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Analysis of selected factors influencing the course of the process in liquid chromatography

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ABSTRACT

This study examined the influence of temperature, eluent modifier type and concentration, and mobile phase flow velocity on the separation efficiency of caffeine (CAF) and phenol (PH) in Hypersil GOLD HILIC and Shodex SILICA 5NH 4D chromatographic columns. A thermodynamic analysis demonstrated that the adsorption processes of these compounds are primarily exothermic and depend on the eluent type. Acetonitrile-water (ACN:H₂O) systems provided superior selectivity between caffeine and phenol compared to methanol-water (MeOH:H₂O) systems, with selectivity improving as temperature increased in ACN:H₂O systems. Variations in eluent modifier concentration significantly impacted retention times, suggesting diverse interaction mechanisms between the analysed compounds and the stationary phase. Furthermore, studies on column efficiency indicated that faster eluent flow reduced separation efficiency, as evidenced by HETP values. The optimal flow rates were determined to range between 0.2 and 1.25 ml/min, depending on the column tested.

Keywords: adsorption, eluent modifier, liquid chromatography, HILIC, selectivity, retention mechanism, column temperature, process efficiency.

INTRODUCTION

Liquid chromatography (LC) is an advanced analytical technique, essential both in science and industry. Due to its versatility and reliability, it plays a crucial role in pharmaceutical analysis, food quality control, environmental research, as well as chemical and biochemical engineering. Liquid chromatography in its various forms is used not only as an analytical method, but also, increasingly often, as a method of separation/ purification of various mixtures of compounds on a preparative and industrial scale. The main advantage of the liquid chromatography process (both under analytical and industrial conditions) is the ability to replace many unit operations with a single process that additionally does not destroy the separated sample. Therefore, understanding chromatographic the mechanisms and factors that influence separation efficiency is crucial for further development of this technique [1, 2] both in analytical and larger scale of this process. This was also the aim of this work. LC separation operates on the basis of the differences in the interactions of the separated compounds with the mobile and stationary phases, resulting in selective retention of chromatographed components [3–5].

Various modes of liquid chromatography exist, each tailored to specific chemical compound properties. The most widely used LC modes are: normal-phase (NP) LC and a variation of this technique – Hydrophilic Interaction Liquid Chromatography (HILIC), enabling effective separation of highly polar compounds such as glycoproteins or nucleic acids [6, 7], reversed-phase (RP) LC used for analysis of non-polar substances [8, 9], ion-exchange chromatography, which is particularly useful in the analysis of ions, amino acids and proteins [10, 11] as well as gel chromatography, also known as size-exclusion chromatography, allowing for the separation of compounds (such as proteins, polymers, or biomolecules) based on their size [12, 13].

Each type of liquid chromatography has applications in chemical analysis, and the proper selection of parameters, such as mobile phase composition, stationary phase type, temperature, and flow rate, is crucial for obtaining precise results. Chromatographic techniques are indispensable tools in laboratories, allowing effective separation and identification of chemical compounds, enabling their wide use in various fields of research and industry [14–16]. Each of these parameters influences the interactions between the mobile phase, stationary phase, and the analysed substances, which in turn determines retention time and separation quality. It should be noted that despite intensive studies, the retention mechanism (including factors and phase interactions influencing it) in different types of HPLC is still not fully understood and requires further research [17–19].

Column temperature is very important in LC and influences the kinetics as well as thermodynamics of the retention process. An increase in temperature can reduce the viscosity of the mobile phase and alter the properties of the stationary phase. Additionally, temperature can change the adsorption equilibrium, affecting the retention time of molecules. Controlling the temperature improves separation selectivity and shortens analysis time, which is particularly important in sequential analysis, when dealing with a large number of samples. Although there are existing studies on the impact of temperature on chromatographic processes, further analysis in this area is still required. In particular, the results of available research show that temperature effects vary depending on the type of compounds analysed and the chromatographic methodology used. Moreover, analysing these relationships in the context of precisely determining optimal experimental conditions remains a challenge. This article examined the influence of temperature, bridging the research gap related to the precise understanding of these relationships, which allows for the optimisation of chromatographic conditions and improvement of analytical efficiency [20-23].

The selection of an appropriate mobile phase composition is one of the key factors influencing the effectiveness of liquid chromatography. The chemical properties of the mobile phase play a decisive role in the interactions between the analytes and the stationary phase, which in turn determines the retention time and the quality of separation. This article examined the impact of the mobile phase composition on the efficiency of liquid chromatography, as the proper selection of its components is crucial for the quality of separation and the precision of the obtained results. Although there is already extensive literature on the role of organic modifiers in liquid chromatography, a research gap remains in the detailed understanding of how varying proportions of water and modifiers (such as acetonitrile or methanol) affect the interactions between the mobile and stationary phases, especially in the case of compounds with similar physicochemical properties that may be difficult to separate. Furthermore, previous studies have not fully addressed the optimisation of these parameters in the context of specific chromatographic applications, where accuracy and analysis speed are crucial. The aim was to provide new insights that would enable a precise adjustment of experimental conditions in liquid chromatography, leading to improved resolution and separation efficiency [24-28].

This article also discussed studies on the efficiency of chromatographic columns, as it is a key parameter determining the quality and effectiveness of the separation of sample components. The efficiency of a column, expressed by the number of theoretical plates (N), significantly affects the width and quality of chromatographic peaks, which is particularly important in the analyses where the precise separation of components is crucial for the accuracy of the results optimisation of parameters such as the flow rate of the mobile phase allows for achieving maximum column efficiency, which translates into better resolution and reduced analysis time. The purpose of conducting these studies is not only to improve separation efficiency, but also to obtain more precise and, reliable results in various analytical fields such as chemistry, biochemistry, pharmacy, and quality control [29-33].

