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Studies on the Effects of Process Conditions on Separation of B1, B2 and B3 Vitamin Mixture Using HILIC and RPLC Chromatography

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Abstract: In this work, a series of experiments on the retention of B1 (riboflavin), B2 (thiamine) and B3 (nicotinic acid) vitamins in the HILIC and RPLC conditions have been performed involving the effects of organic modifier type and content, pH of the eluent and buffering salt (ammonium acetate) concentration in the mobile phase as well as temperature of the system. For the needs of this study, three columns of different features have been chosen: Acclaim™ Mixed–Mode HILIC–1 (Dionex, Sunnyvale, CA, USA), Eurospher II 100–5 HILIC (Knauer, Berlin, Germany) and the RPLC column Nucleodur® C18 Gravity-SB (Macherey-Nagel, Duren, Germany). The influence of acetonitrile and methanol content in the eluent and process temperature have been tested and, based on that, the most promising systems have been selected regarding the possible separation of the vitamin mixture. Both the pH and buffering salt concentrations in the eluent have been adjusted in order to indicate the most effective system, which turned out to be the one involving the Nucleodur column and the eluent with 90% methanol, at pH 6 and C_{buff} = 20 mmol/dm^3, which enables separation of the mixture within a time as short as 2.5 min at a 1.0 mL/s flowrate in isocratic conditions.

Keywords: HILIC chromatography; RPLC chromatography; B1 vitamin; B2 vitamin; B3 vitamin

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1. Introduction

B-complex vitamins are known as both soil and foliar fertilizer additives [1]. Vitamins B1 and B2 are also characterized by their immune-inducing effects in plants [2]. Vitamin B1 is an essential dietary component. The predominant dietary source of vitamin B1 is food of plant origin. Vitamin B1 is also essential for plants themselves [3].

Adequate supplementation of B vitamins is essential for broadly understood health, proper course of labor and lactation, postpartum period and growth of some farm animals, especially cows. From the economic (and also agricultural engineering) point of view, a rational supply of vitamins allows to minimize losses connected with possible disorders of metabolic health, costs of treatment and culling of animals. In the case of deficiencies, but also for herd prevention, supplementation of B vitamins is necessary [4–6]. From the agricultural engineering point of view, it is, therefore, important to indicate methods that enable the quantitative and/or qualitative analysis of various chemical substances, including B-complex vitamins in various biological/environmental systems. One such method is liquid chromatography, widely used as an analytical technique in various fields of engineering, including agricultural engineering. Liquid chromatography is also a very fast and accurate method used to control the effects of implementing/running various agricultural production technologies, fertilization techniques and, finally, animal husbandry. It should be

remembered that the practical application of liquid chromatography requires a number of so-called "basic investigations". Their aim is to analyze the influence of a number of factors on the course of this operation and to select optimal (also from an ecological point of view) conditions for its conduct. It has been the main goal of the research presented in this paper. Diverse physicochemical properties of the B-group vitamins and their occurrence in many biologically active forms pose the issue of vitamin determination as an analytical challenge. The traditional methods of determining the content of the vitamins in food consist in carrying out tedious tests or physicochemical methods for each vitamin [7]. Due to the increased interest in the simultaneous determination of the water-soluble vitamins (WSVs), methods, such as capillary electrophoresis techniques [8–10], and chromatographic techniques, such as thin-layer chromatography [11], micellar electrokinetic chromatography [12–14], micellar liquid chromatography [15], pressurized capillary electrochromatography [16] and high-performance liquid chromatography (HPLC), were used, with the last one being of the greatest interest due to apparatus improvements and a variety of stationary phases and eluents. Among the HPLC techniques, reversed-phase chromatography (RP-HPLC) has been applied most often for simultaneous determination of the VSWs due to simplicity and enhanced efficiency of the stationary phases [7,17–32]. However, the chromatographic separation of the WSVs under isocratic conditions is possible only for a mixture of two or three vitamins [17,18], while the simultaneous chromatography of more complicated mixtures usually requires a gradient elution involving buffer phases [19–27] or specialized detection techniques [28].

Aside from the techniques mentioned above, the WSV mixtures can be analyzed using hydrophilic interaction liquid chromatography (HILIC). The HILIC method proposed by Alpert [29] in 1990 is considered to be a technique enabling the analysis of polar and medium polar compounds and an alternative method to previously used normal phase (NPLC) and reversed-phase (RPLC) liquid chromatography. Within the past few decades, the HILIC technique has filled the gap between the RPLC and NPLC, providing effective separation of the compounds that caused difficulties and were hitherto associated with unsatisfactory results when analyzed under the RP [30,31] or the aqueous conditions [32]. Generally, the HILIC mixed-mode retention mechanism assumes partitioning of the polar solute between the mobile eluent and a layer of the water-enriched mobile phase immobilized on the polar stationary phase [29,33–35]. The separation mechanism differs from that of the RPLC and the NPLC since it may additionally involve chemical interactions (i.e., hydrogen bonding, donor–acceptor), physical interactions (i.e., dipole–dipole interactions, van der Waals forces) and hydrophobic interactions as proposed by the quantitative structure retention relationships (QSRR) approach [36,37]. The final resolution on the HILIC chromatography mechanism requires further work considering the effects of the essential process parameters, i.e., the composition and pH of the mobile phase, the buffering salt concentration, column temperature and type of applied stationary phase on the observed retention of analytes. Application of the hydrophilic interaction chromatography in the separation of the WSV mixtures has been a strong trend in recent years due to good solubility of biologically active compounds in HILIC environments [38–42]. A series of papers by Karatapanis et al. [38–40] have made a valuable contribution to the studies of the effect of variable chromatographic conditions, including the type of organic solvent, pH of the mobile phase and buffer salt type and concentration on the separation of the WSVs using the HILIC-dedicated columns equipped with different stationary phases (i.e., unmodified silica, diol and amino). The experiments under isocratic conditions enabled the separation of a mixture of six vitamins using eluent based on acetonitrile as the organic component, while the gradient elution was found necessary in the analysis of a mixture of eight water-soluble vitamins contained in a vitamin nutrient. In addition, the authors confirmed that the relative contributions of the partitioning and the surface adsorption mechanism are highly connected with the nature of the stationary phase, properties of the solutes and the eluent conditions. As a consequence, another model that considers the solute–solvent–stationary-phase interactions and more precisely describes the multimodal character of the

system should be proposed. In 2013, Yang et al. [41] tested three capillary columns with polar stationary phases: unmodified silica, neutral amide phase and positively charged amine phase. The separation of a mixture of seven WSVs was carried out in a combined system of isocratic and gradient conditions with MS detection. Deep analysis of the effects of the mobile phase composition and its pH on the retention of individual components of the mixture enabled satisfactory separation of the B-group vitamins using a silica column. More recently, Langer et al. [42] focused on optimization of the detection method applied in separation of WSVs using the HILIC technique and proposed UV and fluorescent detection methods as the most suitable for simultaneous determination of group B vitamins.

The mentioned examples of application of the hydrophilic interaction chromatography in the analysis of the water-soluble vitamin mixtures have inspired us to make attempts to separate a mixture of vitamins B1, B2 and B3 in isocratic conditions with at least comparable retention times to the referenced chromatographic systems [38–40]. Therefore, a series of experiments were carried out involving chromatographic columns that have not yet been used in the separation of the B-group vitamins. Our studies are also focused on the attempt to replace acetonitrile with methanol, which is a less toxic solvent of lower price. The effects of the eluent composition, the type of stationary phase and column temperature on the mechanism of the retention of vitamins are also discussed.

2. Materials and Methods

2.1. Chemicals and Solutions

The applied analytical grade chemicals: riboflavin (B1), thiamine (B2), nicotinic acid (B3), ammonium acetate and acetic acid were purchased from Merck, Germany. As the organic components of tested eluents, the HPLC-grade solvents: acetonitrile (ACN) and/or methanol (MeOH) were used (both purchased from Merck, Germany). The deionized water was produced on site using SolPure-78Z (Elkar, Poland) water deionizer. In the first step, the aqueous phase of the eluent was 10 mmol/dm^3 ammonium acetate buffer with controlled pH 5. In the second step, the buffer concentration was changed in the range of 5, 10 and 20 mmol/dm^3, while the aqueous solution pH was kept equal to 4, 5 and 6. The separate vitamin solutions, as well as vitamin mixture with concentrations of 0.2 g/dm^3 for vitamin B1 and B3 and 0.1 g/dm^3 for vitamin B2, respectively, were prepared using the eluent that was used at a given stage of investigations (for more details, see the figures' and tables' captions).

2.2. Columns

Three chromatography columns with stationary phases based on silica gel modifications were used. In addition to two polar columns designed for typical HILIC processes, Nucleodur C18 Gravity-SB dedicated to reversed-phase chromatography (RPLC) has also been selected. Despite the presence of hydrophobic alkyl chains and the absence of modifications with polar groups, this column shows distinct polar selectivity and provides high performance in the case of mobile phases enriched in aqueous phase. The hold-up volume of each column was derived from pycnometric measurements according to Ref. [43] made at 20 °C under atmospheric pressure using acetonitrile and trichloromethane as solvents. The densities of these two solvents at 20 °C are 0.786 and 1.48 g/cm^3, respectively. The detailed parameters of the applied columns are presented in Table 1, together with shortened symbolic names, and the columns are addressed in the paper.

Table 1. Parameters of applied columns.

