times, the influence of the temperature in the density cannot be neglected.. Following, the analyis of the influence of the temperature in the peak shapes and the analysis of the the retention times takes place.

In Figure 4.12 the influence of temperature in the retention times and shapes is presented.

The chromatogram of Caffeine shows a double peak that can be due to contaminants or because of the decay of the sample. At low temperatures, a double peak is observed, though that increase of temperature enhances the shape and reduces the presence of the first peak. So, it is important to highlight the positive influence that the increase of temperature has in the Caffeine peaks.

The other analytes selected, pyridine, naphthalene and p-Cresol don't seem to be affected by the increase of temperatures, maintaining the shape and the times almost constant.

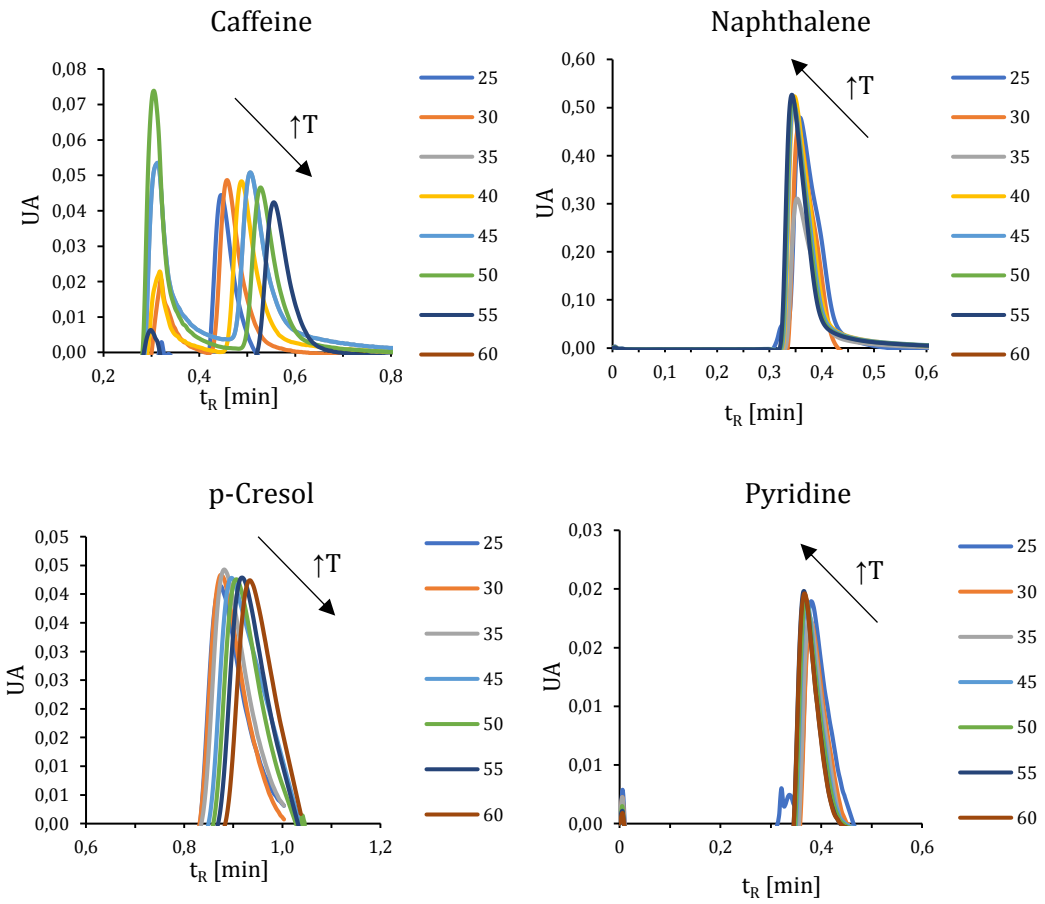


Figure 4.12. Influence of the temperature in peak shape for the column "Sil-k60-ibuprofen". Conditions: 200bar/10% MeOH. (**Caffeine**: strong basic, **Naphthalene**: Non-polar, **p-Cresol**: Acid/basic, **Pyridine**: basic)

Figure 4.13 shows the trend of capacity factors with the increase of temperature for the column "Sil-k60-NH_2" measured at 200 bar with 10% of methanol in the mobile phase and a flow rate of 2 mL min^{-1}.

At first glance, it can say that ln k increases linearly with the temperature. But it is important to highlight the different behaviour of the solutes: While Caffeine and p-Cresol have an increase in the retention times as soon as the temperature is increased, the Naphthalene and the Pyridine have a decrease in the retention times. This similarity can be caused due to the nature of the compounds, considering that Caffeine and p-Cresol are polar compounds, Pyridine is less polar than both of them and Naphthalene is non-polar.

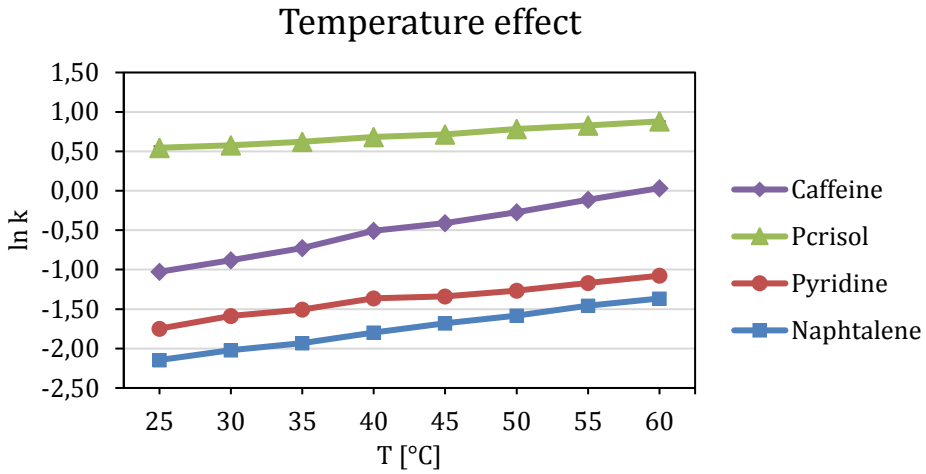


Figure 4.13. Influence of the temperature in retention times for the column "Sil-k60-NH_2". Conditions: 200bar/10% MeOH. (blue: Non-polar, red: basic, violet: strong basic, green: Acid/basic). Standard error: 0.001-0.032

4.4.4. INFLUENCE OF MODIFIER CONCENTRATION

The influence of modifier concentration is explained in following pages.

Taking a look at Figure 4.14, the influence of the concentration modifier in the peak shape can be determined. In a general performing the analysis of selected solutes, a peak tailing behaviour is observed when the concentration of modifier decreases. This can be due to the interaction of the solutes with the stationary phase. This effect is clearly reduced when methanol is added to mobile phase which entails the interaction of the modifier with the functional groups of the stationary phase and reduces the interaction of solutes with it.

Another important fact to emphasize is the presence of a secondary peak in all the chromatograms. After consulting the data collected and considering that it is in all the solutes for each condition at the same time, it may be caused by the methanol used to prepare the solutions of the solutes.

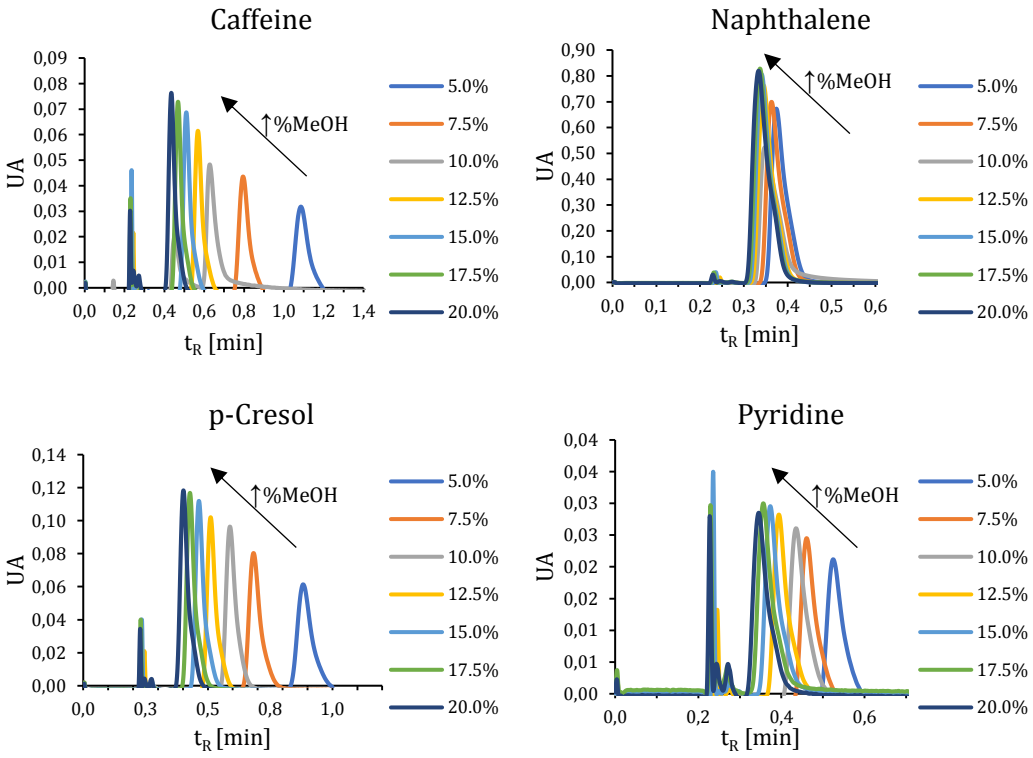


Figure 4.14. Influence of the concentration of modifier in peak shape for the column "Sil-k60-ibuprofen". Conditions: 200bar/10% MeOH. (**Caffeine**: strong basic, **Naphthalene**: Non-polar, **p-Cresol**: Acid/basic, **Pyridine**: basic)

As mentioned above, the peak shape is clearly affected with the presence of methanol, so higher concentrations make the peaks sharper and reduce the tailing for all the analysed solutes.

Figure 4.15 shows the results for the column "Sil-k60-NH_2" measured at 200 bar, 40°C and a flow rate of 2 mL min^{-1}. It gives the plots of ln k versus the increasing modifier concentration for all the representative solutes selected.

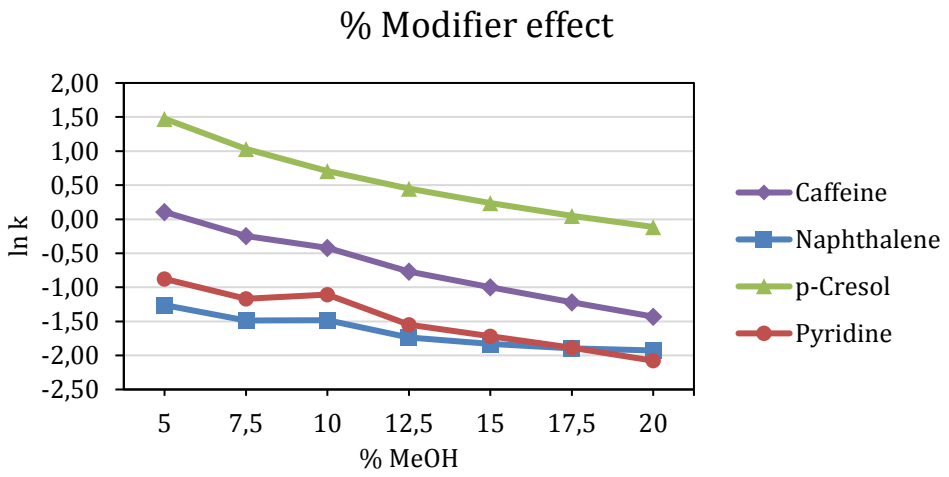


Figure 4.15. Influence of the concentration of modifier in retention times for the column "Sil-k60-NH_2". Conditions: 200bar/40°C. (blue: Non-polar, red: basic, violet: strong basic, green: Acid/basic). Standard error: 0.002-0.358

The general trend is a decrease in retention with the decrease of modifier, obtaining long retention times. This effect is more significant as the modifier concentration is lower, making the elution of certain compounds really slow and affecting significantly the shape of the peaks. For this reason, the analysis of 1% and 3% performed during the experimental phase are not shown in the chart.

4.4.5. THE SPECIAL CASE OF BENZOIC ACID.

When the experimental analysis of "Sil-k60-NH_2" were performed, the benzoic acid represented a particular challenge in working. Initially the decay of the solvent caused the apparition of peaks in the benzene range of times, and after the preparation of new samples it was completely disappeared. The expected times were vacuous, so a long-time set had to be performed to find the benzoic acid peak.

This successful analysis provided us with extremely long retention times even for the higher concentration of modifier, which was expected to present the shortest retention times. The retention time with pressure variation presented times from 11 min to 8 min with increasing pressure and with temperature variation shows 8 min to almost 12 min while the temperature was increased by fives. The variation was still more pronounced for the concentration of modifier, whose data are plotted in the Figure 4.16 to illustrate the case.

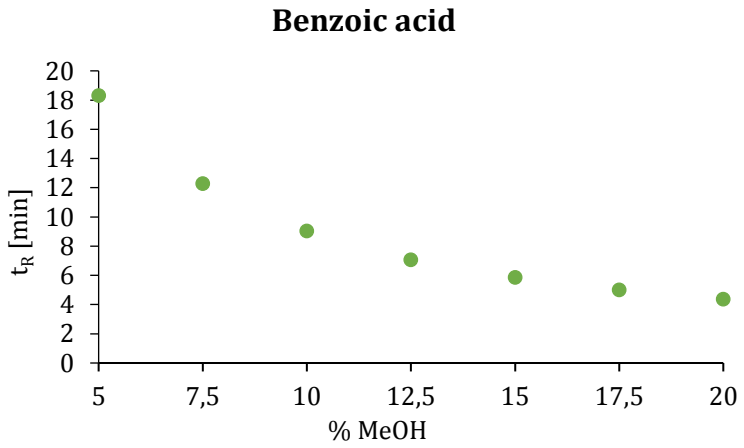


Figure 4.16. Variation of the retention times for the benzoic acid in the concentration of modifier for the column "Sil-k60-NH_2". Conditions: 200bar/40°C.

The retention times of benzoic acid on the four columns were extremley different from each other, which was unexpected, so it was necessary to confirm that the solute that had been analysed was indeed benzoic acid. In order to confirm it, the UV Spectrum was examined.

The UV spectrum used to perform the examination are shown in Figure 4.17. The chart (a) shows the spectrum of benzoic acid for the "Sil-k60-NH_2" column at 200 bar, 40°C and 10% of MeOH in the mobile phase. Charts (b), (c) and (d) represent the UV spectrums on "Sil-k100-comNH_2", "Sil-k60-EPOXY" and "Sil-k60-ibuprofen" respectively. Even with the most similar stationary phase, "Sil-k100-comNH_2", the retention times were completely different.

