UNIVERSITY OF PÉCS

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Investigation of Modifier and Additive Adsorption in Supercritical Fluid Chromatography

PhD thesis

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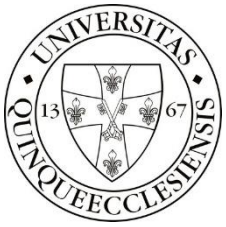

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Dedicated to

*My **Dad,***

who failed to see the completion of this work, he would have been so proud. May Allah almighty have mercy upon him.

*My haven in the tough time, **mother**, even though her interest in the following chapters is nothing, I am stronger with her prayers.*

Anyone who does not give up at the first stumble.

Chapter 1

INTRODUCTION

"I'm an advocate of the use of supercritical fluids not only because of their environmental and cost-saving advantages, but because they provide technical advantages not easily obtained using conventional methods." J. David Pinkston

1.1. Introduction

The power of chromatography as an indispensable tool in the chemistry domain comes from the ability to separate a mixture into individual components, as well as its versatile analytical and preparative applications in several fields like scientific research, chemical industries, and natural products. For example, the pharmaceutical industries rely mainly on analytical chromatography for quality control purpose, as well as on preparative chromatography for the active ingredients purification.

Historically, supercritical fluid chromatography (SFC) had passed through many primary stages of instrumentation development starting its inception 1960s until the packed column SFC system became commercially available by the late 1990s from different vendors [1]. The first complete packed column SFC (pSFC) system came out in the markets at the beginning of 2010s from Waters as ultra-high performance SFC and Agilent supplied the hybrid SFC/UHPLC system, which are included features in the CO_2 pumps equipped with cooling attribute for decreasing the compression ratio, while in the detector the UV cell is improved to resist high pressure values, also electronically controlled back pressure regulator (BPR) for accurately controlling the pressure [2]. Those integrated systems have facilitated a new road for the chromatographers to figure out the advantages and the challenges of SFC that can meet in the sample separation.

In regard of SFC columns, the increased demands to sustainable chemistry encouraged the manufacturing companies to design or develop the chromatographic columns to withstand high pressure environment of SFC with different chemistries to fit a wide range of analyte polarities, such as 2-ethylpyridine bonded silica which was offered by Princeton Chromatography for the use of achiral applications, it is the favorite column for basic analytes separation, it was designed to minimize the interaction between the polar analytes and the accessible silanol groups on the silica.

Also a number of functional groups such as sulfonamide, amide, pyridine and diol-bonded silica stationary phases are available in the markets [3,4]. Above all, it has been shown that the stationary phases developed for high performance liquid chromatography can be used for SFC [5].

The CO_2 based SFC is considered a normal phase chromatographic separation mode due to the adsorption of the mobile phase components onto the stationary phase, the adsorbed layer contains a high concentration of those components comparing to the bulk of mobile phase. Thus, the polar solutes will retain on the polar stationary phase, whereas the nonpolar solutes which are dissolved in CO_2 will elute faster [6]. The technique has not historically been used for the separation of strongly hydrophilic compounds. In the last three decades the SFC specialists have been working to find a convenient way to extend the limits of analytes polarity which can be analyzed with SFC. They have suggested mixing CO_2 with a small amount of polar solvent such as alcohols with a small molecular weight to increase the polarity and solvating power of the mobile phase. Other polar compounds contain ionizable groups require adding a third component called "additive" with low concentration <1% (v/v) for obtaining better chromatographic results. Generally, the additives can be acids, bases, salts or even water [7] [3].

The adsorption study of the concerned components onto the stationary phase by calculating the equilibrium isotherms is very informative tool in different aspects (i) the developing process of a chromatographic instrument, (ii) to characterize the retention mechanism, (iii) to study the influence of the experimental factors on the separation quality of a sample, (iv) to perform purification for the target compounds in nonlinear chromatography. In LC over the past 30 years, the equilibrium isotherms of a wide range of solvents and solutes have been studied to develop the separation method or to investigate the retention mechanism [8] or even to describe the competition between the mobile phase components and solute for adsorption sites [9], but under the supercritical conditions a modest number of studies was observed in the last decade [10].

Concerning the compounds which can be analyzed with SFC, it has been reported that a wide range of compounds can be separated with SFC not only non-polar [11] but also highly polar [12, 13] and polymers [14]. This diversity of applications can be achieved through tuning up the mobile phase composition, which is composed mainly of the supercritical carbon dioxide, and well choosing the stationary phase for the target application.

The supercritical fluid is characterized by "gas-like" viscosity and "liquid-like" density. The low viscosity practically leads to fast diffusivity, which is important feature to achieve fast mass transfer in the separation processes. Generally, the solvation power of the solvent is associated by its density: the higher the density, the greater the solvent strength. The density of supercritical fluid can be increased by raising the pressure and it can become more liquid-like. These main properties of supercritical fluids differentiate the SFC from the LC or GC with its high flow rates and high elution strength for the separation purposes [1].

To perform separation for a sample contains polar analytes (e.g. vitamin D (steroid) [15]), usually it is required to increase the polarity of CO_2 based mobile phase by adding small percentage of a polar solvent called co-solvent or modifier, (such as alcohols with small molecular weight) to improve the solubility of the analytes in the mobile phase, which would give better shape of chromatographic peaks. In addition, for separation of polar analytes (e.g. polyfunctional organic acids [16]) a third component called additive (base, acid, salt or water) is highly recommended to be used in very small amount. It is usually premixed with the modifier then both are transferred to the mobile phase resulting in a better peak shape of the analytes [7].

The possible roles of the modifier and additive compounds on improving the chromatographic results of the analytes will be discussed in Chapter 2.

Our main interest was to clarify the adsorption behavior of both the modifier and the additive on the stationary phases. Also, evaluating the use of water as additive to elute some basic compounds, which is an attempt to use SFC as a green technique with less organic solvent consumption.

1.2. The main objectives

The main objectives in this research were:

- To show how the reversed stationary phase would be affected in presence of methanol as a modifier by calculating the excess adsorption isotherm of methanol under two sets of temperature and back pressure values.

- To evaluate the influence of polar and non-polar sample solvents on the separation efficiency of set of alkylbenzenes on the reversed stationary phases using 100% CO_2 as mobile phase.
- To study the adsorption behavior of water, methanol, and their mixture on the polar stationary phase by calculating the corresponding adsorption isotherms.
- To study the effect of adding water to the methanol-modified carbon dioxide as mobile phase on the efficiency and peak quality of polar solutes eluting from the polar stationary phase.

Chapter 2

LITERATURE REVIEW

"The desire of knowledge, like the thirst of riches,
increases ever with the acquisition of it." Laurence Sterne

2.1. SFC principle and applications

SFC has been described as a chromatographic technique with properties that take place somewhere between liquid chromatography (LC) and gas chromatography (GC). As in LC and GC, separation of solutes is achieved in SFC due to their different affinities (interactions) with the stationary phase. The solutes are driven by a highly compressible dense fluid in the supercritical state represents the mobile phase. However, in case of binary or ternary mobile phases, the mobile phase is high likely at "near critical" or so-called "subcritical" state [17].

Generally, a fluid reaches to the supercritical state when it is exposed to a temperature and a pressure over the critical value (Figure 1). At this state the substance is neither a gas nor a liquid but it can be described as a compressed gas possess liquid-like density. The supercritical state of a compound can be obtained in SFC by utilizing a back-pressure regulator which is installed after the column to keep content of the column above the critical pressure.

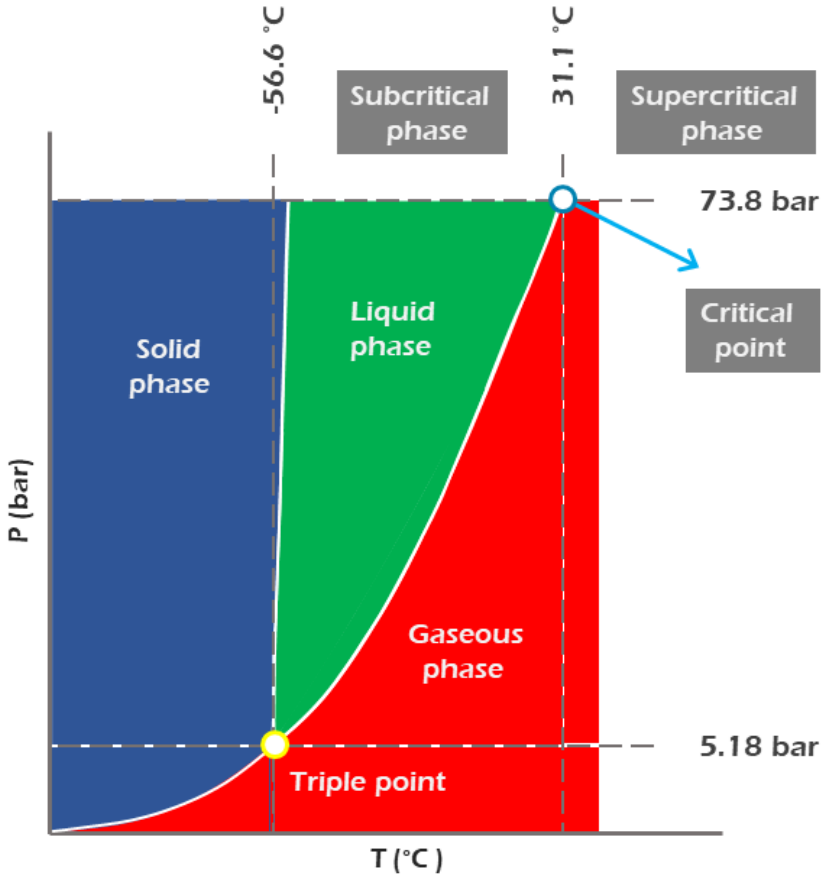


Figure 1. The schematic representation of the phase diagram of CO_2 [18].

It is worth noting that the solvating power of a pure fluid is related to the density of fluid, and near to a critical area in the pressure-density isotherm, as it can be clear from the marked lines with symbols above the pressure value 73.8 bar for carbon dioxide in (Figure 2), the density is very sensitive to the applied pressure, hence small changes in pressure result in large changes in density of the fluid [7]. The pressure-density isotherm was obtained by the REFPROP software Ver. 8 from the National Institute of Standards and Technologies (NIST).

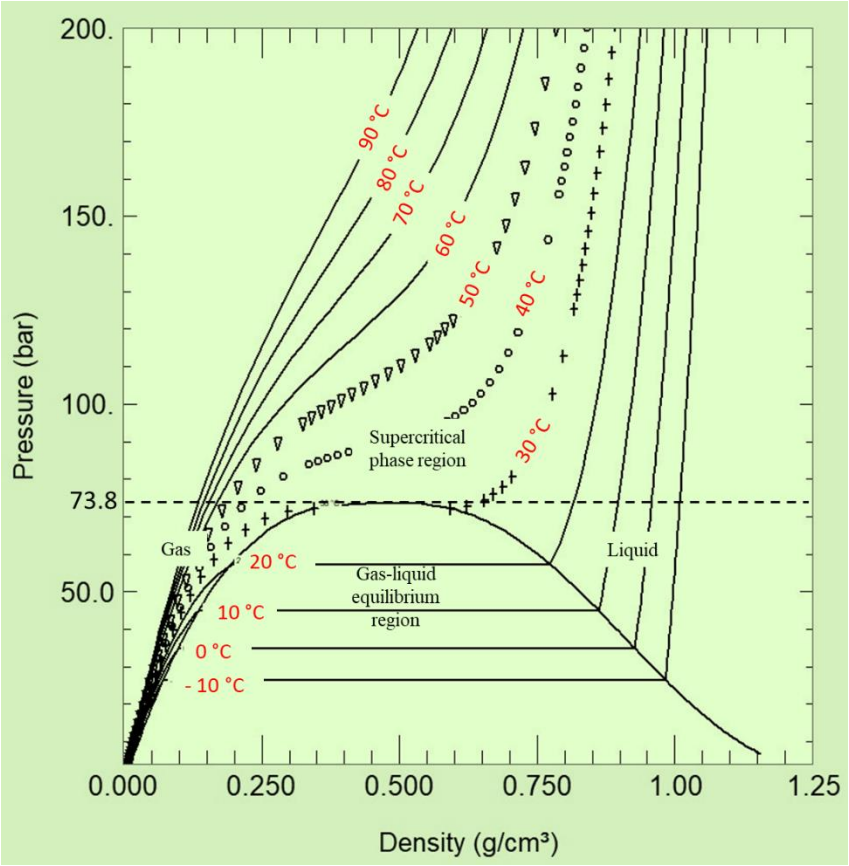


Figure 2. Pressure-density behavior of carbon dioxide at various temperatures (the diagram obtained by REFPROP software).

The unique properties of supercritical fluids comparing to other liquids, such as low viscosities and high diffusivities give the power of SFC, and as a consequence the kinetics will be better than HPLC [7].

Carbon dioxide has become most widely utilized, because of its convenient critical temperature, cheapness, minimal interference with spectrometric detection, chemical stability, inertness, non-flammability, stability in radioactive applications and low-toxicity [7]. Not only CO_2 used as a mobile phase in SFC but also other substances have been investigated as well such as ammonia and nitrous oxide [19].

Lesellier pointed out the benefit of the CO_2 use in preparative chromatography instead of non-polar solvents which ensure the ease of obtaining the target fractions without additional process

for eliminating the solvent. On the other hand, he mentioned about the applicability of SFC to perform separation of the polar pharmaceutical compounds on polar stationary phases instead of the normal-phase liquid chromatography (NPLC). Moreover, SFC can be a very good option to replace reversed-phase liquid chromatography (RPLC) for separation of hydrophobic compounds by using non-polar stationary phases (i.e. alkyl bonded silica based column), where the dipole-dipole and dispersion interactions play a significant role for the retention of hydrophobic compounds. In this case, the retention behavior is similar to that in RPLC, the compounds are separated by the differences in distribution coefficient of the solute to the stationary and mobile phases [20].

The feature of SFC is not only the unique properties of supercritical fluids but also its compatibility with various detectors used with GC and LC [21], that confirms its capability with a wide range of applications in different areas like food, natural products [22], bioanalysis [23], environmental [4] and pharmaceutical [24 - 26].

2.2. Advantages of supercritical fluids in separation

The distinctive properties of the supercritical fluids mainly include [27]:

- High diffusion coefficients or high mass transfer: this ensures to apply higher velocity of mobile phase in SFC comparing to LC, hence a short analysis time can be achieved with high efficiency.
- Low viscosity: which results in a low pressure drop along the chromatographic column, thus longer columns can be installed to get higher separation efficiency without reaching an overpressure. On the other hand, supercritical fluids penetrate into the solid substances more readily than liquids.
- The solvating power of supercritical fluids relates to their densities, so it can be tuned easily by adjusting the values of both temperature and pressure.
- Availability and low cost of CO_2 in comparison to HPLC grade solvents, give it a merit to be used widely in SFC as a main component in the mobile phase.

These factors allowed SFC to contribute considerably in the analytical and preparative separation processes.

A comparison of the properties of liquids and gases with supercritical fluids are presented in Table 1 [28].

Table 1 Comparison of typical physical properties of liquids, gases and supercritical fluids.

	Gas (NTP)*	**Supercritical fluid**	**Liquid**
Density ($g.cm^{-3}$)	10^{-3}	0.3	1
Diffusion coefficient ($cm^2.s^{-1}$)	10^{-1}	$10^{-3} – 10^{-4}$	$< 10^{-5}$
Viscosity ($g.cm^{-1}.s^{-1}$)	10^{-4}	$10^{-4} – 10^{-3}$	10^{-2}

* *under standard conditions of temperature and pressure*

Despite the advantages of the supercritical fluids over liquids, due to the compressibility of supercritical fluids it is more reliable to measure the actual parameters of the mobile phase inside the column (e.g. a mass flow rate, density and pressure) using external meters, whereas these measurements are not needed in LC, see the section 21.3.4.

2.3. The use of modifiers in the mobile phase

Modifiers are small polar organic molecules such as alcohols. Typically, they are added to the mobile phase in amounts range from a few percent to some 20%. The primary alcohols are the most superior group among other small alcohols.

Since the solvent strength of CO_2 is low, similar to that of hexane, therefore the modified-CO_2 with a polar organic solvent has higher solubility for polar compounds. Binary mobile phases are used routinely to extend the polarity range of the compounds that can be separated by packed or capillary columns in SFC. Enhancement of the solubility of polar solutes by modifier addition occurs due to the interaction between them such as hydrogen bonding, dipolar and dispersive interactions [7].

It is worth to briefly refer that there are studies investigated the nature of influence between the supercritical fluid and the modifier based on the spectroscopic measurements of solvatochromic

shifts of various dyes. For instance, the results of solvatochromic shifts measurements of the maximum absorbance peak of the probe compounds [29] based on the Kamlet and Taft scale (which characterizes the interaction of the solvent molecules with electronic transition of a solute by determining three parameters dipolarity/polarizability π*, hydrogen-bond donating (HBD) acidity α, and hydrogen-bond accepting (HBA) basicity β), showed that the increase of methanol content in CO_2 at 35 ℃ increases the polarity of the mixture and its basicity while the acidity does not change. Also, with help of spectroscopic measurements, Nile red compound was used as a probe molecule to estimate the solvation strength of methanol-modified CO_2 mixture. The results referred to a nonlinear relationship between the solvent strength and the modifier percent in the mobile phase, hence, the author assumed that the polar molecules of the modifier are clustering in the surrounded area of the probe compound (the cybotactic region) thus this area would be richer with the modifier molecules than the bulk of mobile phase [30]. Lesellier et al [31], reviewed thoroughly the solubility of compounds in the mixed CO_2 with different modifiers.

When a second component is added to the main fluid of mobile phase it would rise the critical values of total mobile phase. As a consequence, if the applied back pressure on the column is higher than the critical value and the temperature is below the critical point such a case called "subcritical" [1]. The main distinction between SFC and SubFC is that the density of fluids in the subcritical region is higher than in supercritical, whereas the mobile phase compressibility is lower in the subcritical region, consequently the retention in SubFC is not mainly affected by the mobile phase density changes [32].