Column	Acclaim™ Mixed-Mode HILIC-1 (Dionex, Sunnyvale, CA USA)	Eurospher II 100–5 HILIC (Knauer, Berlin, Germany)	Nucleodur ® C_{18} Gravity-SB (Macherey-Nagel, Oensingen, Switzerland)
Symbolic name	Column *A*	Column *E*	Column *N*
Stationary phase type	Alkyl diol on silica gel substrate	Zwitterionic (ammonium–sulphonic acid) on silica gel	Monomeric octadecyl on silica gel substrate
Dimensions [mm]	4.6 × 150	4.6 × 150	4 × 125
Particle diameter [µm]	5	5	5
Specific area [m^2/g]	300	320	338
Pore size [Å]	120	100	110
Hold-up time [cm^3]	1.820 ± 0.008	1.495 ± 0.007	0.897 ± 0.004
Total porosity ε_t,	0.73	0.60	0.43
Phase ratio Φ	0.3697	0.6670	1.1320

2.3. Chromatography

Chromatography tests were carried out under isocratic conditions at 20 °C and a flow rate of 1 cm^3/min using Primaide HPLC system (by Merck—Hitachi, Germany) with thermostat and DAD detector operated at wavelength of 275 nm. The effect of the eluent composition on the retention of vitamins B1, B2 and B3 as well as separation of a mixture of these vitamins has been accomplished in two consecutive steps. In the first stage, the optimal aqueous to organic phase ratios of the mobile phase regarding retention factor values have been established for each of the columns using acetonitrile or methanol as an organic component with a volume fraction in the range of 0.95 to 0.40 (v/v), while the aqueous phase of the eluent was ammonium acetate buffer with a concentration of 10 mmol/dm^3 with controlled pH 5. In these conditions, cationic form of vitamin B1, neutral form of vitamin B2 and anionic form of vitamin B3 are dominant according to the data available in the commonly accepted databases for properties of chemical substances (e.g., Chemicalize, ACD Labs). In the second stage of the experiments, the effects of buffer concentration and pH changes on the mixture retention were studied for the most promising systems preselected in the first stage. Solutions of ammonium acetate buffer (5, 10 and 20 mmol/dm^3) of pH equal to 4, 5 and 6 were employed as aqueous phases in this stage. Moreover, the effects of process temperature on the observed retention of solutes were carried out in the range of 20 °C to 50 °C in 5 °C steps for samples of vitamins B1, B2 and B3 and taken into account during chromatographic conditions optimization.

3. Results and Discussion

3.1. Effect of Mobile Phase Composition on Retention of Vitamins

3.1.1. Column A (Acclaim™ Mixed-Mode HILIC-1)

The results of chromatographic studies obtained using column *A* are shown in Figure 1. In the experiments concerning the effect of the eluent composition on retention in diol column *A*, the majority of the applied eluent compositions generated negative retention factors k in the case of the anionic particle of vitamin B3. Therefore, the retention time *t* was used instead of the retention factor k in the analysis (see Figure 1a). The possible explanation of this phenomenon may be an effect of the ionic strength of the mobile phase on retention in the case of stationary phases with silica matrix modified with alkyl chains. The ionic forms of the acids have weaker interactions with the stationary phase and are usually poorly retained, i.e., are washed out within a shorter time compared to the column hold-up time [44].

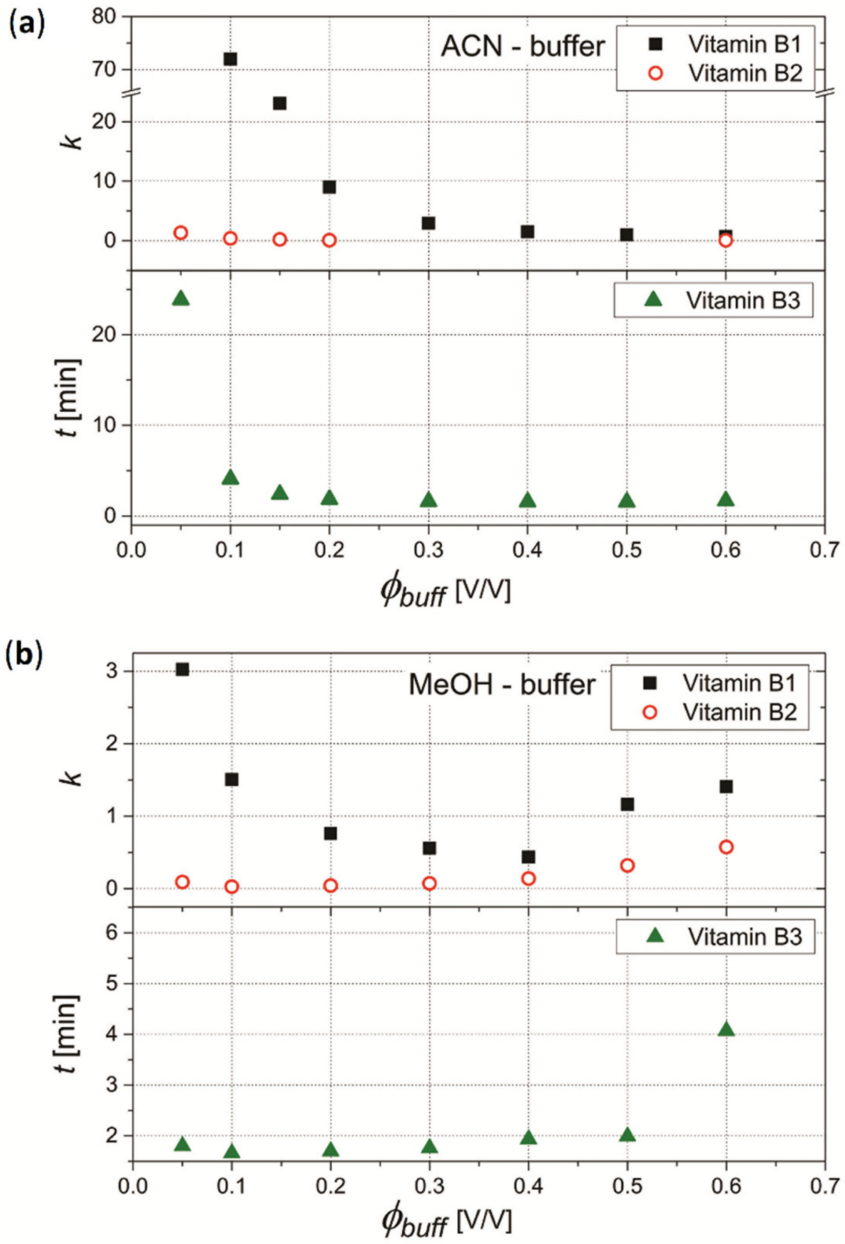

Figure 1. Effects of mobile phase composition φ_{buff} on retention coefficient k (or retention time t) of vitamins obtained using column *A* (Acclaim™ Mixed–Mode HILIC 1) with: (**a**) acetonitrile and (**b**) methanol as mobile phase modifier.

The great importance of proper solvent selection is particularly observed in the case of vitamin B1, which strongly interacts with the polar stationary phase when acetonitrile is used as a component of the eluent (Figure 1a). Due to the cationic form of vitamin B1 under the applied conditions (pH 5) and the negatively charged stationary phase resulting from the deprotonation of free silanol groups present on the surface of the silica matrix, vitamin B1 shows significant retention ($k > 70$ at $\varphi_{buff} \leq 0.1$) caused by electrostatic interactions. When using methanol, a significant reduction in vitamin B1 retention is observed ($k < 2$ at $\varphi_{buff} \leq 0.1$) due to the competitive interaction of the solvent molecules with the stationary phase (Figure 1b). In addition, the hydrophobic nature of the stationary phase surface produced by pronounced methanol adsorption also favors the low retention of hydrophilic vitamin B1. The increase in the buffer content in the acetonitrile containing eluent causes a gradual decrease in the retention of vitamin B1, whereas, in the case of eluent with methanol, a distinct U-shaped dependence was obtained, indicating a possible mixed

adsorption–partition retention mechanism. The neutral particles of vitamin B2 are weakly retained in the case of acetonitrile—buffer eluent mode ($k < 1.35$)—while application of methanol brings about a slight improvement in the retention factor at higher portions of the water-based solvent ($k > 0.32$ at $\varphi_{buff} \geq 0.5$).

3.1.2. Column E (Eurospher II 100–5 HILIC)

The plots of the retention factors of vitamin k versus mobile phase composition φ_{buff} obtained using column E are presented in Figure 2. The presence of both positive and negative charges on the surface of the stationary phase is the reason for the increased retention of vitamins B1 and B3 due to electrostatic interactions, especially when using the buffer–acetonitrile eluent (see Figure 2a).