The results presented in the article may have significant implications for e.g. the pharmaceutical industry, particularly in the context of drug analysis and quality control. They are also relevant to the biotechnology industry, where precise separation of compounds with different polarities is crucial for studying the properties and biological activity of biomolecules. Furthermore, in environmental analysis, chromatographic techniques serve as a fundamental tool for detecting chemical contaminants in water, soil, and air. Optimisation of parameters such as temperature, flow rate, and eluent composition allows for improved efficiency in purification and separation, which has broad applications in various fields. Moreover, proper optimisation of the chromatographic process can lead to a reduction in solvent consumption, contributing to a more environmentally friendly approach to analyses and minimising their impact on the environment [1-4]. Summing up, all of these elements from the effect of the temperature and eluent modifiers to the evaluation of column efficiency are an integral part of the research on HPLC chromatography, aimed at further improving this technique and adapting it to the growing demands in science and industry. Analysis of the relationship between operational parameters and separation efficiency provides not only theoretical data but also practical guidelines for users of chromatographic techniques, supporting the development of new analytical and industrial applications.

MATERIALS AND METHODS

Materials

Phenol (PH) and caffeine (CAF), both sourced from Merck, were selected as test analytes because of their chemical properties, which enable a comprehensive evaluation of the performance chromatographic system. PH is a moderately polar compound, which make it suitable for studying the interactions between the mobile and stationary phases, particularly under the conditions that involve hydrophobic and polar interactions [34]. CAF, which is more polar than PH, allows chromatographic systems to be analysed in the context of substances that interact more strongly with the eluent [35]. The different retention mechanisms of these compounds enable the evaluation of a broad range of chromatographic parameters mentioned in the previous section.

All organic chromatographic solvents, namely methanol (MeOH) and acetonitrile (ACN), were purchased from Merck. Distilled, deionised, and demineralised water was prepared using a SolPure-78Z deionizer (ELKAR). Solvent degassing was performed immediately after mixing with an ultrasonic bath (ULTRON) for 5 minutes. The experiments were conducted with solvent systems comprising MeOH–water and ACN–water, containing 60, 65, 70, 75, 80, 85, 90, 95, and 99% volume fractions (v/v) of the respective organic component.

Columns and instrumentations

Two chromatographic columns were used for the experiments, the parameters of which are presented in Table 1. It should be noted that both the columns can be used in RP, NP and HILIC systems.

Chromatographic analysis was performed on two chromatographs: the first HPLC system consisted of a Primaide Merck–Hitachi pump (model 1110), a Primaide Merck–Hitachi UV detector (model 1410), and a Primaide Merck–Hitachi column oven (model 1310). The second system, LaChrom from Merck-Hitachi, included a pump model L-7100, a column thermostat model L-7360, a UV detector model L-7455, a degasser model L-7612, and an interface model D-7000.

Table 1. Chromatographic column parameters

Chromatographic column name	Hypersil GOLD HILIC	Shodex SILICA 5NH 4D		
Diameter (metric) [mm]	4.6	4.6		
Length (metric) [mm]	150	150		
Max. pressure [bar]	400	200		
Particle size [µm]	5	5		
рН	2 to 8	2 to 7.5		
Pore size [Å]	175	100		
Maximum temperature [°C]	60	60		
Stationary phase	Silica	Aminopropyl		
Total porosity ε [-]	0.694	0.72		
t _o [min]	1.729	1.794		

Methodology of the study

The effect of temperature on retention

In order to gain a deeper understanding of the thermodynamic aspects of retention, the method presented in [22, 36-39] was used, which allows for determining the effect of temperature on the retention constant. This method enables the calculation of the enthalpy and entropy values of the retention process, providing key insights into the nature and intensity of interactions between the analysed compound and the stationary phase. It involves determining the retention factor (k) for the analyte at different temperatures, and then calculating the standard enthalpy (ΔH^0) and entropy (ΔS^{o}) of adsorption based on the lnk=f(1/T) plot (so called van't Hoff plot) [36]. The dependence of retention on temperature can be described thermodynamically using Equation 1.

$$lnk = -\frac{\Delta H^0}{RT} + \frac{\Delta S^0}{R} + ln\theta \tag{1}$$

where: ΔH^o and ΔS^o represent the standard partial molar changes in enthalpy and entropy, *T* is the thermodynamic temperature in Kelvins, and *R* is the gas constant, equal to 8.314 J/(kmol) and θ is the phase ratio [22, 37–39].

The study of compound retention requires the analysis not only of individual values of ΔH^0 and ΔS^{0} , but also consideration of the broader context of thermodynamic phenomena. For a precise understanding of retention mechanisms, it is often necessary to conduct analysis under varying experimental conditions, such as different mobile phase compositions [36]. Theoretically, the plot of the lnk as a function of 1/T should be linear. The slope of this line allows for the determination of the standard enthalpy of the analyte, while the intercept with the lnk axis enables determination of the standard entropy of the analyte [40]. A linear relationship suggests the stability of the separation mechanism responsible for retention within the analysed temperature range, indicating that the processes occurring in the system are consistent with a uniform adsorption mechanism. Nonlinearity in van't Hoff plots may provide important insights into changing separation mechanisms. They may indicate phenomena related to various aspects of interactions between the analyte and the stationary phase, including changes in analyte ionization, solubility in the mobile phase, and potential structural modifications of analyte molecules [41].

In this article, all measurements were made under isocratic conditions, with a constant eluent flow rate of 1 ml/min. All experiments were repeated three times. The arithmetic mean of the obtained values was used for further studies. The eluents consisting of ACN-water and MeOHwater mixtures were analysed. The concentration of the analysed substances was 20 µg/ml, and the solutions were prepared directly in the appropriate eluents to ensure optimal conditions for their analysis. The temperature was varied every 5 °C in the range from 20 °C to 50 °C. The sample injection volume was 20 µL. Detection of chromatographic peaks was carried out at a wavelength of 250 nm for CAF and 270 nm for PH, which was optimal for obtaining clear signals from these compounds. Thermodynamic data analysis was based on the determination of ΔH and ΔS values using the Equation 1. For this purpose, a linear regression method was used, based on experimentally obtained dependencies of lnk = f(1/T), where k is the retention factor and T is the temperature. This type of analysis allows assessing the impact of temperature on the analysed processes. The precision of fitting the experimental results to the theoretical model was evaluated based on the coefficient of determination R².