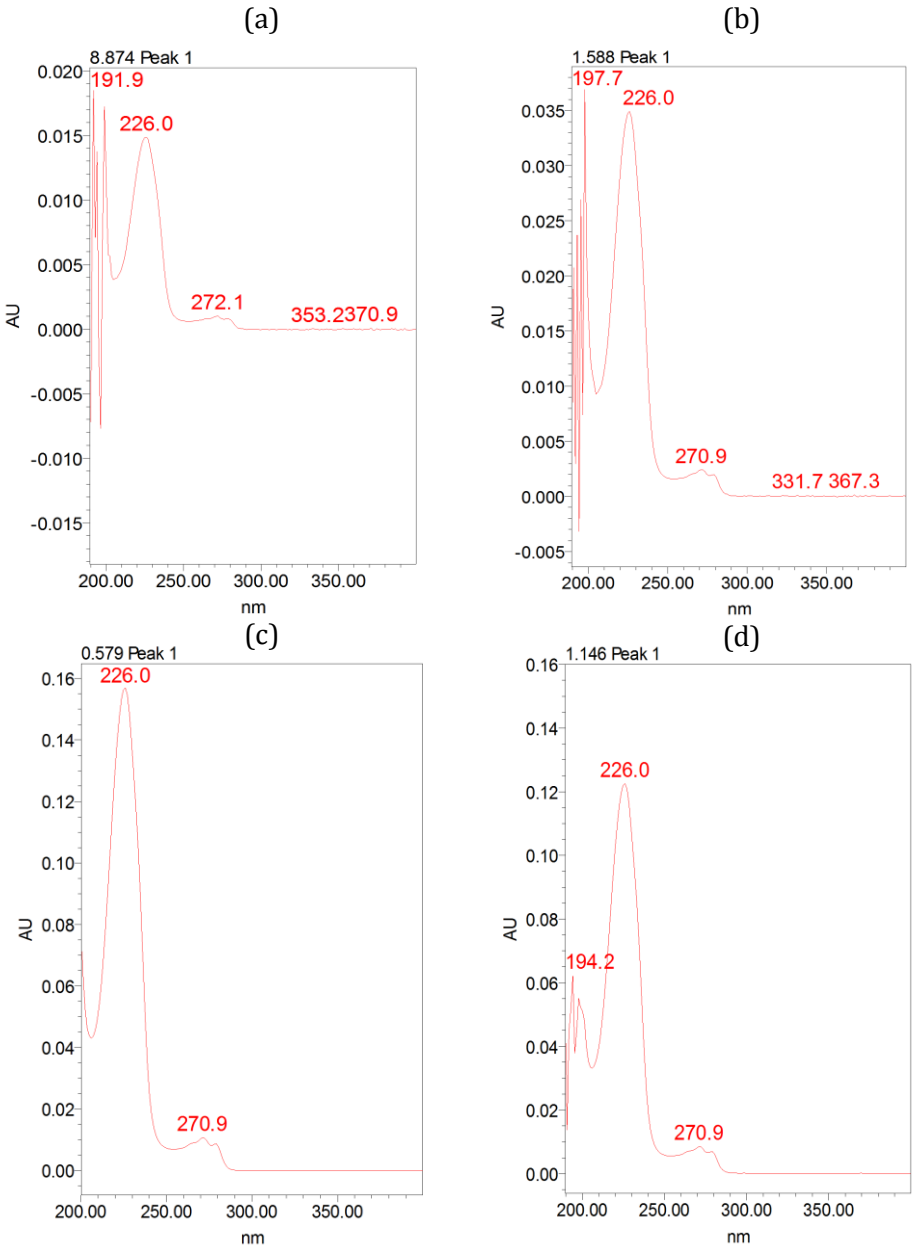


Figure 4.17. UV absortion spectrum of the benzoic acid of the four columns. Columns: Sil-k60-NH_2 (a), Sil-k100-comNH_2 (b), Sil-k60-EPOXY (c), Sil-k60-ibuprofen (d). Conditions: 200bar/40°C/10% MeOH.

The first hypothesis for this strange behaviour is the possibility of an extensive interaction between the stationary phase and the solute, that according to *Tozuka* (Y. *Tozuka et al.,* 2005), explains the adsorption and possible entrapment on the benzoic acid on mesoporous silica. The study claims that the great interactions between the benzoic acid and the silica can be caused due to a kind of Van der Waal dimer-like behaviour that it is facilitated by the small size of the pores where the benzoic acid will be trapped.

The behaviour of the "Sil-k100-comNH_2" supports this hypothesis, because although it is the same modification, the interactions between the stationary phase and the solute are smaller, having shorter retention times than with the self-package column.

However, the studies explain the dimer-like interaction strengthen by the small pore size, the review mentioned certain sections back about the pore size influence allows us to claim that the influence of the pore size was not the key-issue, so, another hypothesis had been proposed.

The new hypothesis explains these interactions using the hydrogen bonding concept. The hydroxyl of the carboxyl group of benzoic acid interacts with the hydrogens of the amine group of the stationary phase, creating a hydrogen bond interaction that affects the retention times. These bounds should be strong enough to enlarge the retention times and retain the solutes.

After analysing the benzoic acid, the full set was checked, finding that all the solutes belonging to the Basic/Acid group had significantly larger retention times at lower modifier concentration. The retention times for all the set, using "Sil-k60-NH_2"as stationary phase, are shown in the Figure 4.18.

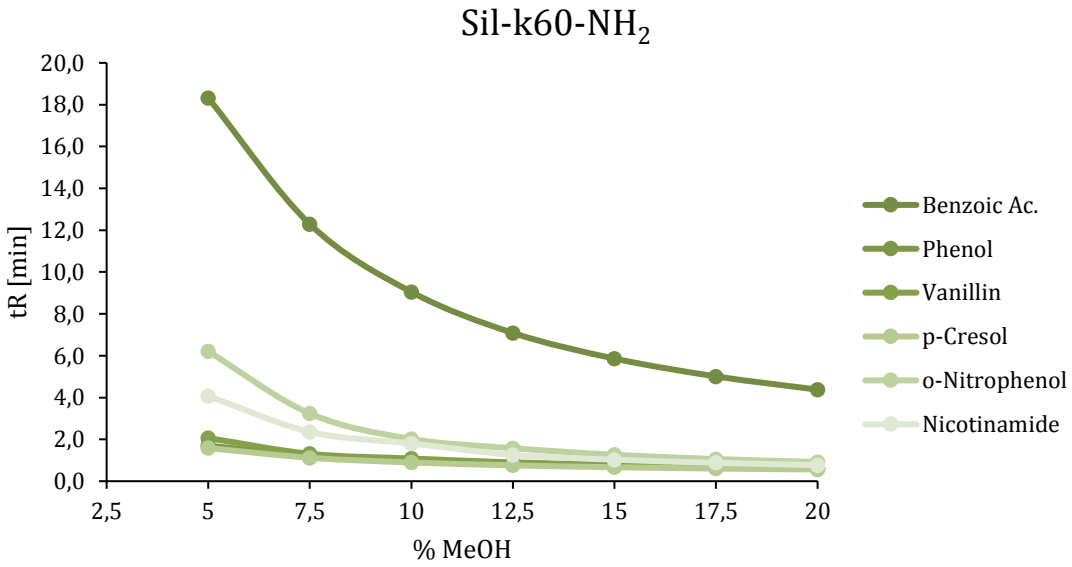


Figure 4.18. Variation of the retention times for the Basic/Acid group in the concentration of modifier for the column "Sil-k60-NH_2". Conditions: 200bar/40°C.

The Hydrogen bonding hypothesis is applicable to all these solutes due to the presence of hydroxyl groups that are suitable to interact with the amine group of the stationary phase.

4.5. ANALYSIS BASED ON LSER MODEL

Linear solvation energy methods, based on *Kamlet a*nd *Abrahams* models (*J. Kamlet et al.,* 1985), have provided lot of information for a wide range of compounds performing the analysis in commercial columns. One of the main advantages of this model is the extensive research that it has been submit, resulting in a large collection of scientific texts dealing about it. The other great benefit is the amount of information that LSER provides about the interaction between solutes and stationary phase, polarity and other properties.

According to the LSER model, the relationship between a chromatographic property, expressed by the logarithm of capacity factor generally, and a certain number of parameters can be written as:

$$Ln\ k = c + eE + sS + aA + bB + vV \qquad (12)$$

As was presented before, the parameters are represented by a pair of letters: one uppercase letter that is named descriptor and represents the solutes properties and a lowercase letter that represents the interaction between those solutes and the stationary phase.

Although the description of these parameters was performed in Chapter 2, a brief overview to refresh the memory is included for the interested reader: E, S, A, B, and V represent the solutes for excess molar refraction, dipolarity/polarizability, hydrogen bond donor ability (acidity), hydrogen bond acceptor ability (basicity), and volume, respectively. On the other hand, the coefficients c, e, s, a, b and v describe the net effect of the interaction between the mobile and stationary phases with the solutes. It is critical to remember that those coefficients are obtained by multilinear regression of the retention factors obtained in the SFC experimental process.

In following pages, the data obtained during the experimental phase are applied to perform the LSER analysis and the subsequent study of the results.

4.5.1. STATISTICS AND GOODNESS OF THE REGRESSIONS.

The LSER model requires a multilinear regression to obtain the coefficients that represent the interaction between the mobile phase, stationary phase and the solutes. The statistics of the regressions for each column are shown in Table 4.3. The coefficient of determination (R^2) are between 0.72 and 0.79 in all the selected cases.

Table 4.3. Statistics of the regression at 200bar, 40 °C, 10% MeOH for all the selected columns.

STATISTICS OF THE REGRESSION

STATIONARY PHASE	R SQUARED	ADJUSTED R SQUARED	F	F CRITIC
SIL-K60-EPOXY	0.8556	0.7900	13.04	0.000258
SIL-K60- IBUPROFEN	0.8290	0.7513	10.66	0.0006298
SIL-K60- NH_2	0.8052	0.7166	9.093	0.001245
SIL-K100- comNH_2	0.8425	0.7709	11.76	0.0004093
SIL-K100- C18	0.8196	0.7376	9.994	0.0008341

The R^2 obtained is not a criterion enough to determine the validity of the regressions, so to this end, other statistic parameters like F-value and P-value are going to be analysed.

The small values of the F critic comparing with the F-value allow us to reject the null hypothesis. The F critic for each column is much lower the value of F, so it can be considered that at least one of the descriptors used for that purpose is significant.

The significant of the F-value does not assure us the significant of all the coefficients obtained with the regression. So, a second criterion, should be applied: p-value. This hypothesis stablishes that every coefficient which has a p-value that is higher than alpha level, has this chance of being a random result, so they can be excluded. The alpha level selected to our determination was 5%, so each coefficient which has a p-value higher than this amount was considered not significant.

All the coefficients of determination obtained during the statistical study that have a significance higher than 95% are available in the Appendix B. Those regressions that are not significant have an asterisk in the cell instead.

One of the key aims of the application of LSER is the use of the model to predict the behaviour of the solutes with a fixed stationary phase. In Figure 4.19, the predicted ln k is plotted against experimental ln k. Predicted ln k was obtained from the regressions of the retention data of the 17 solutes, applying the equation of the LSER model, while the experimental ln k was obtained during the experimental research in the laboratory.

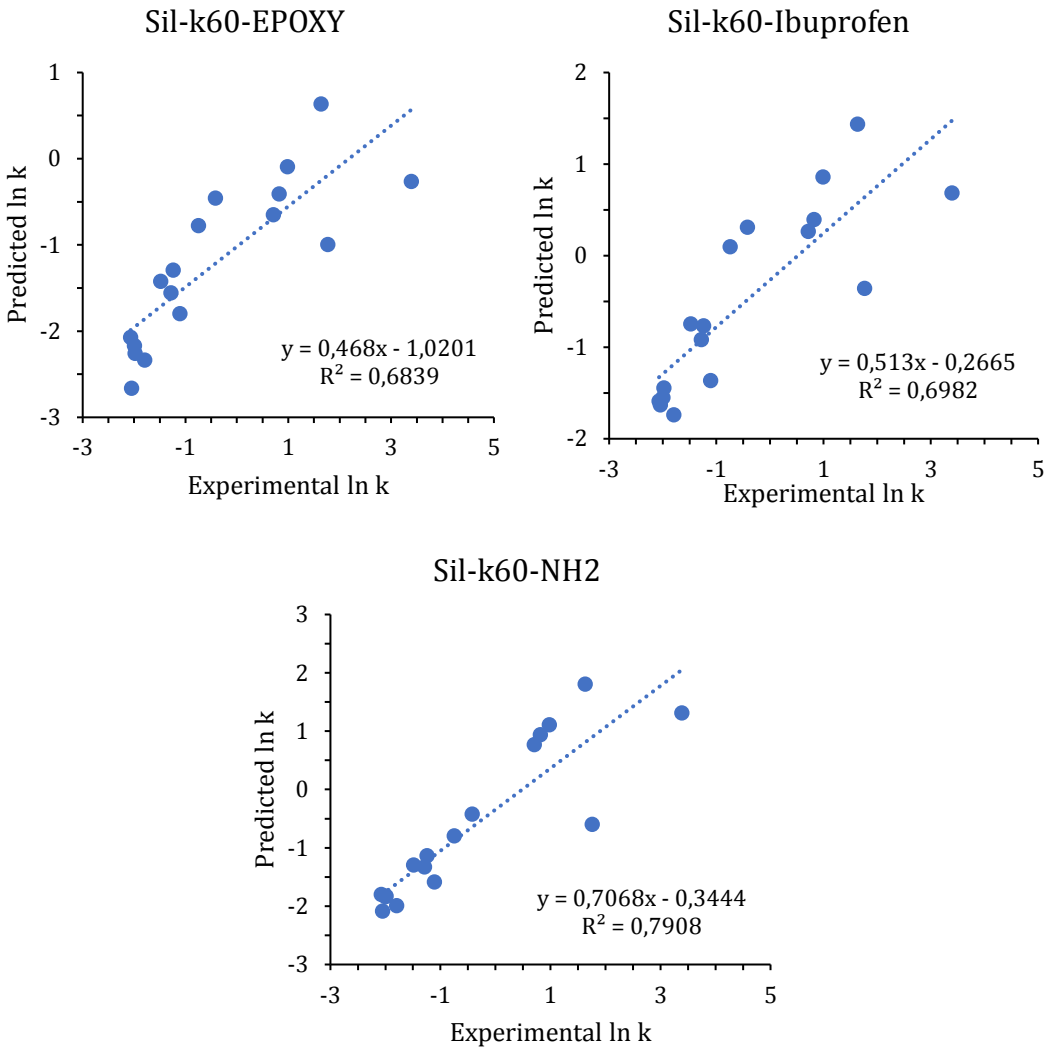


Figure 4.19. Predicted ln k vs experimental ln k for the functionalized self-packed stationary phases. Conditions: 200bar/40°C/10% MeOH.

The coefficients of determination, R^2, for these cases have values relative small, that means that the approximation between theoretical and experimental values is not very suitable. The "Sil-k60-NH_2" shows a bit higher R^2, that means that in that case the adjustment of the model is quite better, but still not enough.

Noting the charts, some points seem to be deviated from the set. After checking the values of these points, it was noticed that they belong to o-Nitrophenol and Nicotinamide. During the performance of the experimental, the determination of the retention times of some solutes had certain hiccups and the chromatographic analysis was performed repeatedly. This special condition could affect the values of retention times and consequentially the performance of the regressions.

On one hand, deleting the two compounds provides a better adjustment of the data and avoids likely errors caused by the measures. On the other hand, the rejection of these compounds makes the regression less robust due to the decrease in the number of solutes evaluated.

Figure 4.20 shows the comparison of the regressions using 15 solutes and 17 solutes to predict the ln k. "Sil-k60-NH_2" has been taken as example of how the regression changes. A slightly improvement was observed, changing from 0.7908 for 17 solutes to 0.8922 for 15 solutes.

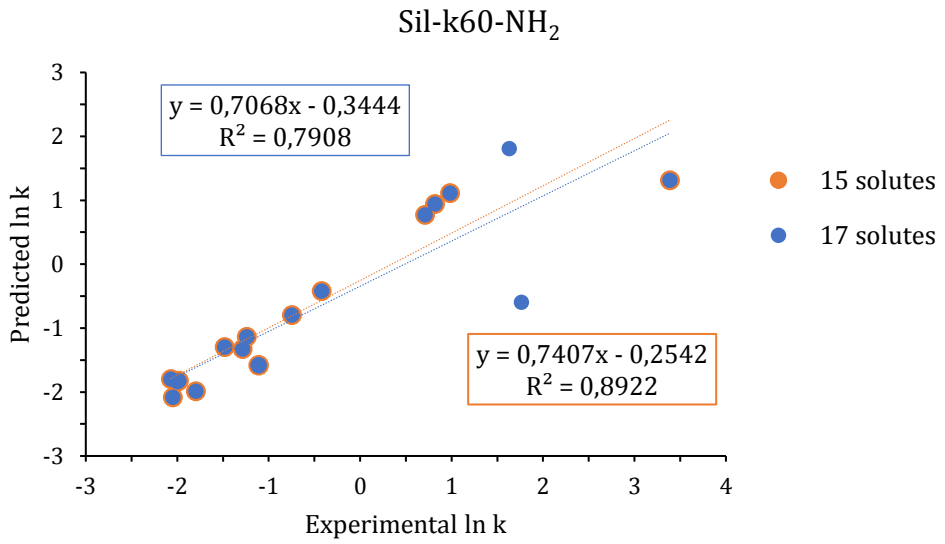


Figure 4.20. Predicted ln k vs experimental ln k for "Sil-k60-NH_2" comparison between regressions with 17 and 15 solutes. Conditions: 200bar/40°C/10% MeOH.

This substantial improvement reveals that the performance of the solute analysis has a very strong influence in the model. Some differences in the conditions and the retention times have a large influence in the adjustment and the goodness of the model.

After the analysis of the statistical parameters obtained with the multilinear regression, it can be concluded that the regressions shall be carefully treated to predict the behaviour of the retention. The analysis of the solutes to obtain the retention times should be performed carefully to minimize the errors; however, the LSER model can be still used to characterize the stationary phase due to the large amount of information that it provides, the relative high values of R^2 obtained and the significance obtained for the model.

In following pages, an in-depth analysis of the coefficients, their significance, the differences between the columns and their trends with the variation of pressure, temperature and concentration of modifier will be developed.