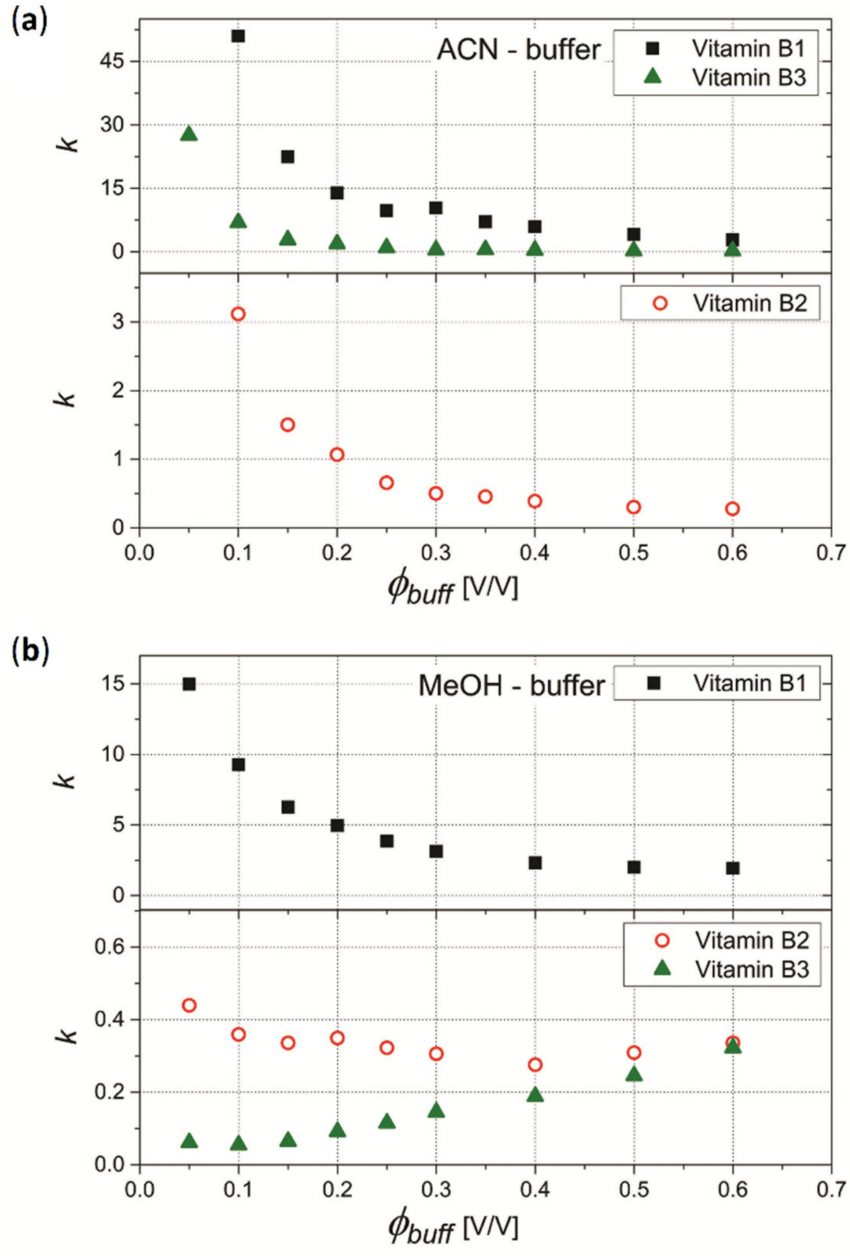

Figure 2. Effects of mobile phase composition φ_{buff} on retention coefficient k of vitamins obtained using column E (Eurospher II 100–5 HILIC) with: (**a**) acetonitrile and (**b**) methanol as mobile phase modifier.

The characteristics of the applied zwitterionic phase indicate an increased retention of analytes with increased charge; hence, the highest values of retention factors are observed for vitamin B1 with two cationic centers on nitrogen atoms ($k > 70$ at $\varphi_{buff} \leq 0.1$ with ACN). An increase in the buffer volume fraction φ_{buff} has shortened the retention times of vitamins B1 and B3 ($k < 6$ for B1 and $k < 0.4$ for B3 at $\varphi_{buff} \geq 0.4$ with ACN). Replacing acetonitrile with methanol (see Figure 2b) affected the retention of these vitamins in a different way. A strong retention at low buffer contents ($k > 9$ at $\varphi_{buff} \leq 0.1$) followed by a decrease in retention ($k < 2.5$ at $\varphi_{buff} \geq 0.4$) is observed in the case of vitamin B1, while vitamin B3 shows poor retention for low concentrations of the buffer ($k < 0.07$ at $\varphi_{buff} \leq 0.1$), and a slight increase occurs with increasing buffer content ($k > 0.18$ at $\varphi_{buff} \leq 0.4$). Therefore, application of methanol for analysis of vitamin B3 is connected with a negligible contribution of electrostatic interactions in the global retention mechanism. The neutral vitamin B2 molecule shows much lower retention in the system with acetonitrile as compared to vitamins B1 and B3 (from $k = 3.1$ at $\varphi_{buff} = 0.1$ to $k = 0.28$ at $\varphi_{buff} = 0.6$). In the system with methanol, the interaction of vitamin B2 with the adsorbent is much weaker than in the system with acetonitrile; therefore, the residence time of the analyte in the column is very short. Regardless of the organic solvent used, an increase in the concentration of the buffer in the composition of the eluent further reduces the retention of vitamin B2 (from $k = 0.44$ at $\varphi_{buff} = 0.05$ to $k = 0.33$ at $\varphi_{buff} = 0.6$).

3.1.3. Column N (Nucleodur C18 Gravity-SB)

When using column *N* in the analysis of vitamin B1 in the system with acetonitrile, a strong retention is observed, which decreases with the increasing content of the aqueous part in the eluent composition (from $k = 15$ at $\varphi_{buff} = 0.05$ to $k = 1.9$ at $\varphi_{buff} = 0.6$; see Figure 3a).

Similar to the previous HILIC-type columns, vitamin B1 interacts more weakly with the stationary phase because of the more hydrophobic character of the adsorbent achieved due to methanol interactions with the stationary phase (from $k = 2.2$ at $\varphi_{buff} = 0.05$ to $k = 0.6$ at $\varphi_{buff} = 0.6$; see Figure 3b). The increase in the aqueous phase content in the system affects the slight growth of poor retention of vitamin B3 regardless of the organic modifier used ($k = 0.32$ at $\varphi_{buff} = 0.6$ using ACN and $k = 0.52$ at $\varphi_{buff} = 0.6$ using MeOH). However, the neutral vitamin B2 is retained more strongly in the system with methanol and the retention gradually rises as the mobile phase is enriched by water buffer (from $k = 0.53$ at $\varphi_{buff} = 0.05$ to $k = 2.65$ at $\varphi_{buff} = 0.6$), while, in the system with acetonitrile, the retention factor dependence is slightly U-shaped (from $k = 0.44$ at $\varphi_{buff} = 0.05$ to $k = 0.28$ at $\varphi_{buff} = 0.4$, and $k = 0.34$ at $\varphi_{buff} = 0.6$), which may indicate the dualistic adsorption–partition mechanism of retention.

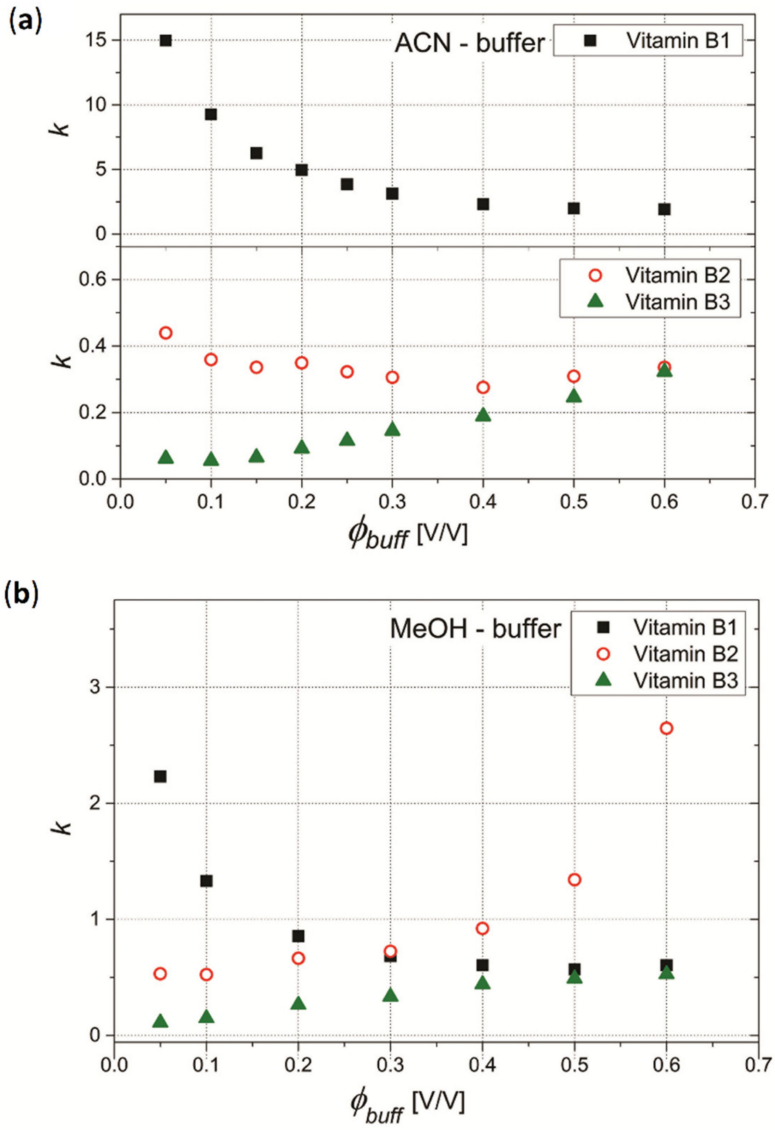

Figure 3. Effects of mobile phase composition φ_{buff} on retention coefficient k of vitamins obtained using column N (Nucleodur C18 Gravity–SB) with: (**a**) acetonitrile and (**b**) methanol as mobile phase modifier.

3.2. Effect of Temperature on Retention of Vitamins

Prior to the indication of optimal conditions for separation of the mixture of vitamins B1, B2 and B3, a series of experiments have been accomplished aiming at the determination of effects of process temperature on the retention. For this purpose, the systems containing 90% and 70% of acetonitrile (except for column A due to excessive retention) as well as 90% and 70% of methanol in the eluent have been selected as representatives.

3.2.1. Column A (Acclaim™ Mixed- Mode HILIC-1)

The van't Hoff plots for vitamins B1 and B2 and for selected compositions of eluents in the case of the Acclaim™ Mixed-Mode HILIC-1 (column A) diol column are presented in Figure 4. The results for vitamin B3 were omitted due to the negative retention coefficient mentioned in the previous section. The enthalpy values ΔH were calculated using linear regression method based on the van't Hoff Equation (1):

$$lnk = -\frac{\Delta H^0}{R \cdot T} + \frac{\Delta S^0}{R} + ln\Phi, \quad (1)$$

In which: ΔH^0 [J/mol] and ΔS^0 [J/mol·K] are the enthalpy and entropy changes corresponding to the chemical exchange of the solute going from the mobile to the stationary phase, respectively; R is the gas constant (8.314 [J/mol·K]), Φ [-] is the phase ratio and T [K] is temperature.