The modified-mobile phase concept thanks to Jentoft et al. with their exceptional work of separation the polystyrene (Mw= 600) into eighteen oligomer peaks using mixture of methanol/n-pentane (5/95 v/v%) as mobile phase from n-octyl groups bonded silica phase packed column [33]. Further successful applications have been experienced for low thermal stability substances or for high molecular weight compounds which are difficult to be volatile for the GC analysis, or they would have long residence time in LC. Different examples have been studied to investigate the effect of different modifiers on the retention and the peak shapes of PAHs, aromatic amines, alkylbenzenes and phthalates on different stationary phases [34 - 37].

The main question should be asked here, how can the modifier influence the mobile phase to enhance the elution of solutes which could not elute by the supercritical CO_2?

The answer for this question is extracted from numerous studies that have been addressed to give an explanation for the influence of a modifier on the chromatographic retention process, which can be summarized in the following effects:

- Changing the solvent strength of the mobile phase which proved by the linear relation between the retention of a solute and the solvent strength [38].
- Adsorbing on the adsorbent surface which may lead to behave as a component of the stationary phase [39 - 41].
- Deactivating the silica support by covering the active sites, inhibiting adsorption of solutes with uncovered silanol groups [42].
- The volume of the stationary phase increases due to the sorbed modifier resulting in a change in the column phase ratio [38].
- Clustering of a polar modifier around the polar solutes in the bulk of mobile phase forming a solvation sphere which is basically the main reason for the enhanced solubility in SFC [1, 30].

According to a study of Strubinger et al [39], increasing the modifier percentage gives rise to a higher retention time of the solute at a constant density, which might be ascribed to adsorbing the modifier molecules on the stationary phase resulting a decreased mobile phase volume, and consequently decreasing of the phase ratio β. This leads to increasing retention factor k according to the formula: $k = K/\beta$, where K the partition coefficient of solute, thus we usually observe a noticeable change in the retention time with only small amount of modifier. A study made by Terry and his co-workers [38] refers to retention variations when changing the composition of the mobile phase by adding a polar modifier while keeping a constant density. It has been found that those variations correlated with the mobile phase solvent strength, these observations indicate that the role of modifier primarily influencing on the mobile phase solvent strength, while covering the active sites has just a small role.

It should be noted that in case of adding a modifier to the mobile phase, its concentration plays the main role in the retention behavior of analytes (according to a study which was performed with the basic chiral pharmaceuticals [43]) then followed by the pressure and temperature effects [44].

The number of applications and reviews about the advantages of the modified-CO_2 have been increased in the 1990s [45 - 47] most of them referred to an increase in the solvent strength of mobile phase which facilitate the elution of the polar compounds.

The amount and type of a modifier drastically influence the retention process, only small amount of modifier can affect remarkably the retention time and the peak shape of a solute as reported by Upnmoor et. al. [48] by comparing methanol and 2-propanol as modifiers for the xanthines mixture separation. In a similar study [49], the author compared the separation of xanthines mixture using two modifiers, 2-methoxyethanol and 2-propanol with different concentrations, he got different chromatograms even with equal polarities of the mobile phase mixtures. Later Berger et al., studied the effect of various modifiers on separation of polar steroids mixture from different columns [50]. It was found that only when polar modifier used with a polar stationary phase results in good peak symmetry, whereas lower polarity modifiers compared to methanol (such as acetonitrile or tetrahydrofuran) gives poor peak shapes.

Lesellie et al., investigated the influence of adding both methanol and acetonitrile to CO_2 on the retention of alkylbenzene homologues. It is indicated from the results that regardless of the modifier used, a change in the phase ratio results in retention change of the short alkyl chain homologues, but the retention variations for the long alkyl chain were a consequence of modification of the solute solubility in the mobile phase (the solvent strength) [32].

2.4. The use of additives in the mobile phase

The use of additives in SFC can be a very good choice to solve one of the most challenging problems for the analytes that could not elute at all with a modified mobile phase [51] or elute with a low quality peak shape [52].

Generally, the additives are highly polar molecules, they usually are dissolved in modifier in order to avoid immiscibility with the main fluid of the mobile phase which is typically non-polar, and then they (modifier and additive) are delivered by a pump as a single component to converge with a main fluid in the mixer. This arrangement would ensure delivering an accurate and constant amount of additive to the mobile phase, at the same time, it would follow the modifier amount changes.

Many kinds of compounds can be used to function as additive in SFC, such as acids to improve the chromatographic process of the acidic compounds [53], bases to improve the chromatographic properties of the basic solutes [52, 54], ammonia solution which is considered as an advantageous

additive for the ease of removal process of the purified target compounds in the preparative applications [55], salts [56, 57] and water [56, 58, 59].

The role of additive is still point of debate for being comprises different mechanisms [31, 60]. For the ease, we subcategorized the possible influences into the stationary and the mobile phase modifications:

A- Modification of a stationary phase by the additive addition

- Covering the active sites (e.g. residual silanol groups of silica based stationary phases) which are one of the main reasons for poor quality of the peak shapes [61].
- Changing the chemical nature of stationary phase (e.g. polarity) by adsorbing the additives on it [61 - 63].

B- Modification of a mobile phase by the additive addition

- Ion pair formation between ionizable solutes and additives [60, 64].
- Alter the apparent pH of the mobile phase which results in formation of different ionization states of the analytes, hence that could activate or eliminate specific solute – stationary phase interactions [65].
- Solute ionization suppression by using stronger acidic additives than the acidic solutes, and in a similar manner for the basic solutes [66]

Relating to how the additive can influence the chromatographic process of solutes, Raimbault et. al. used linear solvation energy relationships methodology (LSER) to get insight into the effect of the acidic and basic additives with various concentrations on the retention of chiral and achiral solutes in both chiral and achiral stationary phases [67]. They found that the basic additive (isopropylamine) in low concentration affects the retention by its adsorption on the stationary phase, while at higher concentration the effect on the mobile phase becomes more significant. Meanwhile, with the acidic additive (trifluoroacetic acid), the retention influenced by the adsorption effects.

Our concern in the current work is about the use of water as additive for polar analytes separation. This interest for the use of water is ascribed to the unique properties of being eco-friendly solvent, highly polar character (e.g. solvent polarity parameter P = 10.2 for water, P = 5.1 for methanol

and $P = 0$ for pentane [68]) and it is capable to act both as a hydrogen bond acceptor and a hydrogen bond donor.

It was referred in the study of Liu et al. that adding a mixture of water and methanol to CO_2 improves noticeably the peak shapes for variety of hydrophilic analytes on different stationary phases comparing to those obtained without water. The authors are believed that water addition to the methanol-CO_2 mixture increases the solvating power of the mobile phase and enhances the solubility of hydrophilic analytes [59]. That explanation is in agreement with an earlier study on separation of four nucleobases on different columns [63], which also confirmed that addition of water to the methanol-CO_2 mixture enhances the solubility of the studied compounds. But lately, Roy et al., reported that adding 6% water to the CO_2 containing different percentages of methanol (10 ~ 40%) resulted in a very little change in polarity of the mixture when the methanol percentage reached 40%, which means adding water did not contribute to increasing the solvating power of the mixture [69]. According to our results, which would be mentioned later in the sections 4.6.1, 4.6.2, that a very low level of water content 0.06% in the mobile phase, was able to impart influence on the separation process of the basic analytes as a consequence of water adsorption on the stationary phase. Both of these findings (from a study of Roy et al. and our results) support the assumption of employing water as an additive in a ternary mobile phase follows the same concept of HILIC mechanism in the liquid chromatography, wherein CO_2 is used instead of an organic component in the mobile phase. As a result of the preferential adsorption of water (the polar component of mobile phase) on a polar stationary phase, a water rich adsorbed layer will form, then the elution of a polar analyte is presumed to occur as a result of partitioning between the adsorbed layer and the mobile phase [6, 58, 70].

Incorporating the additives with the modified mobile phase can lead to a good separation process for several polar solutes, which were thought previously that SFC is not a proper choice for them.

2.5. Adsorption isotherms

The relation between the concentrations of solute in the liquid phase to that in the stationary phase at a constant temperature is described by the adsorption isotherm. Admittedly, calculating the adsorption isotherm is considered the main tool in analytical or preparative chromatography to determine the adsorption capacity of a chromatographic column for the target compounds, also to

obtain information about the extent of heterogeneity of the column, and to conclude the possible interactions between the mobile phase components and an adsorbent, or adsorbate – adsorbate interactions. It also helps to improve the productivity rates of production for the preparative chromatography applications, or to measure the specific surface area of the adsorbent material.

Different types of adsorption isotherm can be seen according to the adsorption mechanism at the liquid solid interface, which is a useful tool to get knowledge about the adsorption nature of the studied system [71]. The most frequent shape observed in the liquid-solid adsorption cases is L shape or type I according to Giles classification [71] and Brunauer [72], respectively. This type predicts adsorbing each molecule of a solute on one adsorption site of the adsorbent till formation a monolayer of the adsorbed molecules, and further adsorption is prevented. The common adsorption isotherm model which is able to describe this adsorption mechanism is the Langmuir adsorption isotherm model, which assumes the adsorption sites of an adsorbent surface has a uniform energy and the adsorption is limited to solutes – adsorbent only [73]. In this case, the corresponding overloaded elution profile is characterized with a steep front and tailing end. Whereas in many real cases in the linear chromatography, when the elution profile has a long tail (which can be caused by the surface heterogeneity of stationary phase) the Langmuir model is unable to predict the elution profile with a good fit with the experimental profile, thus more developed models are recommended to account for the equilibrium adsorption such as bi-Langmuir [74], Toth, and Freundlich models [8]. The bi-Langmuir adsorption model was used in our study to express the adsorption process for the single component adsorption isotherm measurement, which is given by the following equation:

$$q_i = \frac{a_1 C_i}{1 + b_1 C_i} + \frac{a_2 C_i}{1 + b_2 C_i} \qquad (1)$$

where q_i is concentration of component i in the stationary phase, a_1 is the initial slope of the isotherm, b_1 the equilibrium constant for one site, and a_2 and b_2 are the same parameters for the other site, respectively. Those parameters give the saturation capacities $q_{s,1}$, $q_{s,2}$ for site 1 and site 2 by calculating the ratio a_i/b_i.

The main types of adsorption isotherm used for describing the liquid-solid adsorption of a component to a stationary phase are I, II and III types, as shown in first row in (Figure 3). Type I

is the most frequent and simple type such as Langmuir and bi-Langmuir models. Type II and III are more complex which indicate to that solute-solute interactions between the adsorbed molecules are present. The second row illustrates the corresponding overloaded band profiles for each isotherm type, the last row is a schematic representation of the adsorbed layers of the molecules [75].

2.5.1. Excess and absolute adsorption

The *adsorption* occurs when the intermolecular forces of an adsorbent bind the fluid molecules forming an accumulation at the interface in contact with a surface (liquid or solid). When no adsorption occurs between a surface and the fluid molecules in the bulk fluid, the density of the molecules at the interface is equal to the density of the bulk fluid. On the other hand, by adsorbing molecules on the surface an increase in amount of the molecules (in comparison to non-adsorption case) is observed, this increased amount expressed as a surface excess Γ, which is defined by Kazakevich et al. [76] as "the difference between the amount of the component that would be in a hypothetical system with the same geometrical parameters but without the surface influence and the same system with the influence of the surface". In the real systems (non-idealized system), the surface excess Γ (mol/m^2) of component (i) is given as the amount (in moles) per unit area of a solid surface:

$$\Gamma_i = \frac{n_i}{A} \qquad (2)$$

where n_i (mol) is the excess quantity adsorbed of component (i), A (m^2) is the interfacial area. The absolute adsorbed amount m^a is defined as the sum of two amounts: *reference molecules* which are present (without adsorption effect) within the adsorbed layer and *surface excess molecules* which are the measured quantity of adsorption.

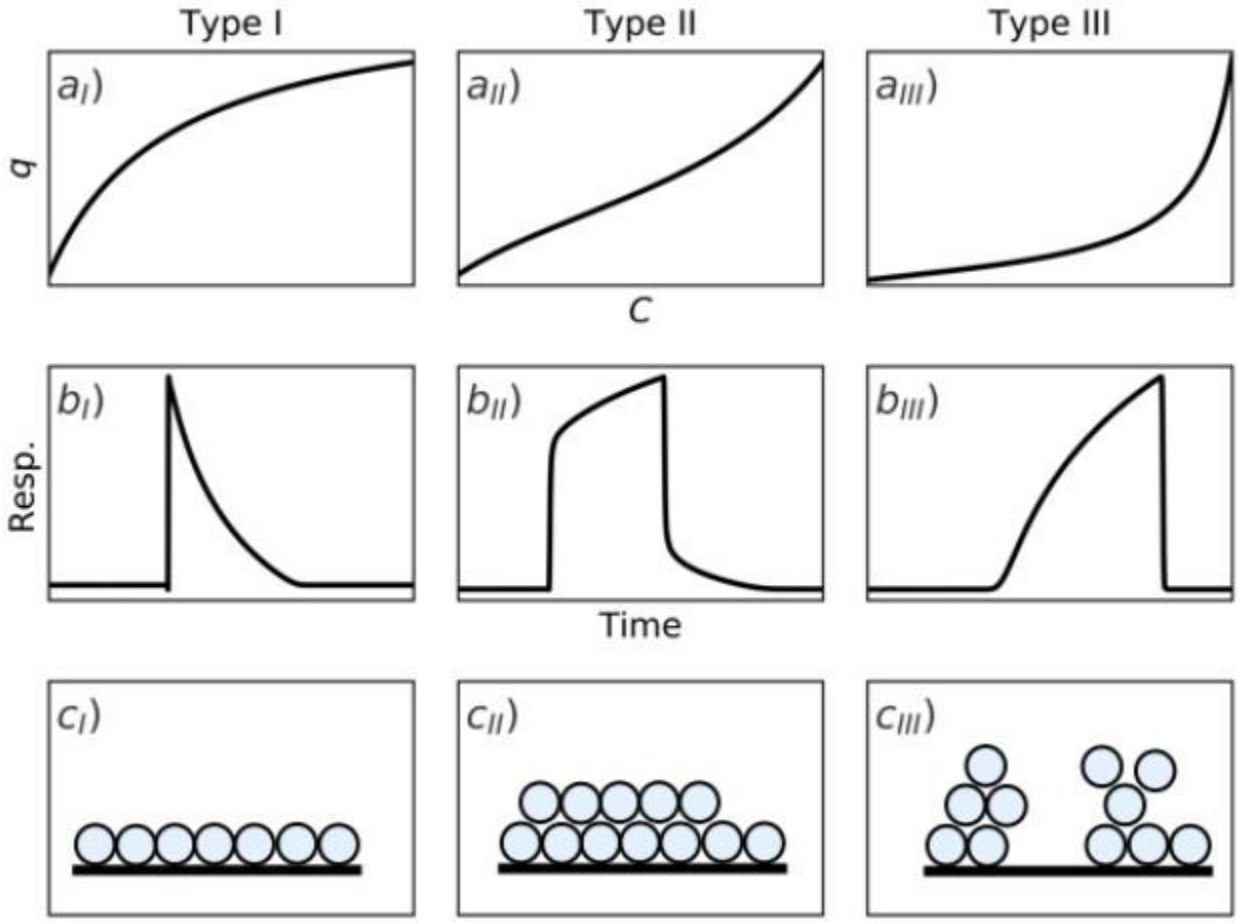


Figure 3. **Top row**: the common types of adsorption isotherm in chromatographic process.
Middle row: the corresponding overloaded elution profiles.
Bottom row: representation of the adsorbed molecules layers with increasing solute concentration [75]

However, Kazakevich et. al. and Vajda et. al. explained thoroughly the excess adsorption isotherm calculation in liquid chromatography using the disturbance method or also known as the perturbation method [76, 77]. According to their studies, the used equation for determining the surface excess isotherms in liquid chromatography depends on the retention volume of the disturbance peak of the studied component in the binary mixture. The formula is derived from the column mass balance equation, it is given as:

$$\Psi_A = \frac{\int_0^{\phi_A} (V_{R,A'}(\phi) - V_0) d\phi_A}{S} \quad (3)$$

where Ψ_A (cm^3/m^2) the surface excess of component A of the binary mixture, $V_{R,A}$ (cm^3) is the retention volume of the perturbation peak, ϕ is the volume fraction of the organic modifier in the mixture, S is the total surface area (m^2) of packing material contained in the column, and V_0 (cm^3) is the thermodynamic void volume of the column which is written:

$$V_0 = \int_0^1 V_{R,A}(\phi) d\phi \quad (4)$$

The excess isotherm is represented by plotting the surface excess amount of a component of the mixture against the mole fraction of the component in the mixture. Accordingly, different shapes of the excess isotherm would be expected as reported by Guiochon et. al. [8].

The surface excess of a component in the binary mixture increases with increasing the mole fraction of the component in the mixture, it reaches its maximum when it fills all the available adsorption sites on the surface of adsorbent, which means a monolayer of the component is established, then gradually goes down to zero value where the density of molecules of the component at the interface is the same as density in the bulk fluid [78].

2.5.2. Competition for Adsorption

The competition occurs between the mobile phase components for the contribution to adsorption on the stationary phase. For example, the mobile phase in SFC usually includes CO_2 the main component, the co-solvent, and the additive, in case the last two components or one of them adsorbs on the stationary phase, they will compete with the solutes which leads to modify the interaction of solute-adsorbent, which in turn modify the retention time of solutes. The competition is attributed to different affinity of each component to the adsorption sites of a stationary phase. On the other hand, it is not always assumptive for the mobile phase components to compete with solutes, because there are cases where adding a modifier to the mobile phase can only change the solubility of solutes in the mobile phase, so it depends on the mechanism of the components which affect the retention of solutes. As a result of the competition, it primarily deforms the shape of a chromatographic peak of the eluted compounds and modify the retention time [8], and this effect is more significant when at least one component adsorbs more strongly to the stationary phase than the solute does [79]. To describe this competitive adsorption, we need to use an adsorption model which considers a multicomponent adsorption such as the competitive Langmuir model for a

homogeneous surface of adsorbent, or the competitive bi-Langmuir model for a heterogeneous surface [8]. The latter is used in this study, and it is given with the formula:

$$q_i = \frac{a_{i,1} C_i}{1 + b_{A,1} C_A + b_{B,1} C_B} + \frac{a_{i,2} C_i}{1 + b_{A,2} C_A + b_{B,2} C_B} \quad (5)$$

where C_A and C_B and concentrations of component A and B in the mobile phase**.**

2.6. Band profile simulation

The numerical simulation offers an alternative approach for investigations with more saving of chemicals, time, and money, it can provide deep insight about a specific problem which is difficult and tedious to study by the conventional practical methods, so it can provide the best parameters to perform the target experiment with a very good empirical results [8].