The effect of temperature on the selectivity

Understanding the values of ΔH and ΔS° and their impact on the separation mechanisms provides important information about the selectivity of chromatographic processes, showing a significant dependence on temperature. In liquid chromatography, the selectivity coefficient, α , (Equation 2) is a key parameter for evaluating the effectiveness of analyte separation. It is typically defined as the ratio of retention factors of two separated substances, which allows for an accurate evaluation of the ability of the chromatographic system to selective for the selective separation of those compounds [37–38, 42].

$$\alpha = \frac{\kappa_n}{\kappa_{n-1}} = \frac{\kappa_n}{\kappa_{n-1}} \tag{2}$$

where:
$$K$$
 – thermodynamic partition coefficient, k retention factor-, n – first analyte number, n -1 – second analyte number [37–38, 42].

The change between two compounds as a function of temperature (Equation 3) can be derived by substituting Equation 1 into Equation 2. In Equation 1, it is assumed that θ is essentially invariant with temperature.

$$ln\alpha = \frac{lnK_n}{lnK_{n-1}} = \frac{lnk_n}{lnk_{n-1}} = -\frac{\Delta H_n^0 - \Delta H_{n-1}^0}{RT} + \frac{\Delta S_n^0 - \Delta S_{n-1}^0}{R} = -\frac{\Delta (\Delta H^0)}{RT} + \frac{\Delta (\Delta S^0)}{R}$$
(3)

where: $\Delta(\Delta H^o)$ and $\Delta(\Delta S^o)$ are the differences in enthalpy and entropy of adsorption for the more and less retained compounds in the stationary phase [42, 43].

The effect of temperature on the selectivity of the test substances was analysed using the same methodology as for the van't Hoff analysis. Retention factors (k) for both compounds were calculated at each temperature, and then lnk was calculated as a function of the 1/T. Using Equation 1, the values of Δ H⁰ and Δ S⁰ were determined. Similarly, for the selectivity analysis, retention factors for the two compounds compared were determined at each temperature, and then the selectivity coefficient (α) was calculated using the Equation 2. The temperature dependence of the selectivity coefficient (α) was calculated using Equation 3.

The effect of the mobile phase modifier on retention

Measurements of the effect of the mobile phase modifier on the retention of the analysed substances were carried out isocratically, with a constant mobile phase flow rate of 1 ml/min and a temperature of 20°C. All experiments were repeated three times. The arithmetic mean of the obtained values was used for further studies. ACN-water and MeOH-water mixtures were used for the studies. The percentage of organic components in these mixtures was 60, 65, 70, 75, 80, 85, 90, 95, and 99 [%v/v]. The concentration of the chemical substances analysed was 20 µg/ ml, and the solutions were prepared directly in the appropriate eluents, ensuring optimal conditions for their analysis. The sample volume was 20 µL, which was a standard dosing parameter in chromatographic analyses. Chromatographic peaks were detected at wavelengths of 250 nm for CAF and 270 nm for PH, which providing the appropriate sensitivity for the identification of both compounds. On the basis of experimental results, graphs of the retention factor (k) as a function of the composition of the mobile phase i.e. the volume fraction of water in the mobile phase (ϕ H₂O) were created. The value of ϕ H₂O represents the percentage of water in the mixture

with the organic modifier, such as ACN or MeOH. The resulting graph allows for a detailed analysis of how changes in the mobile phase composition affect the retention of the analysed chemical compounds. The $k=f(\phi H_2 O)$ relationship provides valuable information on the interactions between the substances analysed and the stationary phase, which is crucial for determining optimal analytical conditions. An increasing proportion of water in the mobile phase can alter the polarity of the chromatographic system, which in turn affects the retention time of the individual compounds [27–28].

Chromatographic column efficiency

One of the most commonly used methods for evaluating the efficiency of a column is the van Deemter method, which allows the determination of the height of the theoretical plate (HETP) and resulting number of theoretical plates (N) and thus the chromatographic column efficiency. The general form of the method is presented as Equation 4, with the expansion of the components (Equations 5–7) [31–33].

$$HETP = A + \frac{B}{u} + Cu \tag{4}$$

$$A = 2\lambda d_p \tag{5}$$

$$\frac{B}{u} = 2\gamma \frac{D_M}{u} \tag{6}$$

$$Cu = (C_s + C_M)u = f_S k d_f^2 \frac{u}{D_S} + f_M k d_p^2 \frac{u}{D_M}$$
 (7)

where: HETP is the height of the theoretical plate, *u* is the linear velocity, *A* is the term associated with eddy diffusion, which is related to the diameter of the packing material (d_n) , λ is the packing factor related to the structure of the packing material and the uniformity of the packing state, *B* is the term associated with longitudinal diffusion, which reflects the dispersion of the analyte molecules in the longitudinal direction, γ is a constant also related to the structure and morphology of the packing material and the uniformity of the packing state, C is the mass transfer resistance term, which describes the mass transfer resistance of the analyte in the stationary and mobile phases, and is related to the square of the particle size (d_p) , D_s and D_M is the diffusion coefficient of the analyte in the stationary and mobile phases, respectively, d_{c} is the thickness of the stationary phase layer [33].

A high value of N translates into higher column efficiency, resulting in narrower and better-separated chromatographic peaks. In particular, it shows how the particle size of the packing material, the flow rate of the mobile phase, the structure of the packing material, and interactions between the stationary and mobile phases influence the separation efficiency. Analysing the individual components of the equation allows for optimisation of the chromatographic conditions to achieve the best separation results. The equations above show that the particle size of the packing material is a key factor influencing the column efficiency through its impact on the A and C terms. Reducing the particle size leads to a decrease in both the A and C terms, which results in a reduction of the height of the theoretical plate, and ultimately increases the column efficiency. Moreover, from the equations, it follows that uniform particle size, regular morphology, and packing state leads to small components of eddy diffusion and longitudinal diffusion, which favour the column efficiency. Therefore, the ideal packing material used in high-efficiency separations with high velocity in liquid chromatography (LC) should be characterized by small, uniform particles with a well-defined morphology and structure [44]. Equation 4 is a fundamental tool in chromatography, allowing for analysis of the influence of various factors on the efficiency of the chromatographic column. In particular, it indicates how the size of the packing material, the flow rate of the mobile phase, the structure of the packing material, as well as the interactions between the stationary and mobile phases affect the efficiency of separation. Analysing the individual components of the equation enables the optimisation of chromatographic conditions to achieve the best separation results [29–33].