The enthalpy values ΔH and the adjusted determination coefficients R^2 are summarized in Table 2. In almost every analyzed system, the increase in temperature caused a decrease in the retention of analytes. Only in the case of vitamin B1, in the eluent containing 90% methanol, the increase in temperature (up to 313 K) initially caused the growth of the retention and then its decrease, which indicates the inversion from an exothermic ($\Delta H = -4.65$ kJ/mol) to endothermic ($\Delta H = 4.66$ kJ/mol) process at this point. In the case of other systems, the enthalpy values ΔH had negative values in the range from -4.73 to -31.74 kJ/mol (see Table 2), with high linearity confirmed by the determination factors (adj.$R^2 > 0.92$).

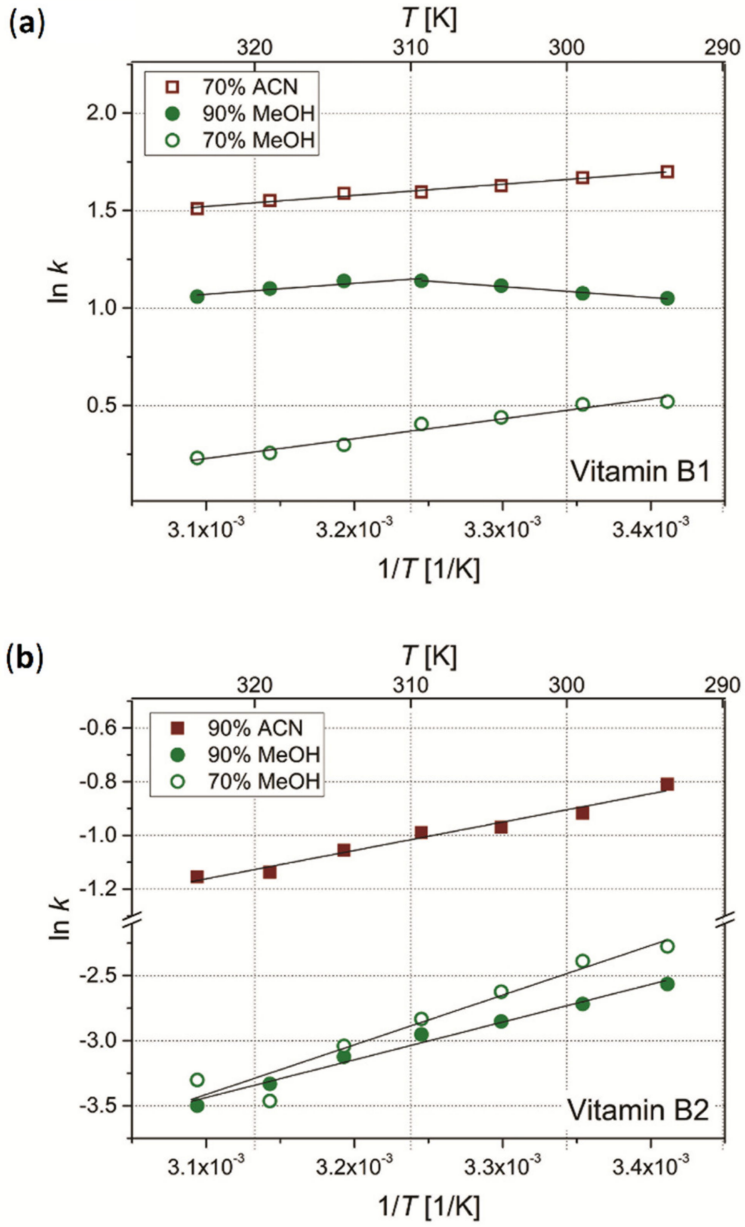

Figure 4. Effect of temperature on retention coefficient of: (**a**) vitamin B1 and (**b**) vitamin B2 in column *A* (Acclaim™ Mixed–Mode HILIC 1).

Table 2. Van't Hoff equation regression data for analytes in column *A* for different mobile phase compositions.

	ACN–Buffer 90:10			ACN–Buffer 70:30		
	Δ*H* [kJ/mol]	**Adj.R^2**	**Prob > *F***	**Δ*H* [kJ/mol]**	**Adj.R^2**	**Prob > *F***
Vitamin B1	-	-	-	−4.73	0.9808	1.1×10^{-5}
Vitamin B2	−8.78	0.9663	4.52×10^{-5}	-	-	-
	MeOH–buffer 90:10			**MeOH–buffer 70:30**		
	Δ*H* [kJ/mol]	**Adj.R^2**	**Prob > *F***	**Δ*H* [kJ/mol]**	**Adj.R^2**	**Prob > *F***
Vitamin B1	−4.65/4.66	0.8255/0.9867	$5.99 \times 10^{-2}/4.44 \times 10^{-2}$	−8.46	0.9606	6.73×10^{-5}
Vitamin B2	−24.10	0.9827	8.32×10^{-5}	−31.74	0.9293	2.92×10^{-5}

3.2.2. Column E (Eurospher II 100–5 HILIC)

The van't Hoff's dependences of the analyzed vitamins in column *E* are presented in Figure 5, while the enthalpy values and corresponding regression data are gathered in Table 3. All the vitamins in the system with 90% acetonitrile content initially showed a decrease in retention, followed by an increase with rising temperature. In other cases, heating up the system caused a decrease in retention. For all tested systems, the transfer of the analyzed substance from the mobile phase to the stationary phase appeared exothermic, and the calculated enthalpy values (Equation (1)) were in the range from −0.27 to −23.92 kJ/mol. The good ($R^2 > 0.9$) or acceptable ($R^2 > 0.8$) linearity of the van't Hoff's plots were obtained for vitamin B2 in every tested system, for vitamin B1 in both systems with methanol and with 70% of acetonitrile and for vitamin B3 in the system with 70% methanol content only. The data for the other systems demonstrated significant dissipation from linearity.

Table 3. Regression data of Van't Hoff equation for analytes in column *E* for different mobile phase compositions.

	ACN–Buffer 90:10			ACN–Buffer 70:30		
	Δ*H* [kJ/mol]	**Adj.R^2**	**Prob > *F***	**Δ*H* [kJ/mol]**	**Adj.R^2**	**Prob > *F***
Vitamin B1	−0.27	−0.195	0.8903	−10.01	0.8790	1.14×10^{-3}
Vitamin B2	−9.35	0.9112	5.19×10^{-4}	−5.11	0.9478	1.36×10^{-4}
Vitamin B3	−3.23	0.2099	0.1682	−4.55	0.3058	0.1146
	MeOH–buffer 90:10			**MeOH–buffer 70:30**		
	Δ*H* [kJ/mol]	**Adj.R^2**	**Prob > *F***	**Δ*H* [kJ/mol]**	**Adj.R^2**	**Prob > *F***
Vitamin B1	−7.80	0.9923	1.11×10^{-6}	−3.70	0.9864	4.71×10^{-6}
Vitamin B2	−5.22	0.9157	4.56×10^{-4}	−5.88	0.9214	3.82×10^{-4}
Vitamin B3	−8.36	0.3506	0.0946	−23.92	0.9833	7.79×10^{-6}

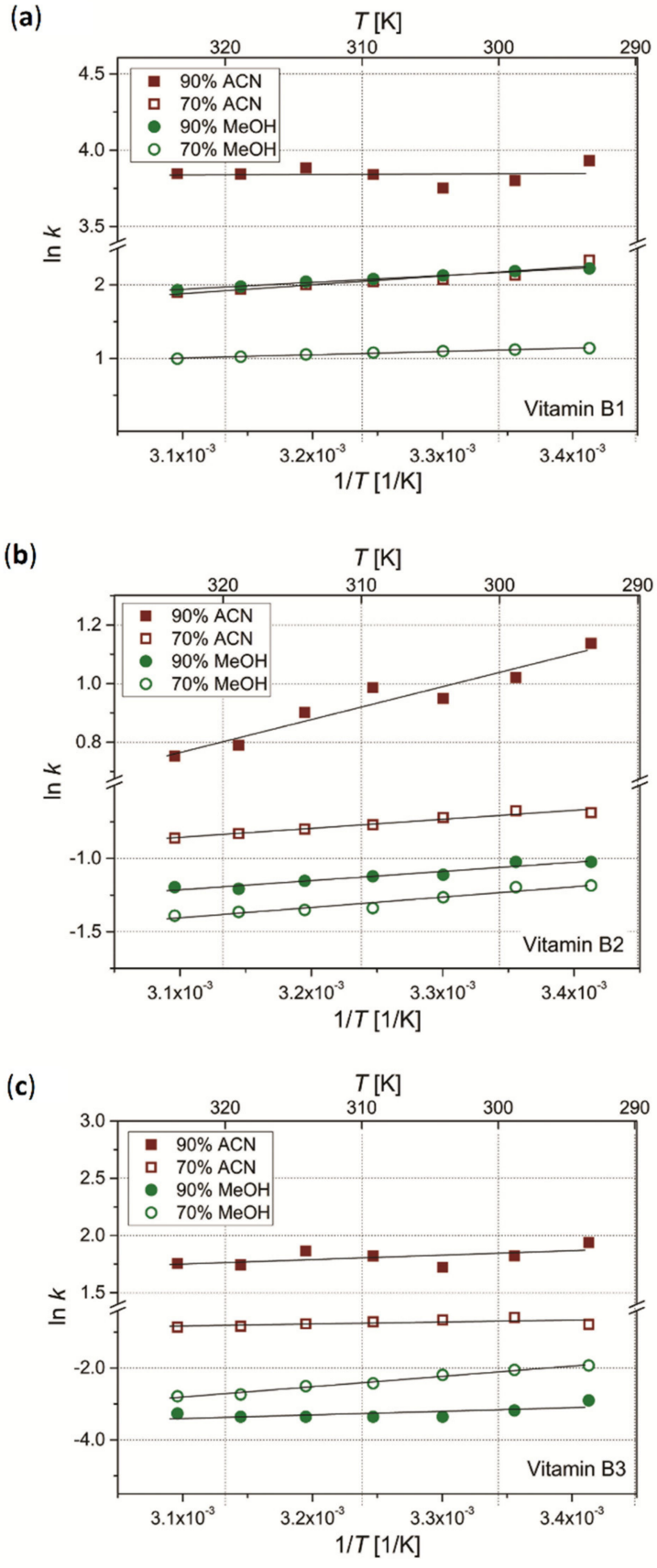

Figure 5. Effect of temperature on retention coefficient of: (**a**) vitamin B1, (**b**) vitamin B2 and (**c**) vitamin B3 in column *E* (Eurospher II 100–5 HILIC).