In nonlinear chromatography, the experimental elution profile can be simulated by using algorithm which is a numerical solution of the partial differential equation of the chosen model, such as the Equilibrium-Dispersive (ED) model which is the simplest model used to describe the chromatographic behavior of a solute within the column. This model assumes that the mobile and the stationary phases are in equilibrium instantaneously, it takes into account the contributions of both axial dispersion effect and mass transfer kinetics which are gathered in a single apparent dispersion coefficient, (two main sources contribute to axial dispersion: molecular diffusion in the inter-particle pores and eddy diffusion). When the mass transfer kinetics are fast but not infinitely fast, the mass balance equation of the ED model is written:

$$\frac{\partial C_i(z,t)}{\partial t} + F \frac{\partial q_i(z,t)}{\partial t} + u \frac{\partial C_i(z,t)}{\partial z} = D_a \frac{\partial^2 C_i(z,t)}{\partial z^2} \quad (6)$$

where C_i and q_i are the concentrations of component i in the mobile and the stationary phases, respectively, z is the length of the column, t the time, u the mobile phase linear velocity and F the phase ratio, wherein $F = (1 - \varepsilon_t)/\varepsilon_t$ where ε_t is the total porosity of the column, and the apparent dispersion coefficient relates to the column efficiency (theoretical plates N) with the following equation:

$$D_a = \frac{uL}{2N} \quad (7)$$

where L is the column length.

The purpose of the elution band simulation is to deeply understand the separation process, to validate the calculated parameters of the adsorption isotherm that is used in the simulation program

that could account well for the adsorption process at the equilibrium. By predicting the elution profile in a good agreement with an experimental profile we would be able to select the best conditions to carry out the chromatographic separation process [8, 74].

Chapter 3

EXPERIMENTAL AND METHODS

"It doesn't matter how beautiful your theory is, it doesn't matter how smart you are. If it doesn't agree with experiment, it's wrong." Richard P. Feynman

3.1. Equipment

The Waters UPC^2 SFC system (Waters Corporation, Milford, MA, USA) was used in this study. The controller and data acquisition software of the system was Empower provided by Waters. The instrument includes: a binary pump which is a cooled pump for the carbon dioxide and a modifier pump with as many as 4 channels with integrated vacuum degassing, an auto sampler with a 10 μL sample loop, a column compartment provides temperature from 20.0 to 90.0 °C for up to 3 columns, a pressure regulator (the convergence manager unit) to monitor and regulate the pressure of carbon dioxide and maintain the set back-pressure value, a diode array UV/VIS detector in the range of wavelength 190 – 800 nm.

A mini CORI-FLOW mass flow meter from Bronkhorst High-Tech B.V. (Ruurlo, Netherlands), Model No. M13-ABD-11-0-S used for measuring the CO_2 mass flow. The inlet and outlet pressures of the column measured with an external digital pressure meter (Norwalk, Connecticut, USA), Model No. DPG 4000 series.

3.2. Columns

The surface excess measurements were performed on two end capped reversed phase columns, (Figure 4) shows a schematic for the chemical structure of the used stationary phases, the first column is Supelcosil ABZ+plus HPLC column (Sigma-Aldrich, Germany) 150 × 4.6 mm is embedded a polar group (amide), packed with 3 μm spherical particles, specific surface area S_{BET}

=170 m^2/g (all values of S_{BET} for the used columns in this work were provided by the vendor), the carbon load 12% and pore size 120 Å which is characterized by a high bonded phase density and by the presence of amide group (electrostatic shielding agent) within the bonded alkyl chain. The second column is Symmetry C_{18} (Waters, US) 150 × 4.6 mm packed with 5 µm spherical particles, specific surface area S_{BET} =335 m^2/g, the carbon load 19% and pore size 100 Å, which is classified under a moderate polarity and high hydrophobicity group among the other common C_{18} phases [80].

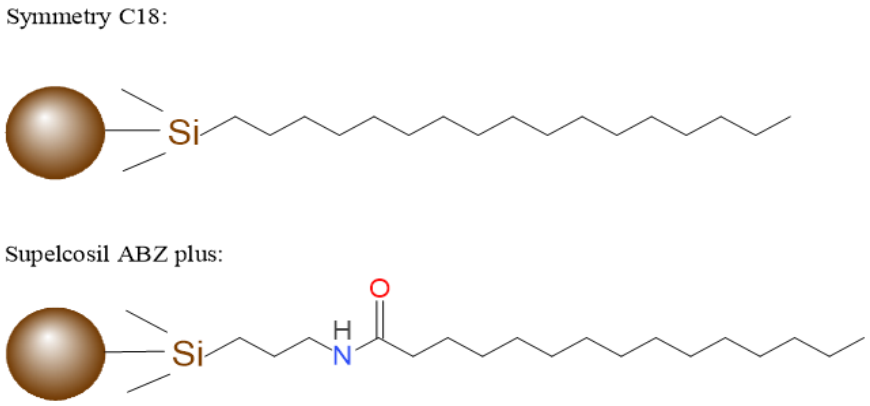
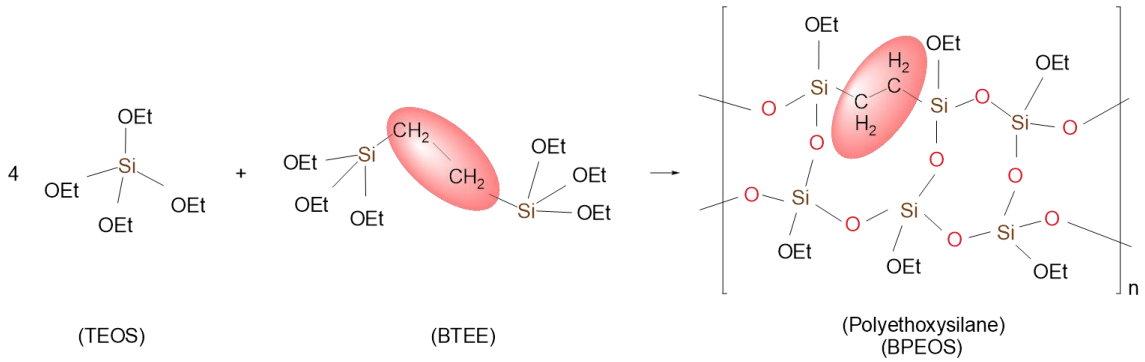


Figure 4. Chemical structure of the stationary phases used in this study [81].

Also, a hybrid silica Viridis BEH column (Waters, US) has been used for the multicomponent adsorption isotherm experiments and separation of the polar solutes, the dimensions are 50 × 3 mm packed with 1.7 µm spherical particle, specific surface area S_{BET} =340 m^2/g and 130 Å pore size.

The BEH silica stationary phase is a hybrid (organic –inorganic) material prepared by reacting two organosilanes, tetraethoxysilane (TEOS) and 1,2-bis(triethoxysilyl) ethane (BTEE) with the ratio 4:1 mole according to the following reaction:



This type of packing materials ensures performing chromatographic experiments under a wide pH range 1 -12 [82].

3.3. Chemicals and analytes

A liquefied carbon dioxide filled in a pressurized cylinder with purity 99.5% used as the main mobile phase was purchased from (Linde Group, Hungary). Methanol, water, and heptane with HPLC grade purity were purchased from (Fisher chemicals, UK) and benzene from (VWR, France). The analytical standards with a purity at least 99% benzene, ethylbenzene, butylbenzene, hexylbenzene, octylbenzene, decylbenzene, dodecylbenzene, tetradecylbenzene, octadecylbenzene, aniline, uracil, propranolol HCl, sulfamethazine and sulfamethizole were all purchased from (Sigma-Aldrich, Germany), caffeine anhydrous from (Fluka, Germany). Chemicals used as an additive in the chromatographic experiments diethylamine, triethylamine were obtained from (Fluka, Germany) and ammonia solution 32% from (VWR, France).

3.4. SFC Operation conditions

3.4.1. Estimation of the actual volumetric flow rate of the mobile phase

The actual volumetric flow rate of the mobile phase is different from the set value due to the compressibility of CO_2, so it should be measured under the experimental conditions using external pressure and mass flow meters. The actual mass flow rate was measured using a Coriolis mass flow meter, which is installed after the CO_2 pump outlet and before the mobile phase mixer. We assumed that the actual and set flow rates of methanol are same. The pressure at the inlet and outlet

of the column was measured by an external pressure meter, the values were 184.57 and 154.3 bar, respectively. The density of the mobile phase inside the column was determined by the REFPROP software Ver. 8 from NIST at the set temperature of the column 26 °C and the measured pressure values at inlet and outlet of the column. By input the values of the measured mass flow rate of the mobile phase F_m = 0.943 g/min and the calculated density of the mobile phase ρ = 0.89891 and 0.87466 g/cm^3 for inlet and outlet of the column, respectively, in the formula (8) we could obtain the average of the two volumetric flow rates as F_v = 1.065 mL/min, which represents the actual volumetric flow rate (100% CO_2) inside Viridis column which will be used for calculation of the single component and the competitive adsorption isotherms.

$$F_v = F_m/\rho \qquad (8)$$

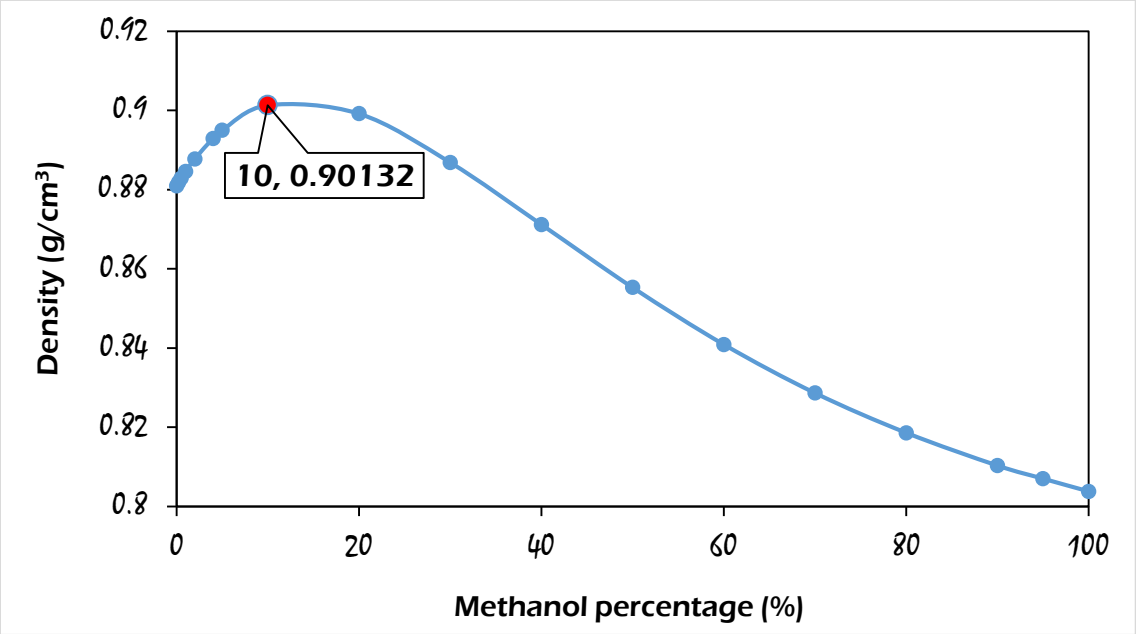


Figure 5. Variations of the density of the mobile phase inside the ABZ+plus column over the full range of methanol concentration in the mobile phase under the experimental conditions: 1 mL/min, 26 °C and 150 bar.

The density changes of mobile phase inside the ABZ+plus column are shown in (Figure 5), the maximum density of a mobile phase was recorded with 10% methanol in CO_2. The density drop

along the column was 2.7%. The density of 100% CO_2 mobile phase under the subcritical (26 °C, 145 bar) and the supercritical (40 °C, 250 bar) conditions were 0.875, 0.887 g/cm^3, respectively.

3.4.2. The surface excess adsorption isotherm

Measurement of the surface excess isotherm of methanol was carried out using the minor disturbance method [8], according to this method, the chromatographic column equilibrated with a pure organic modifier, with a pure carbon dioxide and with binary mixtures of carbon dioxide and organic modifier covering the whole range from pure carbon dioxide to pure organic modifier, after the equilibrium is completed at the chosen composition a small amount 2 µL of an organic modifier is injected into the chromatographic column, which generates minor disturbance peaks which were recorded at wavelength 210 nm. The whole procedures have been described by Kazmouz et. al. [41].

The void volume of each column which is required for excess amount calculation, it can be calculated from the pattern of the disturbance peak retention, according to Eq. (4). The resulting average thermodynamic void volumes in sub- and supercritical conditions are 1.486 cm^3 and 1.671 cm^3 for the Symmetry C_{18} and ABZ+plus columns, respectively. Also the void volume of the columns was determined by injecting 1µL of nitrous oxide gas dissolved in methanol as an un-retained solute eluted with 100 % CO_2 giving very close results to the previous ones: 1.476 cm^3 and 1.579 cm^3, respectively.

The experiments for measuring the surface excess of methanol on the reversed phases were performed under two conditions of temperature and back pressure, the subcritical (26 °C, 145 bar) and the supercritical (40 °C, 250 bar), the mobile phase flow rate was set at 1 mL/min, the wavelength of detector was set to 210 nm. All the retention volumes were corrected by taking in account the extra-column volume of the instrument, which was measured by replacing the column with a zero-volume connector, it was 54 µL between the injector loop and the detector cell.

3.4.3. Alkylbenzenes sample

Elution of the alkylbenzenes mixture sample (the nine alkylbenzenes homologous are mentioned in section 3.3) was performed under subcritical and supercritical conditions, the mixture was dissolved in two sample solvents, methanol and heptane with concentrations 0.5, 0.7, 1.1, 1.8, 2.2,

3.4, 4.5, 5.0 and 5.4 g/L, respectively. 2µL of the mixture was injected into both the Supelcosil ABZ+plus HPLC and Symmetry C_{18} columns using 100% CO_2 as mobile phase. The chromatograms were recorded at 210 nm wavelength of the detector.

3.4.4. The single-component and competitive adsorption isotherms

The chromatographic experiments were performed at 1 mL/min, 26 ℃ and 150 bar of a flow rate, a column temperature and a back pressure, respectively. The injection volume into the Viridis BEH column was 1 µL of each pure methanol, pure water and (10:90 v/v, MeOH:H_2O) mixture sample. For the mixed sample we have chosen a high percentage of water in respect of methanol because the corresponding intensity of water signal of the detector was very small comparing to the methanol signal. All the chromatograms recorded at 195 nm wavelength. The void volume (the occupied volume by a mobile phase in the column) V_0= 0.266 mL was determined with pentane as an un-retained solute using 100% CO_2, its retention time was corrected by subtracting the required time of the injection profile t_{sys}=0.07 min. The column was equilibrated for 90 minutes with 100% CO_2 before the sample injection. The adsorption isotherms were calculated by the inverse method (IM) [8]. The procedures of IM can be summarized as following:

1. Recording the overloaded band profiles of each studied component.
2. Selecting an a priori model for the adsorption isotherm, which is able to represent the distribution of a solute between the two phases accurately. The isotherm model is usually predictable (whether it is a convex, a concave, or S-shaped) form the shape of the recorded overloaded band profile.
3. Calculating the chromatograms using the initial estimated values of the isotherm parameters (the initial values were determined using the elution by characteristic point ECP method) and an appropriate model for describing the chromatographic process, we used in the calculation the equilibrium-dispersive model (the properties of this model were mentioned in somewhere in Section 2.6).
4. The use of the following formula to compare the experimental and calculated profiles:

$$\min \sum_i r_i^2 = \min \sum_i \left(C_i^{\mathrm{sim}} - C_i^{\mathrm{meas}}\right)^2 \qquad (9)$$

where C_i^{sim} and C_i^{meas} are calculated and measured concentrations at point i and r_i is their difference.

5. Optimizing the isotherm parameter values by obtaining the minimum of the residual sum of squares (SSR) between the experimental and calculated curves using an optimization routine.

3.4.5. Transformation from absorbance to concentration profile

In order to get the overloaded profile of an analyte, which is required for determining the adsorption isotherm by the IM method, we need a high concentration sample, but this will be out of the linear range of the detector response, additionally each component of our sample (methanol and water) has different response of the detector, so it is required to calibrate the detector according to the following steps:

- The amount of the injected sample can be calculated by integrating the elution profile:

$$m = \int C(V)dV = \overline{F_v} \int C(t)dt \quad (10)$$

- Absorbance is proportional to concentration in the linear range of detector response, thus the relation between the absorbance A with sensitivity factor k is expressed as follows:

$$A\,(t) = kC(t) \quad (11)$$

$$A_T = \int A(t)dt \quad (12)$$

$$A_T = k \int C(t)dt = \frac{km}{\overline{F_v}} \quad (13)$$

$$k = \frac{A_T \overline{F_v}}{m} \quad (14)$$

where A_T is the peak area, $\overline{F_v}$ the mean volumetric flow rate and m the amount of the injected sample (mg). By calculating k from Eq. (14) the detector calibration is achieved, so we can get the concentration profiles from the raw chromatographic data (absorbance vs time) by applying Eq. (15):

$$C(t) = \frac{A(t)}{k} \quad (15)$$

Those steps are applied to transform the elution profile of each studied sample (methanol, water and the mixture), with different UV response factors, to get the concentration profiles (concentration vs time).

3.4.6. Standards preparation

The standards used in this study are: aniline, uracil, caffeine, sulfamethaznie, sulfamethizole and propranolol HCl ranging from weak (aniline) to strong (propranolol) basic compounds, which they

or their derivatives are widely used in the pharmaceutical industry. The standards were chosen to exhibit a distinctive elution behavior from a Viridis BEH column. The samples were prepared by dissolving 0.5 mg of each compound in a glass vial containing 1.5 mL methanol, except the amount of aniline was 1 µL. The amount of sample injection was 1 µL, different mobile phase compositions were used, the flow rate at 2 mL/min, the column temperature at 26 °C, the back pressure 150 bar and UV detection at 204 nm.