Efficiency studies were carried out at a temperature of 20 °C for eluent flow rates of 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.40, 0.50, 0.65, 0.80, 1.0, 1.25, 1.50, 1.75, 2.00, and 3.0 [ml/min]. The injection volume of the sample was 20 μ L. The concentrations of the samples for PH and CAF were 20 μ g/mL. All experiments were repeated three times. The arithmetic mean of the obtained values was used for further studies. The calculations were performed using moment analysis, described in more detail in e.g. [30–33], using the freeware computer program Chromatographic-Column.exe. by prof. Krzysztof Kaczmarski from Rzeszow University of Technology. Among other

things, the method of moments was implemented in this program. The analysis of chromatographic data using moment analysis is an advanced tool that enables a precise evaluation of key chromatographic parameters based on the detector signal. This method is based on treating the chromatographic peak as a probability distribution function, which allows for the calculation of successive statistical moments. Owing to this method, it is possible to independently evaluate various aspects of the chromatographic peak shape, which is particularly useful in the cases where the peaks are not perfectly symmetrical or when disturbances arise from overlapping components. The method of moments also allows for the assessment of the influence of variable experimental conditions, such as temperature, eluent composition, and flow rate, on separation parameters, thereby contributing to a better understanding of retention mechanisms. The implementation of this method in the specialised software Chromatographic Column.exe enables the automation of calculations, thus increasing the precision and reproducibility of the results [30–32].

RESULTS AND DISCUSSION

Analysis of the effect of temperature on retention

The studies carried out analysed the behaviour of the test substances, PH and CAF, on two chromatographic columns: Hypersil GOLD HIL-IC (Fig. 1, 2) and Shodex SILICA 5NH 4D (Fig. 3, 4). The aim was to determine thermodynamic properties such as enthalpy (ΔH) and entropy (Δ S) using lnk=f(1/T) plots, which were constructed based on the data obtained from various eluent compositions. The mobile phases analysed were methanol with water (MeOH:H₂O [%v/v]) in proportions of 70:30, 90:10, and 99:1, as well as acetonitrile with water (ACN:H₂O [%v/v]) in the same proportions. On the basis of the obtained results (Figs. 1–4), the values of ΔH and ΔS were determined, together with an evaluation of the model fit quality using the coefficient of determination R² (Table 2).

Analysing the enthalpy values reveals that for both test substances (PH and CAF), for different eluents and chromatographic columns, the enthalpy is generally negative (e.g., -5.95 kJ/mol for CAF in a MeOH:H₂O 90:10 [%v/v] eluent



Figure 1. lnk = f(1/T) plot for CAF on the Hypersil GOLD HILIC chromatographic column



Figure 2. lnk = f(1/T) plot for PH on the Hypersil GOLD HILIC chromatographic column



Figure 3. lnk = f(1/T) plot for CAF on the Shodex SILICA 5NH 4D chromatographic column



Figure 4. lnk = f(1/T) plot for PH on the Shodex SILICA 5NH 4D chromatographic column

using the Hypersil GOLD HILIC column). Negative ΔH values suggest that the retention process is exothermic, meaning that it involves the release of energy during the interactions between the analyte molecules and the stationary phase. The causes of this phenomenon may result from the nature of the interactions between the adsorbate and the adsorbent surface, such as: (*) intermolecular interactions during adsorption, van der Waals forces (physical adsorption) or chemical bonds (chemical adsorption) forming between the adsorbate molecules and the adsorbent surface. These processes lead to a decrease in the energy of the system, which results in the release of heat, (**) a decrease in the free energy of the system – during adsorption, the freedom of movement of the adsorbate molecules is reduced, which results in a decrease in the energy of the system and the release of heat, (***) energy stabilisation of the system - adsorbate molecules, interacting with the adsorbent surface, reach a lower energy state compared to the state in the liquid phase, which causes the release of excess energy in the form of heat, (****) entropy of the system – in the case of physical adsorption, entropy may decrease because the molecules move from a more chaotic fluid condition to a more ordered state on the adsorbent surface. For a process to be spontaneous, the enthalpy must be negative enough to compensate for the decrease in entropy. Changes in ΔH under different conditions can indicate varying degrees of adsorption of PH and CAF on the adsorbent, which depend on the chemical and physical interactions between these substances and