3.2.3. Column N (Nucleodur C18 Gravity-SB)

The obtained effects of temperature on retention of vitamins B1, B2 and B3 in column *N* are presented in the form of van't Hoff relationships in Figure 6, while the enthalpy ΔH values calculated from Equation (1) with statistics are shown in Table 4. Although the adj.R^2 values do not confirm a strict linear correlation, the increase in temperature influenced the growth in retention of vitamin B1 with acetonitrile as co-solvent in the entire temperature range as well as the retention of vitamin B2 up to 313 K, but only in the case of 90% ACN as eluent. The sorption processes in these cases are, therefore, endothermic, which may result from the contribution of adsorption interactions to the global retention mechanism. In systems with methanol, the retention of vitamins B1 and B2 decreased with increasing temperature. The diverse effect of temperature change on retention was observed for vitamin B3. To be specific, in a system with 90% acetonitrile content, the increase in temperature initially caused an increase and then a decrease in retention. In a system with 70% acetonitrile, the effect of temperature turned out to be negligible in the retention of vitamin B3, for which the van't Hoff's relationship was almost constant. In the systems with methanol, however, a slight decrease in retention was observed. The range of enthalpy values ΔH obtained for column *N* was twice smaller as compared to the diol (*A*) and zwitterionic (*E*) columns and ranged from 8.21 to −3.98 kJ/mol. In none of the tested systems, highly linear runs of the $\ln k = f(1/T)$ relationship were observed; therefore, a mixed complex mechanism of retention of vitamins B1, B2 and B3 in the Nucleodur C18 Gravity-SB column can be concluded.

Summarizing, the temperature increase affected the retention of the analyzed vitamins for most of the analyzed chromatographic systems by reducing the retention. The endothermic nature of sorption processes, which was observed especially for column *N*, may indicate the contribution of adsorption interactions in the global retention mechanism. Under certain conditions, van't Hoff's nonlinear (close to parabolic) relationships were also observed, i.e., for a given chromatographic system in a certain temperature range, the sorption processes changed from exothermic to endothermic or the opposite way. In addition, the dependence of $\ln k = f(1/T)$ diverged from the linear course, indicating the complexity of the retention mechanism in the studied systems. The general conclusion from the temperature studies is that shifting temperature of separation to the levels above room temperature is not justified since the energy expenditure would not be balanced by possible improvement in the separation quality.

Table 4. Regression data of Van't Hoff equation for analytes in column *N* for different mobile phase compositions.

	ACN–Buffer 90:10			**ACN–Buffer 70:30**		
	ΔH [kJ/mol]	**Adj.R^2**	**Prob > *F***	**ΔH [kJ/mol]**	**Adj.R^2**	**Prob > *F***
Vitamin B1	8.21	0.8672	1.44×10^{-3}	5.28	0.5164	0.0417
Vitamin B2	0.23	0.4094	0.0723	1.66/−0.98	0.9494/0.9217	3.18×10^{-3}/0.126
Vitamin B3	−0.74	0.7150	0.0102	−0.05	0.2260	0.158
	MeOH–buffer 90:10			**MeOH–buffer 70:30**		
	ΔH [kJ/mol]	**Adj.R^2**	**Prob > *F***	**ΔH [kJ/mol]**	**Adj.R^2**	**Prob > *F***
Vitamin B1	−3.98	0.9001	6.85×10^{-4}	−0.93	0.7688	5.97×10^{-3}
Vitamin B2	−1.31	0.7528	7.09×10^{-3}	−2.48	0.5578	0.033
Vitamin B3	−0.43	0.5957	0.0258	−1.61	0.4938	0.0472

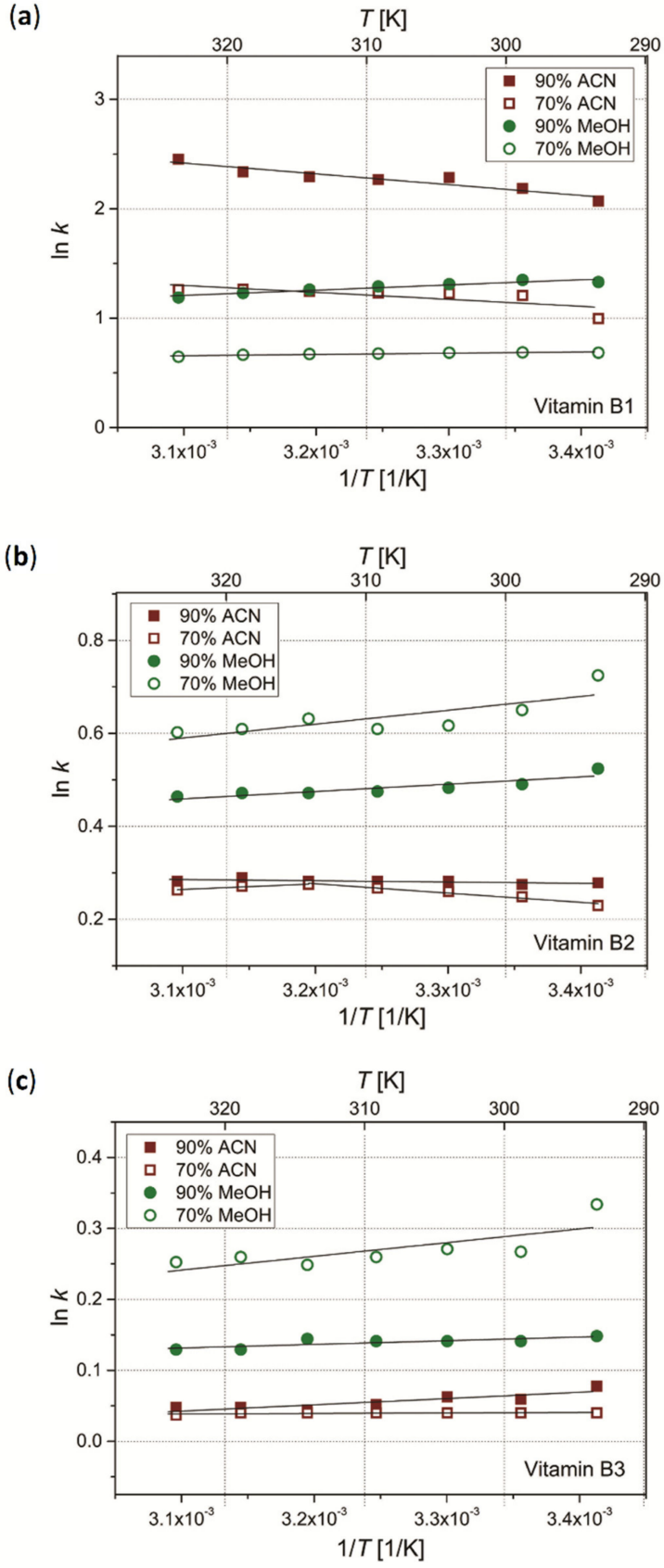

Figure 6. Effect of temperature on retention coefficient of: (**a**) vitamin B1, (**b**) vitamin B2 and (**c**) vitamin B3 in column *N* (Nucleodur C18 Gravity–SB).

3.3. Preliminary Separation of the Mixture of Vitamins

As shown in the previous sections, the volume fraction of an organic modifier in the eluent composition clearly affects the retention of vitamins B1, B2 and B3 and, as one may expect, would also impact the quality of separation of their mixture. In the preliminary stage, the mixture separation was studied in the conditions of constant pH 5 and buffer salt concentration of 10 mmol/dm^3. The process thermal conditions were maintained at room temperature. The comparison of chromatograms showing the results of the separation in the selected systems containing 90, 70 and 40% of organic solvent are shown in Figures 7–9 for the diol (column *A*), zwitterionic (column *E*) and RP (column *N*), respectively. Regardless of the stationary phase used for most of the analyzed systems, apparently shorter times required for proper separation were obtained as the content of the aqueous phase in the eluent composition was increased. It was caused by a significant shortening of the retention time of vitamin B1 along with the decreasing content of the organic solvent in the eluent. The effect of the mobile phase composition on the retention of riboflavin and nicotinic acid was the decisive factor in the quality of the separation of the vitamin mixture. The substitution of acetonitrile with methanol brought about shorter separation times. The tendency of methanol to form hydrogen bonds, and, consequently, the more hydrophobic nature of the stationary phases, resulted in a significant reduction in retention, especially in the case of vitamins B1 and B3. The values of the selectivity coefficients α gathered in Table 5 were calculated according to (2):

$$\alpha_{i-j} = \frac{k_i}{k_j} = \frac{t_{ri} - t_0}{t_{rj} - t_0}, \tag{2}$$

where: t_0—the column hold-up time, t_{ri} and t_{rj}—the retention times of substance *i* and *j*, respectively, k_i and k_j—the retention coefficients of substance *i* and *j*, respectively.