Chapter 4

RESULTS AND DISCUSSION

"Science is wonderfully equipped to answer the question 'How?' but it gets terribly confused when you ask the question 'Why?'." Erwin Chargaff

4.1. The surface excess isotherms of methanol on the reversed stationary phases

The adsorption of the eluent components on the stationary phase has a big influence on the retention of the solutes. The surface excess adsorption isotherm in chromatography provides us information about the amount of the mobile phase components that could be adsorbed on the stationary phase revealing the extent of packing material affinity to the polar and nonpolar molecules. Also it gives information about the maximum level that the adsorption could reach in the whole composition range.

In order to obtain accurate results about the adsorption of the eluent components on the stationary phase in SFC, it is advisable to measure the actual volumetric fraction of the studied component. In this study we used the molar fraction unit X_{MeOH} to express the real amount of modifier (methanol) used in the mobile phase. For that purpose, the actual mass flow rate of methanol and CO_2 was measured separately at different fractions of methanol in the mobile phase 0.1, 0.2, 0.5, 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100% (v/v) using a mass flow meter. Then, the amount of substance for both methanol and CO_2 were calculated by dividing the actual mass flow of the component by its molar mass. After that, the molar fraction of methanol obtained by dividing its amount by the combined amount of methanol and CO_2.

The surface excess isotherm of methanol is determined by forming the disturbance peaks as a result of injecting a small amount of methanol in the column, then the retention volume of the disturbance

peaks and the elution peak of pure carbon dioxide are used for the calculation of the surface excess isotherm using Eq. (3).

(Figure 6) shows the retention volumes of the recorded disturbance peaks over the full range of methanol with the reversed stationary phases. All the patterns have similar shape in either sub- or supercritical conditions. When the mobile phase composition is close to pure carbon dioxide, a sharp increase of the retention volumes can be observed. The amplitude of this increase is smaller for the ABZ+plus column than for Symmetry C_{18}.

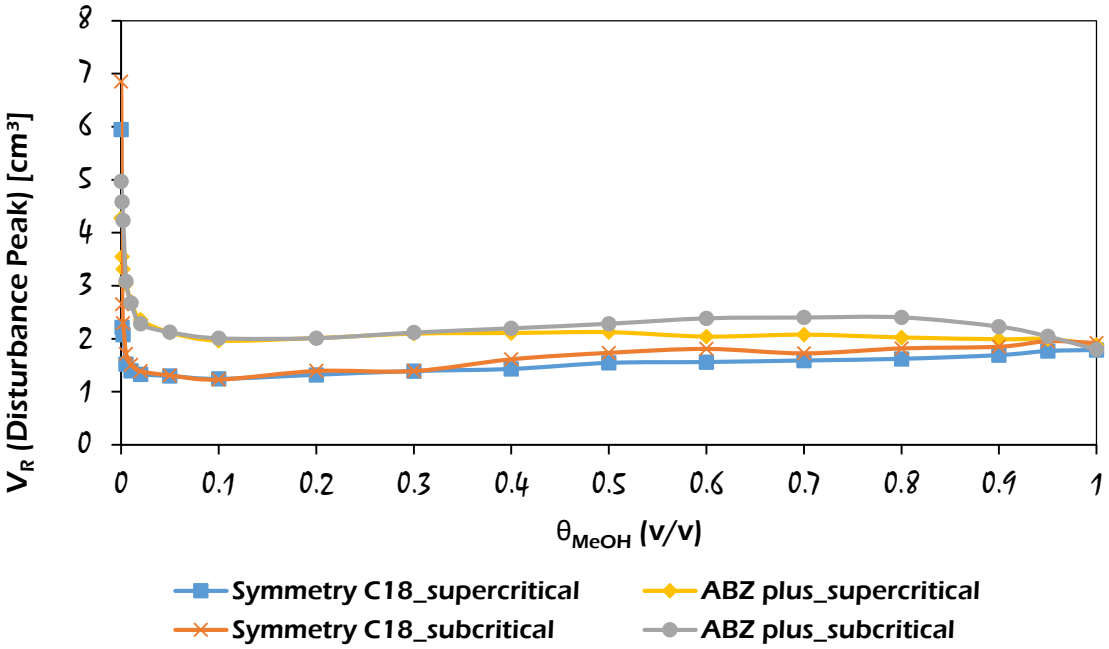


Figure 6. Retention pattern of the minor disturbance peaks of methanol on ABZ+plus, Symmetry C_{18} columns in sub- and supercritical conditions using methanol as the organic modifier with carbon dioxide at concentrations between 0 and 100%. The set conditions were used for calculating the volume fraction of methanol.

It can be noticed in (Figure 6) that the retention volumes of ABZ+plus column are higher than those of Symmetry C_{18} due to the ability of the embedded amide group in ABZ+plus column to form hydrogen bonding with a hydroxyl group in methanol, resulted in higher retention volumes. In (Figure 7), the methanol's surface excess isotherms are presented. It is shown similar trends for the curves under both the subcritical and supercritical conditions.

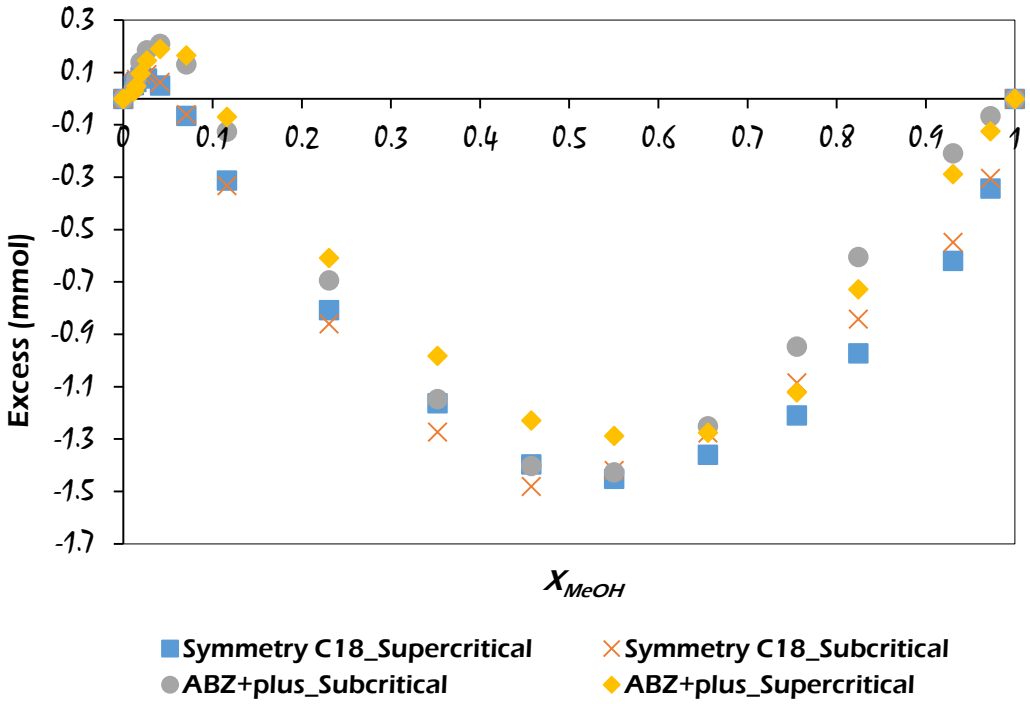


Figure 7. Methanol surface excess isotherms with mixture of methanol and carbon dioxide on ABZ+plus, Symmetry C_{18} columns in sub- and supercritical conditions. The molar fractions of methanol are calculated based on measuring the actual mass flow of CO_2 and methanol separately.

A pronounced wide negative part is observed for the two columns, which is related to the preferential adsorption of carbon dioxide onto hydrophobic bonded ligands of the stationary phase surface. While methanol adsorption appears much more lesser, which is represented by a positive small part at a low concentration of methanol in the mobile phase, it also indicates to a small amount of methanol could adsorb on the accessible silanols and other polar groups, if they are present in the structure of stationary phase (such as amide group in the ABZ+plus column).

The maximum of surface excess for methanol was at very small set volume fractions 0.02 and 0.005 of the organic modifier in the mobile phase for ABZ+plus and Symmetry C_{18} columns, respectively, which corresponds to the actual molar fractions X_{MeOH} = 0.041 and 0.019, as it is

presented in (Figure 8). This maximum value for Symmetry C_{18} was smaller than for ABZ+plus column.

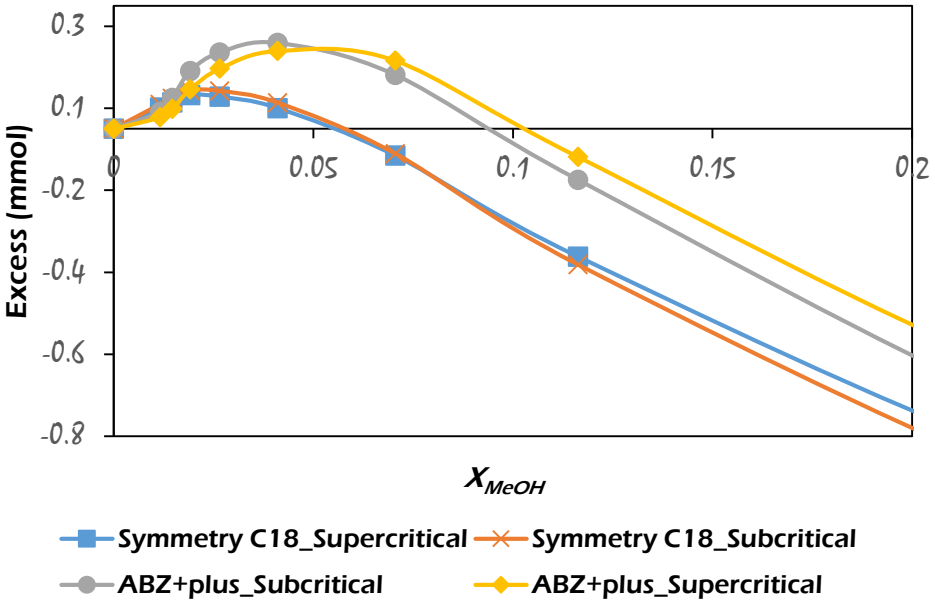


Figure 8. Methanol surface excess isotherms with mixture of methanol and carbon dioxide, the plot was scaled between 0 – 10% of the organic modifier.

4.2. Alkylbenzene Separation on the reversed stationary phases

This separation was used as an indicator for the possible effect of the use of methanol or heptane as a sample solvent on the separation process of the alkylbenzenes mixture on the reversed stationary phases with 100% CO_2 as mobile phase.

Some of the chromatograms obtained for the two columns in sub- and supercritical conditions are shown in (Figure 9). Baseline resolution was achieved for all the components of the mixture under the subcritical conditions (Figs. 9 A, C). By comparing A with B and C with D, it is obvious that the components elute faster (smaller retention times) under supercritical conditions than under subcritical conditions which may due to the increased mixture solvation by higher dense CO_2. The elution order of the compounds from the short alkyl chain compound to the long, the longer the alkyl chain the higher retention time.

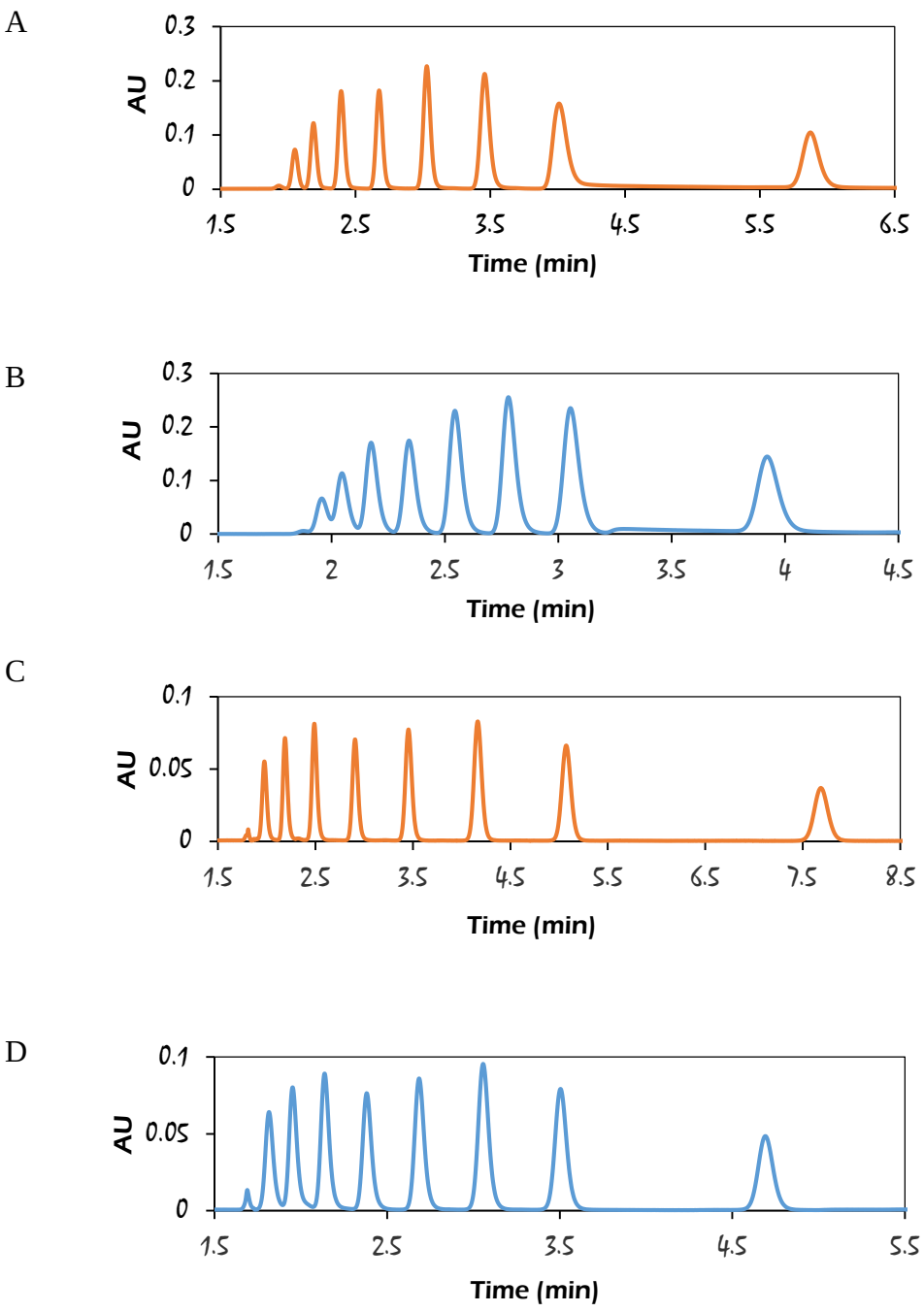


Figure 9. Chromatograms of alkylbenzenes: a) ABZ+plus column, methanol solvent under subcritical conditions, b) ABZ+plus column, methanol solvent under supercritical, c) Symmetry C_{18} column, heptane solvent under subcritical conditions, d) Symmetry C_{18} column, heptane solvent under supercritical conditions. The experimental conditions: injection volume: 2µL, flow rate: 1 mL/min, wavelength: 210 nm.

The separation efficiency of the compounds was determined using PeakFit software v4.12, the calculation of number of theoretical plates was an average of two injections for each sample.

(Figure 10) demonstrates similar trend of an increasing separation efficiency starting from the component with short alkyl chain to the long one, under the studied experimental conditions and columns, except for the case of methanol solvent on the ABZ+plus column under the subcritical conditions, where there is an abrupt drop in the curve after the decylbenzene peak. This may arise from the influence of the adsorbed methanol on the surface of stationary phase where a competition between the abundant methanol (sample solvent) and alkylbenzenes for adsorption introducing a combined displacement and tag-along effects for the various alkylbenzenes in turns reducing the *N* values of these components.

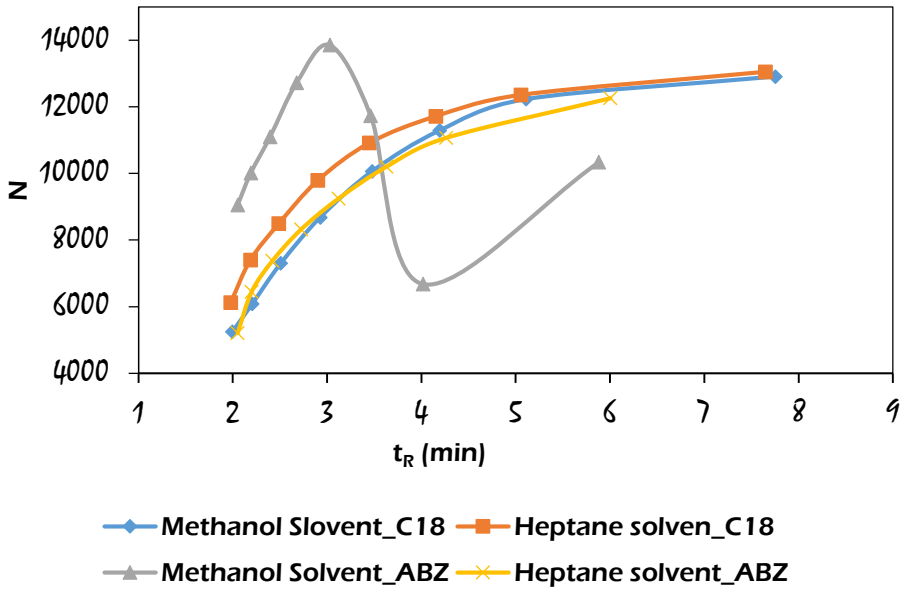
Also it is shown that the efficiency value for each component was higher under the subcritical conditions than the supercritical conditions, which was as consequence of the higher retention times which were obtained in the former case than in the latter one.

For comparing the performance of the use of methanol and heptane as a sample solvent with the studied columns, we calculated the difference of the retention times (Δt_R) for each analyte of the alkylbenzenes sample:

$$\Delta t_R = t_R(\text{methanol}) - t_R(\text{heptane}) \qquad (16)$$

where t_R(methanol), t_R(heptane) are the retention times of a given analyte dissolved in methanol solvent and heptane solvent, respectively.