	CAF	MeOH:H ₂ O 70:30 [%v/v]		MeOH:H ₂ O 90:10 [%v/v]			MeOH:H ₂ O 99:1 [%v/v]			
Hypersil GOLD HILIC		ΔH [kJ/mol]	∆S [J/mol·K]	R ²	∆H [kJ/mol]	∆S [J/mol·K]	R ²	ΔH [kJ/mol]	∆S [J/mol·K]	R ²
		-4.12	-30.56	0.68	-5.95	-35.58	0.93	-7.37	-39.19	0.83
		ACN: H ₂ O 70:30 [%v/v]		ACN: H ₂ O 90:10 [%v/v]			ACN: H ₂ O 99:1 [%v/v]			
		ΔH [kJ/mol]	∆S [J/mol·K]	R ²	ΔH [kJ/mol]	∆S [J/mol·K]	R ²	ΔH [kJ/mol]	∆S [J/mol·K]	R ²
		-5.02	-34.23	0.88	-8.20	-40.48	0.87	-7.14	-35.51	0.96
		MeOH:H ₂ O 70:30 [%v/v]			MeOH:H ₂ O 90:10 [%v/v]			MeOH:H ₂ O 99:1 [%v/v]		
		∆H [kJ/mol]	∆S [J/mol·K]	R ²	∆H [kJ/mol]	∆S [J/mol·K]	R ²	ΔH [kJ/mol]	∆S [J/mol·K]	R ²
	рц	-5.18	-28.96	0.98	-5.75	-31.70	0.98	-16.56	-66.29	0.83
		ACN: H ₂ O 70:30 [%v/v]		ACN: H ₂ O 90:10 [%v/v]			ACN: H ₂ O 99:1 [%v/v]			
		∆H [kJ/mol]	∆S [J/mol·K]	R ²	∆H [kJ/mol]	∆S [J/mol·K]	R ²	ΔH [kJ/mol]	∆S [J/mol·K]	R ²
		21.65	47.93	0.76	-2.93	-23.42	0.86	-11.28	-42.74	0.99
	CAF	MeOH:H ₂ O 70:30 [%v/v]		MeOH:H ₂ O 90:10 [%v/v]			MeOH:H ₂ O 99:1 [%v/v]			
		∆H [kJ/mol]	∆S [J/mol·K]	R ²	∆H [kJ/mol]	∆S [J/mol·K]	R ²	ΔH [kJ/mol]	∆S [J/mol·K]	R ²
		-6.20	-36.45	0.97	-10.82	-50.01	0.85	-10.10	-48.78	0.99
Shodex SILICA 5NH 4D		ACN: H ₂ O 70:30 [%v/v]		ACN: H ₂ O 90:10 [%v/v]			ACN: H ₂ O 99:1 [%v/v]			
		∆H [kJ/mol]	∆S [J/mol·K]	R²	∆H [kJ/mol]	∆S [J/mol·K]	R ²	ΔH [kJ/mol]	∆S [J/mol·K]	R ²
		-4.67	-32.00	0.87	-5.75	-23.28	0.98	-8.97	-33.36	0.99
		MeOH:H ₂ O 70:30 [%v/v]		MeOH:H ₂ O 90:10 [%v/v]			MeOH:H ₂ O 99:1 [%v/v]			
	PH ·	∆H [kJ/mol]	∆S [J/mol·K]	R²	∆H [kJ/mol]	∆S [J/mol·K]	R ²	ΔH [kJ/mol]	∆S [J/mol·K]	R ²
		-4.45	-29.03	0.96	-13.80	-62.21	0.81	-6.54	-38.65	0.87
		ACN: H ₂ O 70:30 [%v/v]		ACN: H ₂ O 90:10 [%v/v]			ACN: H ₂ O 99:1 [%v/v]			
		∆H [kJ/mol]	∆S [J/mol·K]	R ²	∆H [kJ/mol]	∆S [J/mol·K]	R ²	ΔH [kJ/mol]	∆S [J/mol·K]	R ²
		8.15	1.93	0.88	-1.97	-25.44	0.09	-9.83	-42.80	0.99

Table 2. The values of enthalpy and entropy of adsorption, as well as the determination coefficients for the HypersilGOLD HILIC and Shodex SILICA 5NH 4D columns

the stationary phase (e.g., van der Waals forces, hydrogen bonding). The values of ΔS are also mostly negative, indicating a decrease in disorder during interactions between the analyte molecules and the stationary phase. Changes in ΔS can reflect how the degree of molecular organisation is altered as a result of adsorption. Negative ΔS values may indicate that the system is becoming more ordered. Reasons of this may include: (*) a decrease in temperature - a decrease in temperature reduces the kinetic energy of molecules, limiting their mobility and reducing the number of available energy states, (**) intermolecular interactions - the formation of hydrogen bonds, complexes or strong interactions between molecules restricts their freedom of movement, which reduces entropy, (***) a decrease in the freedom of movement of molecules - in solution, molecules move freely in a large volume. After adsorption,

their movement is restricted to the surface of the adsorbent, (****) an increase in order – adsorbed molecules are arranged on the surface of the adsorbent in a more orderly manner than in the liquid phase, (*****) a decrease in the number of possible configurations – in solution, molecules can be in different positions and orientations, but after adsorption, the available configurations are restricted by the surface of the adsorbent.

For PH, being a smaller molecule, interactions with the stationary phase may induce greater structural changes in the stationary phase, leading to a more significant decrease in entropy. In the case of CAF, which has larger molecules, changes in Δ S may be less pronounced. The chromatographic columns Hypersil GOLD HILIC and Shodex SILICA 5NH 4D exhibit differences in the values of Δ H and Δ S, suggesting that they influence interactions with the substances studied differently. For Hypersil GOLD HILIC, the enthalpy is more negative compared to Shodex SIL-ICA 5NH 4D, which may indicate that PH and CAF are more strongly adsorbed on the stationary phase. When comparing the effects of different eluents, it was observed that in the ACN:H2O system, the enthalpy values are less negative compared to the MeOH:H2O system. MeOH, as a more polar modifier, interacts more effectively with the stationary phase and the substances analysed, which can enhance hydrogen bonding and other molecular interactions, leading to more negative ΔH values. In contrast, ACN, because of its lower polarity and different interaction characteristics with the stationary phase, weakens the retention of test substances. This results in less negative ΔH values and weaker energetic effects during the adsorption process.

For PH, in both columns, in the ACN:H₂O 70:30 [%v/v] system, the values of ΔH and ΔS are positive, indicating an endothermic reaction. Positive ΔH values can be caused by: (*) disruption of the adsorbent structure - if adsorption leads to significant changes in the surface structure of the adsorbent (e.g. pore expansion, change in the arrangement of adsorbate layers), it may be necessary to supply energy to carry out the process, (**) overcoming intermolecular interactions in the adsorbate - if the adsorbate molecules are strongly bound in the liquid phase (e.g. by hydrogen bonds), their separation during adsorption requires energy, (***) change in the structure of adsorbates - in some cases, adsorption can lead to a change in the conformation of the adsorbate molecules or the breaking of bonds, which requires energy, (****) effect of temperature on adsorption - for some systems, an increase in temperature can increase the amount of the adsorbed substance, suggesting an endothermic nature of the process, (*****) entropy of the system - if adsorption leads to an increase in disorder (e.g. when the adsorbate molecules diffuse freely over the adsorbent surface), the process may be endothermic to compensate for the increase in entropy. Positive ΔS values may result from several factors: (*) desorption of solvent molecules - if adsorption occurs from solution, solvent molecules may be displaced from the adsorbent surface into the volume, which increases the total entropy of the system, (**) adsorbate restructuring – in some cases, especially in multicomponent systems, the adsorbate may become more free on the surface, for example by loosening its structure, (***)

increased mobility on the surface – in some cases, adsorbed molecules may gain greater freedom of movement along the surface, instead of being immobilised in one place, (****) conformational effects – for large organic molecules, adsorption may lead to the adoption of a more flexible structure, which increases the entropy of the system, (****) reduced order in the adsorbent crystal lattice – in some cases, adsorption may cause a loosening of the adsorbent surface structure, which increases the entropy of the system.