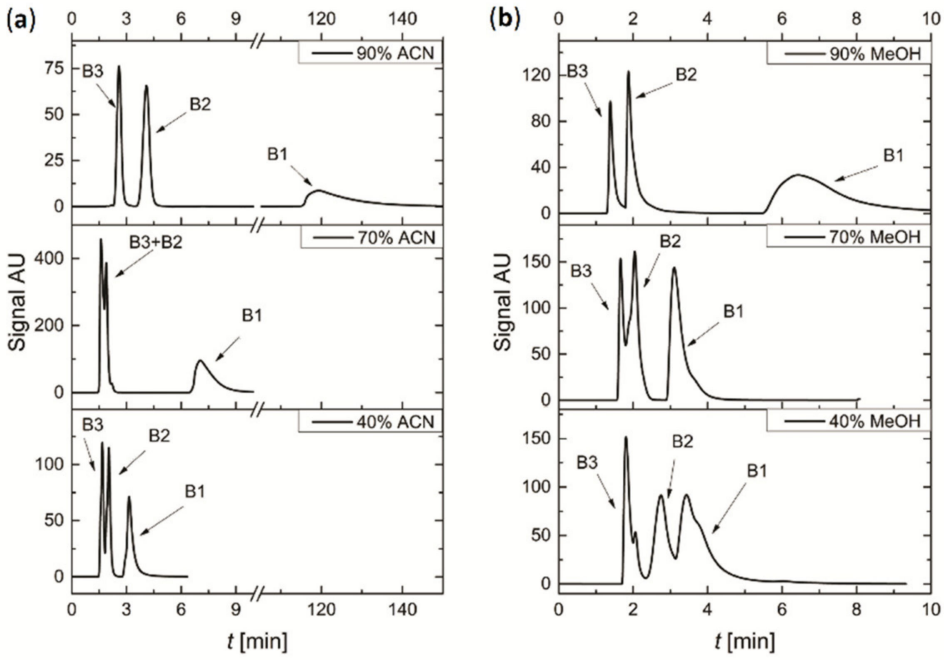

Figure 7. Preliminary results of separation of vitamin mixture obtained using column *A* (Acclaim™ Mixed–Mode HILIC 1) with: (**a**) acetonitrile–ammonia acetate buffer (10 mmol/dm^3, pH 5) and (**b**) methanol–ammonia acetate buffer (10 mmol/dm^3, pH 5) as mobile phase.

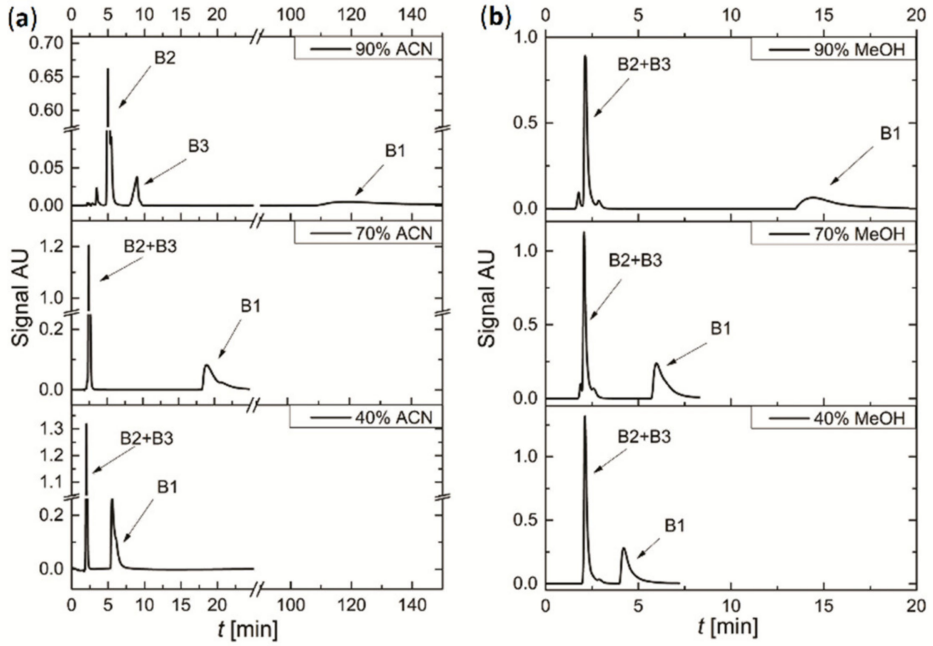

Figure 8. Preliminary results of separation of vitamin mixture obtained using column *E* (Eurospher II 100–5 HILIC) with: (**a**) acetonitrile–ammonia acetate buffer (10 mmol/dm^3, pH 5) and (**b**) methanol–ammonia acetate buffer (10 mmol/dm^3, pH 5) as mobile phase.

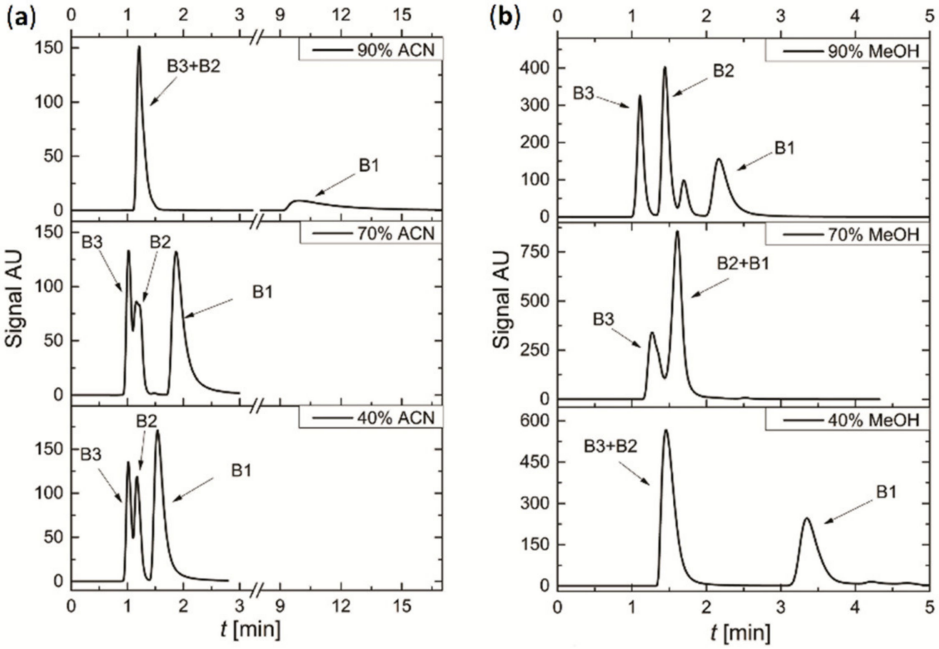

Figure 9. Preliminary results of separation of vitamin mixture obtained using column *N* (Nucleodur C18 Gravity–SB) with: (**a**) acetonitrile–ammonia acetate buffer (10 mmol/dm^3, pH 5) and (**b**) methanol–ammonia acetate buffer (10 mmol/dm^3, pH 5) as mobile phase.

The column resolution coefficients *Rs*, also shown in Table 5, were obtained as follows:

$$Rs_{i-j} = \frac{t_{ri} - t_{rj}}{\frac{S_i - S_j}{2}} = \frac{2 \cdot (t_{ri} - t_{rj})}{S_i + S_j}, \quad (3)$$

where i is the species with the longer retention time, and t_r and S are the retention time and elution peak width, respectively. On the basis of the best values of the coefficients,

the following, most promising systems in terms of the eluent composition have been selected: (a) column *A*: 90 and 40% of acetonitrile and 80 and 50% of methanol, (b) column *E*: 80% of acetonitrile and 80% of methanol, (c) column *N*: 60% of acetonitrile and 90% of methanol (bolded in Table 5). For the selected systems, further experimental studies were carried out aiming at the determination of the effect of pH and buffer ammonium acetate concentration on the retention of individual vitamins. The investigations on the impact of these parameters on the separation of the vitamin mixture were also accomplished.

Table 5. Selectivity coefficient α and column resolution coefficient *Rs* values in the chromatographic systems applied in the first stage of experiment.

					ACN–Ammonium Acetate Buffer (pH 5)			
	φ_{buff} [*v*/*v*]	**0.05**	**0.10**	**0.20**	**0.30**	**0.40**	0.50	0.60
Column *A*	α_{1-2}	9.13	**3.07**	-	-	-	-	**-**
	Rs_{1-2}	9.24	**2.14**	0.65	0.89	0.89	0.96	**1.18**
	α_{2-3}	-	**184**	102	-	-	-	**10.44**
	Rs_{2-3}	-	**8.79**	7.60	4.94	8.87	3.27	**2.10**
Column *E*	α_{1-2}	-	2.23	**1.79**	1.10	1.03	1.22	1.22
	Rs_{1-2}	-	12.7	**4.10**	0.21	0.05	0.28	0.27
	α_{2-3}	-	1.54	**13.0**	20.6	15.1	13.5	10.1
	Rs_{2-3}	-	2.52	**4.10**	7.44	10.2	6.54	7.15
Column *N*	α_{1-2}	0.11	1.35	4.19	5.72	**6.98**	4.89	5.18
	Rs_{1-2}	0.18	0.36	0.93	0.89	**1.12**	0.79	0.77
	α_{2-3}	287	32.9	8.56	4.34	**3.18**	3.69	2.98
	Rs_{2-3}	7.30	3.53	2.68	2.08	**1.58**	1.70	1.25
					MeOH–ammonium acetate buffer (pH 5)			
	φ_{buff} [*v*/*v*]	0.05	0.10	0.20	0.30	0.40	0.50	0.60
Column *A*	α_{1-2}	-	-	**-**	-	7.77	**-**	2.07
	Rs_{1-2}	0.79	0.75	**0.82**	0.80	0.62	**2.07**	2.09
	α_{2-3}	32.37	51.70	**17.29**	7.60	3.11	**3.63**	2.45
	Rs_{2-3}	7.73	1.11	**0.89**	0.93	0.67	**1.78**	2.08
Column *E*	α_{1-2}	7.14	6.55	**3.81**	2.11	1.46	1.35	1.47
	Rs_{1-2}	1.77	1.38	**1.45**	0.84	0.44	0.27	0.06
	α_{2-3}	34.07	25.75	**14.18**	10.22	8.38	6.44	5.69
	Rs_{2-3}	5.99	5.98	**5.91**	4.87	4.28	3.67	3.18
Column *N*	α_{1-2}	4.79	**3.53**	2.52	2.05	1.37	1.16	1.15
	Rs_{1-2}	1.71	**1.53**	1.36	0.98	0.58	0.21	0.21
	α_{2-3}	4.20	**2.54**	1.29	1.06	1.52	2.36	4.37
	Rs_{2-3}	2.96	**2.07**	0.44	0.13	1.03	2.01	4.26