A



B

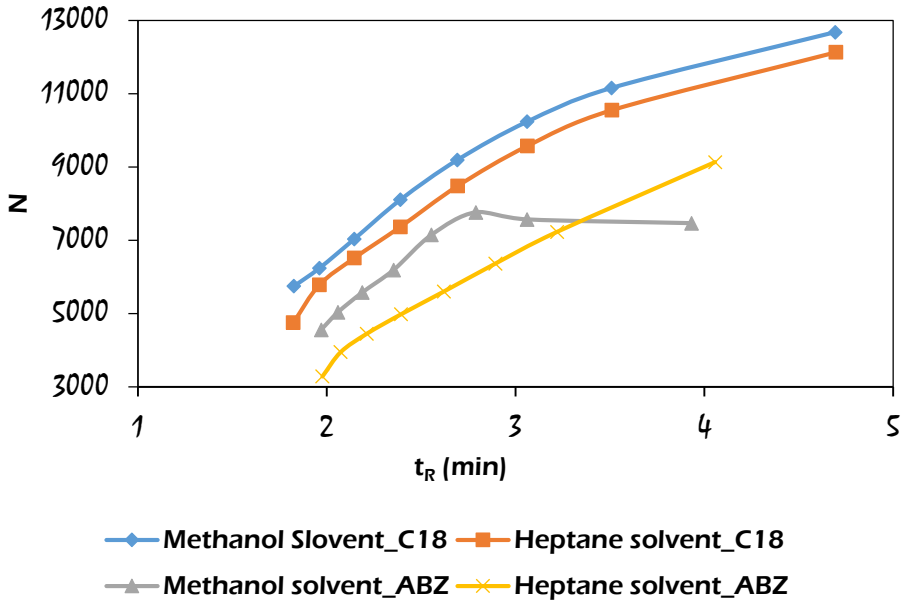


Figure 10. Alkylbenzene separation efficiency of Symmetry C_{18} & Supelcosil ABZ+plus columns with different solvents in **a)** subcritical conditions, **b)** supercritical conditions.

It is noticed from (Figure 12, Figure **14**) that for the ABZ+plus column there is an increasing trend of Δt_R values in the negative direction of the Y axis for each analyte. This means, the analytes eluted with the use of methanol faster than with heptane, that explained by a higher amount of the adsorbed methanol on ABZ+plus column compared to Symmetry C_{18}, which can modify the polarity of the stationary phase surface, in turn it forces the hydrophobic analytes to elute faster from the column. Additionally, the displacement effect of methanol has an influence on shifting the retention of solutes to shorter times comparing to heptane which has not showed such an effect. For the Symmetry C_{18} column, the values of Δt_R does not differ significantly in case the use of methanol or heptane as sample solvent due to the high hydrophobic character of this column.

(Figure 11, Figure **13**) are presented to show methanol peak shape, while how it could affect the chromatographic results which are shown in (Figure 10, 12, 14). It is observed from the strong adsorbed methanol peak, that the front part of its elution profile coincides with elution of the sample components leading to displacing the less retained alkylbenzenes to shorter retention times and increasing their efficiency. This is called displacement effect. While the long tail of the methanol peak would disturb the subsequent eluting alkylbenzenes to reach the adsorption sites, this effect is the tag-along which results in decreasing efficiency.

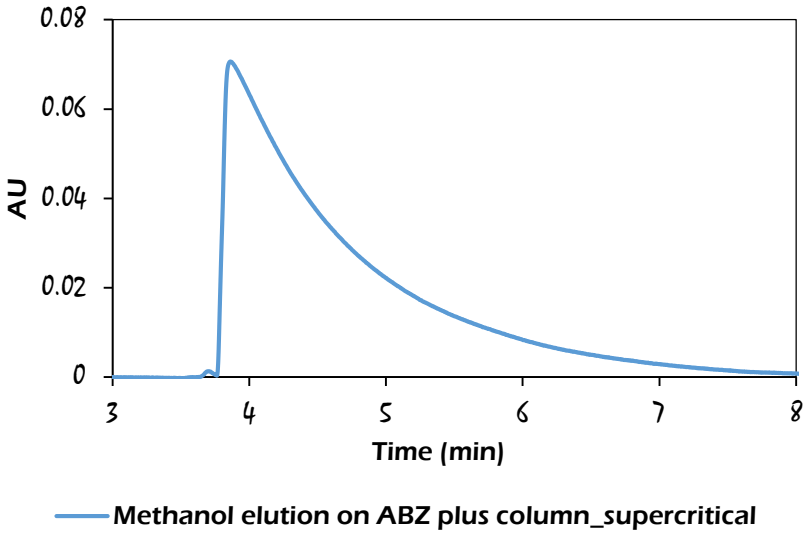


Figure 11. Broad tailing of methanol elution on ABZ+plus column in **supercritical conditions**.

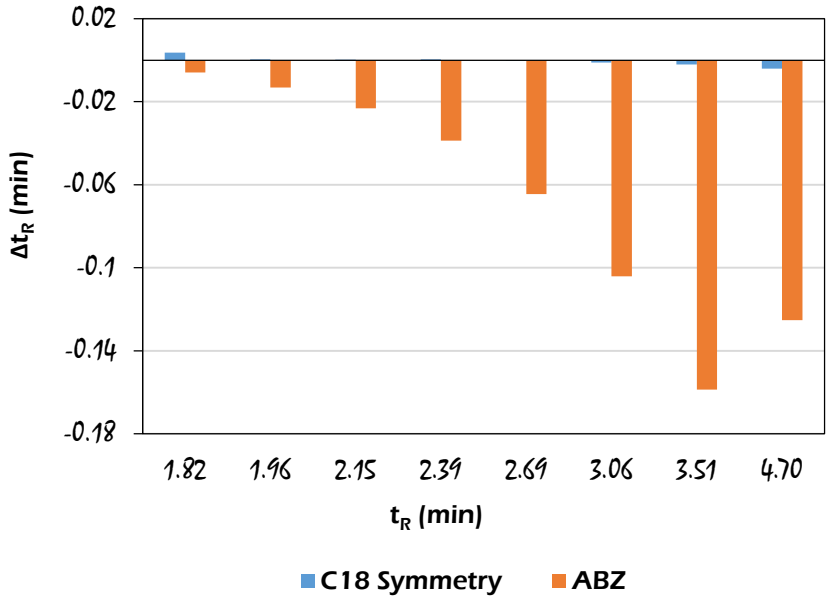


Figure 12. The difference in alkylbenzenes retention time between methanol and heptane solvents of Symmetry C_{18} & Supelcosil ABZ+plus columns in **supercritical conditions**.

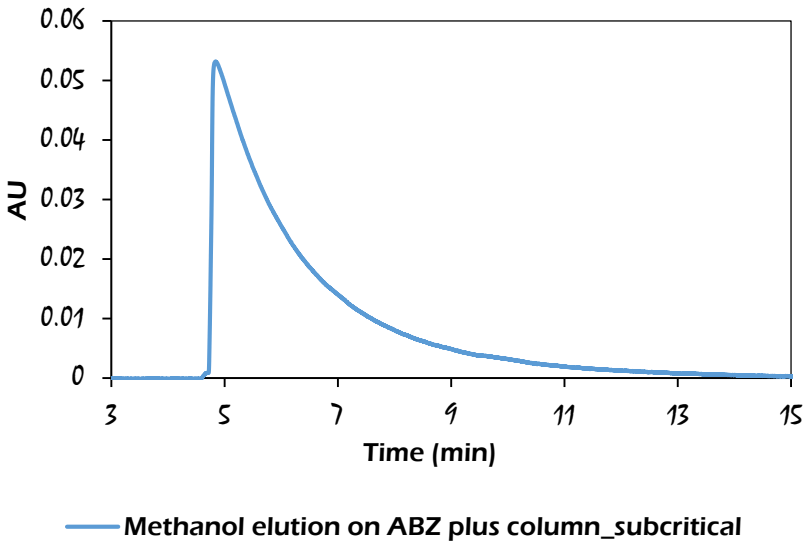


Figure 13. Broad tailing of methanol elution on ABZ+plus column in **subcritical conditions**.

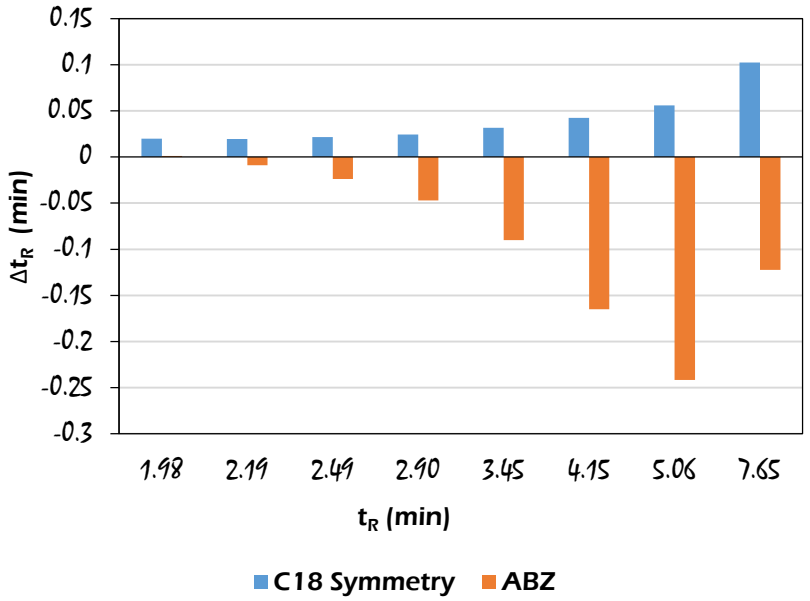


Figure 14. The difference in alkylbenzenes retention time between methanol and heptane solvents of Symmetry C_{18} & Supelcosil ABZ+plus columns in **subcritical conditions**.

4.3. Surface heterogeneity

Despite they are characterized as end-capped columns, the surface of the tested bonded stationary phases is heterogeneous, which can be noticed from the shape of the excess isotherms in (Figure 7), where the dominant negative part indicates the adsorption of CO_2 on the bonded ligands, and the small positive part of the isotherm shows the polar organic modifier (methanol) adsorption on the available polar groups (the residual silanols or on the embedded amide groups in case ABZ+plus column).

As a verification step, we performed fitting for our experimental excess adsorption amounts of methanol on both columns under sub- and supercritical conditions to the theoretical model of excess amount of Eq. (17). This model assumes that the adsorbent surface is made of two different nature of adsorbent sites, one is the bonded hydrophobic ligands, and the complementary surface is the accessible unreacted silanols after the surface derivatization. Therefore, two constants were assumed, K_1 and K_2 representing the hydrophobic (alkyl chains), the hydrophilic (residual silanols and other available polar groups) adsorption sites, respectively, the ε parameter represents the surface heterogeneity.

$$n_1^e = At\left(\varepsilon\frac{(K_1-1)x_1^l(1-x_1^l)}{K_1a_1^*x_1^l+a_2^*(1-x_1^l)}+[1-\varepsilon]\frac{(K_2-1)x_1^l(1-x_1^l)}{K_2a_1^*x_1^l+a_2^*(1-x_1^l)}\right) \quad (17)$$

The value of surface area of the column packing (A) was determined according to the equation:

$$A = V_{ads}\rho_{silica}S_{BET} \quad (18)$$

where V_{ads} is the volume of the adsorbent (cm^3), ρ_{silica} density of the stationary phase (g/cm^3), S_{BET} the specific surface area.

The value of molar fraction of the organic modifier x_1^l in Eq. (17) was determined based on the actual mass flow rate measurements, as it is mentioned in section 4.1.

The number of adsorbed monolayers t can be calculated using the following equation [83, 84]:

$$t = -\frac{1}{A}\left(\left[\frac{dn_1^e}{dx_1^l}\right]_I\left(x_1^la_1^*+[1-x_1^l]a_2^*\right)+(a_2^*-a_1^*)[n_1^e]_I\right) \quad (19)$$

where *I* is the location of the inflection point of the excess adsorption isotherm observed in its decreasing part, x_1^l is the molar composition, $[n_1^e]_I$ is the excess adsorbed amount, and $[dn_1^e/dx_1^l]_I$ is the derivative of the excess adsorbed amount with respect to the molar fraction of component 1 at the inflection point *I*. Parameters a_1^* and a_2^* are the surface requirements per one molecule of

methanol and CO_2, respectively, when they adsorbed on the hydrophobic surfaces. We have got their numerical values a_1^*= 133 m^2/mol, a_2^*= 166.8 m^2/mol by applying Eq. (32) in [84].
Generally, the homogeneous surfaces give type I excess isotherm where the inflection point of decreasing branch can be observed at composition $x_1^l = 1$ [85]. In our experiments we observed type V excess isotherms, the inflection points of the curves in (Figure 15) take place at the molar composition values 0.1124, 0.1121 for the Symmetry C_{18} and at 0.1715, 0.1899 for the ABZ+plus column in subcritical and supercritical, respectively. This clearly demonstrates that the surfaces of the tested stationary phases are heterogeneous, but the small values of ε under all the studied conditions indicates a minor heterogeneity on the stationary phases, (see the values of parameter ε in Table 2).

Table 2 Best fitting parameters (ε, K_1, K_2) of Eq. (17) to the experimental excess amount.

Column – Conditions The number of adsorbed monolayers *t*	**ε**	**K_1 (Hydrophobic sites)**	**K_2 (Hydrophilic sites)**	**R^2**
ABZ+plus – Subcritical $t = 0.0026$	0.012	89.64	0.83	0.95344
ABZ+plus – Supercritical $t = 0.0022$	0.0197	37.22	0.805	0.98884
Symmetry C_{18} – Subcritical $t = 0.0012$	0.0065	185.4	0.839	0.98437
Symmetry C_{18} – Supercritical $t = 0.001$	0.011	58.88	0.813	0.99147

Under subcritical conditions the values of K_1 (which represents the hydrophobic sites) for both Symmetry C_{18} and ABZ+plus columns were higher than those in supercritical due to the preferential adsorption of CO_2 on the bonded ligands in the subcritical conditions. Also, the K_1 values of Symmetry C_{18} are higher than for ABZ+plus, which is explained by the higher carbon loading of the former column. While the K_2 values for the two columns are very close to each other, which means that methanol adsorption has very similar extent for both columns under the

two different conditions. The R^2 coefficient indicates the goodness of fitting of the model to the experimental data. However, the results in Table 1 are relative because those results were obtained using Eq. (17) which is based on the presumption of ideal bulk and adsorbed liquid mixtures, which does not match the real systems as it was reported by Gritti et. al. [85].

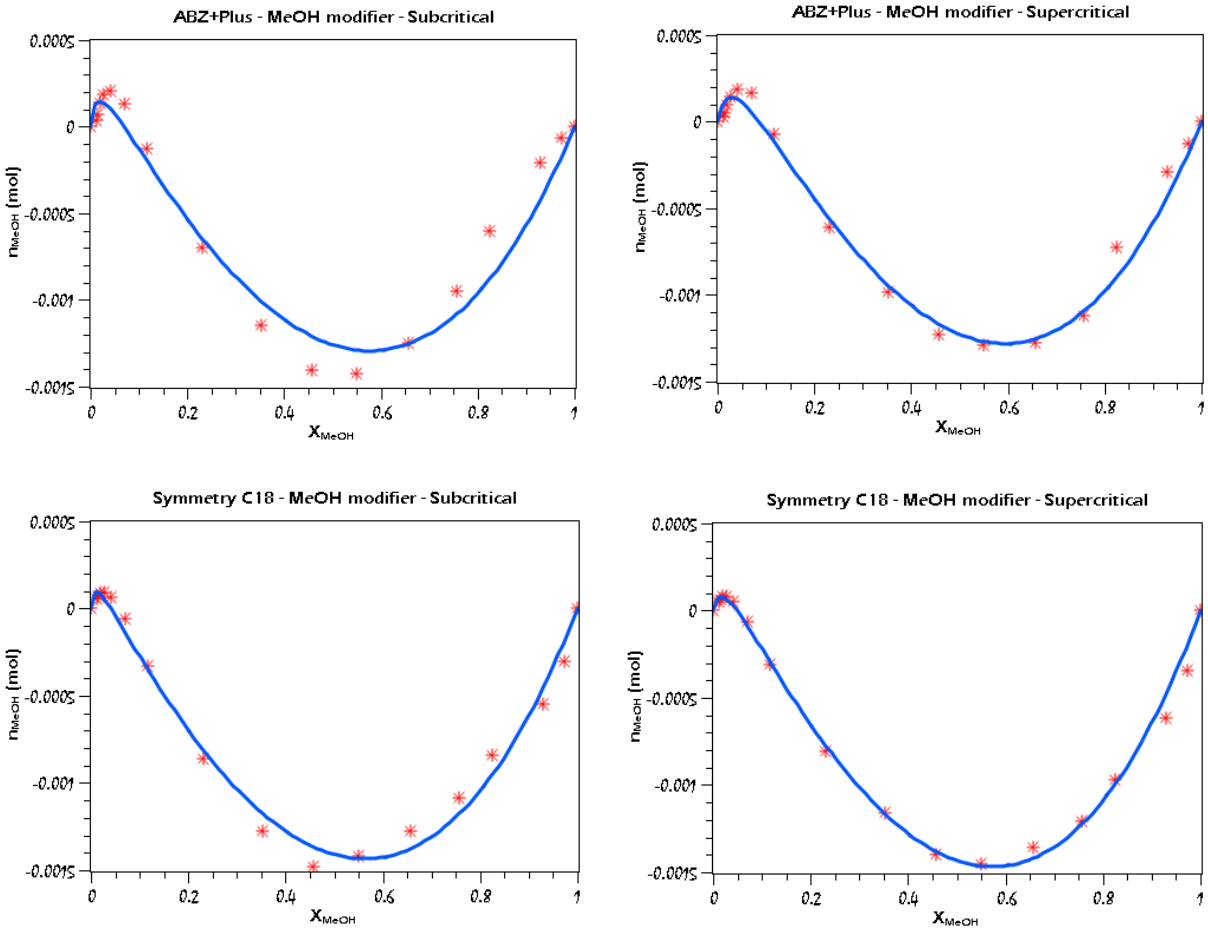


Figure 15. Fit of the excess amount of methanol to Eq. (17), which assumes ideal bulk and adsorbed liquid mixtures. (*) Experimental data, (blue line) best fit model.