For Hypersil GOLD HILIC: $\Delta H = 21.65 \text{ kJ/}$ mol, $\Delta S = 47.93$ J/mol·K. For Shodex SILICA 5NH 4D: $\Delta H = 8.15 \text{ kJ/mol}$, $\Delta S = 1.93 \text{ J/mol} \cdot \text{K}$. The positive values of ΔH indicate an endothermic reaction, which means that PH adsorption in on both stationary phases requires energy input. Such endothermic reactions are typical for processes in which molecules adsorb on to the surface of the stationary phase, and the process absorbs energy from the surroundings. The positive ΔS values also indicate an increase in disorder during adsorption. This result suggests that the process may lead to greater freedom of movement for phenol molecules after adsorption or may indicate the loosening of the stationary phase structure during the adsorption of PH. This could be due to the dynamic interaction between PH and the mobile phase. The higher ΔS value on the Hypersil GOLD HILIC column suggests a more dynamic interaction in this system.

The value of the coefficient R^2 is an indicator of how well Eq. 1 fits the experimental data. High values (close to 1, for example, 0.96–0.99) suggest a good fit of the model, indicating that the chromatographic behaviour of PH and CAF in the given eluent and stationary phase is stable. However, low R^2 values in some cases (for example, for PH in the ACN:H₂O 90:10 [%v/v] mixture on the Shodex SILICA 5NH 4D column) may indicate the presence of additional factors not considered in the model, such as more complex interactions with the stationary phase.

Analysis of the effect of temperature on the selectivity

For the Hypersil GOLD HILIC column in the 70:30, 90:10, and 99:1 [%v/v] MeOH:H₂O systems, the selectivity between CAF and PH is very weak (Fig. 5). The values of ln α decrease with increasing temperature, indicating that the selectivity between CAF and PH decreases further. High



Figure 5. The effect of temperature on the selectivity of test substances for Hypersil GOLD HILIC

 $\Delta\Delta$ H values (Table 3) at the 99:1 [%v/v] MeO-H:H₂O ratio suggest that more energy is required for CAF adsorption compared to PH. The increase in $\Delta\Delta$ S indicates greater mobility of CAF in the system with a high concentration of MeOH. On the other hand, in the 70:30 [%v/v] ACN:H₂O system, ln α sharply with increasing temperature, indicating that the selectivity between CAF and PH increases. High negative values of $\Delta\Delta$ H and $\Delta\Delta$ S are observed, suggesting more stable and ordered interactions between PH and the stationary phase. At the 90:10 [%v/v] ACN:H₂O ratio, the curve is flatter, suggesting smaller differences in the interactions between the two analytes. At the 99:1 [%v/v] ACN:H₂O ratio, the graph suggests that CAF is retained more compared to PH in the less aqueous system, and selectivity is high. For the Shodex SILICA 5NH 4D column (Fig. 6) in the 70:30 [%v/v] MeOH:H₂O system, very low selectivity between CAF and PH is observed. However, for the 90:10 [%v/v] MeOH:H₂O eluent, the selectivity of the test substances is good and decreases sharply with increasing temperature. For the 99:1 [%v/v] MeOH:H₂O eluent, selectivity is good and increases with temperature. The curve indicates a greater retention of CAF compared to PH at higher temperatures. In the 70:30 [%v/v] ACN:H₂O system, clear differences in selectivity between CAF and PH are visible. The curve is steep, suggesting a strong influence



Figure 6. The effect of temperature on the selectivity of test substances for Shodex SILICA 5NH 4D

Hypersil GOLD HILIC	CAF:PH	MeOH:H ₂ O 70:30 [%v/v]		MeOH:H ₂ O 90:10 [%v/v]		MeOH:H ₂ O 99:1 [%v/v]		
		ΔΔΗ [kJ/mol]	∆∆S [J/mol·K]	ΔΔΗ [kJ/mol]	∆∆S [J/mol·K]	ΔΔΗ [kJ/mol]	ΔΔS [J/mol·K]	
		1.06	-1.6	-0.2	-3.88	9.19	27.1	
		ACN: H ₂ O 70:30 [%v/v]		ACN: H ₂ O 90:10 [%v/v]		ACN: H ₂ O 99:1 [%v/v]		
		ΔΔΗ [kJ/mol]	ΔΔS [J/mol·K]	ΔΔΗ [kJ/mol]	ΔΔS [J/mol·K]	ΔΔΗ [kJ/mol]	∆∆S [J/mol·K]	
		-26.67	-82.16	-5.27	-17.06	4.14	7.23	
Shodex SILICA 5NH 4D	CAF:PH	MeOH:H ₂ O 70:30 [%v/v]		MeOH:H ₂ O 90:10 [%v/v]		MeOH:H ₂ O 99:1 [%v/v]		
		ΔΔΗ [kJ/mol]	ΔΔS [J/mol·K]	ΔΔΗ [kJ/mol]	ΔΔS [J/mol·K]	ΔΔΗ [kJ/mol]	ΔΔS [J/mol·K]	
		-1.75	-7.42	2.98	12.2	-3.56	-10.13	
		ACN: H ₂ O 70:30 [%v/v]		ACN: H ₂ O 90:10 [%v/v]		ACN: H ₂ O 99:1 [%v/v]		
		ΔΔΗ [kJ/mol]	ΔΔS [J/mol⋅K]	ΔΔΗ [kJ/mol]	ΔΔS [J/mol·K]	ΔΔΗ [kJ/mol]	ΔΔS [J/mol·K]	
		-12.82	-33.93	-3.78	2.16	0.86	9.44	

Table 3. Values of $\Delta\Delta H$ and $\Delta\Delta S$ for the Hypersil GOLD HILIC and Shodex SILICA 5NH 4D columns in different

systems

of temperature on selectivity. As the temperature increases, the selectivity improves significantly. Similarly, in the 90:10 [%v/v] ACN:H₂O system, the selectivity of the test substances is high and increases with temperature, but at a more stable rate. In the 99:1 [%v/v] ACN:H2O system, the selectivity is also high, but decreases with increasing temperature. Positive $\Delta\Delta H$ values (Table 3) in this system suggest more dynamic interactions in systems rich in ACN. In conclusion, both the Hypersil GOLD HILIC and Shodex SILICA 5NH 4D columns show that ACN-water systems lead to better selectivity for the test substances, CAF and PH, compared to MeOH-water systems. This is especially evident on the Shodex SILICA 5NH 4D column, where selectivity is very high.