4.5.2. LSER COEFFICIENTS

The system constants were obtained using the multilinear regression considering all the descriptors abovementioned: E, S, A, B and V. The methodology applied allow us to obtain the coefficients from a regression that considers all the descriptors and then, using the p-value criterion, analyse the significance of each coefficient for each column. This methodology provides us a general equation, that considers all the significant coefficients.

4.5.2.1. Analysis of the parameters according the stationary phase.

Hereafter, the coefficients obtained using each k60 functionalized column are shown in Table 4.4. The meaning of these values without their error and their significance does not mean anything for a properly analysis, so the corresponding error and p-values were added in the Appendix C.

Table 4.4. LSER coefficients obtained at 200bar, 40 °C, 10% MeOH for all the selected columns.

	SYSTEM COEFFICIENTS					
STATIONARY PHASE	c	e	s	a	b	v
SIL-K60-EPOXY	-2.5171	1.0706	1.3078	2.4776	0.9313	-1.4729
SIL-K60- IBUPROFEN	-2.4615	1.0035	1.1819	3.1072	0.0850	-0.7036
SIL-K60- NH_2	-2.2733	0.6811	1.1360	4.8177	-0.0410	-0.9621
SIL-K100- comNH_2	-3.1364	0.3841	1.2249	4.0326	0.4575	-0.7270
SIL-K100- C18	-2.7275	1.1714	-0.9584	-1.4988	-1.4455	1.4468

In the view of the results, the different nature of "Sil-k100-C18" is obvious now. While the other compounds have almost every coefficient positive, the reverse stationary phase has a completely disparate. Following, each coefficient is going to be carefully analysed and then more information will be provided.

According to *West* and *Lesellier*, *2005,* the coefficients can be understood as the difference between the interactions of the solute with the stationary phase and the interaction of the solute with the mobile phase. Following this criterion, the meaning of the results obtained is going to be explained in next paragraphs.

The first parameter that takes the attention is the c coefficient. This term represents the relation between the stationary phase and the mobile phase and it is inherent of each column. It should remain almost constant in all the regressions performed with a column; despite this fact the small variations that can be detected could be caused due to the variation in the ratio V_{SP}/V_{MP}.

The e term represents the π-π interactions between the solutes and the surface of the modified silica or the mobile phase. As in all the cases it has a positive value for e, that allows us to think that the interaction with the mobile phase has little impact. However, it is important to underscore the amine columns cases, where the e has clearly decreased.

The s term depicts the strength of the dipolarity/polarizability capacity. The "Sil-k100-C18" is the only one that has a negative value for this term and this is caused due to its non-polar nature. Along all the columns, this parameter has a special significance in almost each condition, so it can be considered as one of the characteristic coefficients for our model.

The a term represents the proton donating capacity of the solute. So, in certain way, it can be used to approach a measured of the capacity of the mobile or the stationary phase to accept those protons. Its high values, the highest of all the coefficients, show this term as the main and most important coefficient in all the columns but the non-polar. The change of this parameter across the columns is clearly observed and as it was expected, the amine stationary phase, as a base, have the largest values.

As the previous one represents the donating capacity, the b term represents the donor capacity. This term has not special statistical value, because of the nature of the selected functionalized silica; however, it is important to notice that, with the coefficient a, b term is the parameter that has the large deviation along the columns.

The v term represents the difference in dispersion interactions and cavity effects. There are negative values in all the columns, but the non-polar once again. This negative value demonstrates that the interactions between mobile phase and solutes govern this coefficient.

Figure 4.21 corresponds to a summary of all these ideas, representing only our modified stationary phases. In general terms, the greatest changes were revealed for the b and a term. This fact is specially noted in the amine stationary phase, which basic nature of the amine group explains the high a values obtained. Undoubtedly, the column that has the most profound changes regarding all the coefficients is the amine column whose parameters can be clearly distinguished at the chart in purple.

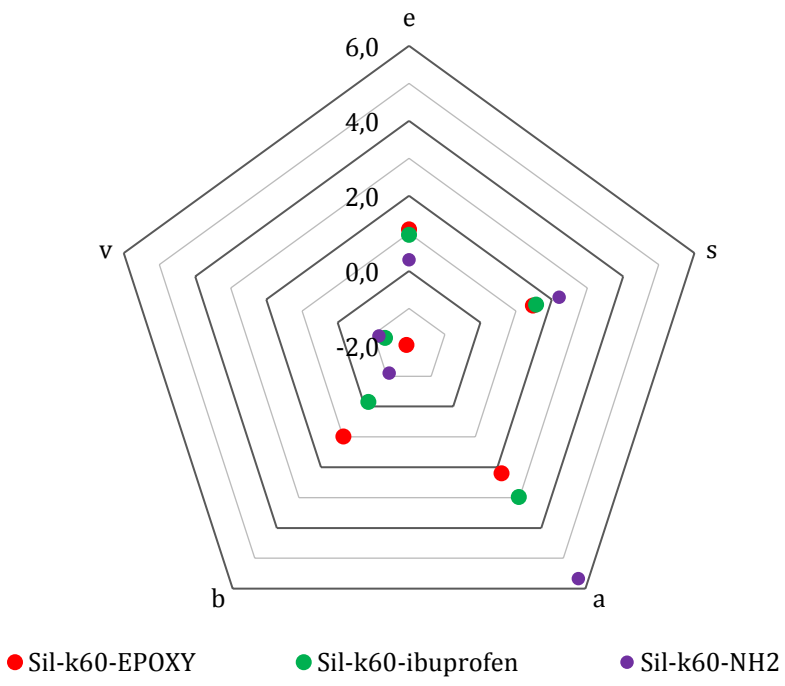


Figure 4.21. Comparison of LSER coefficients between the different modified columns selected. Conditions: 200bar/40°C/10% MeOH.

4.5.2.2. Improvement of the approximation: Introduction of a simplified LSER model.

After analysing the Figure 4.21, the idea of making a second regression set raised.

According to the regressions performed with five descriptors based on equation 2, the only parameter that it is presented in all of the performance is the a, correspond with the hydrogen donor capacity factor, and this is why an extensive series of regressions were performed. As it was mentioned in "Statistics and goodness of the regressions" the Appendix B shows all the regressions of possible combinations and the determination coefficients (R^2) of the significative ones. In following pages, the general model with five descriptors is compared with a second model with only two significant descriptors.

Because of the significance of parameters and the goodness of the regressions, that can be shown in Appendix C, the equation selected by this second approach was:

$$Ln\ k = c + sS + aA \tag{13}$$

"Sil-k100-C18" will not appear in the subsequent studies because the selected equation does not fit the stationary phase due to its singular nature. To obtain a second approach for this stationary phase the next equation should be used instead; however, the study for this column has not been performed.

$$Ln\ k = c + eE + vV \tag{14}$$

The new equation presents generally adjustments that are not so far from the determination coefficients obtained using five descriptors. In Table 4.5 a summary of the statistical parameters of the new regressions in the modified stationary phases for the 2 parameters model is shown.

Table 4.5. Statistics of the second approach at 200bar, 40 °C, 10% MeOH for all the selected columns.

	STATISTICS OF THE REGRESSION			
STATIONARY PHASE	**R SQUARED**	**ADJUSTED R SQUARED**	**F**	**F CRITIC**
SIL-K60-EPOXY	0.7627	0.7288	22.49	4.25×10^{-5}
SIL-K60- IBUPROFEN	0.7547	0.7197	21.53	5.34×10^{-5}
SIL-K60- NH_2	0.7708	0.7381	23.54	3.32×10^{-5}
SIL-K100- comNH_2	0.8123	0.7855	30.29	8.21×10^{-6}

Comparing with the previous model, this multilinear regression provides a slightly worse goodness of fitting than the initial one. The coefficient of determination, R^2, are between 0.7197 and 0.7855 in all the selected cases, so the new goodness of fitting is not significantly worse than the case of the model with five descriptors.

As in the previous case, the R^2 is not a criterion sufficient to determine the validity of the regressions, so F-value and P-value were compared too. The F critic is smaller than F for all the stationary phases, and the p-values of each parameter is less than 0.05, what ensures the significance of the parameters used and the coefficients obtained.

Finally, to check it the new equation can be used to predict the retention times, the ln k calculated for the second regression against the experimental data obtained during the experimental phase. Figure 4.22 illustrates the relation between the predicted ln k and the experimental ln k.

These charts show as the regressions, a slightly worsening; however, if it is applied the same criterion as in the previous one and a new regression with 15 solutes is performed, the improvement for this second model is much better than the general equation, obtaining even adjustments between experimental and predicted like 0.89 for amine or 0.98 for ibuprofen stationary phase.

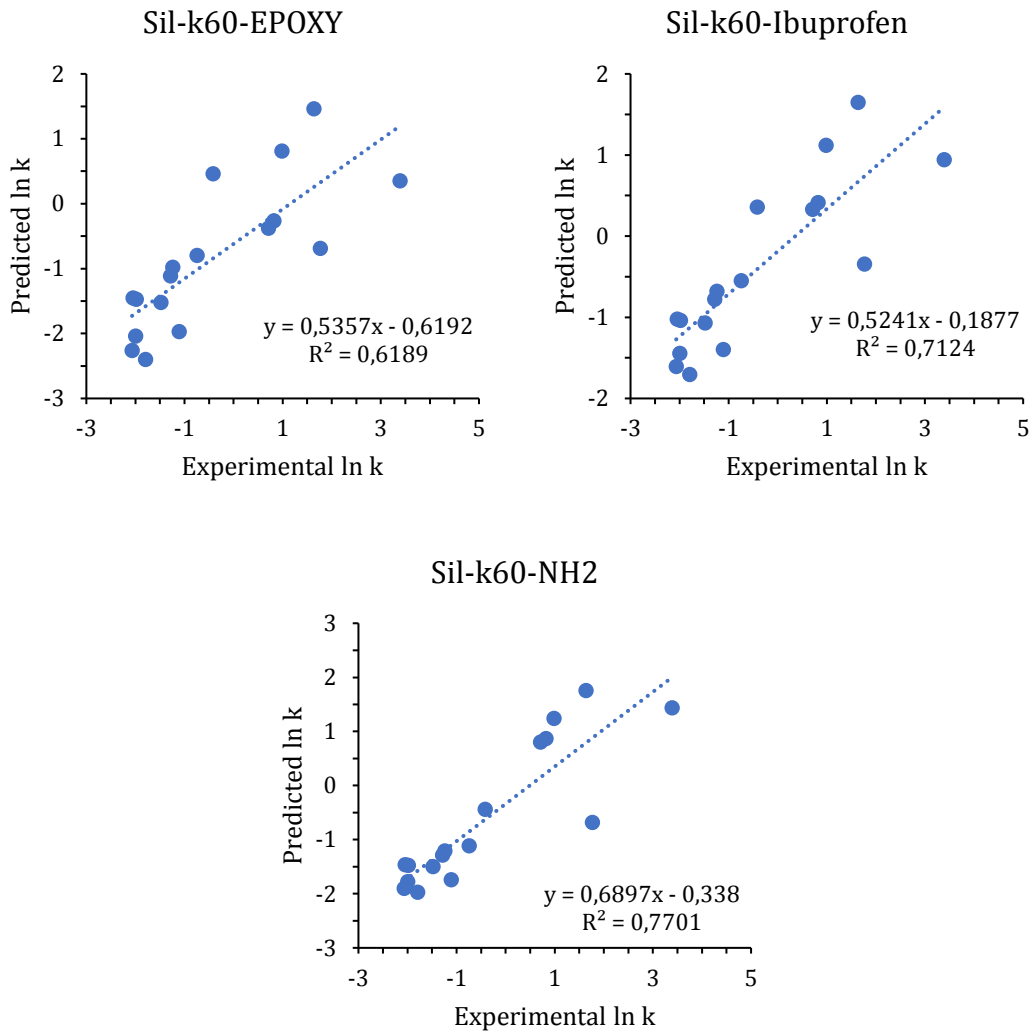


Figure 4.22. Predicted ln k vs experimental ln k for the functionalized self-packed stationary phases with the 2 descriptors model. Conditions: 200bar/40°C/10% MeOH.

The meaning of the coefficients was explained in the previous regression, so it would not be explained again. In general terms the variation in the value of c can be caused due to the change in the ratio V_{SP}/V_{MP} characteristic of each stationary phase. The positive values of s and a determine the domination of the solute-stationary phase interactions against the solute-mobile-phase interactions.

The new coefficients and the error calculated during the regression are shown in the following table.

Table 4.6. LSER coefficients obtained at 200bar, 40 °C, 10% MeOH by 2 descriptors model.

	SYSTEM COEFFICIENTS					
STATIONARY PHASE	**c**	**Sdev**	**s**	**Sdev**	**a**	**Sdev**
SIL-K60-EPOXY	-3.581	0.411	2.859	0.356	2.156	0.525
SIL-K60- IBUPROFEN	-2.612	0.499	1.637	0.433	3.157	0.639
SIL-K60- NH_2	-2.733	0.375	1.206	0.325	5.024	0.480

Comparing the new coefficients with the obtained using the general regression (available in Appendix C), the errors found during these second regressions are quite similar. So, it is thought that the goal of this second regression is to obtain as similar accurate coefficient values as the general model and know the trend of the most significant coefficients.

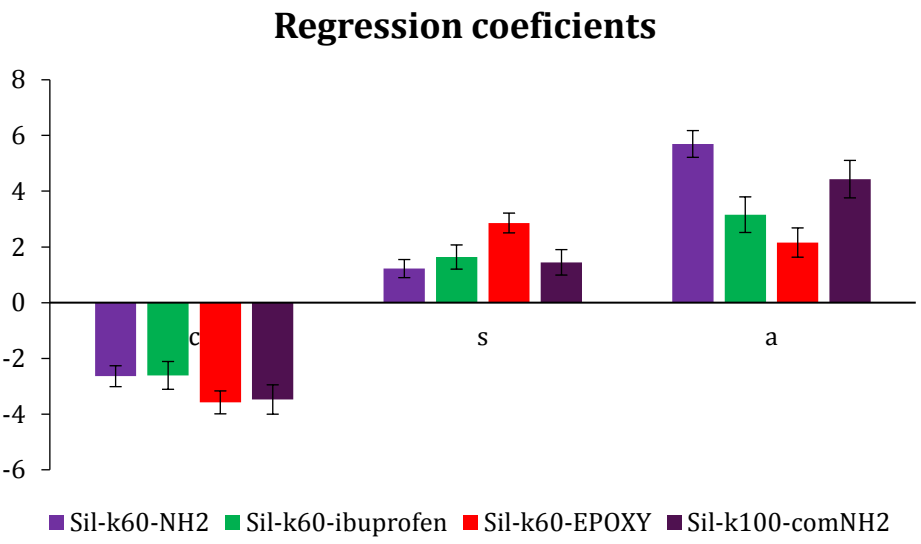


Figure 4.23. LSER regressions coefficients obtained by the 2 descriptors model. Conditions: 200bar/40°C/10% MeOH.

Hereunder, an analysis of the influence of pressure, temperature and concentration of modifier in the coefficients for both regressions will be performed.

4.5.3. INFLUENCE OF OPERATIONAL PARAMETERS.

After analysing the influence of the stationary phase selected, the aim of this paragraph is the analysis of the influence of the variation of pressure, temperature a concentration of modifier across the columns.

To perform an appropriate analysis, the operating scheme to follow is: A general overview of all parameters, shown in a selected column; and an in-depth analysis focusing in significative coefficients. The presence of the a term in all the columns (but "Sil-k100-C18") marks this coefficient as the essential one, as it was settled in the previous section "Improvement of the approximation".

4.5.3.1. Pressure influence

The LSER coefficient were evaluated at different pressures, from 120 to 300 bar. The results for the all stationary phases are plotted in Figure 4.24.

For all the stationary phases, it is shown that the strongest interactions are given by a, as it was predicted, and s, due to the polarity of the stationary phase.

In the case of amine columns, the value of a is clearly higher than the other coefficients showing the dominance of the acidity capacity above the other interactions. This fact is less obvious in the case of "Sil-k60-EPOXY" whose values of a are really near the other coefficients.

The effect of polarity and dipolarity, represented by coefficient s seems to be almost constant for all the columns and the values are around 2. The coefficient e has values around cero and one, what means that the interaction produced by π-π relations with the solute is almost equal for the stationary phase and the mobile phase, it is important to highlight that the apparent rise in the value of e that appears in the case of "Sil-k60-NH_2" and "Sil-k60-ibuprofen" is an small variation and may be caused due to the inner error of the values.

Finally, parameters b and v have negative values, what means that the influence nature of these coefficients is more related with the mobile phase than with the stationary phase. However, the "Sil-k60-EPOXY" presents a positive value and slightly higher of b term.