4.4. Determination of the adsorption isotherms on the hybrid silica column

4.4.1. Methanol and water adsorption from a binary system

As it has been indicated in the theoretical part about the importance of determination of adsorption isotherms of a compound for developing its production rate through a chromatographic process or understanding the separation process from a mixture it is required to perform adsorption study by determining the single component adsorption isotherms of both pure methanol and water by the inverse method (IM), as one of the main aims of this study.

The IM helps to optimize the adsorption isotherm parameters of each component studied by using the super modified simplex algorithm to minimize the differences between the experimental and the simulated elution band profiles. The bi-Langmuir adsorption model Eq. (1) used to express the adsorption process of the single component on the stationary phase.

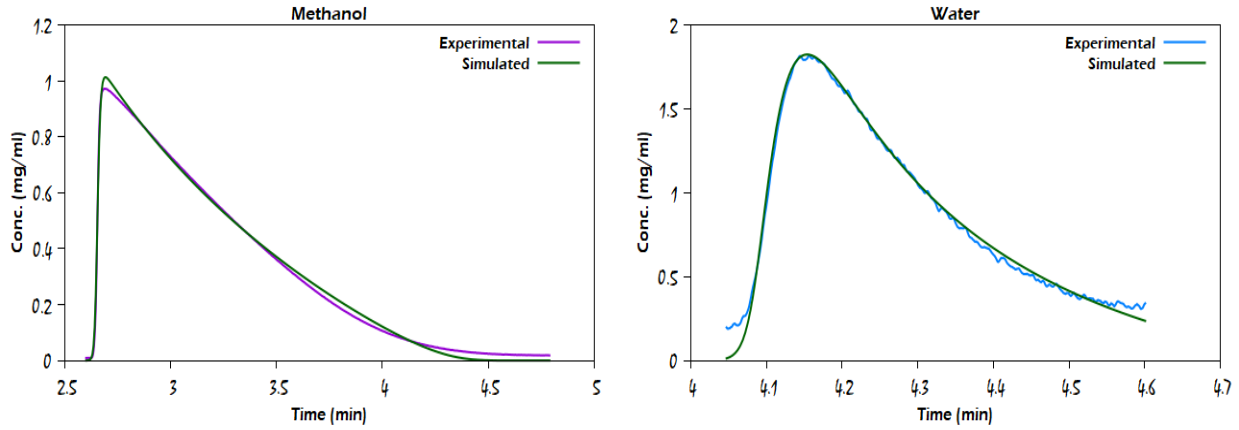


Figure 16. The experimental and simulated concentration profiles for the single component sample of both methanol and water elution from the hybrid silica column with 100% CO_2 mobile phase at 1 mL/min, 26 ℃, 150 bar and 195 nm.

The results of the optimized isotherm parameters by the IM are summarized in Table 3. In (Figure 16) an overlay of the simulated and experimental concentration profiles of 1 µL sample of both methanol and water is shown.

Table 3 Best-fitting parameters of the isotherms for the single component of both methanol and water, and the competitive (mixture) cases determined by the inverse method.

Single component bi-Langmuir isotherm parameters							
Case 1 (one of the adsorption sites is linear for methanol and the other site is linear for water)							
	a_1	b_1 (L/g)	$q_{s,1}$ (g/L)	a_2	b_2 (L/g)	$q_{s,2}$ (g/L)	**SSR**
Methanol *N= 1700	7.58	Negligible	__	29.48	0.39	75.59	0.76
Water N= 2700	6.81	0.68	10.01	35	Negligible	__	1.38
Case 2 (the sites 1 and 2 for water in reverse order compared to case 1)							
Methanol N = 1700	7.58	Negligible	__	29.48	0.39	75.59	0.76
Water N = 2700	35	Negligible	__	6.81	0.68	10.01	1.38
Competitive bi-Langmuir isotherm parameters							
Methanol N = 3000	2.85	Negligible	__	34.1	0.72	47.36	0.824
Water N = 3000	9.09	1.3	6.99	35.77	Negligible	__	

*N values refer to the column efficiency which are used in the simulation program.

It can be noticed from the comparison of the profiles that there is a very good agreement between the two profiles, especially, at the front part, and although the end of the tail does not exhibit a full consistency, the bi-Langmuir model is still considered a suitable choice to model the adsorption for both methanol and water on the hybrid silica column. (Figure 17) represents the single component bi-Langmuir isotherms determined with the IM for both methanol and water from the binary mixtures (methanol / CO_2) and (water / CO_2), respectively. The *SSR* value is an indicator to the goodness of fit.

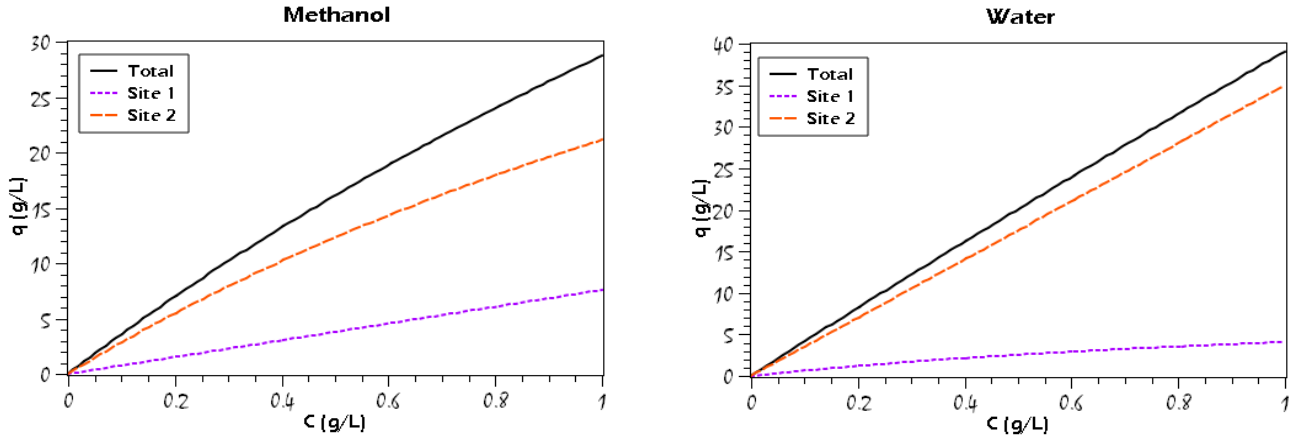


Figure 17. The single component bi-Langmuir isotherms determined by IM for adsorption of both methanol and water on the hybrid silica column with 100% CO_2 as mobile phase. The best isotherm parameters are presented in case 1 of table 3.

The bi-Langmuir model has been used to account for the single component adsorption assuming the stationary phase is nonhomogeneous covered with two different adsorption energy sites. Moreover, it was suggested that one of the adsorption sites on the stationary phase can be characterized with a linear isotherm (where *b* parameter is negligible) because the preliminary results pointed out that one of the sites has a large saturation capacity for methanol and the other one has also a large capacity for water. The curvature of the corresponding isotherms was not observed within the used concentration of the injected sample.

The best-fit parameters of the single component bi-Langmuir isotherm are presented in case 1 of table. 3, where one of the adsorption sites is linear for methanol and the other one is linear for water. Moreover, for the sake of comparison, the case 2 was proposed where the sites 1 and 2 for water are swapped for finding out how the simulated band profile in (Fig. 19) looks like if the two linear adsorption sites for a studied compound were swapped.

The best-fit parameters show that the methanol adsorbs strongly on site 2 with contribution 79.5% of the total adsorption (which might be the reason for the tailing peak shape of methanol elution from the column), whereas site 1 did not reach to the saturation limit. However, the adsorption site 1 contributes for retention of water only 16.3%.

Also, it is obvious from the isotherm parameters that water molecules prefer to bind to the site of the highest amount on the surface (which is characterized with a linear isotherm) more than the methanol molecules do.

4.4.2. Competitive adsorption between methanol and water from a ternary system

The IM has been used to calculate the adsorption isotherm of the binary mixture methanol and water from the ternary system (methanol, water and CO_2) on the hybrid silica stationary phase with 100% CO_2 as mobile phase, the recorded data fitted well to the competitive bi-Langmuir model Eq. (5) which assumes a heterogeneous surface with two types of non-cooperative independent adsorption sites. The results of fitting the competitive bi-Langmuir model to the experimental chromatogram are summarized in Table 3. The competitive bi-Langmuir isotherms are shown in (Figure 18).

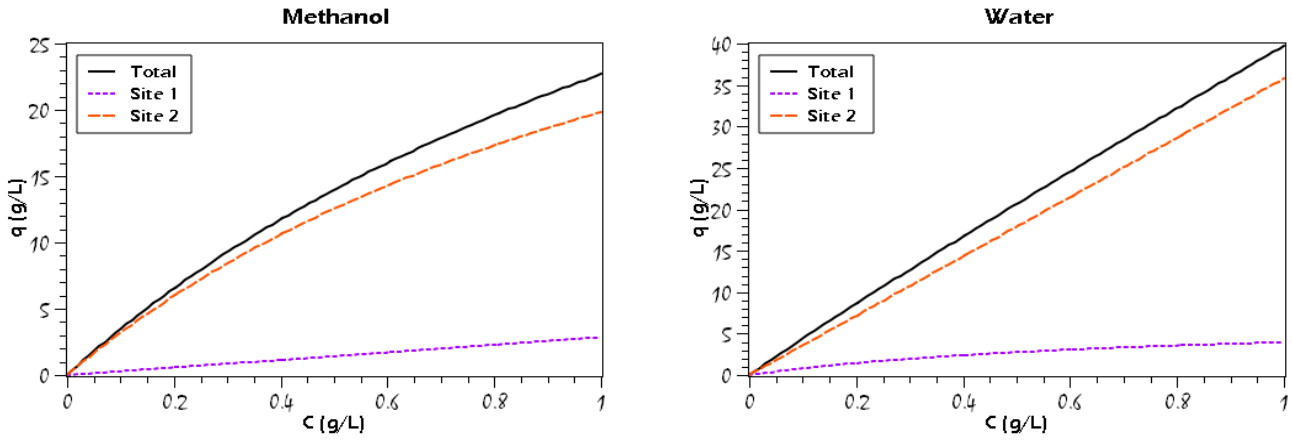


Figure 18. The competitive bi-Langmuir isotherms determined by IM for adsorption the mixture (10:90 v/v, methanol:water) on the hybrid silica column with 100% CO_2 as mobile phase .

Tendency of the competitive isotherm values is similar to that obtained with the single component isotherm case, except there is a change in the saturation capacity values ($q_{s,\,2\text{methanol}}, q_{s,\,1\text{water}}$) which are decreased for adsorption of both methanol and water in the mixture compared to the single component sample, emphasizing the competition between methanol and water to adsorb on the same site.

By comparing the values of the isotherm parameter a_1 in Table 3 (7.58, 2.85) for methanol (the site which has a linear isotherm), we found that the methanol amount adsorbed on the stationary phase decreases in presence of water giving a less steep slope, see site 1 in (Figure 17, Figure **18**) (methanol). Whereas the slope of the linear adsorption isotherm of water on site 2 did not change significantly.

4.5. Prediction of the band profile

It is important to perform simulation of the experimental elution profile to find out how accurate the calculated isotherm parameters are. The chromatographic simulation process was based on the (ED) model as described in Section 2.6. The simulation was performed with the isotherm parameter values obtained from (i) the bi-Langmuir isotherm of single component of both methanol and water, and (ii) the competitive bi-Langmuir isotherm of the mixture, for predicting the elution profile of (10:90 v/v, methanol:water) mixture sample.

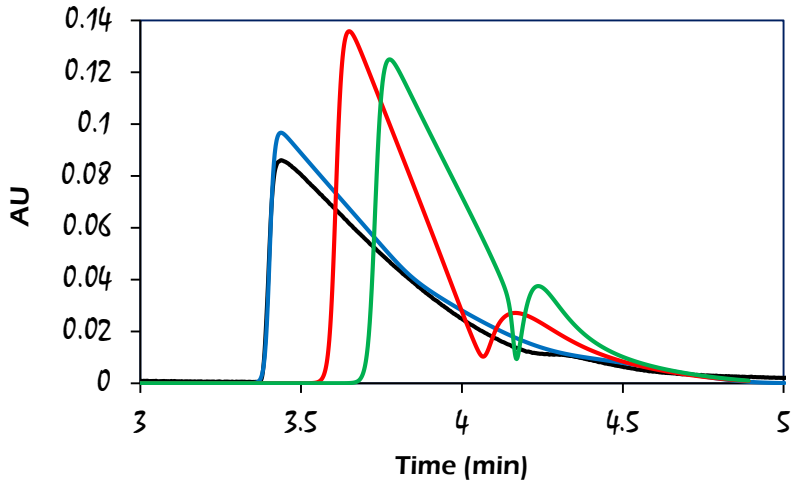


Figure 19. Overlay of the experimental and simulated absorbance profiles of (10:90 v/v, methanol:water) mixture on the VIRIDIS BEH column. The simulated profiles are obtained using three cases of the isotherm parameters as following: **Blue**: The mixture profile simulated using competitive bi-Langmuir isotherm parameters; **Red**: The mixture profile simulated using single component bi-Langmuir isotherm parameters – case 1 (see Table. 3); **Green**: The mixture profile simulated using single component bi-Langmuir isotherm parameters – case 2 (see Table. 3); **Black**: The experimental profile of the mixture sample.

The overlay of measured and calculated band profiles is shown in (Figure 19). The simulated band profile which utilized the values from the competitive bi-Langmuir isotherm (blue curve) remarkably gave better agreement with the experimental profile (black curve) than those obtained from the single component isotherms (red and green curves). Despite there is a good agreement between the measured and simulated profiles, the rear diffuse part of the calculated band profile has less conformity with the measured profile than the front part of the profile.

Additionally, it was possible to simulate the concentration profile of the individual components of the mixture, (Figure 20), which corresponds the simulated absorbance profiles in (Fig. 19) with blue and red curves, respectively. It can be obvious from (Figure 20) that apparently the concentration peaks of water are larger than methanol peaks which is reverse for the absorbance peaks in (Figure 19). This could be explained by the different responses of the detector for the two components of the mixture as it was obvious from the two different sensitivity factors $k_{\text{methanol}} = 0.208$, $k_{\text{water}} = 0.0048$ which were obtained during the detector calibration (see Eq. 14).

A) B)

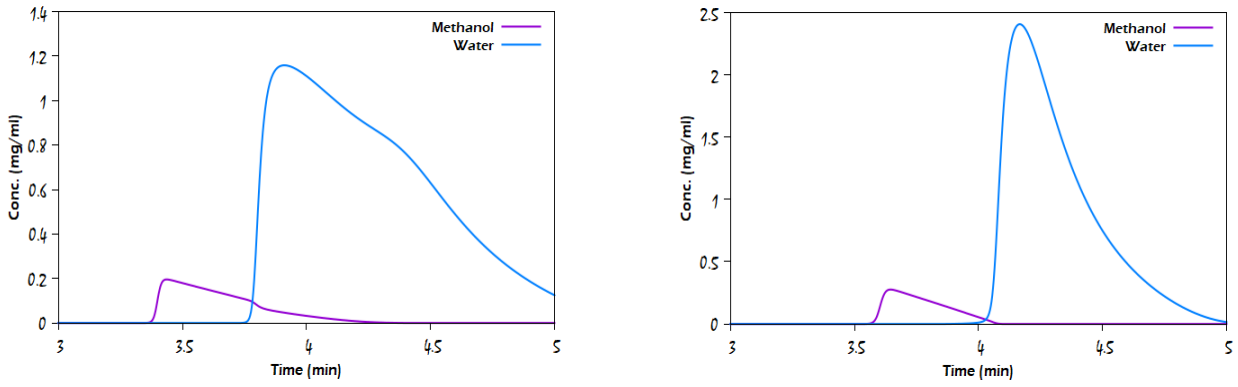


Figure 20. Overlay of the simulated concentration profiles of the individual components of the (10:90 v/v, methanol:water) mixture on the VIRIDIS BEH column, (A) using best-fit parameters of the competitive bi-Langmuir isotherm, (B) using best-fit parameters of the single component bi-Langmuir isotherm, determined by IM.

4.6. Elution of polar analytes from the hybrid silica column with employing water as additive

The aforementioned observations revealed that both methanol and water are able to adsorb strongly on the hybrid silica column. So the consequences of the use of water as additive in the methanol modified mobile phase to elute polar analytes from that column should be investigated, that is the main goal for this this part.

The structures of the nitrogen containing compounds used in this study are shown in (Figure 21) vary from weak (e.g. aniline) to strong (e.g. propranolol) basic compounds which they or their derivatives have notable uses in the pharmaceutical industry. The analytes were chosen to exhibit distinctive behavior on Viridis BEH column as it will be shown in the next sections. The experimental conditions are referred on the caption of the related figures.

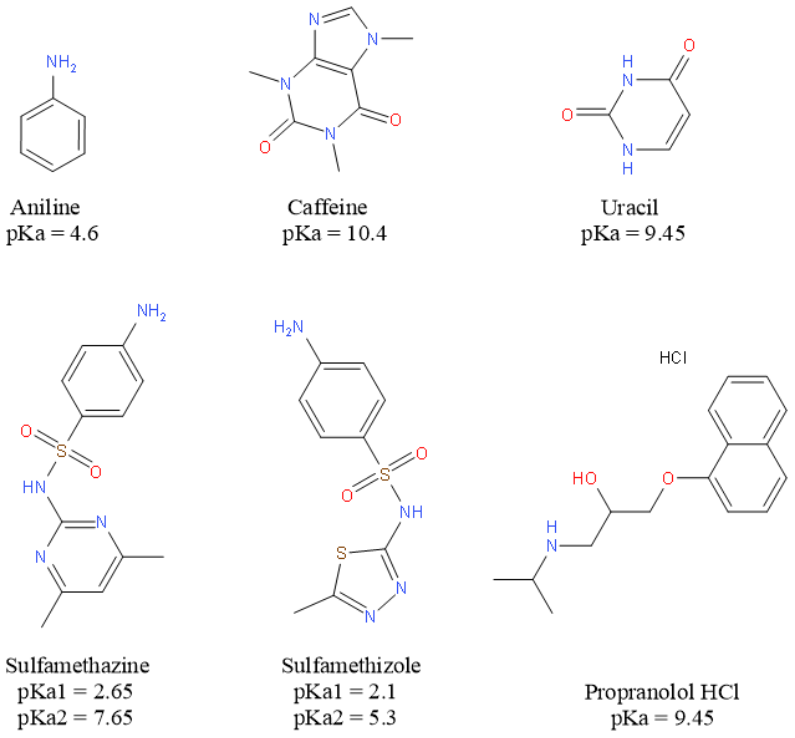


Figure 21. Analyte structures and corresponding pKa values.