The $\Delta\Delta H$ vs. $\Delta\Delta S$ plot (Fig. 7) allows the analysis of the relationship between the differences in enthalpy and entropy for CAF and PH under different chromatographic conditions, taking into account the effect of the eluent modifiers (MeOH and ACN) and the use of Hypersil GOLD HILIC and Shodex SILICA 5NH 4D columns. Increasing the proportion of MeOH (\geq 90%v/v) shifts the points toward positive $\Delta\Delta H$ and $\Delta\Delta S$, indicating that endothermic reactions are occurring. These reactions can negatively impact the selectivity of the substances studied. On the other hand, in more aqueous systems (70:30 [%v/v] ACN:H₂O), the most negative $\Delta\Delta H$ and $\Delta\Delta S$ values are observed, which may lead to more pronounced changes in selectivity with increasing temperature. The points in the graph that align in a straight line (linear correlation) suggest that the changes in $\Delta\Delta H$ and $\Delta\Delta S$ are proportional. This may indicate the dominance of a single interaction mechanism (e.g. hydrogen bonding and van der Waals forces). In contrast, scattered points may suggest that different adsorption mechanisms dominate under different conditions. Similar conclusions can be drawn based on mixed values of entropy and enthalpy differences, such as for the Shodex SILICA 5NH 4D column in the 99:1 [%v/v] AC-N:H₂O system, where $\Delta\Delta H = -3.78$ [kJ/mol] and $\Delta\Delta S = 2.16 [J/mol \cdot K].$

Analysis of effect of the eluent modifier on the retention

The graphs showing influence of the eluent modifier on PH (Fig. 8, 9) and CAF (Fig. 10, 11) retention for the Hypersil GOLD HILIC and Shodex SILICA 5NH 4D chromatographic columns



Figure 7. The relationship between the entropy and enthalpy differences for the Hypersil GOLD HILIC and Shodex SILICA 5NH 4D columns under different chromatographic conditions



Figure 8. The effect of the eluent modifier on the retention of PH in Hypersil GOLD HILIC and Shodex SILICA 5NH 4D chromatographic columns for mobile phase: ACN:H₂O [%v/v]



Figure 9. The effect of the eluent modifier on the retention of PH in Hypersil GOLD HILIC and Shodex SILICA 5NH 4D chromatographic columns for mobile phase: MeOH:H₂O [%v/v]



Figure 10. The effect of the eluent modifier on the retention of CAF in Hypersil GOLD HILIC and Shodex SILICA 5NH 4D chromatographic columns for mobile phase: ACN:H₂O [%v/v]



Figure 11. The effect of the eluent modifier on the retention of CAF in Hypersil GOLD HILIC and Shodex SILICA 5NH 4D chromatographic columns for mobile phase: MeOH:H₂O [%v/v]

indicate significant differences in the behaviour of these substances in systems with different mobile phases, i.e., mixtures of ACN:H₂O [%v/v] and MeOH:H₂O [%v/v]. Interestingly, in most analysed systems, no clear U-shaped dependences of the retention factor versus eluent composition were observed (which is quite typical for HILIC systems). The presence of the U-shape k=f(ϕ) dependences usually show the combined HILC/ RPLC retention mechanism, with a minimum corresponding to the transition point from the RPLC to the HILIC mechanism [45]. This may indicate the presence of a different (unexpected/ of the $k=f(\phi)$ relationships presented for Hypersil GOLD HILIC with MeOH as eluent modifier (see Figs. 9 and 11) may indicate that in these systems the retention mechanism is a compilation of various, difficult to define interactions between the components of the chromatographic system. Reducing the concentration of ACN in the mobile phase decreases the retention time of PH on both chromatographic columns. For CAF, reducing the concentration of ACN leads to a significant decrease in retention on both columns, although this effect is more pronounced on the Shodex SILICA 5NH 4D column. In the MeOH-water system, reducing the MeOH concentration results in a decrease in the retention of PH and CAF in the Shodex SILICA 5NH 4D column. For the Hypersil GOLD HILIC column, PH the retention gradually decreases as the MeOH concentration in the system is reduced. However, a sharp increase in retention factor is observed at 85 [% v/v]MeOH in the mixture, followed by a subsequent decrease in retention at MeOH concentrations of 60–65 [%v/v]. A similar pattern is observed for CAF, with an increase in the retention factor at 80 [%v/v] MeOH in the mixture, followed by a further decrease in retention at MeOH concentrations of 60–70 [%v/v]. Differences in PH and CAF retention depending on the concentration of the eluent modifier may result from various mechanisms of interaction between the analytes and the stationary and mobile phases. ACN and MeOH differ in polarity, which influences the strength of interactions between the analytes and the stationary phase. The increase in retention observed for the Hypersil GOLD HILIC column at certain MeOH [%v/v] concentrations may indicate specific interactions, such as the formation of hydrogen bonding networks. CAF, being a more polar substance than PH, responds more distinctly to changes in the concentration of the eluent modifier, particularly on the Shodex SILICA 5NH 4D column.

more uniform) retention mechanism in the stud-

ied systems. Besides, the rather unusual courses

Analysis of chromatographic columns efficiency

Figures 12 and 13 show sample results of the efficiency tests for the chromatographic columns, namely Hypersil GOLD HILIC and Shodex SILI-CA 5NH 4D, with a 95:5 [%v/v] MeOH:H₂O eluent. For the Hypersil GOLD column, the HETP



Figure 12. Efficiency of Hypersil GOLD HILIC and Shodex SILICA 5NH 4D chromatographic columns with a 95:5 [%v/v] MeOH:H₂O eluent: for CAF