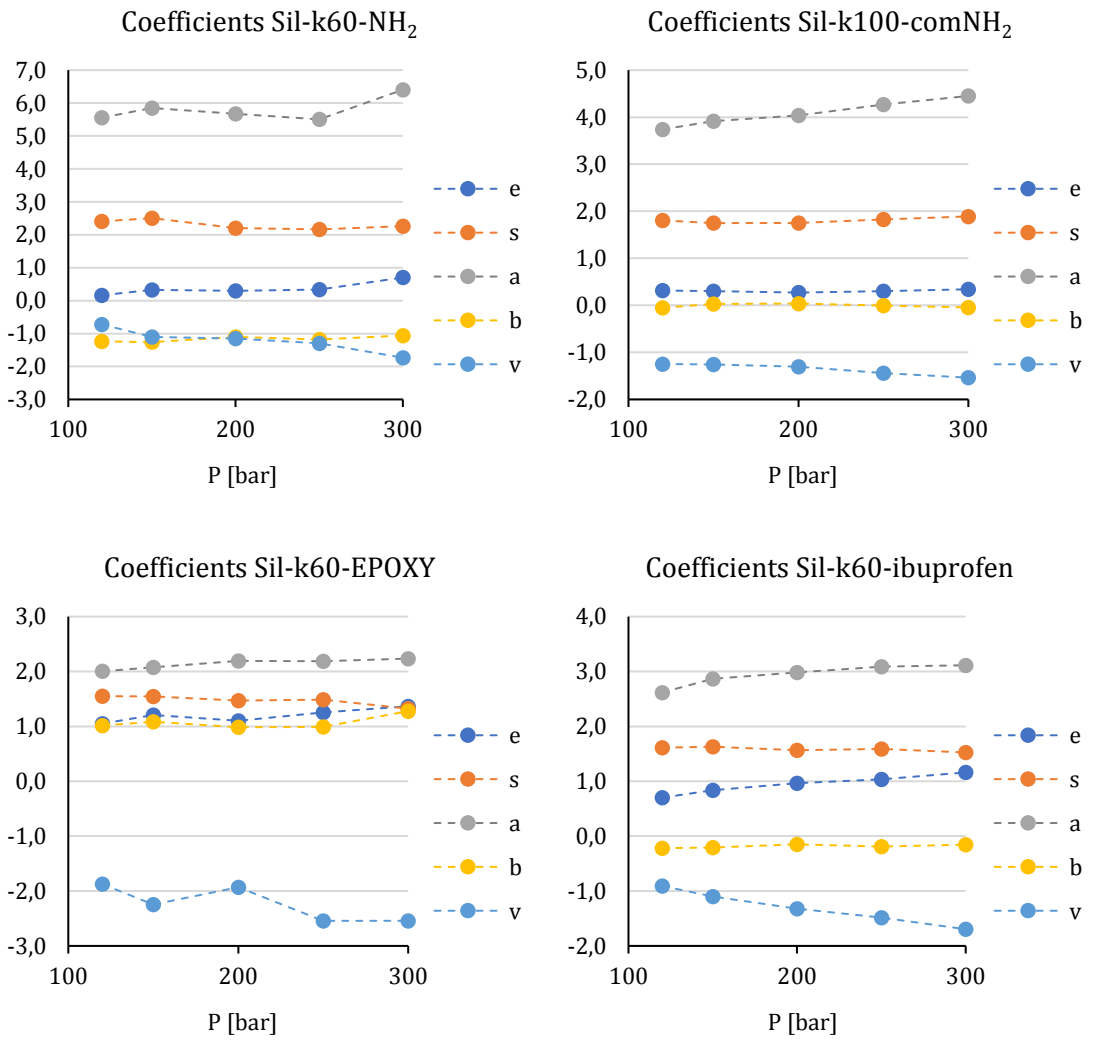


Figure 4.24. LSER regressions coefficients obtained in all selected stationary phases for pressure variation. Conditions40°C/10% MeOH.

In the Appendix D the comparison for all the modified columns its shown. Analysing these charts, it is shown that pressure does not have special influence in the coefficients values. The coefficients seem to be almost constant or with a really small change across the pressure variation. In spite this fact the most affected coefficient is the v term. Due to the increase of pressure, an increase of density in the system is done. This variation in the density decrease the dispersion interactions between the solutes and the mobile phase, what means a decrease in the value of v.

LSER with 2 descriptors

The comparative results for all the stationary phases can be consulted in the Appendix E, and the results using the second approach are plotted in Figure 4.25.

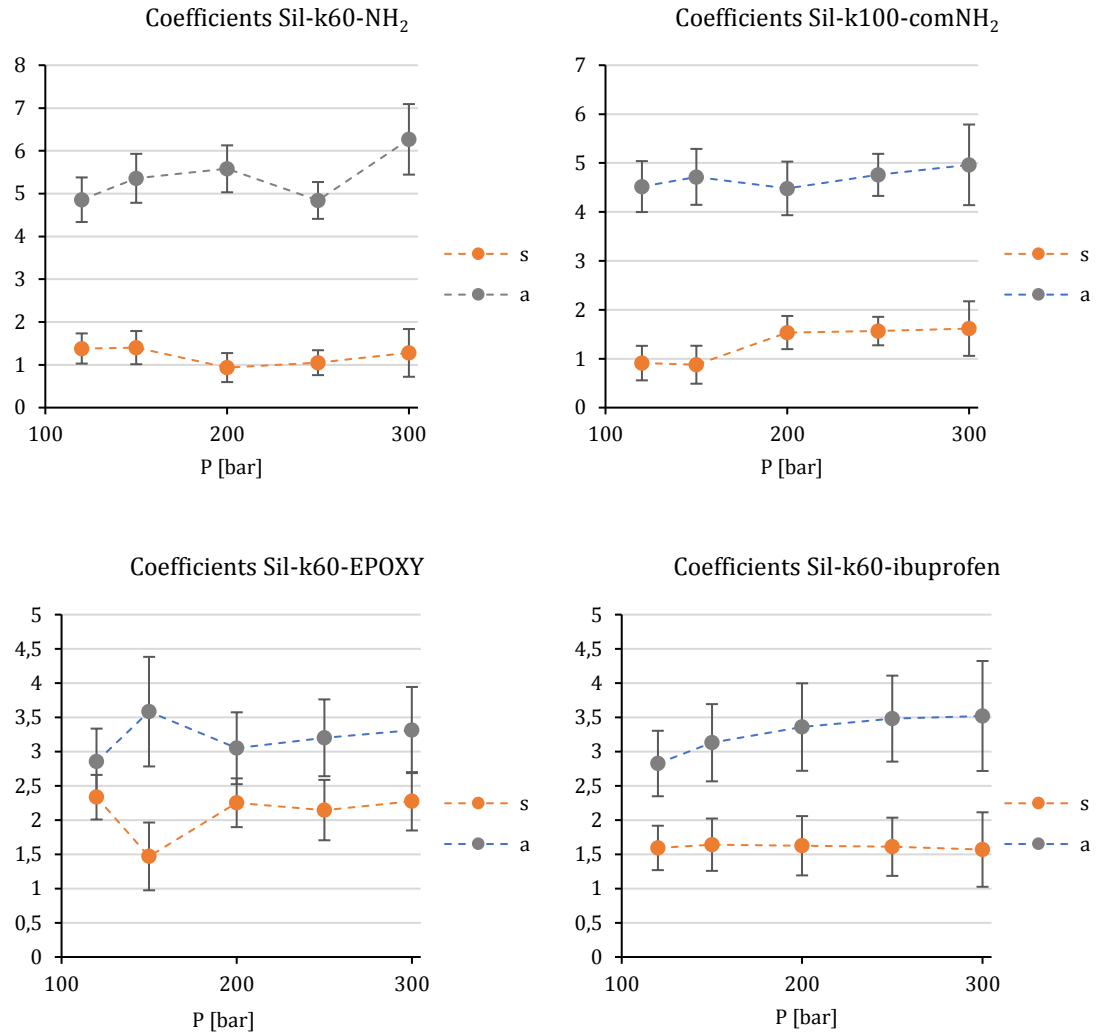


Figure 4.25. LSER regressions coefficients obtained for pressure variation using the 2 descriptors model. Conditions40°C/10% MeOH.

As in the general case, the strongest interaction is given by the a term that has similar values as the obtained with the general equation, being the "Sil-k60-NH_2" the stationary phase that presents the greatest variation in the value of a term. On the other hand, s remains almost constant for all the stationary phases.

All the columns maintain the trend that was seen using the general equation, but the values of a and s coefficient has slightly change. This variation is small enough to consider that it is concomitant to the method.

The trend of both parameters is clear enough now and corroborates the behaviour previously shown. The value of an increases with a rise of the pressure, what means that the capacity to accept protons of the stationary is moderately affected by a pressure increase. While the s term remains constant regardless the stationary phase and the pressure conditions.

4.5.3.2. Temperature influence

The LSER coefficient were evaluated at different temperatures, from 25 to 60 °C by fives. The results for the selected stationary phases are shown in Figure 4.26. It shows that the strongest interactions are given by a and s again. Coefficients b and v, has negative values, what means that the influence of them is more related with the mobile phase than with the stationary phase. And the e term seems to reduce his influence in the stationary phase-solutes interaction while the temperature is increased.

Even so, the influence of temperature in the parameters seem to be not significant, due to the almost constant values of the coefficients except in the case of a term for "Sil-k60-NH_2". Once again, the largest variations are detected in the a term and the v.

The v coefficient variation is explained because of the density variation caused by the temperature effect. As in the pressure case, the interactions with the mobile phase and solute determine this parameter, so any increase in temperature leads a decrease in the density and consequently a increase in the dispersion interactions, as it is shown in the Appendix D figures and the previous chart.

The a coefficient has a perceptible decrease due to the growth of temperature and the reason of this change is the effect of temperature in the pH of the media. Although the influence of temperature is slightly, it is well-known that a temperature rise causes more molecular vibrations, making easy to break the bonds between them. If the broken bonds contain hydrogen, the acidity of the bulk increases, reducing the hydrogen donor capacity. This action goes unnoticed for ibuprofen and epoxy stationary phases; however, the amine stationary phase liberates enough protons to make this effect significant and affects the a value.

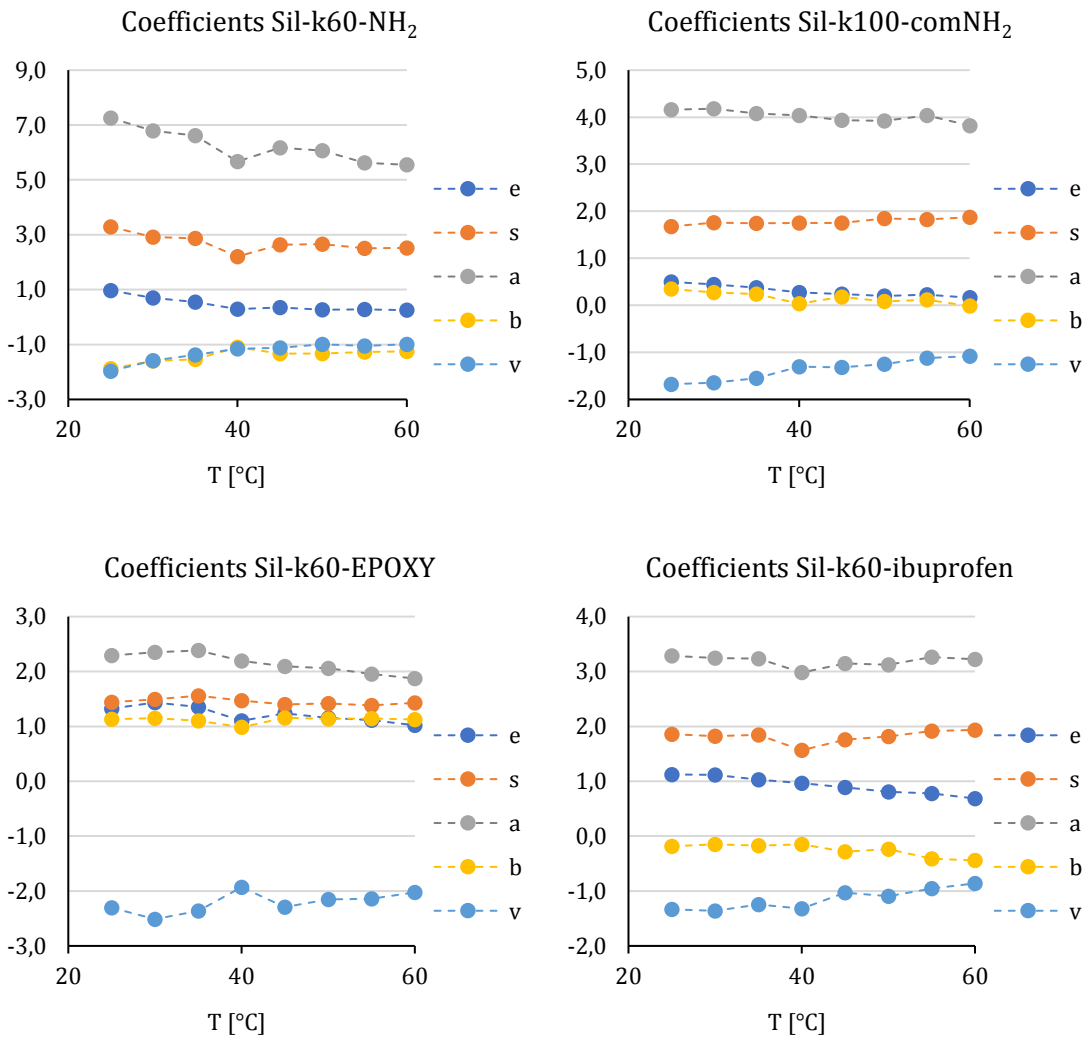


Figure 4.26. LSER regressions coefficients obtained in all selected stationary phases for temperature variation. Conditions: 200bar/10% MeOH.

LSER with 2 descriptors

The following figure shows the results obtained using the second equation for all the stationary phases. The error bars represent the standard error obtained during the regression for each coefficient in each condition. As in the previous case, the a term is the highest coefficient and comparing with the general equation regressions, the values obtained are contained in the range of the preceding ones.

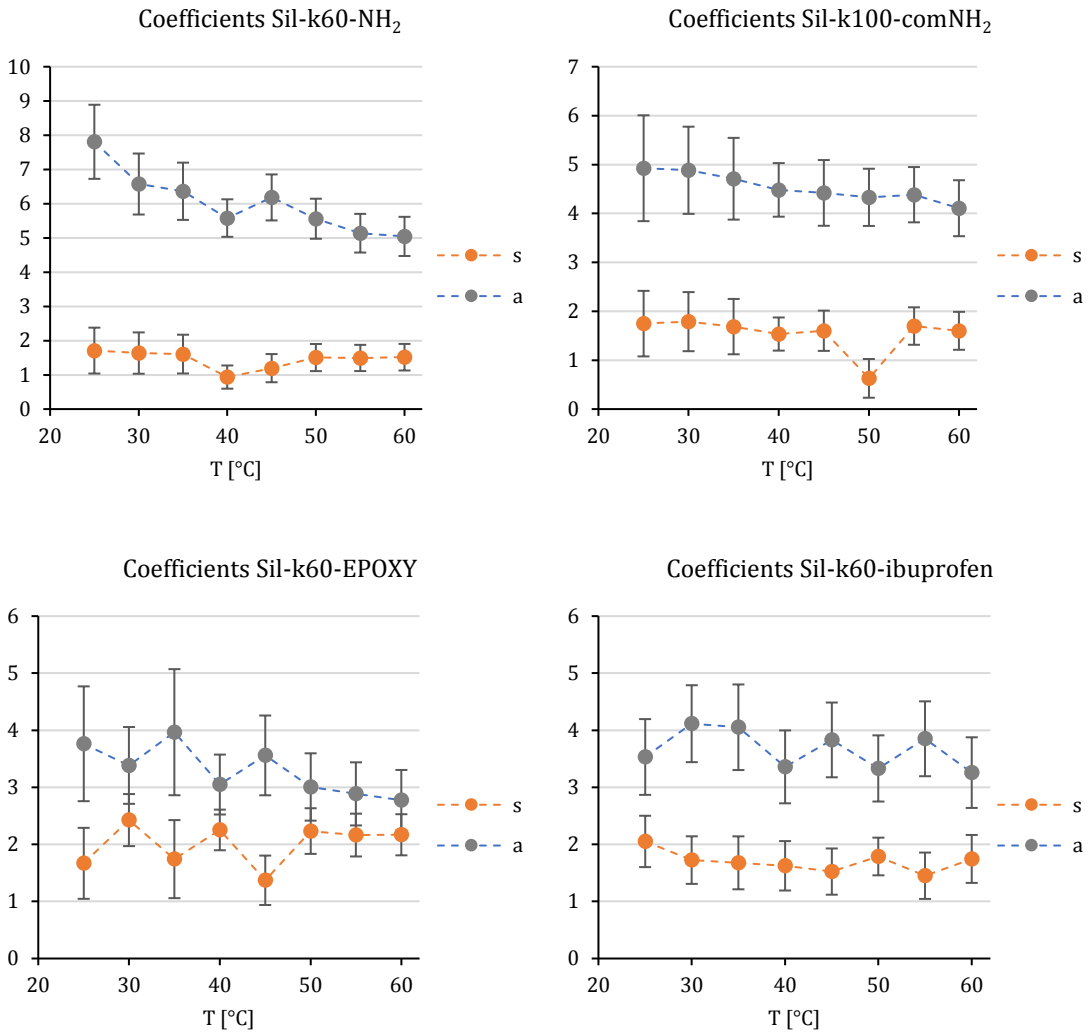


Figure 4.27. LSER regressions coefficients obtained for temperature variation using the 2 descriptors model. Conditions: 200bar/10% MeOH

It is observed that the temperature has a great influence in the coefficients obtained with "Sil-k60-NH_2" and that effect can be observed in the "Sil-k100-comNH_2" as well. On the other two stationary phases, "Sil-k60-EPOXY" and "Sil-k60-ibuprofen" the variation of the a term is barely appreciated and the high values of deviation, represented in the error bars make difficult the analysis of the trend.