3.4. Separation of Mixture of Vitamins

3.4.1. Effect of pH and Concentration of Buffer Salt on the Retention of Vitamins B1, B2 and B3

Figures 10 and 11 illustrate the effects of pH and the concentration of ammonium acetate buffer on the retention coefficient *k* of the analyzed vitamins in diol column *A* with acetonitrile as a component of the mobile phase. Due to retention times exceeding 140 min in the case of 90% acetonitrile, it is not possible to analyze the effect of pH of the eluent as well as the buffer salt concentration on the retention in the entire range. However,

at lower organic solvent content (40% ACN), the retention is definitely weaker and the analysis of pH and buffer salt concentration effects is possible (see Figure 10b). The applied changes in pH and the buffer salt concentration have an ambiguous effect on the observed retention coefficients and it is difficult to conclude on a general trend. The increase of pH enhances the retention of the positively charged vitamin B1 molecules under the conditions of analysis (e.g., k = 0.94 at pH 4 to k = 4.11 at pH 6, for C_{buff} = 10 mmol/dm^3). In view of the fact that, at pH 4 and higher, the deprotonation process of silanol groups bound to the surface of the matrix occurs, the stationary phase becomes more negatively charged. The observed stronger retention of vitamin B1 may result, therefore, from augmenting electrostatic interactions along with increasing pH as was noticed in Ref. [45]. The opposite trend is observed for vitamin B3, especially when operating in the HILIC mode (Figure 10a, e.g., t_R = 2.473 at pH 4 to t_R = 1.07 at pH 6, for C_{buff} = 5 mmol/dm^3). The anionic particles of vitamin B3 are washed out faster from the column as the pH increases due to stronger repulsive electrostatic interactions with the residual silanol groups. The pH value affected the inert vitamin B2 molecules to the smallest extent since any dominant trend of retention change along with pH of the eluent has not been observed, both in the case of 90% and 40% acetonitrile content in the eluent. The presence of the buffer salt in the mobile phase composition confirms its role in controlling electrostatic interactions between charged analytes and the stationary phase. On the other hand, the effect of buffer salt concentration on the retention of neutral compounds is manifested by the modification of the water layer thickness on the surface of the stationary phase [13]. The increase in the salt concentration in the case of organic phase rich eluents leads to the development of the thick water layer on the surface of the adsorbent, resulting in stronger retention due to the partition process equilibrium. Therefore, in the HILIC mode, an increase in the buffer salt concentration from 5 to 20 mmol/dm^3 caused a moderate increase in retention observed for all the analytes (e.g., vitamin B3, k = 1.07 for C_{buff} = 5 mmol/dm^3 to k = 1.95 for C_{buff} = 20 mmol/dm^3, at pH 6). The application of water-rich eluent results in the absence of the above mentioned observations as in the case of the eluent with 40% acetonitrile content (Figure 10b).

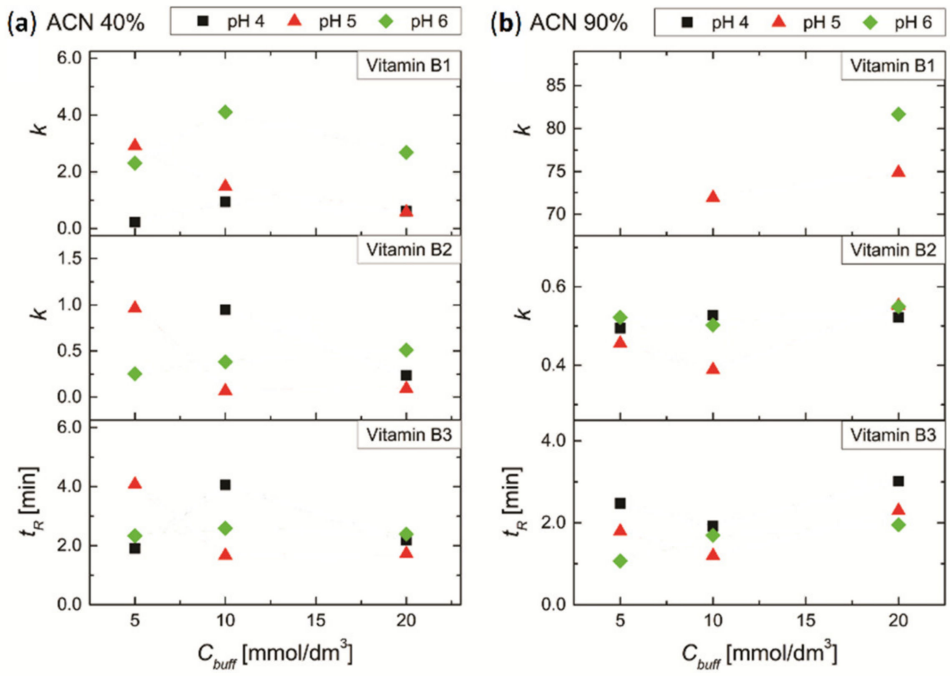

Figure 10. Effect of buffer pH and concentration on retention of vitamins in column *A* (Acclaim™ Mixed–Mode HILIC 1) with: (**a**) 90% and (**b**) 40% content of acetonitrile in mobile phase.

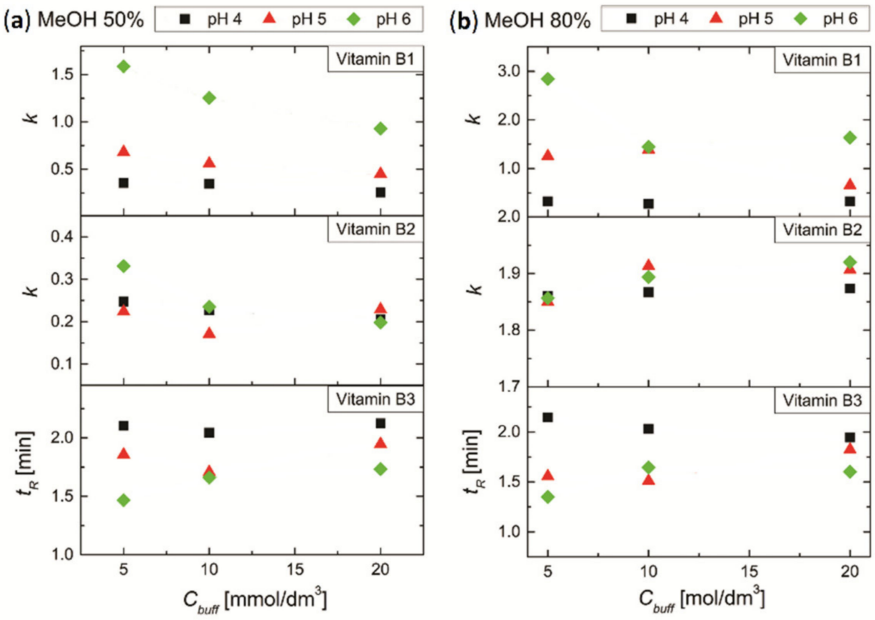

Figure 11. Effect of buffer pH and concentration on retention of vitamins in column *A* (Acclaim™ Mixed–Mode HILIC 1) with: (**a**) 50% and (**b**) 80% content of methanol in mobile phase.

The results of experiments involving systems with methanol in diol column *A* are shown in Figure 11. The effect of pH on vitamin retention when using 80% of methanol is analogous to that of acetonitrile. The increase in pH causes a growth in vitamin B1 retention (e.g., $k = 0.326$ at pH 4 to $k = 2.84$ at pH 6, for $C_{buff} = 5$ mmol/dm^3) and a decrease in vitamin B3 retention (e.g., $t_R = 2.14$ at pH 4 to $t_R = 1.35$ at pH 6, for $C_{buff} = 5$ mmol/dm^3) due to electrostatic interactions of the attracting and repulsive type, irrespective of the composition of the mobile phase. Vitamin B2 retention with increasing pH is only moderately changed for 80% content of methanol in the eluent (k comprised in the range 1.85–1.92, Figure 11b). However, this kind of trend is not observed when the composition of the eluent is enriched in the aqueous phase (Figure 11a). Generally, the effect of buffer salt concentration growth was associated with a gradual decrease in retention of vitamin B1 (e.g., $k = 2.84$ for $C_{buff} = 5$ mmol/dm^3 to $k = 1.635$ for $C_{buff} = 20$ mmol/dm^3 at pH 6) and slight fluctuations in the case of other vitamins.