Figure 13. Efficiency of Hypersil GOLD HILIC and Shodex SILICA 5NH 4D chromatographic columns with a 95:5 [%v/v] MeOH:H₂O eluent: for PH

values for CAF vary depending on the flow rate of the mobile phase. The HETP values increase at higher flow rates, indicating that at higher flow velocities, the resolution deteriorates, and the column becomes less efficient. The maximum flow rate for this column was 1.2 ml/min due to pressure limitations. The optimal flow rate measurements is in the range of 0.2–0.9 ml/min. For PH, similarly to CAF, the HETP values increase with higher flow rates. The increase in HETP values at higher flow rates indicates a reduction in the separation efficiency. The optimal flow rate in this case is between 0.15–0.5 ml/min. For the Shodex SILICA 5NH 4D column, the HETP values for CAF also increase at higher flow rates, but the values are lower compared to the Hypersil GOLD HILIC column. The optimal flow rate is between 0.25–1.25 ml/min. For PH on this column, the HETP values are also higher at greater flow rates, again indicating a decrease in the column efficiency at higher flow rates. The optimal flow rate is between 0.25–1.25 ml/min.

CONCLUSIONS

Analysis of the effect of temperature on retention of PH and CAF shows that the values of ΔH are generally negative, suggesting an exothermic nature of the retention process, which means that energy is released during the interaction of the analytes with the stationary phase. Changes in ΔH may indicate varying degrees of adsorption, depending on chemical and physical interactions, such as van der Waals forces or hydrogen bonding. The values of ΔS are generally negative, indicating a decrease in disorder during the interaction with the stationary phase, and changes in ΔS may reflect the molecular organisation resulting from adsorption. PH, being a smaller molecule, causes greater changes in the structure of the stationary phase, leading to a stronger decrease in entropy, while for CAF, these changes are less pronounced. The Hypersil GOLD HILIC and Shodex SILICA 5NH 4D columns show differences in the values of ΔH and ΔS , suggesting that they interact with the substances studied in different ways. For the Hypersil GOLD HILIC column, the enthalpy is more negative, which may indicate a stronger adsorption of PH and CAF. In the ACN:H₂O system, the enthalpy is less negative compared to the MeOH:H₂O system, suggesting that MeOH, being a more polar modifier, interacts more effectively with the stationary phase, enhancing the strength of interactions. In contrast, ACN, being less polar, weakens retention, resulting in lower negative ΔH values. For PH in the ACN:H₂O 70:30 [%v/v] system, both Δ H and ΔS values are positive, indicating an endothermic reaction. Low R² values (eg., for PH in the mixture of ACN:H₂O 90:10 [%v/v] the Shodex SILI-CA 5NH 4D column) can suggest the presence of additional factors not taken into account for in the model, such as more complex interactions with the stationary phase.

The studies showed that ACN-water systems provide better selectivity between CAF and PH than MeOH-water systems. For the Shodex SIL-ICA 5NH 4D column, selectivity in ACN-based systems was particularly high. In more aqueous systems, such as 70:30 [%v/v] ACN:H₂O, an increase in selectivity was observed with increasing temperature, accompanied by negative $\Delta\Delta H$ and $\Delta\Delta S$ values. In systems with a high concentration of ACN (99:1 [%v/v]), positive $\Delta\Delta H$ values suggest more dynamic interactions, and selectivity decreased with increasing temperature. In MeOH-water systems, a high concentration of MeOH (\geq 90% v/v) was associated with positive $\Delta\Delta H$ and $\Delta\Delta S$ values, indicating endothermic reactions that reduced selectivity. For the Hypersil GOLD HILIC column, selectivity in MeOH:H2O systems was low, and its value decreased with temperature. However, in the 70:30 [%v/v] ACN:H2O system, selectivity increased sharply with temperature, highlighting greater differences in the interactions of CAF and PH with the stationary phase.

Changes in PH and CAF retention depending on the concentration of the eluent modifier, can result from various interaction mechanisms between the analytes and the stationary and mobile phases. Reducing the concentration of ACN shortens the retention time of PH on both columns, with a more pronounced effect on the Shodex SILICA 5NH 4D column. For CAF, lowering the ACN concentration leads to a significant decrease in retention, particularly on the Shodex SILICA 5NH 4D column. In the MeOH-water system, decreasing the concentration of MeOH results in a decrease in the retention of PH and CAF in the Shodex SILICA 5NH 4D column. For the Hypersil GOLD HILIC column, the retention of PH gradually decreases as the MeOH concentration is reduced, but at 85 [%v/v] MeOH, a marked increase in retention is observed, followed by a decrease at MeOH concentrations of 60 to 65 [%v/v]. A similar trend is observed for CAF, where retention increases at 80 [%v/v] MeOH, followed by a decrease at MeOH concentrations of 60 to 70 [%v/v]. Differences in the retention of PH and CAF may arise from different interactions with the stationary phase. ACN and MeOH differ in polarity, which affects the strength of interactions with the analysed substances. The increase in retention observed on the Hypersil GOLD HILIC column at certain concentrations of MeOH [%v/v] can indicate specific interactions, such as the formation of hydrogen bonds. CAF, being a more polar substance than PH, reacts more distinctly to changes in the eluent modifier concentration, particularly on the Shodex SILICA 5NH 4D column.

On the basis of the studies conducted on the efficiency of the Hypersil GOLD HILIC and Shodex SILICA 5NH 4D chromatographic columns, it was found that the HETP values for CAF and PH increase with higher mobile phase flow rates, indicating a deterioration in separation efficiency and broader chromatographic peaks. For the Hypersil GOLD HILIC column, the optimal flow rate range is between 0.2 and 0.9 ml/min, with a maximum flow rate of 1.2 ml/min due to pressure limitations. For PH, the optimal flow rate is 0.15 to 0.5 ml/min. On the Shodex SILICA 5NH 4D column, the HETP values for CAF are lower than those for the Hypersil GOLD HILIC column, and the optimal flow rate range is between 0.25 and 1.25 ml/min. For PH on this column, an increase in HETP values is also observed at higher flow rates, indicating a decline in separation efficiency. The optimal flow rate for PH in this column is 0.25 to 1.25 ml/min. These conclusions highlight the importance of selecting the appropriate mobile phase flow rate to achieve the best separation efficiency in chromatography.

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