So, it can be concluded that the a coefficient is affected by temperature variation, decreasing when the temperature rises for all the stationary phases. It is important to note that this variation is largest as soon as the polarity of the modified stationary phase increases.

The s term remains constant for all the stationary phases, meaning a difference with the general equation in the "Sil-k60-NH_2" case, in which the variation of the s term is almost one unit.

4.5.3.3. Modifier concentration influence

The LSER coefficient were evaluated at different concentrations of methanol in the mobile phase from 5% to 20% with increments of 2.5%. The results for all the stationary phases are plotted in Figure 4.28.

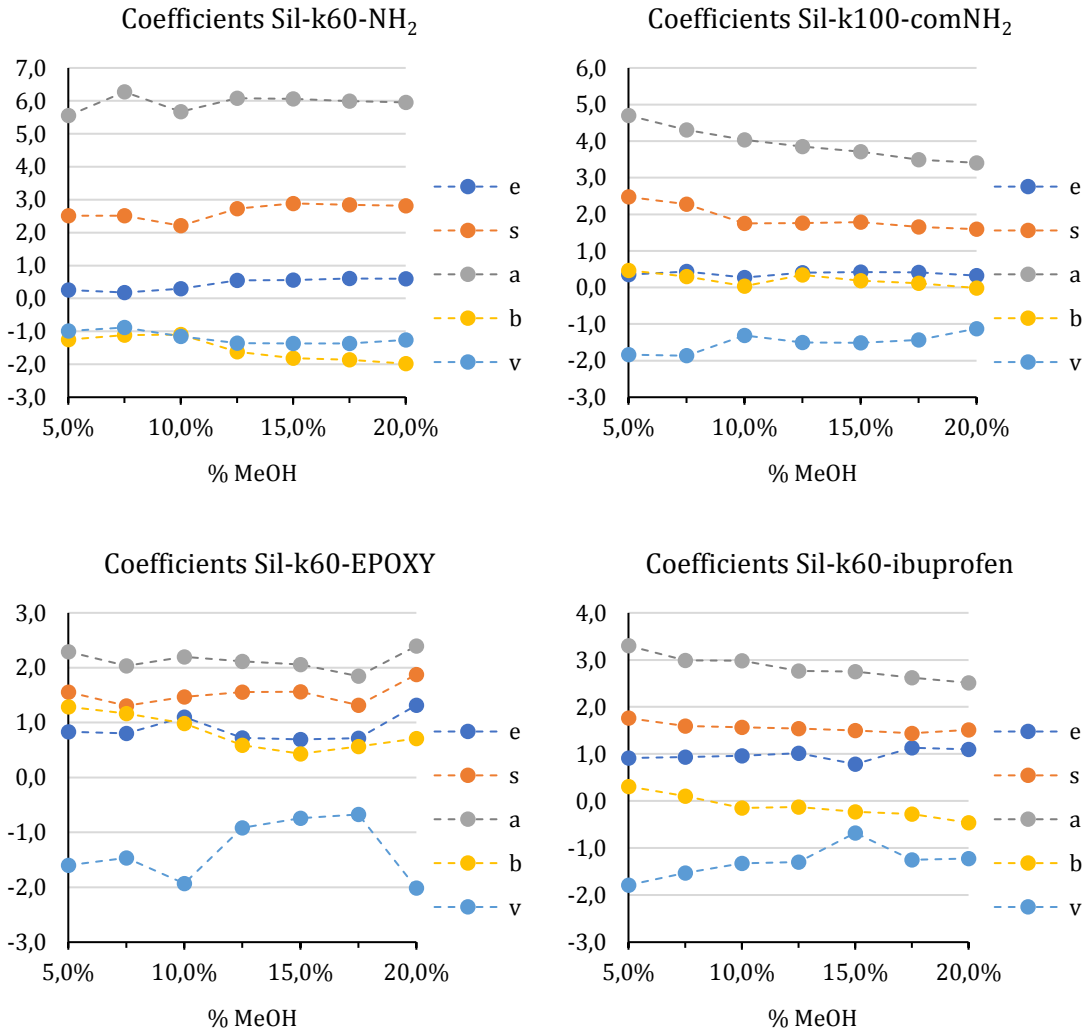


Figure 4.28. LSER regressions coefficients obtained in all selected columns for concentration of modifier variation. Conditions: 200bar/40°C.

It shows that the strongest interactions are given by a, because of the hydrogen donor capacity, and s, due to the polarity of the stationary phase. According with literature, the a term should present the largest variation with the rise of modifier; however, it only presents a variation of 25%. This small variation allows us to think that the methanol is not much adsorbed in the surface, fact that according with *West & Lesellier*, causes a great increase in

the a term. Besides the high values of a obtained for "Sil-k60-NH_2" reveal a great interaction between the stationary phase and the solutes, instead.

The coefficient e has values similar to cero that means that the interaction produced by π-π relations with the solute is almost equal for the stationary phase and the mobile phase and the charge-transfer between them is compensated.

Parameters b and v have negative values, what means that the influence nature of these coefficients is more related with the mobile phase than with the stationary phase. The b parameter decreases with the increase of concentration of methanol in the mobile phase, as it was expected. It is important to highlight here the changes in b: The rapid decrease in b after adding 10% methanol is due to an increase in the acidity of the mobile phase.

Finally, the addition of methanol in the mobile phase produces a increasement in the mobile phase polarity, which causes a decrease in the dispersion interaction between the solute and the mobile phase

LSER with 2 descriptors

As it was mentioned before, the a term should present the largest variation with the rise of modifier. However, the change presented in the a term with the modifier is still far to be the greatest. The variation has increase from 25% to 43% for the "Sil-k60-NH_2" but he medium variation of the other stationary phases is about 60% of the initial value.

Although the behaviour is not exactly as it was expected, the trend in the a term is clear and it leads a decrease while the concentration of modifier is increasing.

The s term has a clear constant trend, but two mayor differences has been noticed: The value of s for "Sil-k60-NH_2" have decrease dramatically, what can be cause for the lack of the other terms to explain the interactions and with "Sil-k60-EPOXY" a rise parallel to the increasing of concentration can be observed.

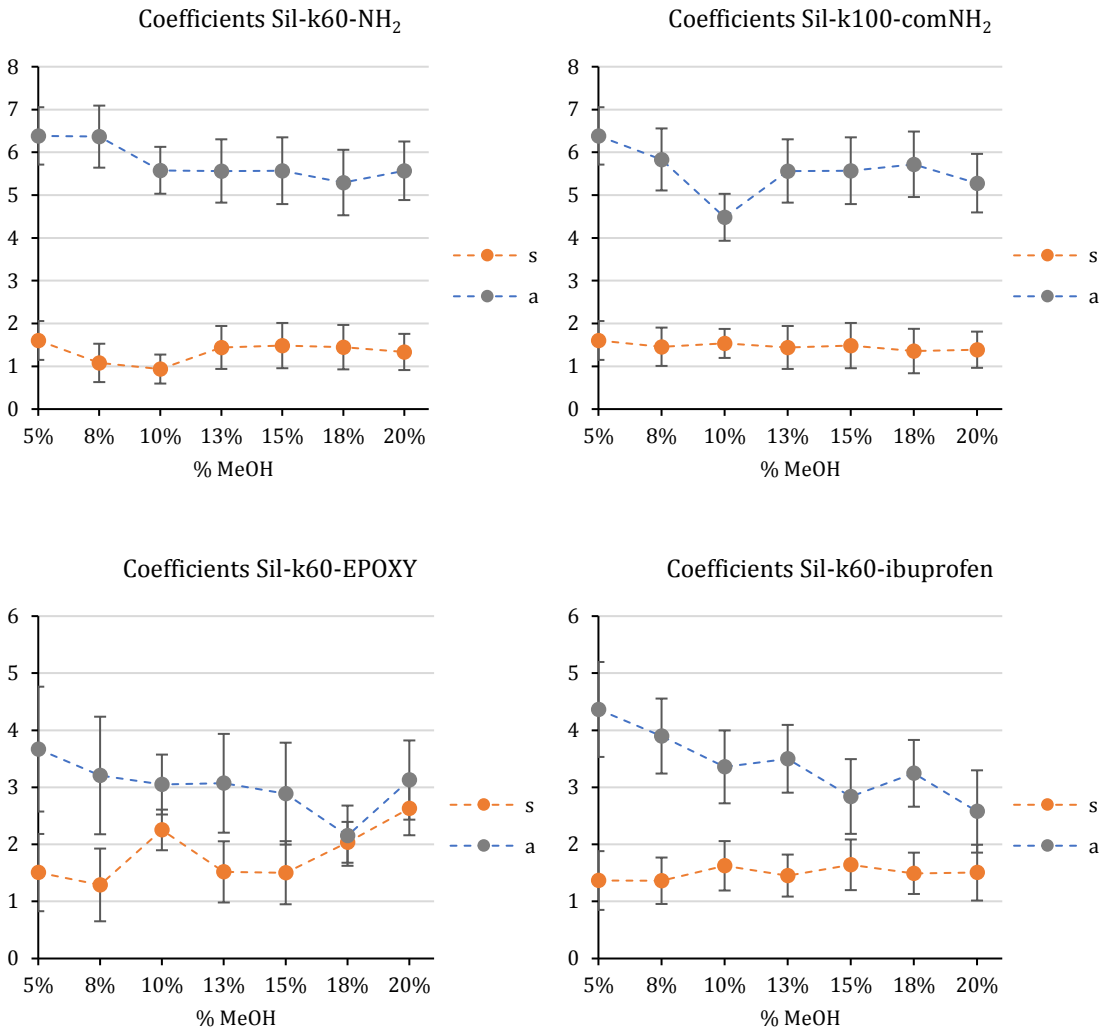


Figure 4.29. LSER regressions coefficients obtained for Concentration of modifier variation using the 2 descriptors model. Conditions: 200bar/40°C.

After analysing the variation of the coefficients with pressure, temperature and concentration of modifier; the study reveals an unexpected result: The greatest variations in coefficients, specially the a parameter, is shown with the temperature variation. This result is in striking contradiction with previous studies which state the insignificance of the temperature and the slightly significance of pressure in the coefficients behaviour.

The new approach, obtained with the 2 descriptors regression, has been discussed and after that, it can be concluded that: On one hand, this second approach represent an improvement compared to the general equation due to the accuracy of the new coefficients and the great enhancement in the prediction of retention times. On the other hand, the amount of information provided performing the regression with all the parameters cannot be compared with the more succinct information provided for the specific equation.

4.5.4. TRANSFERABILITY OF SILICA-MODIFIED COLUMNS

The transferability, according with the IUPAC, is the assumption that a chemical property that is associated with an atom or a functional group in a molecule will have a similar, but not necessary identical, value in certain circumstances.

Comparing two materials with a fixed pressure, temperature and modifier concentration, a method to find the transferability between them is obtained. The equation 15 represents the linear relation between the adsorption constant, K_{ads}, of two different materials, X and Y, where α and β are constants:

$$Ln\ K_{ads}(X) = \alpha\ \ Ln\ K_{ads}(X) + \ \beta \tag{15}$$

This relation can be written for the capacity factor, k, considering that:

$$k = \ K_{ads}\ \left(\frac{V_{SP}}{V_{MP}}\right) \tag{16}$$

Trying to quantify the transferability and modification of the new stationary phases, after performing the chromatographic analysis and the LSER modelization, the predicted logarithm of capacity factor obtained for each column was plotted against the predicted logarithm of capacity factor obtained for bare silica. The condition selected as reference for all the stationary phases were 200bar, 40°C and 10% of methanol in the mobile phase.

Results are shown in Figure 4.30, where the success of the modifications is obvious due to the deviation of the new data from the y=x line supposed for bare silica. Where the equation of regression obtained for each stationary phase are represented in the same colour as the data plotted.

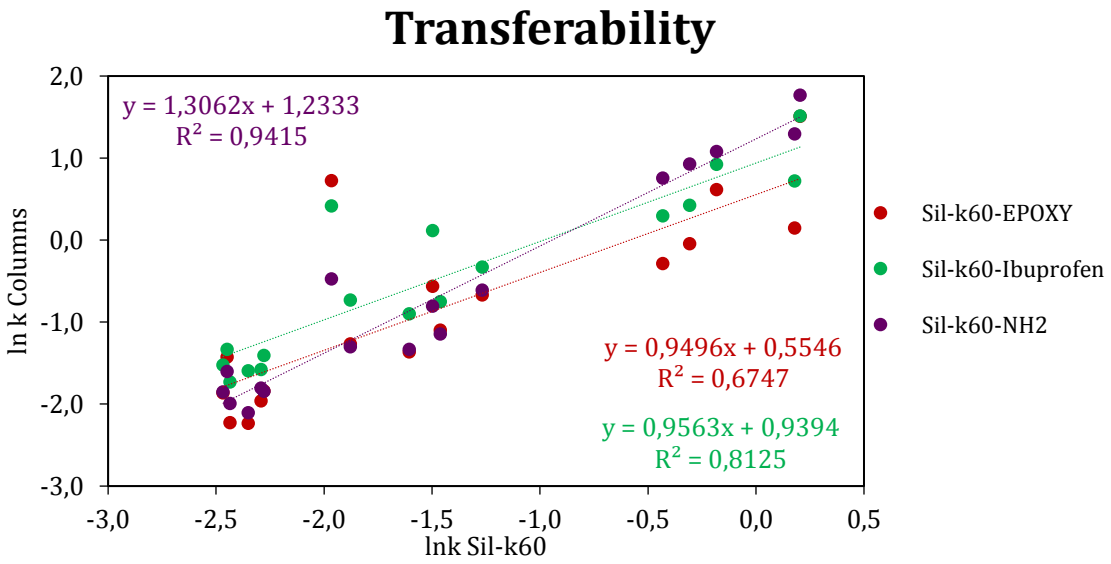


Figure 4.30. Transferability of capacity factors for modified silica columns and bare silica. Reference material: Bare silica (Sil-k60). Conditions: 200bar/40°C/10% MeOH.

The slop α in the equation 15 serves to quantify and compare the overall "strength" of the retention. Thus, a slope of α>1 means that the retention is larger in the compared stationary phase than in the reference material and if α<1, the retention of the reference material is larger than the compared one.

In the case that α is equal 1, the materials should have identical retention properties and characteristics.

As per the figure, only the "Sil-k60-NH_2" has more retention power towards more polar solutes the bare silica (α= 1.3062). On the other hand, the "Sil-k60-ibuprofen" and "Sil-k60-EPOXY" have smaller values than the bare silica (α= 0.9562 and α= 0.9496, respectively). But those values are quite similar from bare silica which allow us to think that the retention power of them are more or less the same than the "Sil-k60"

Although, determine the exact degree of modification of the stationary phases will need a more extensive research, the current data can conclude that the functionalization has been successfully performed, being the APTES modification the most successful one.

5. CONCLUSIONS

In this work SFC analysis were performed in order to validate the LSER model to characterize the modified stationary phases.