4.6.1. Influence of the use of water as additive on peak shape and symmetry factor

The elution of analytes from Viridis BEH (hybrid silica) column using the pure supercritical carbon dioxide as mobile phase, as it was expected, undoubtedly indicates to that the solvent strength of CO_2 was not able to elute any of the analytes, except the aniline was eluted early (approximate three times void time) with a tailed peak shape as shown in (Figure 22). That can be caused by the low probability of aniline to form a hydrogen bond with the adsorption sites of the stationary phase because the only lone pair on the nitrogen atom in aniline molecule will be involved with the pi electrons in the benzene ring, thus it can be eluted easily with CO_2.

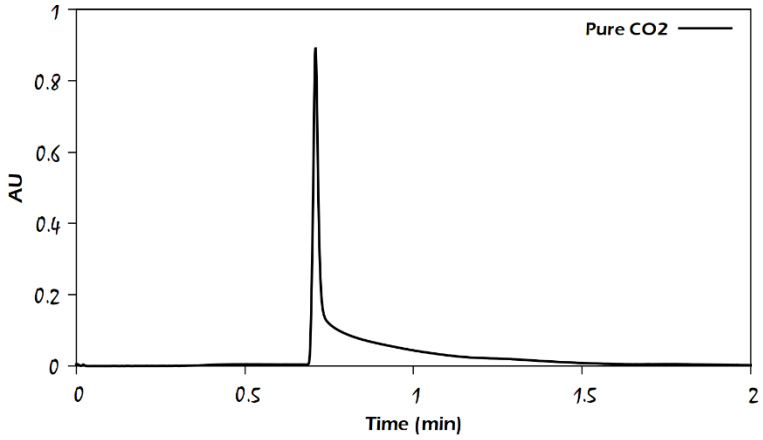


Figure 22. Aniline elution chromatogram from the hybrid silica column using 100% CO_2 as mobile phase at 26 °C column temperature, back pressure 150 bar and flow rate: 2 mL/min. These analytical conditions are same for figures 23–31.

It can be noticed from the comparison in (Figure 23) that the peak shapes improved for the analytes uracil (URA), sulfamethazine (ZIN) and sulfamethizole (ZOL) by adding 1-2% water as additive to the modifier (methanol), furthermore, as we showed through the adsorption isotherm determination that this stationary phase has a large affinity to water, thus it reduces the undesired interactions of solutes with silanol groups. The use of higher water percentage in the mobile phase led to higher retention times of the analytes owing to the increase of hydrophilicity character of the analytes, in turn interact much stronger to the adsorbed water molecules on the stationary phase.

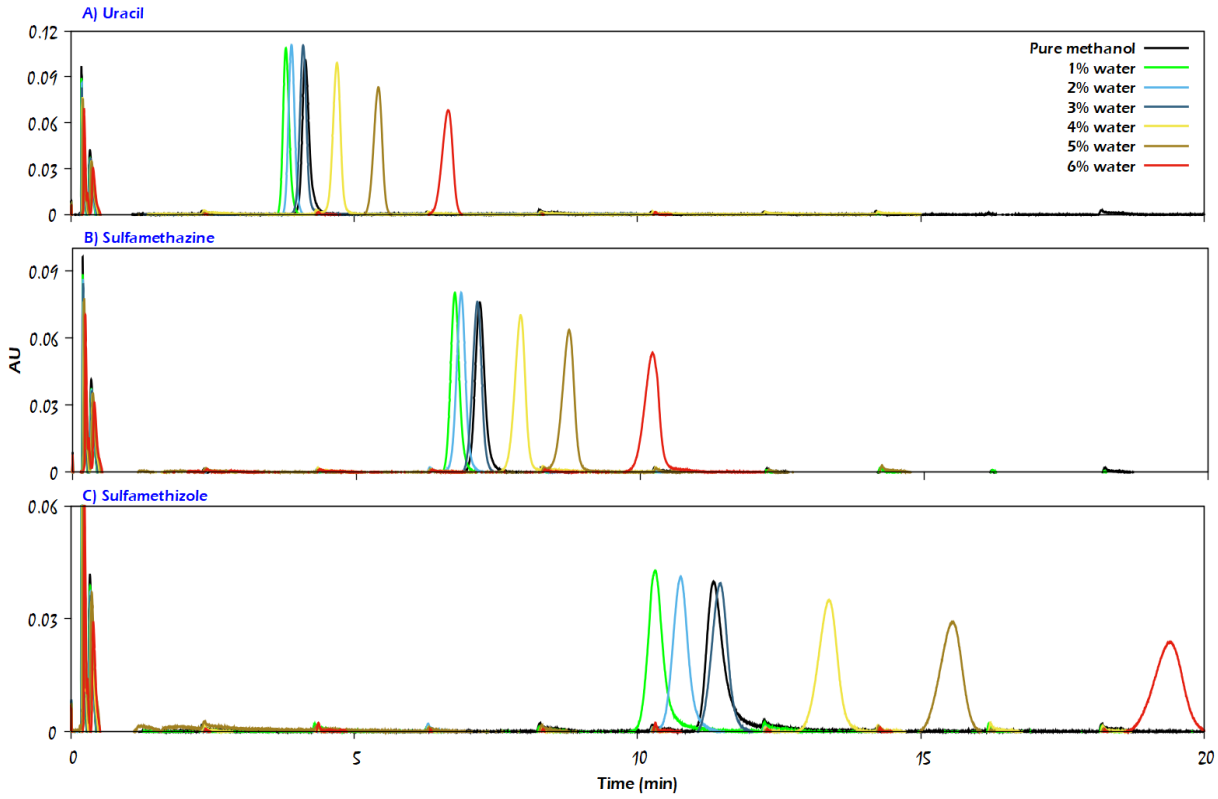


Figure 23. Chromatograms comparison for uracil, sulfamethaznie and sulfamethizole elution from the hybrid silica column with CO_2/modifier (97:3, v/v), with different water amounts.

(Figure 24) shows the caffeine (CAF) chromatograms at different water percentages. The water addition slightly improved the peak shape and decreased the retention time in a very little extent that is similar to the elution with only methanol – CO_2 mobile phase. This could be a result of absence of the hydrogen bond donors, and only 3 acceptors available in the CAF molecule, in addition, the weakly hydrated flat faces of the CAF molecule. Therefore, adding water to the mobile phase could not influence largely on the caffeine retention. Furthermore, increasing water amount (higher than 3%) in the modifier, resulted in split peaks for CAF because of the disturbance peak of the strong adsorbed additive (water), which elutes after the CAF from the column, in turn it affects the CAF elution band resulting in unusual peak shapes. The deformation in the CAF peak shape caused the abnormal results of a symmetry factor and plates number, as it will be seen in the later section.

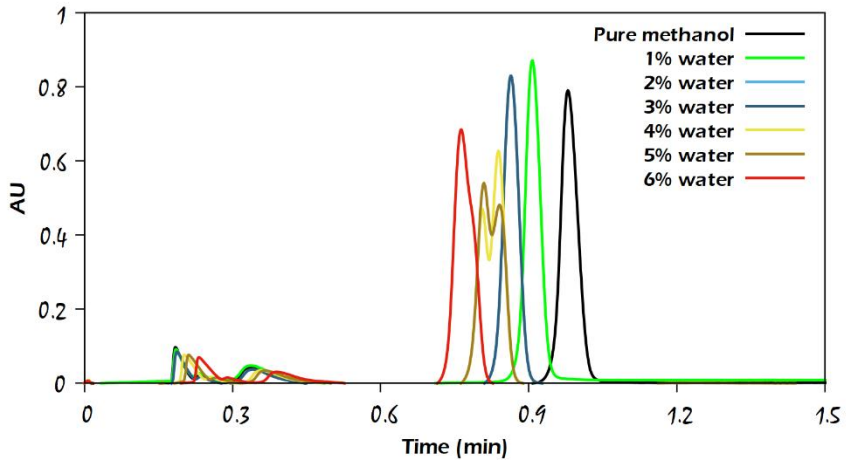


Figure 24. Chromatograms comparison for caffeine elution from the hybrid silica column with CO_2/methanol (97:3, v/v), with different water amounts.

The experiments of eluting a racemic mixture of (±) propranolol HCl (PRO) from the used column revealed that adding a basic additive (for an ion suppression purpose) along with, at least 10%, methanol is necessary (incorporating methanol / water mixture as modifier in the mobile phase was not enough to elute PRO with an acceptable peak shape), therefore 15% of modifier has been used to give a convenient retention time. The influence of three common basic additives have been invistegated diethylamine (DEA), ammonium hydroxide (NH_4OH) and trimethylamine (TEA) with / without water for the PRO elution. The results showed different trends as a response for the water use with a basic additive, as it is presented in (Figure 25). Adding a combined additive of 0.5% water and 0.1% ammonium hydroxide solution to the methanol as modifier improved the peak shape, signal intensity and retention time in comparison to the case of 0.1% ammonium hydroxide without water, this mixture of the additives was indicated by Liu and his co-workers [86] as a chaotropic agent, which helps to disrupt the unfavorable hydrogen bonding and the analyte solvation shell.

When we incorporated an amine salt with water as an additive, the results showed different effects for the water use. For the combination 1% water and 0.1% TEA didn't influence on the elution of PRO comparing to the case without water, while the use of DEA instead of TEA at the same concentration, adding water resulted in reducing the signal intensity of the PRO peak to the half, and negatively influenced on the peak shape comparing to the use of DEA without water, which

is probable due to the slow mass transfer of the analyte in presence of the adsorbed water on the stationary phase. Those findings of PRO separation emphasize that water played propitious and unpropitious roles in the elution process in accordance to the mobile phase components.

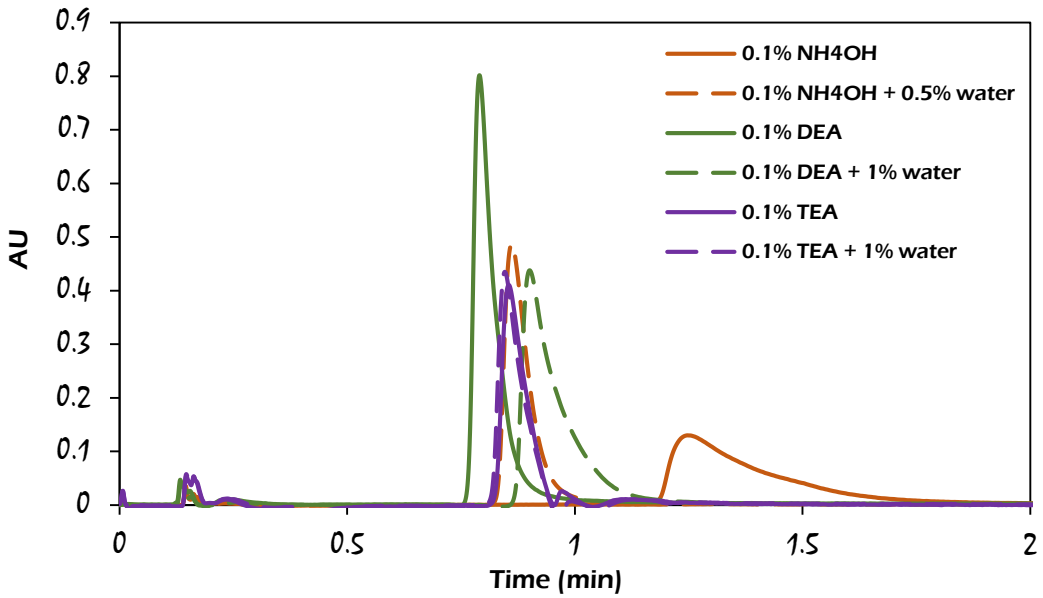


Figure 25. Comparison for the influence of different mobile phase additives on elution of (±) PRO HCl with CO_2/methanol (85:15, v/v) from the hybrid silica column, dashed lines: modifier with water; solid lines: modifier without water.

For evaluating the influence of water on the quality of peaks of the standards, we calculated the symmetry factor A_s from the recorded chromatograms of CAF, URA, ZIN and ZOL in (Figure 23Figure 24) according to the formula:

$$A_s = \frac{w_{0.05}}{2d} \qquad (20)$$

$w_{0.05}$: width of the peak at one-twentieth of the peak height.

d : distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at one-twentieth of the peak height.

The values of symmetry factor of the four analytes were close to each other (except for CAF there was an anomalous trend when the water amount is higher than 2% due to the deformed peaks), as it is shown in **(**Figure 26**)** they approximately decreased from 1.4 to 0.8 when water percentage in

the modifier was increasing from 1% to 5%, referring that 2-3% water in the modifier was enough to treat the peak tailing for the analytes studied.

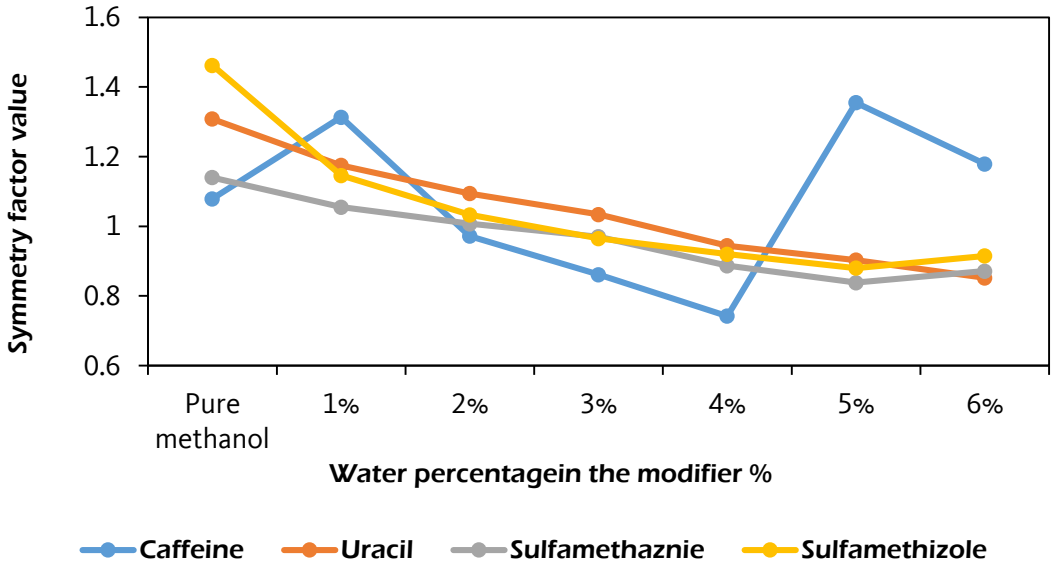


Figure 26. Effect of the water percentage on the symmetry factor values for four analytes elution from the hybrid silica column with mobile phase composition: CO_2/methanol (97:3, v/v).

4.6.2. Influence of the use of water as additive on retention time and peak efficiency

The CAF chromatograms in (Figure 24) show rapid elution from the column using the modified CO_2 with methanol in presence of water or even without, also, the CAF analyte elutes earlier as the water percentage in the modifier increases, which has not been observed with rest of the analytes, that can be due to the relatively low hydrophilic character of CAF.

It can be noticed from (Figure 23) that the retention times of URA, ZIN and ZOL decreased at 1% water, then started to increase as water percentage in the modifier increases, which may attribute to that the extra water amount could result in higher extent of the analyte molecules surrounded by the water molecules, which basically increases the analyte attraction toward the adsorbed water molecules on the stationary phase, as mentioned earlier in section 4.6.1.

Regarding the signal intensity of the peaks, they have been increased at water content 1% then they got less as the water percentage in modifier increased due to the increased retention times.
For the influence of water on the PRO retention times, it can be favorable or unfavorable according to the used additive in the mobile phase, as it is seen in (Figure 25).
In case of adding a mixture of water and DEA to the mobile phase, it would affect negatively on the role of DEA as additive to suppress the ionization of the basic PRO molecules, because there is possibility to form positively charged amine in presence of water (the lone pair of electron on the nitrogen atom can accept the proton from water to form nitrogen with a positive charge), in turn it leads to a tailing peak of PRO with higher retention time.
In case of combining water with TEA as additive, that has less basicity than DEA due to the steric hindrance of TEA molecule, this combination did not produce influence on the PRO retention time comparing to the case of the use TEA alone. While in case of the use of NH_4OH solely, it could not provide total ion suppression of PRO because the $pKa_{(NH4OH)}$ (9.26) is slightly smaller than $pKa_{(propranolol)}$ (9.45), consequently, a long tail for the analyte peak produced as a result of unfavorable adsorption of the protonated PRO. While the addition of water beside NH_4OH to the mobile phase could result in the carbonate/bicarbonate anion under the SFC conditions, as it was mentioned earlier in section 4.6.1, which is responsible for the improvement in the chromatographic results of the hydrophilic analytes.
In the light of the foregoing, we can conclude that the use of DEA alone gave the best result for the propranolol peak among the other trials.
By a quick look to the previous chromatograms, we can see that the ZOL was the most retained compound among the analytes, even with or without water addition, that may be attributed to the high number of hydrogen bond donors and acceptors in the ZOL molecule enabling the molecule to interact strongly with stationary phase.
The plots of the water amount against the efficiency of the analyte peak are represented in (Figure 27,Figure **28**). Since the water use resulted in different effects on the elution of the studied analytes, thus different trends of the efficiency values are expected as well. For example, the calculated efficiency of the URA peaks are very close to each other under the used range of water amount, while with the CAF peaks introducing a higher amount of 3% water in the mobile phase a loss in the efficiency was observed because of the deformed chromatographic peaks. For

the ZIN and ZOL analytes, the efficiency trend was increasing in accordance with the water content up to 2% then followed by decreasing due to the wide peak shapes.