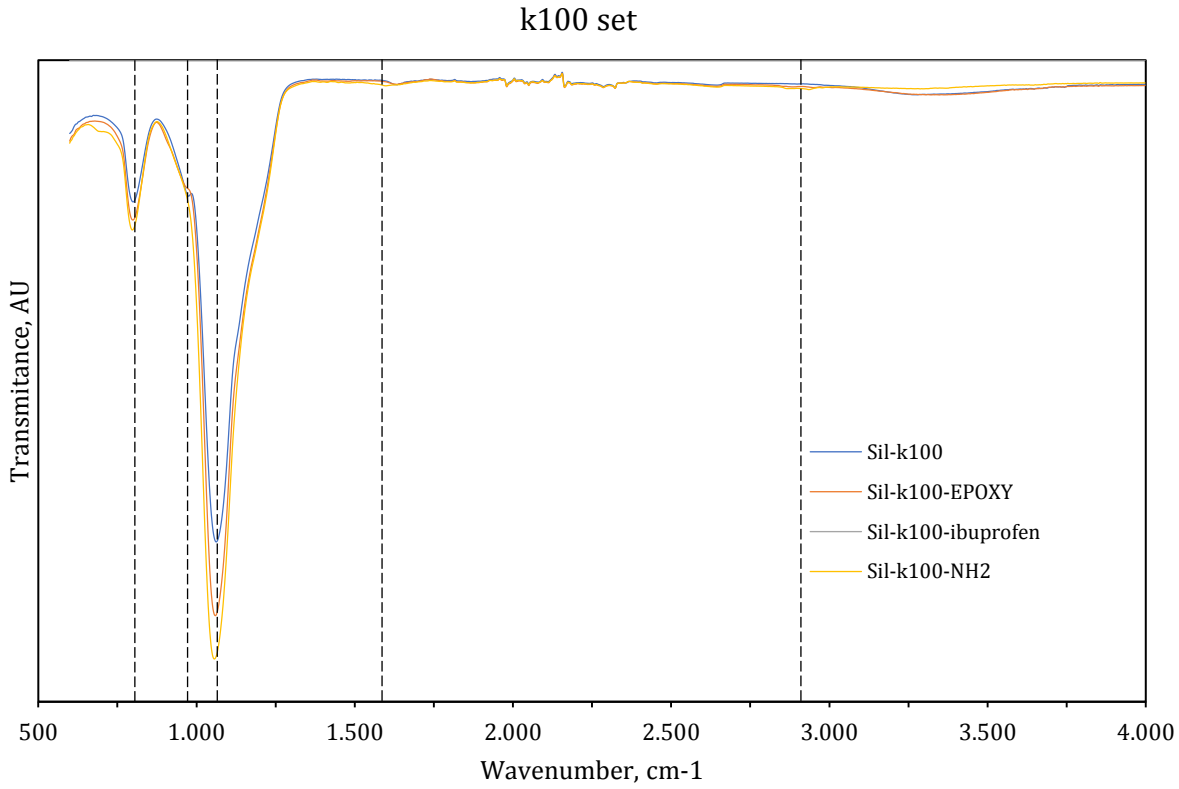
After completing the study, following conclusions has been reached:

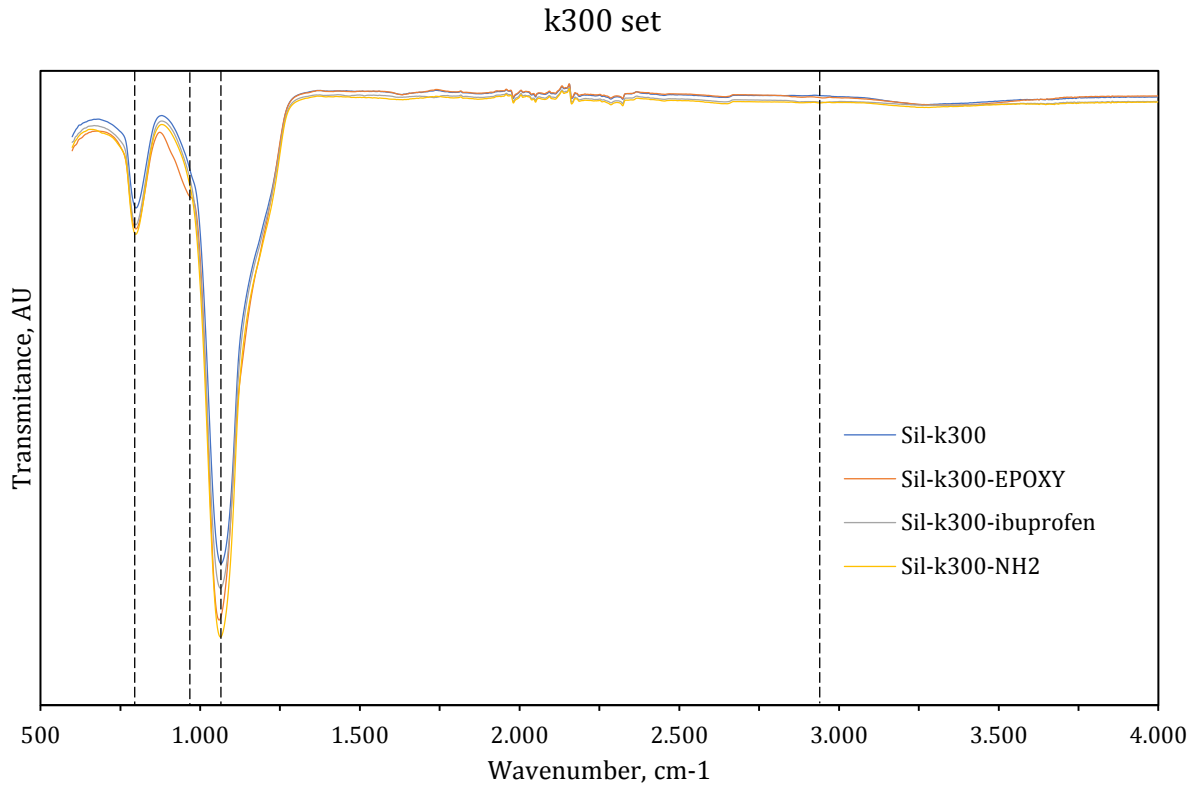
a) The functionalization of the bare silica has been successful following the proposed methods, which was confirmed by elementary analysis and FTIR. The main drawback of the current system is the difference in the loading of the columns that, in some cases, does not allow us to compare one stationary phase with another adequately. The improvements and changes to this fact will results in an improve of the yields and ideally a better quality in the determination of regression times; besides the enhancement in the coefficients determination as well.
b) The comparison of "Sil-k100-C18" and "Sil-k100-comNH_2" has proven the importance of the polarity of the stationary phase on the retention of the solutes.
c) To group the solutes base on their LSER descriptors and the chemical structures makes the analysis much easier. The fact that the solutes that belong to one group behave similarity allows us to analyse a greater amount of solutes.
d) LSER analysis was successfully carried out and comparison of columns with different modification based on the information obtained from the model was carried out. It has been found the biggest difference between the columns is reflected on a and b coefficients which are related to hydrogen bonding interaction. The regressions obtained values of R^2 between 0.7166 and 0.7900. A better selection of solutes and a carefully analysis of retention times may minimize the errors and reach better adjustments.
e) A simplification of the model was carried out based on significance analysis. A model of 2 descriptors, a and s, was proposed and validated. The simplified model improved the sensitivity of the analysis. So, present format of the LSER model can provide a lot of information about the stationary phase and its interactions with the mobile phase and the solutes. But even in this case it is inappropriate to predict the retention of solutes in the silica modified stationary phases. The enhancement of the descriptors will provide a better adjustment to obtain correct and accurate time values.
f) The coefficients obtained for the "Sil-k60-NH_2" are strongly changed with the influence of temperature. This is different comparing to the information that it is stablished in previous literature. One possible reason for the significant influence is the density change caused by temperature.

6. APPENDIX

APPENDIX A

FTIR charts for the modified silica Kromasil 100 and 300.





APPENDIX B

Summary of goodness of the regressions calculated by a multilinear regression using a python programme for pressure variation conditions.

Conditions: 17solutes, 40°C/10%MeOH, 2mL min^{-1}.

	Sil-k60-NH_2					Sil-k60-EPOXY					Sil-k60-ibuprofen				
Parameters	**120**	**150**	**200**	**250**	**300**	**120**	**150**	**200**	**250**	**300**	**120**	**150**	**200**	**250**	**300**
E, S	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E, A	0.7154	0.713	0.7162	0.7193	0.7291	0.578	0.565	0.5751	0.5689	0.569	0.6923	0.6992	0.6989	0.699	0.6977
E, B	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E, V	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
S, A	0.7387	0.7375	0.7271	0.7232	0.7387	*	0.7389	0.7372	0.6996	0.7058	0.7562	0.7443	0.7284	0.7192	0.7062
S, B	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
S, V	*	*	*	*	*	0.6555	0.6521	0.6116	*	0.6173	*	*	*	*	*
S, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
A, B	*	*	*	*	*	0.6367	0.6215	0.6227	0.5968	0.6166	0.5772	0.5807	0.5788	*	*
A, V	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
A, L	0.7044	0.6953	0.6995	*	*	0.539	0.5193	0.5366	0.4914	0.5022	0.6479	0.6428	0.6295	0.6199	0.6066
B, V	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
B, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
V, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E, S, A	*	*	*	*	*	*	*	*	*	*	0.7528	0.7426	0.734	0.7321	0.7236
E, S, B	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E, S, V	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E, S, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E, A, B	*	*	*	*	*	0.6952	0.6902	0.6927	0.7032	0.7095	0.7167	0.7092	*	*	*
E, A, V	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E, A, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E, B, V	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E, B, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E, V, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
S, A, B	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
S, A, V	*	*	*	0.7262	*	*	*	*	*	*	*	*	*	*	*
S, A, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
S, B, V	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
S, B, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
S, V, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
A, B, V	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
A, B, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
A, V, L	0.7113	0.7113	0.7143	0.7243	0.7431	0.7848	*	0.7761	0.792	0.7946	0.7658	0.7606	0.7645	0.7638	0.7748
B, V, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E, S, A, B	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E, S, A, V	*	0.7017	0.7015	0.7123	0.7216	0.8074	0.8152	*	*	*	*	*	*	0.7512	0.7655
E, S, A, L	*	0.7023	0.7078	0.7148	0.7308	*	0.8129	*	0.7989	0.7908	*	*	*	0.7511	0.7655
E, S, B, V	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E, S, B, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E, S, V, L	0.2089	0.214	0.2276	0.1966	0.2323	*	*	*	*	*	0.4656	0.4679	0.4692	0.4712	0.4739
E, A, B, V	*	*	*	*	*	*	*	*	*	*	*	*	*	*	0.7374
E, A, B, L	*	*	*	*	*	*	0.7728	*	0.7579	0.7768	*	*	*	*	*
E, A, V, L	*	*	*	*	*	0.8057	*	0.7676	*	*	*	*	*	*	*
E, B, V, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
S, A, B, V	*	*	*	*	*	0.8002	0.8078	0.7808	0.7968	0.8093	*	*	*	*	*
S, A, B, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
S, A, V, L	*	*	*	*	*	0.8112	*	*	*	*	*	*	*	*	*
S, B, V, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
A, B, V, L	*	*	*	*	*	0.8241	0.8272	*	*	0.8172	*	*	*	*	*
E, S, A, B, V	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E, S, A, B, L	*	*	*	*	*	*	*	*	0.8201	0.8172	*	*	*	*	*
E, S, A, V, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E, S, B, V, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E, A, B, V, L	0.6768	0.67674	0.663	*	*	*	*	*	*	*	0.7143	0.7067	*	0.7115	*
S, A, B, V, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E, S, A, B, V, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*

Summary of goodness of the regressions calculated by a multilinear regression using a python programme for temperature variation conditions. Part I.

Condition: 17solutes, 200bar/10%MeOH, 2mL $min^{-1.}$

	Sil-k60-NH_2							Sil-k60-EPOXY						
Parameters	**25**	**30**	**35**	**45**	**50**	**55**	**60**	**25**	**30**	**35**	**45**	**50**	**55**	**60**
E, S	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E, A	0.7421	0.7386	0.73	0.718	0.7094	0.6505	0.6412	0.5924	0.5885	0.5885	0.5548	0.5543	0.5349	0.5209
E, B	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E, V	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*
S, A	0.7457	0.75	0.7505	0.7419	0.7396	0.6793	0.6737	0.7162	0.7095	0.7158	0.7252	0.7339	0.7358	0.7476
S, B	*	*	*	*	*	*	*	*	*	*	*	*	*	*
S, V	*	*	*	*	*	*	*	0.6091	0.6139	0.6098	0.6345	0.6374	0.6467	0.6595
S, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*
A, B	*	*	*	*	*	*	*	0.6085	*	0.6023	0.623	0.6305	0.634	0.6436
A, V	*	*	*	*	*	*	*	*	*	*	*	*	*	*
A, L	*	*	*	0.6951	0.6913	0.6388	0.6319	0.5166	0.5111	0.5191	0.5102	0.5167	0.5056	0.5119
B, V	*	*	*	*	*	*	*	*	*	*	*	*	*	*
B, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*
V, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E, S, A	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E, S, B	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E, S, V	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E, S, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E, A, B	*	*	*	*	*	*	*	0.7098	0.7177	0.6968	0.709	0.7034	0.6877	0.7116
E, A, V	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E, A, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E, B, V	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E, B, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E, V, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*
S, A, B	*	*	*	*	*	*	*	*	*	*	*	*	*	*
S, A, V	*	*	*	*	*	*	*	*	*	*	*	*	*	*
S, A, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*
S, B, V	*	*	*	*	*	*	*	*	*	*	*	*	*	*
S, B, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*
S, V, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*
A, B, V	*	*	*	*	*	*	*	*	*	*	*	*	*	*
A, B, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*
A, V, L	0.7651	0.7509	0.739	0.7183	0.7086	0.6569	0.6486	0.7899	0.7981	0.7878	0.8079	0.7918	0.7999	*
B, V, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E, S, A, B	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E, S, A, V	0.7548	0.7405	0.7272	0.7037	*	*	*	0.7808	0.7892	0.7833	*	*	*	*
E, S, A, L	0.7594	*	*	0.7068	*	*	*	0.7783	0.7829	0.7796	0.805	0.798	0.8098	*
E, S, B, V	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E, S, B, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E, S, V, L	0.2845	0.2457	0.2259	0.1982	0.1978	0.2259	0.2276	*	*	*	*	*	*	*
E, A, B, V	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E, A, B, L	*	*	*	*	*	*	*	0.7607	0.7634	*	0.7753	0.7716	0.7761	0.7742
E, A, V, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E, B, V, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*
S, A, B, V	*	*	*	*	*	*	*	0.7906	0.7964	0.7842	0.8083	0.8035	0.8088	0.8065
S, A, B, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*
S, A, V, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*
S, B, V, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*
A, B, V, L	*	*	*	*	*	*	*	*	*	*	0.8252	0.823	0.8278	*
E, S, A, B, V	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E, S, A, B, L	*	*	*	*	*	*	*	*	*	*	0.8271	0.8233	0.8311	0.834
E, S, A, V, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E, S, B, V, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E, A, B, V, L	*	*	*	0.6858	0.6735	*	*	*	*	*	*	*	*	*
S, A, B, V, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*
E, S, A, B, V, L	*	*	*	*	*	*	*	*	*	*	*	*	*	*

Summary of goodness of the regressions calculated by a multilinear regression using a Python programme for temperature variation conditions. Part II.

Condition: 17solutes, 200bar/10%MeOH, 2mL $min^{-1.}$

	Sil-k60-ibuprofen						
Parameters	**25**	**30**	**35**	**45**	**50**	**55**	**60**
E, S	*	*	*	*	*	*	*
E, A	0.7011	0.6998	0.6942	0.68	0.6853	0.6821	0.6738
E, B	*	*	*	*	*	*	*
E, V	*	*	*	*	*	*	*
E, L	*	*	*	*	*	*	*
S, A	0.7446	0.7449	0.7472	0.7305	0.7657	0.7401	0.7386
S, B	*	*	*	*	*	*	*
S, V	*	*	*	*	*	*	*
S, L	*	*	*	*	*	*	*
A, B	0.5675	0.5709	0.573	*	0.5836	0.5637	0.5655
A, V	*	*	*	*	*	*	*
A, L	0.6315	0.6318	0.6303	0.6306	0.6414	0.6397	0.64
B, V	*	*	*	*	*	*	*
B, L	*	*	*	*	*	*	*
V, L	*	*	*	*	*	*	*
E, S, A	0.7513	0.7512	0.7524	0.7267	0.7423	0.7288	0.7226
E, S, B	*	*	*	*	*	*	*
E, S, V	*	*	*	*	*	*	*
E, S, L	*	*	*	*	*	*	*
E, A, B	*	*	*	*	*	*	*
E, A, V	*	*	*	*	*	*	*
E, A, L	*	*	*	*	*	*	*
E, B, V	*	*	*	*	*	*	*
E, B, L	*	*	*	*	*	*	*
E, V, L	*	*	*	*	*	*	*
S, A, B	*	*	*	*	*	*	*
S, A, V	*	*	*	*	*	*	*
S, A, L	*	*	*	*	*	*	*
S, B, V	*	*	*	*	*	*	*
S, B, L	*	*	*	*	*	*	*
S, V, L	*	*	*	*	*	*	*
A, B, V	*	*	*	*	*	*	*
A, B, L	*	*	*	*	*	*	*
A, V, L	0.7779	0.7807	0.7749	0.7438	0.7579	0.7379	0.7273
B, V, L	*	*	*	*	*	*	*
E, S, A, B	*	*	*	*	*	*	*
E, S, A, V	*	*	*	*	*	*	*
E, S, A, L	*	*	*	*	*	*	*
E, S, B, V	*	*	*	*	*	*	*
E, S, B, L	*	*	*	*	*	*	*
E, S, V, L	*	*	*	*	0.469	0.4293	0.4158
E, A, B, V	*	*	*	*	*	*	*
E, A, B, L	*	*	*	*	*	*	*
E, A, V, L	*	*	*	*	*	*	*
E, B, V, L	*	*	*	*	*	*	*
S, A, B, V	*	*	*	*	*	*	*
S, A, B, L	*	*	*	*	*	*	*
S, A, V, L	*	*	*	*	*	*	*
S, B, V, L	*	*	*	*	*	*	*
A, B, V, L	*	*	*	*	*	*	*
E, S, A, B, V	*	*	*	*	*	*	*
E, S, A, B, L	*	*	*	*	*	*	*
E, S, A, V, L	*	*	*	*	*	*	*
E, S, B, V, L	*	*	*	*	*	*	*

Summary of goodness of the regressions calculated by a multilinear regression using a python programme for concentration of the modifier variation conditions. Part I.

Condition: 17solutes, 200bar/40°C, 2mL $min^{-1.}$

	Sil-k60-NH_2						Sil-k60-EPOXY					
Parameters	**5%**	**7.50%**	**12.50%**	**15%**	**17.50%**	**20%**	**5%**	**7.50%**	**12.50%**	**15%**	**17.50%**	**20%**
E, S	*	*	*	*	*	*	*	*	*	*	*	*
E, A	*	0.7067	0.7242	0.7275	0.7182	0.7186	0.5322	0.5482	0.5735	0.5702	0.5792	0.5971
E, B	*	*	*	*	*	*	*	*	*	*	*	*
E, V	*	*	*	*	*	*	*	*	*	*	*	*
E, L	*	*	*	*	*	*	*	*	*	*	*	*
S, A	0.7352	0.7373	0.7349	0.7342	0.7224	0.7192	0.7267	0.7467	0.7507	0.7392	0.7494	0.7252
S, B	*	*	*	*	*	*	*	*	*	*	*	*
S, V	*	*	*	*	*	*	*	*	*	*	*	0.6073
S, L	*	*	*	*	*	*	*	*	*	*	*	*
A, B	*	*	*	*	*	*	0.6541	0.6689	0.6006	*	*	0.5599
A, V	*	*	*	*	*	*	*	*	*	*	*	*
A, L	0.6882	0.6969	0.6922	*	0.6855	0.6829	0.529	0.5395	0.576	0.5752	0.5866	0.5312
B, V	*	*	*	*	*	*	*	*	*	*	*	*
B, L	*	*	*	*	*	*	*	*	*	*	*	*
V, L	*	*	*	*	*	*	*	*	*	*	*	*
E, S, A	*	*	*	*	*	*	*	*	*	*	*	*
E, S, B	*	*	*	*	*	*	*	*	*	*	*	*
E, S, V	*	*	*	*	*	*	*	*	*	*	*	*
E, S, L	*	*	*	*	*	*	*	*	*	*	*	*
E, A, B	*	0.731	0.7104	0.7108	0.6675	0.6888	0.6887	*	*	*	*	*
E, A, V	*	*	*	*	*	*	*	*	*	*	*	*
E, A, L	*	*	*	*	*	*	*	*	*	*	*	*
E, B, V	*	*	*	*	*	*	*	*	*	*	*	*
E, B, L	*	*	*	*	*	*	*	*	*	*	*	*
E, V, L	*	*	*	*	*	*	*	*	*	*	*	*
S, A, B	*	*	*	0.7575	0.7659	0.7636	*	*	*	*	*	*
S, A, V	*	*	*	*	*	*	*	*	*	*	*	*
S, A, L	*	*	*	*	*	*	*	*	*	*	*	*
S, B, V	*	*	*	*	*	*	*	*	*	*	*	*
S, B, L	*	*	*	*	*	*	*	*	*	*	*	*
S, V, L	*	*	*	*	*	*	*	*	*	*	*	*
A, B, V	*	*	*	*	*	*	*	*	*	*	*	*
A, B, L	*	*	*	*	*	*	*	*	*	*	*	*
A, V, L	0.7019	0.6991	0.7317	0.7336	0.7311	0.7249	*	*	0.7142	0.7014	0.7137	0.7683
B, V, L	*	*	*	*	*	*	*	*	*	*	*	*
E, S, A, B	*	*	*	*	*	*	*	0.7134	*	*	*	*
E, S, A, V	*	*	0.7174	*	0.7212	0.7132	*	*	*	*	*	*
E, S, A, L	*	*	0.7215	0.725	0.7251	0.7191	*	*	*	*	*	*
E, S, B, V	*	*	*	*	*	*	*	*	*	*	*	*
E, S, B, L	*	*	*	*	*	*	*	*	*	*	*	*
E, S, V, L	0.175	0.1845	0.2353	0.2495	*	*	*	*	*	*	*	*
E, A, B, V	*	*	*	*	*	*	*	*	*	*	*	*
E, A, B, L	*	*	*	*	*	*	*	*	*	*	*	*
E, A, V, L	*	*	*	*	*	*	*	*	*	*	*	*
E, B, V, L	*	*	*	*	*	*	*	*	*	*	*	*
S, A, B, V	*	*	*	*	*	*	*	*	*	*	*	0.7421
S, A, B, L	*	*	*	*	*	*	*	*	*	*	*	*
S, A, V, L	*	*	*	*	*	*	*	*	*	*	*	0.7589
S, B, V, L	*	*	*	*	*	*	*	*	*	*	*	*
A, B, V, L	*	*	*	*	*	*	*	*	*	*	*	*
E, S, A, B, V	*	*	*	*	*	*	*	*	*	*	*	*
E, S, A, B, L	*	*	*	*	*	*	*	*	*	*	*	*
E, S, A, V, L	*	*	*	*	*	*	*	*	*	*	*	*
E, S, B, V, L	*	*	*	*	*	*	*	*	*	*	*	*
E, A, B, V, L	*	*	0.7127	0.7235	0.7224	0.721	*	*	*	*	*	*
S, A, B, V, L	*	*	*	0.7244	0.7243	0.7233	*	*	*	*	*	*
E, S, A, B, V, L	*	*	*	*	*	*	*	*	*	*	*	*

Summary of goodness of the regressions calculated by a multilinear regression using a python programme for concentration of the modifier variation conditions. Part II.

Condition: 17solutes, 200bar/40°C, 2mL $min^{-1.}$

	Sil-k60-ibuprofen					
Parameters	**5%**	**7.50%**	**12.50%**	**15%**	**17.50%**	**20%**
E, S	*	*	*	*	*	*
E, A	0.6448	0.6717	0.7053	0.698	0.7171	0.7183
E, B	*	*	*	*	*	*
E, V	*	*	*	*	*	*
E, L	*	*	*	*	*	*
S, A	0.7295	0.7325	0.737	0.7386	0.7071	0.6936
S, B	*	*	*	*	*	*
S, V	*	*	*	*	*	*
S, L	*	*	*	*	*	*
A, B	0.6424	0.5985	0.5661	0.5601	*	*
A, V	*	*	*	*	*	*
A, L	0.6033	0.6096	0.6236	0.6504	0.6086	0.5985
B, V	*	*	*	*	*	*
B, L	*	*	*	*	*	*
V, L	*	*	*	*	*	*
E, S, A	*	0.7254	0.7445	0.7528	*	0.7414
E, S, B	*	*	*	*	*	*
E, S, V	*	*	*	*	*	*
E, S, L	*	*	*	*	*	0.4652
E, A, B	0.6846	0.6978	*	*	*	*
E, A, V	*	*	*	*	*	*
E, A, L	*	*	*	*	*	*
E, B, V	*	*	*	*	*	*
E, B, L	*	*	*	*	*	*
E, V, L	*	*	*	*	*	*
S, A, B	*	*	*	*	*	*
S, A, V	*	*	*	*	*	*
S, A, L	*	*	*	*	*	*
S, B, V	*	*	*	*	*	*
S, B, L	*	*	*	*	*	*
S, V, L	*	*	*	*	*	*
A, B, V	*	*	*	*	*	*
A, B, L	*	*	*	*	*	*
A, V, L	*	0.764	0.7826	0.7507	0.7858	0.7902
B, V, L	*	*	*	*	*	*
E, S, A, B	*	*	*	*	*	*
E, S, A, V	*	0.7567	0.7706	*	0.7771	0.777
E, S, A, L	*	*	0.7708	*	0.7779	0.779
E, S, B, V	*	*	*	*	*	*
E, S, B, L	*	*	*	*	*	*
E, S, V, L	0.4716	0.4699	0.4986	*	0.5178	*
E, A, B, V	*	*	*	*	*	*
E, A, B, L	*	*	*	*	*	*
E, A, V, L	*	*	*	*	*	*
E, B, V, L	*	*	*	*	*	*
S, A, B, V	*	*	*	*	*	*
S, A, B, L	*	*	*	*	*	*
S, A, V, L	*	*	*	*	*	*
S, B, V, L	*	*	*	*	*	*
A, B, V, L	*	*	*	*	*	0.7734
E, S, A, B, V	*	*	*	*	*	*
E, S, A, B, L	*	*	*	*	*	*
E, S, A, V, L	*	*	*	*	*	*
E, S, B, V, L	*	*	*	*	*	*
E, A, B, V, L	*	*	0.7244	*	*	*
S, A, B, V, L	*	*	*	*	*	*
E, S, A, B, V, L	*	*	*	*	*	*

APPENDIX C

Summary of coefficients calculated by a multilinear regression using a Python programme at 200bar, 40°C, 10% of MeOH.

Sil-k60-EPOXY		**Coef.**	**S_{dev}**	**p-value**
E S A B V	**c**	-2.5171	0.508	0.000
R^2_{adj} 0.7953	**e**	1.0706	0.398	0.007
	s	1.3078	0.625	0.037
	a	2.4776	0.464	0.000
	b	0.9313	0.587	0.113
	v	-1.4729	0.530	0.005

Sil-k60-ibuprofen		**Coef.**	**S_{dev}**	**p-value**
E S A B V	**c**	-2.4615	0.341	0.000
R^2_{adj} 0.7350	**e**	1.0035	0.267	0.000
	s	1.1819	0.420	0.005
	a	3.1072	0.312	0.000
	b	-0.0850	0.216	0.829
	v	-0.7036	0.356	0.048

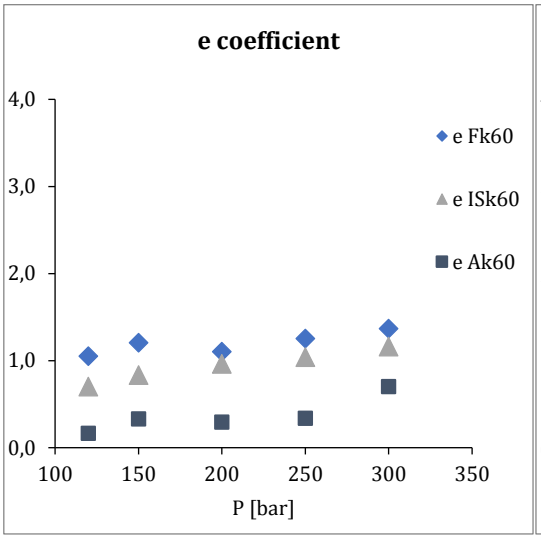
Sil-k60-NH_2		**Coef.**	**S_{dev}**	**p-value**
E S A B V	**c**	-2.2733	0.367	0.000
R^2_{adj} 0.6790	**e**	0.6811	0.288	0.000
	s	1.1360	0.453	0.012
	a	4.5177	0.336	0.018
	b	-0.0410	0.425	0.923
	v	-0.9621	0.384	0.012

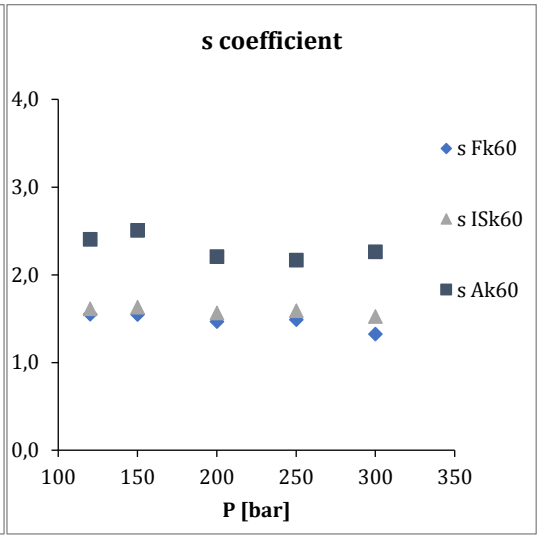
Sil-k100-comNH_2		**Coef.**	**S_{dev}**	**p-value**
E S A B V	**c**	-3.1364	0.302	0.000
R^2_{adj} 0.7709	**e**	0.3841	0.236	0.104
	s	1.2249	0.372	0.001
	a	4.0326	0.276	0.000
	b	0.4575	0.349	0.190
	v	-0.7270	0.315	0.021

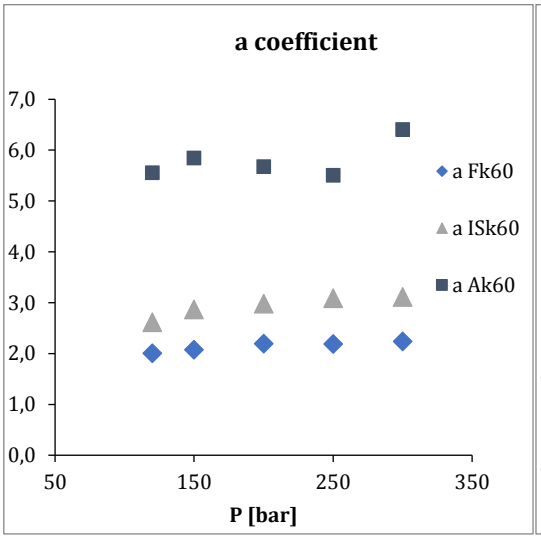
Sil-k100-C18		**Coef.**	**S_{dev}**	**p-value**
E S A B V	**c**	-2.7275	0.302	0.000
R^2_{adj} 0.7364	**e**	1.1714	0.257	0.000
	s	-0.9584	0.404	0.018
	a	-1.4988	0.300	0.000
	b	-1.4455	0.379	0.000
	v	1.4468	0.343	0.000

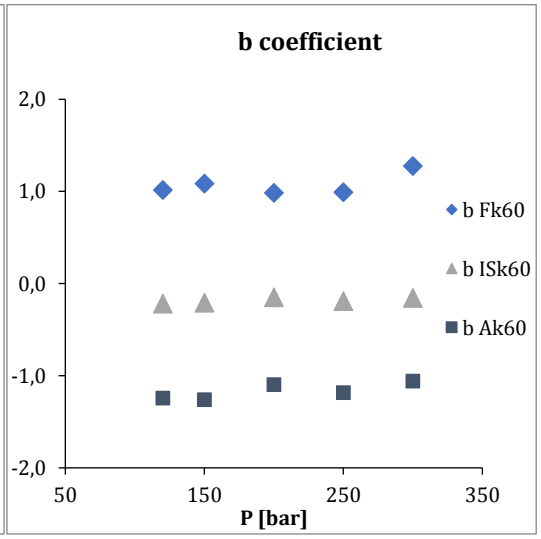
APPENDIX D

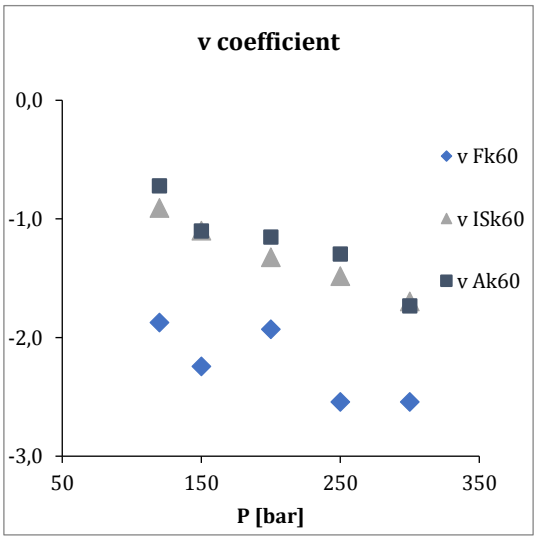
Comparison of coefficients for pressure variation. General equation.