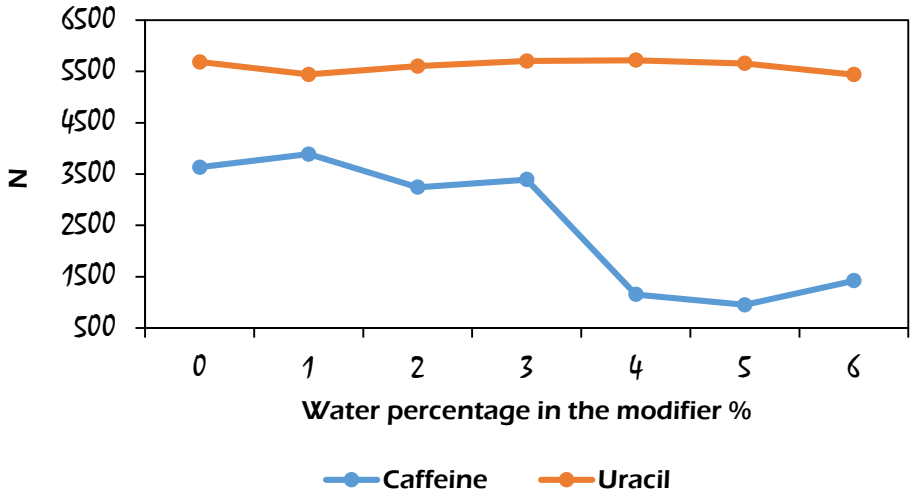


Figure 27. Effect of the water percentage on the calculated efficiency values for caffeine and uracil elution from the hybrid silica column with CO_2/methanol (97:3, v/v) mobile phase.

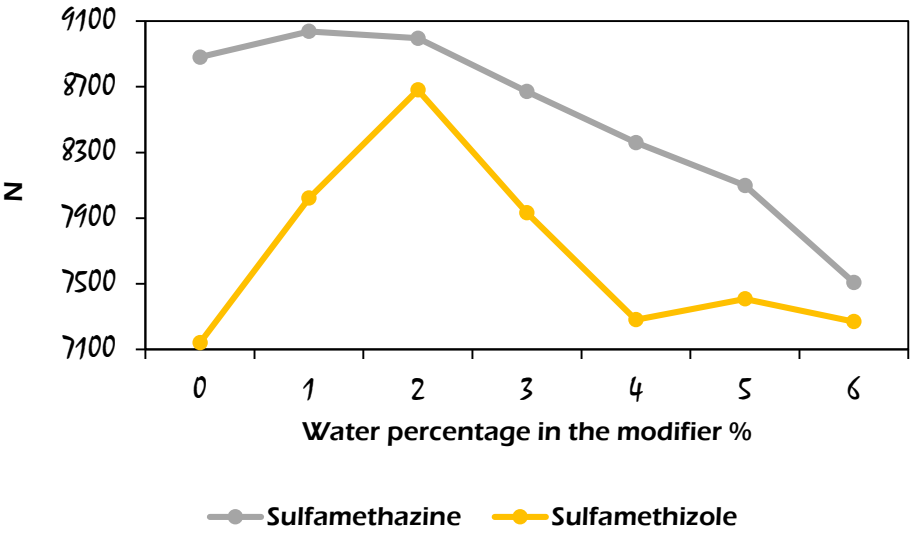


Figure 28. Effect of the water percentage on the calculated efficiency values for sulfamethaznie and sulfamethizole elution from the hybrid silica column with CO_2/methanol (97:3, v/v) mobile phase.

Also, it is noticed that the impact of water percentage on the efficiency of the ZOL peaks was larger than those for the other analytes (considering the difference in N values between two consecutive points), which can be explained by the high number of the hydrogen bond acceptor / donor sites (7/2) in the molecule of ZOL.

4.6.3. Effect of the modifier percentage on elution of polar analytes

Generally, increasing the polar solvent (modifier) percentage in the mobile phase in SFC, even with a small amount, would enhance the solvent strength, thus increasing the analyte – mobile phase interactions leading to change in the retention of solutes. In this regard, we investigated the influence of two proportions A: 3% and B: 5% of the modifier in the mobile phase on elution of polar analytes. The obtained chromatograms from the two cases are overlaid in (Figure 29 –Figure **31**) for the URA, ZIN and CAF, respectively. The same series of the water amount was added in both cases A and B.

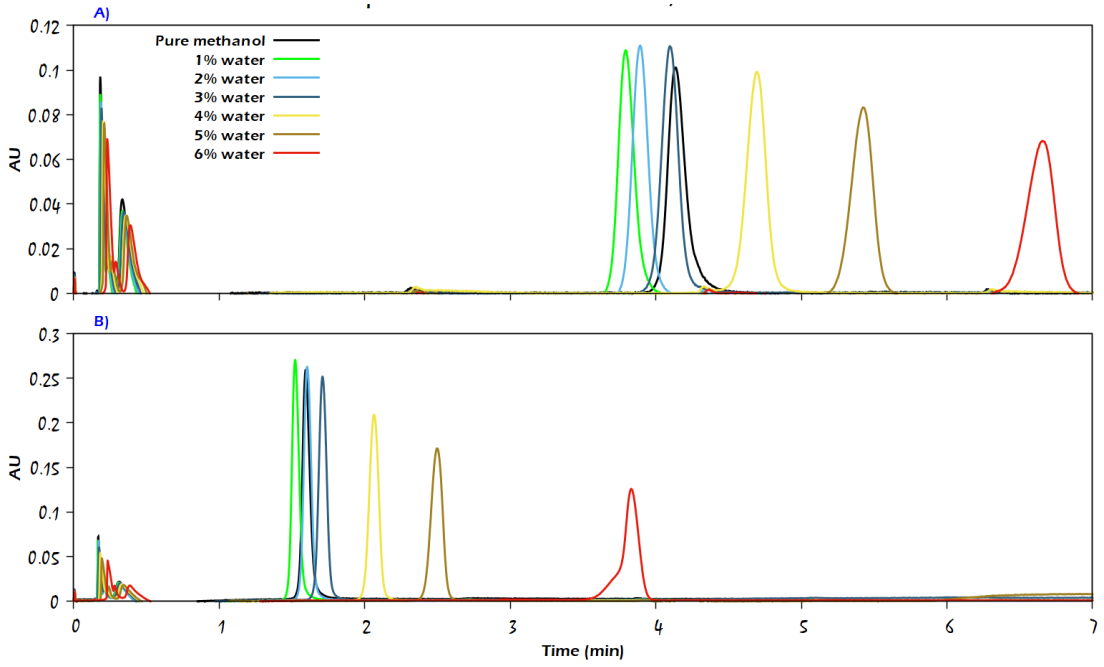


Figure 29. Comparison for the influence of increasing modifier amount on elution of URA from the hybrid silica column, A: CO_2/methanol (97:3, v/v); B: CO_2/methanol (95:5, v/v).

The results show that the use of a higher amount of the modifier reduces the retention of analytes to less than half time, and increases the intensity of detector signal more than twofold, and narrow peak shapes were obtained. Noting that the deformation in the analyte peak can be observed due to the competition between the analyte and methanol to adsorb on the stationary phase such as in the case of URA at CO_2/methanol (95:5, v/v) with 6% water.

Also, it is observed that a higher amount of the modifier substantially diminishes the importance of the water role in improving the chromatographic peaks, herein, the role of water can be noticeable in improving the properties of chromatographic peaks when it is employed in the mobile phase at a low amount of modifier.

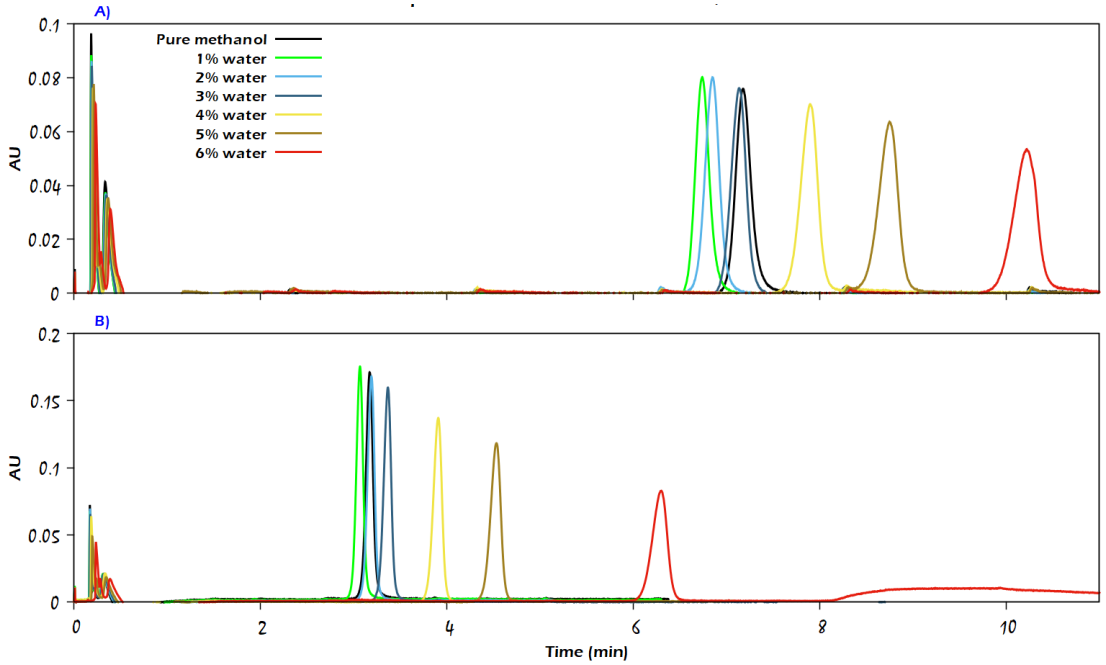


Figure 30. Comparison for the influence of increasing modifier amount on elution of ZIN from the hybrid silica column, A: CO_2/methanol (97:3, v/v); B: CO_2/methanol (95:5, v/v).

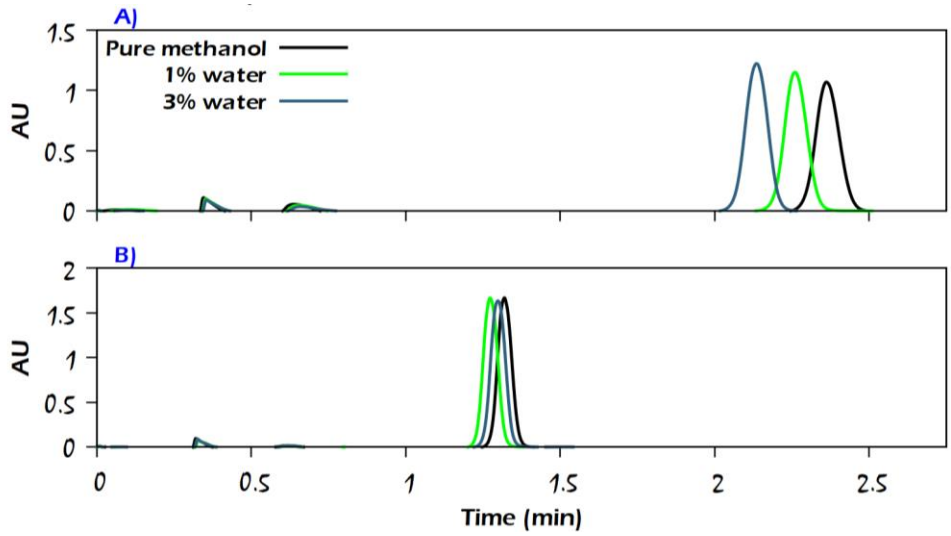


Figure 31. Comparison for the influence of increasing modifier amount on elution of CAF from the hybrid silica column with flow rate: 1mL/min, A: CO_2/methanol (97:3, v/v); B: CO_2/methanol (95:5, v/v).

Chapter 5

CONCLUSION

"Gravity explains the motions of the planets but it cannot explain who sets the planets in motion." Isaac Newton

This work was performed to investigate the adsorption behavior of methanol on the reversed stationary phases, and its consequences on the elution of non-polar analytes. In addition, the single component adsorption isotherms on the hybrid silica column were calculated for both methanol and water from the binary systems (methanol and CO_2) and (water and CO_2), respectively, and the competitive adsorption isotherms for both methanol and water from the ternary system (methanol - water - CO_2). Then, the influence of incorporating water in the methanol modified mobile phase on elution of some polar compounds has been evaluated.

Based on the research results, a few conclusions can be drawn:

- The end-capped alkyl bonded silica phases, especially the stationary phase embedded with a polar group, showed surface excess adsorption of a small amount of methanol from the methanol – CO_2 mixture, which in turn negatively effects on the peak shape and the efficiency of non-polar compounds which coincide with the elution of methanol.
- The surface excess isotherm of methanol on the studied reversed phases under two sets of the operational conditions (temperature and set back pressure) gave rise to very similar results.
- The maximum value of surface excess adsorption of methanol from the methanol – CO_2 mixture on the studied reversed phases was recorded at small set volume fractions 2% and 0.5% (v/v) of the organic modifier in the mobile phase for the alkylamide and the C_{18} stationary phases, respectively.

- The sample solvent should be chosen carefully in which it should not be adsorbed on the stationary phase because it can impact on the peak shape of the analytes which elutes in coincidence with the solvent molecules leading to a low peak efficiency as a result of the tag-along effect.
- The addition of water to the methanol modified mobile phase in SFC could be one of the green alternatives to improve the separation of some polar analytes, noting that this improvement won't be obvious with an amount of modifier higher than 3% in the mobile phase.
- The band profile simulation emphasizes that the competitive bi-Langmuir isotherm parameters gave better agreement with the experimental band profile than those obtained from the single component isotherms, confirming the competition occurring between methanol and water to adsorb on the hybrid silica stationary phase.
- The more hydrogen bonding acceptor / donor sites in the analyte molecule, the more favorable the use of water as additive for improving the chromatographic results.
- The best chromatographic results of the analytes have been obtained at water percentage 0.03 ~ 0.06% in the total mobile phase, noting that, an excess water amount would have a negative impact on the efficiency, the retention time, and the peak symmetry.
- The use of water alone as an additive is ineffective for eluting such a strong basic compound PRO with a good peak quality from the hybrid silica column.

Chapter 6

THESIS STATEMENT

1. I determined the surface heterogeneity of two end-capped reversed stationary phases by calculating the excess adsorption isotherm for methanol from the methanol – CO_2 mixture under two sets of temperature and back pressure values using the minor disturbance method. The obtained results of the excess adsorption isotherms are very similar under the studied conditions for each column. Additionally, the surface excess adsorption amount of methanol for the embedded polar function stationary phase (amide group) is higher than the amount for C_{18} alkyl bonded stationary phase.
2. I studied the influence of methanol and heptane as a sample solvent to elute the alkylbenzenes mixture from the reversed stationary phases with 100% CO_2 as mobile phase. I concluded that the analyte peaks are influenced negatively as a result of the tag-along effect of methanol on the alkylamide column.
3. I investigated the adsorption behavior of both methanol and water on the hybrid silica column by determining the single component adsorption isotherms by the inverse method and using bi-Langmuir adsorption model equation, which was a suitable choice to account for this adsorption.
4. I used the inverse method to determine the competition between methanol and water as components in the CO_2 mobile phase by calculating the competitive bi-Langmuir adsorption isotherms. The competitive bi-Langmuir adsorption isotherm parameters showed decrease in the saturation capacity for methanol and water comparing to the saturation capacity values obtained from the single component bi-Langmuir adsorption parameters, emphasizing the competition between methanol and water to adsorb on the stationary phase.
5. I performed simulation for the band profile of 10:90 v/v methanol:water mixture sample, by using the equilibrium-dispersive model. It was found, that the simulated band profile calculated by using the competitive bi-Langmuir isotherm parameters gives better fitting

with the experimental band profile than those obtained from the single component isotherms.

6. I evaluated the influence of employing water in the methanol modified mobile phase to elute polar compounds from the hybrid silica column. The results showed different behaviors for the use of water as additive in the mobile phase, which depends on the target analyte structure. Some of the studied polar analytes exhibited improvement in the symmetry factor and the efficiency when the water was added within the range of 0.03 ~ 0.06% in the total mobile phase. On the other hand, an excess water amount gives a negative impact on the separation properties.

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Publications related to the thesis

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2. M. Y. Kazmouz, A. Felinger, Competitive Adsorption of Water and Methanol on a Hybrid Silica Stationary Phase in Supercritical Fluid Chromatography (submitted for publication).
3. M. Y. Kazmouz, A. Felinger, Impact of Water Use as Additive on Elution Some Basic Compounds in Supercritical Fluid Chromatography (submitted for publication).

Posters

1. M. Y. Kazmouz, A. Felinger, The Competitive Adsorption of Water and Methanol on a Hybrid Silica Stationary Phase in Supercritical Fluid Chromatography; METT25, Egerszalók, Hungary (2021).
2. M. Y. Kazmouz, A. Felinger, Improved peak shape of basic analytes analyzed by supercritical fluid chromatography; 12th Balaton symposium, Siófok, Hungary (2019).
3. M. Y. Kazmouz, A. Felinger, Competitive adsorption of the mobile phase modifiers in supercritical fluid chromatography; HPLC conference, Milan, Italy (2019).
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5. M. Y. Kazmouz, Sz. László, Cs. Rédei, I. Bacskay, A. Felinger, Surface excess isotherms of organic modifier and carbon dioxide mixture in sub- or supercritical fluid chromatography; Applications of supercritical fluids 2018 conference, Budapest University of Technology and Economics, Hungary (2018).

GLOSSARY

SFC	Supercritical fluid chromatography
pSFC	Packed column supercritical fluid chromatography
UHPLC	Ultra-high performance liquid chromatography
CO_2	Carbon dioxide
UV	Ultraviolet
BPR	Back pressure regulator
HPLC	High performance liquid chromatography
LC	Liquid chromatography
GC	Gas chromatography
NPLC	Normal-phase liquid chromatography
HBD	Hydrogen-bond donating
HBA	Hydrogen-bond accepting
SubFC	Subcritical fluid chromatography
Mw	Molecular weight
PAHs	Polycyclic aromatic hydrocarbons
LSER	Linear solvation energy relationships
HILIC	Hydrophilic interaction chromatography
ED	Equilibrium-Dispersive
BEH	Bridged ethylene hybrid

TEOS	Tetraethoxysilane
BTEE	1,2-bis(triethoxysilyl) ethane
URA	Uracil
CAF	Caffeine
PRO	Propranolol HCl
ZIN	Sulfamethazine
ZOL	Sulfamethizole
DEA	Diethylamine
TEA	Trimethylamine

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