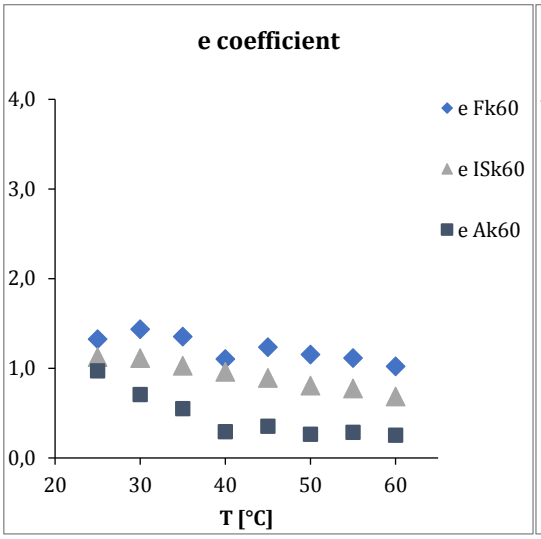


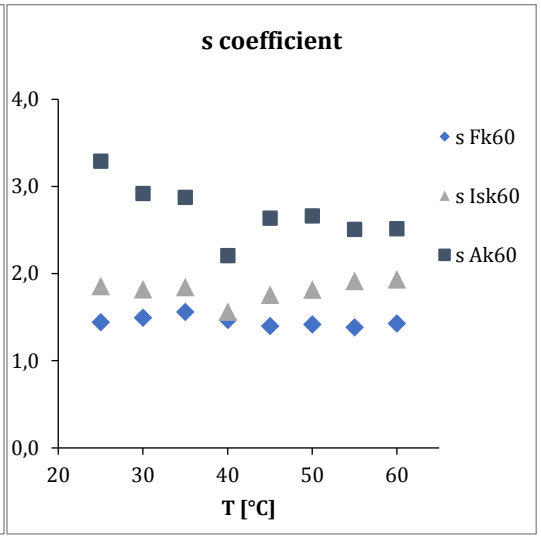


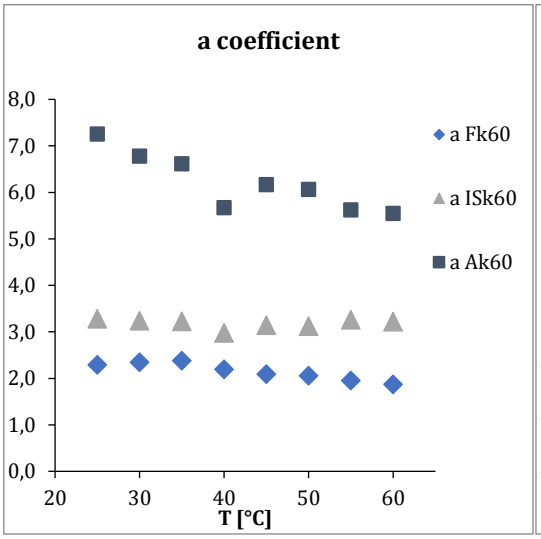


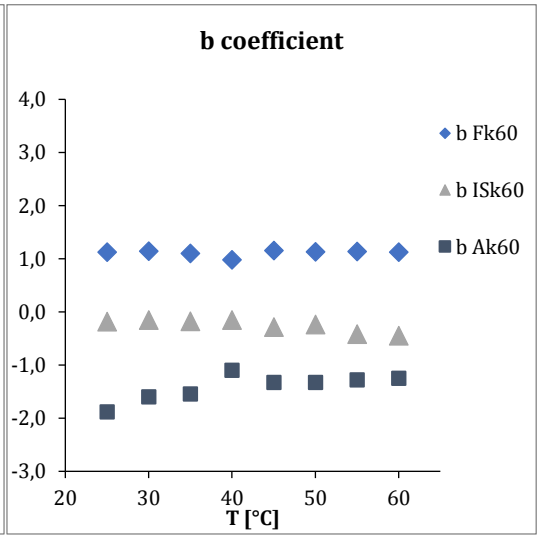


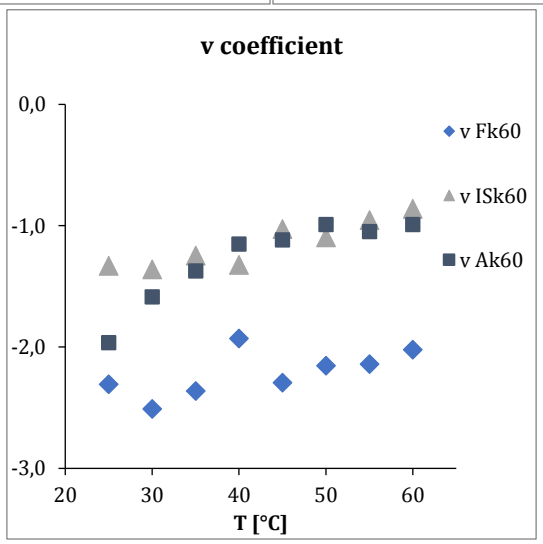
Comparison of coefficients for temperature variation. General equation.



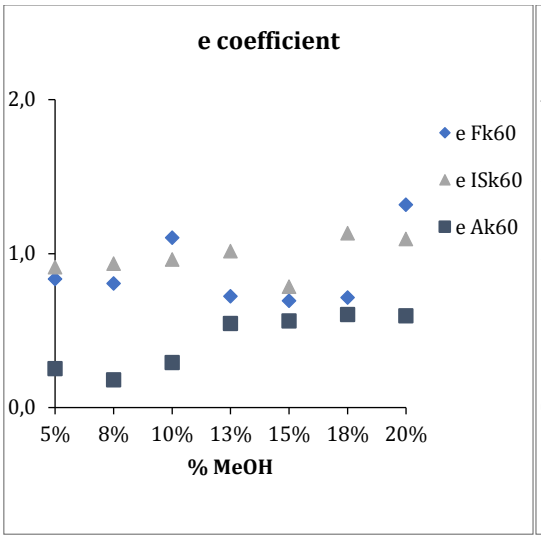


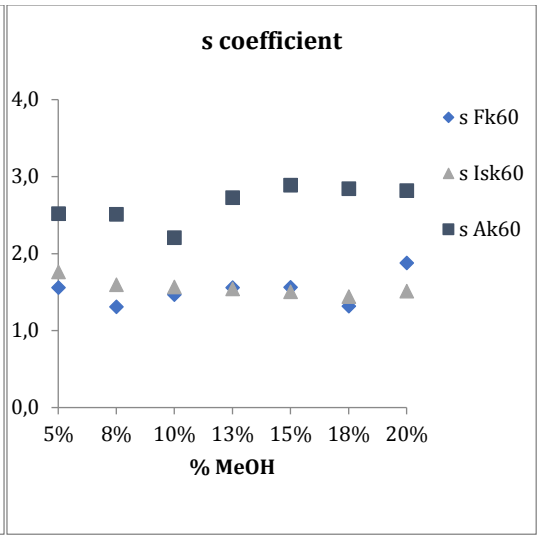


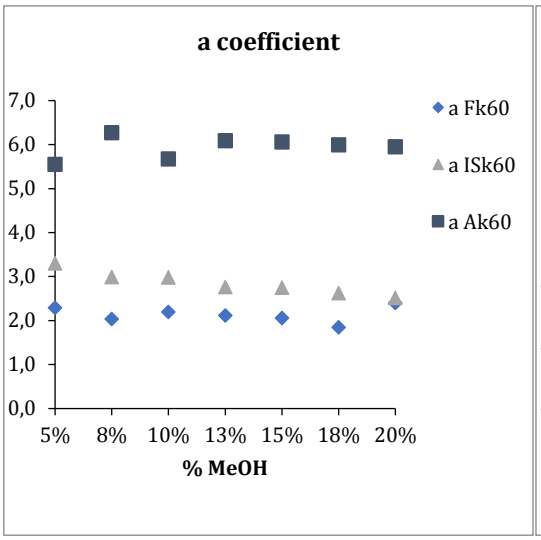


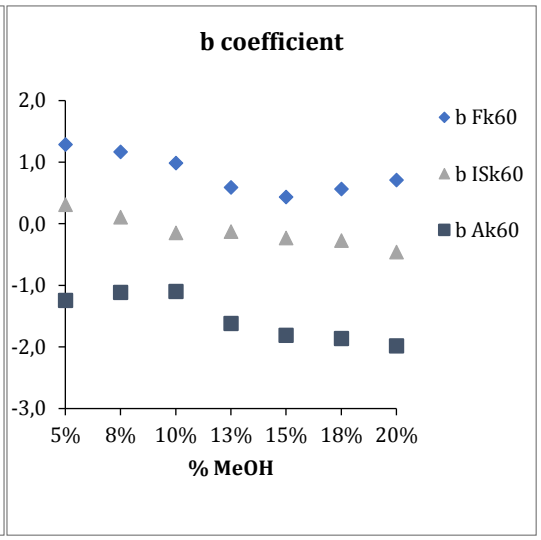


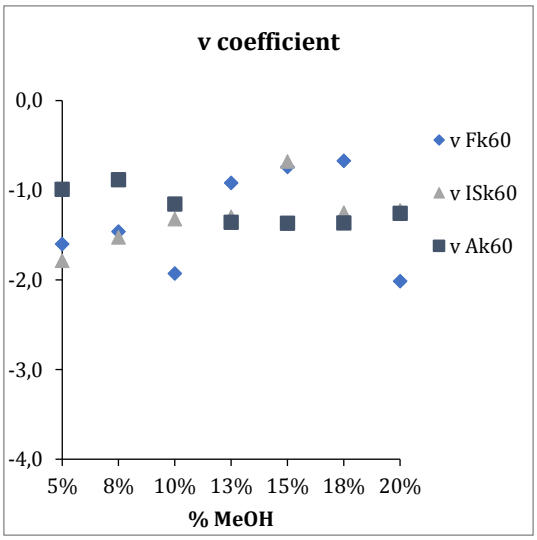
Comparison of coefficients for concentration of modifier variation. General equation.





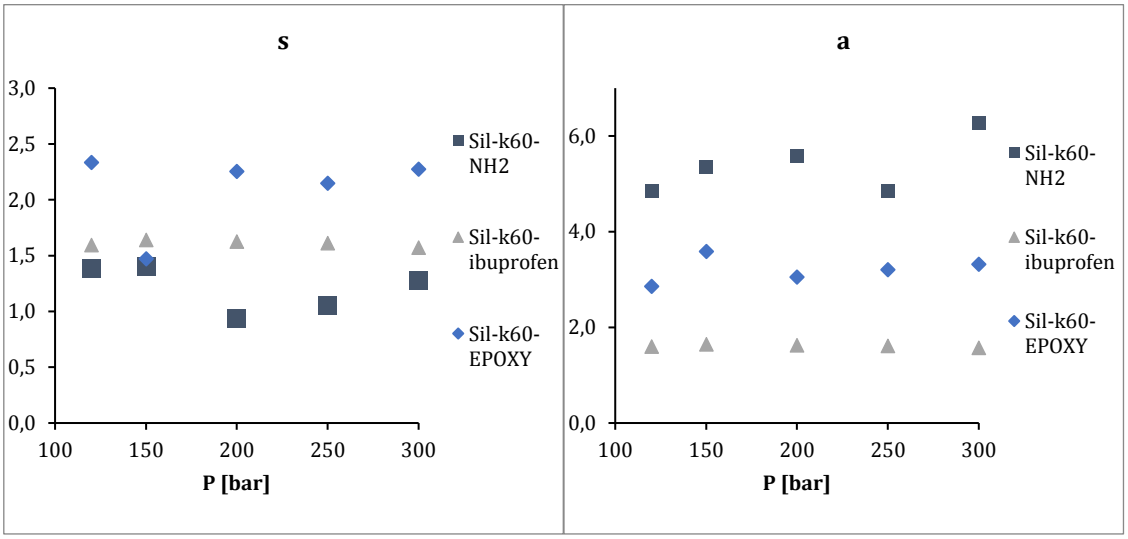




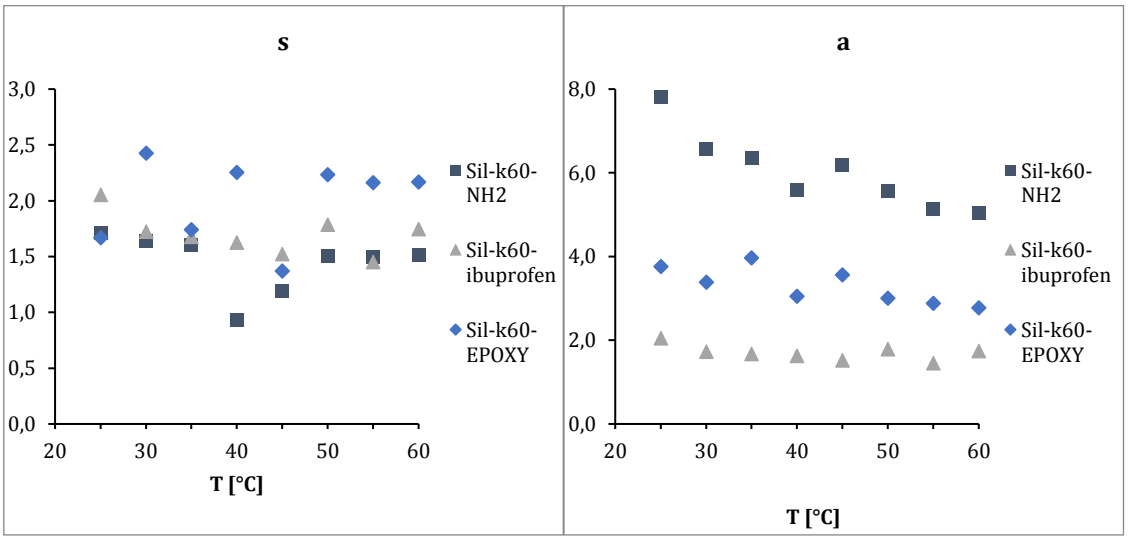


APPENDIX E

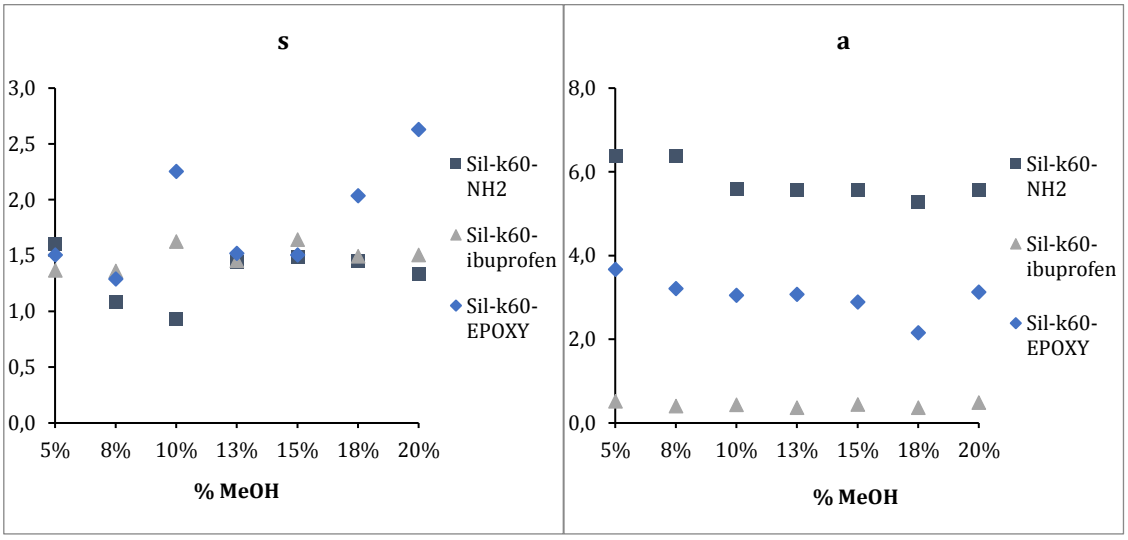
Comparison of coefficients for pressure variation. Simplified equation.



Comparison of coefficients for temperature variation. Simplified equation.



Comparison of coefficients for temperature variation. Simplified equation.



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