The increase in pH of the eluent in the case of zwitterionic column *E* (Figure 12) and the Nucleodur column *N* (Figure 13) in an analogous manner affects the retention of vitamins, regardless of the organic solvent used. However, the effect of pH in column *E* is of a special nature. Vitamin B1 molecules occurring in the solution in the form of cations and vitamin B3 particles in the form of anions simultaneously interact with charges of the sulfobetaine group, and the resultant of manifold electrostatic interactions determines the retention of the analyte. For the increasing pH value, in addition to the deprotonation of residual silanol groups, deprotonation of the sulphone group could also be expected. Thus, the higher the pH of the mobile phase, the stronger the attraction of the vitamin B1 cations with the negative charges of the adsorbent surface and the more pronounced the retention in column *E* (e.g., $k = 20.1$ at pH 4 to $k = 57.8$ at pH 6, for $C_{buff} = 5$ mmol/dm^3 using acetonitrile as the eluent component (Figure 12a), $k = 0.95$ at pH 4 to $k = 13.5$ at pH 6, for $C_{buff} = 5$ mmol/dm^3 using methanol as the eluent component (Figure 12b)). The opposite interactions are observed in the case of vitamin B3 anion together with reduction in the retention. The range of changes in the retention coefficient for vitamin B3 are similar for all the adsorbent types used (e.g., $k = 1.88$ at pH 4 to $k = 0.66$ at pH 6, for $C_{buff} = 5$ mmol/dm^3 using column *E* and acetonitrile as the mobile phase component; see Figure 12a, and $k = 0.47$ at pH 4 to $k = 0.081$ at pH 6, for $C_{buff} = 5$ mmol/dm^3 using column *N* and methanol as the mobile phase component; see Figure 12b). Vitamin B2 retention confirms the moderate

influence of pH on the retention of neutral compounds. It should be noted, however, that, despite a small change in the retention coefficients, the increase in the pH value in the case of the zwitterionic column *E* increases, while, in the case of the Nucleodur column *N*, it reduces the retention of vitamin B2.

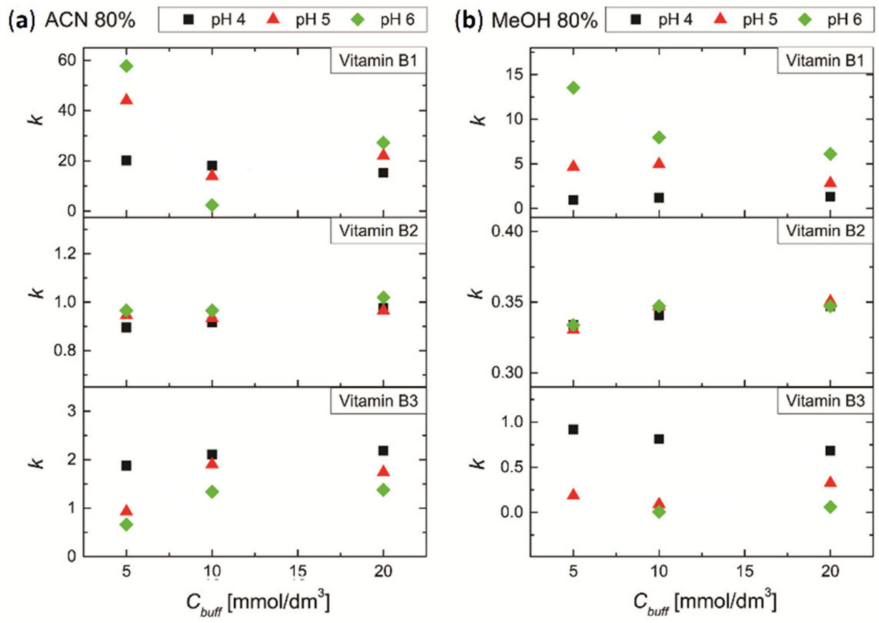

Figure 12. Effect of buffer pH and concentration on retention of vitamins in column *E* (Eurospher II 100–5 HILIC) with 80% content of (**a**) acetonitrile and (**b**) methanol in mobile phase.

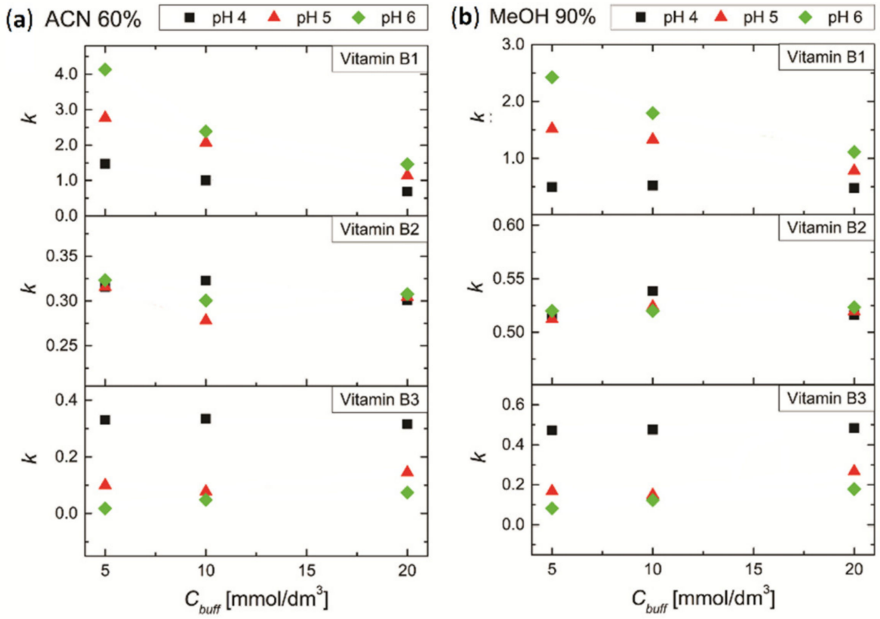

Figure 13. Effect of buffer pH and concentration on retention of vitamins in column *N* (Nucleodur C18 Gravity–SB) with: (**a**) 60% content of acetonitrile and (**b**) 90% content of methanol in mobile phase.

The slight effect of the buffer salt concentration on growth of the water-rich adsorption layer hydrophilicity, and, thus, the role of the partition equilibrium in overall retention, is clearly seen in the case of vitamin B2 in the zwitterionic column *E* for both the applied solvents (Figure 12a,b) and to a smaller degree in column Nucleodur *N* in the system with methanol (Figure 13b). The application of eluent with 60% acetonitrile results in the lack of similar phenomenon in the Nucleodur column *N* (Figure 13a). Vitamin B1 retention

decreases with increasing ammonium acetate concentrations from 5 to 20 mmol/dm^3 for both column *E* (e.g., k = 57.8 for C_{buff} = 5 mmol/dm^3 to k = 27.2 for C_{buff} = 20 mmol/dm^3 at pH 6, Figure 12) and column *N* (e.g., k = 2.43 for C_{buff} = 5 mmol/dm^3 to k = 1.11 for C_{buff} = 20 mmol/dm^3 at pH 6, Figure 13) despite the solvent organic modifier used. Shifting the concentration of the buffer salt to higher levels leads to an increase in the concentration of counter-ion (ammonium) in the mobile phase, decreasing the number of active anion groups able to interact with vitamin B1 particles, thus reducing its retention time. Both the retention of vitamin B3 and vitamin B2 in columns *E* and *N* is only slightly dependent on the concentration of the buffer salt regardless of the column and the organic solvent applied (e.g., vitamin B3, column *N*, 60% ACN: k = 0.33 for C_{buff} = 5 mmol/dm^3 to k = 0.316 for C_{buff} = 20 mmol/dm^3 at pH 4, Figure 13a, and Vitamin B2, column N, 60% ACN: k = 0.316 for C_{buff} = 5 mmol/dm^3 to k = 0.3 for C_{buff} = 20 mmol/dm^3 at pH 4, Figure 13a), except for the case of vitamin B3 studied in the acetonitrile–buffer system in column *E*, where a gentle growth of retention coefficients is noted.

3.4.2. Effect of pH and Concentration of Buffer Salt on the Separation of Mixture of Vitamins B1, B2 and B3

Based on the conducted experiments, in which the pH range varied from 4 to 6, and the ammonium acetate concentration was 5, 10 or 20 mmol/dm^3, the most promising mobile phase compositions regarding the buffer salt pH and concentration values were selected for each column and gathered in Table 6. Chromatograms illustrating the separations are shown in Figures 14–16 for the diol (column *A*), the zwitterionic (column *E*) and Nucleodur (column *N*) column, respectively. The values of selectivity coefficients α (Equation (2)) and column resolution coefficients *Rs* (Equation (3)) obtained for the most effective systems are also presented in Table 6.

Table 6. The systems selected for optimization of vitamin mixture separation and their performance regarding selectivity and resolution coefficients.

	Column Type	**Column A**		**Column E**		**Column N**	
Mobile phase properties	Eluent indication	A1	A2	E1	E2	N1	N2
	Organic modifier	ACN	MeOH	ACN	MeOH	ACN	MeOH
	Buffer content fbuff [-]	0.6	0.5	0.2	0.2	0.4	0.1
	Buffer salt concentration Cbuff [mmol/dm^3]	10	20	20	10	10	20
	Buffer pH [-]	5	6	4	6	6	6
Selectivity coefficient	α_{1-2}	-	-	2.23	57.86	6.27	4.24
	α_{2-3}	22.36	4.89	6.95	22.93	7.97	3.45
Column resolution coefficient	Rs_{1-2}	3.02	1.49	4.44	1.29	1.37	1.27
	Rs_{2-3}	12.48	1.76	18.21	8.65	3.48	1.41

The zwitterionic column *E* turned out to be the least advantageous for the separation of the mixture as the analysis time exceeded 15 min in both analyzed cases. Moreover, the peaks are distorted and separated insufficiently. However, diol column *A* and Nucleodur column *N* appear to be much better alternatives. The former case offers reasonable peak separation, with analysis times below 6 min regardless of the organic modifier applied. Nevertheless, incomplete separation of B2 and B3 and the peak distortion may be regarded as drawbacks of the method. Despite the fact that column *N* is dedicated to the RPLC, its outcomes regarding separation of B-group vitamins have proven to be the most effective among the tested ones. Although the use of acetonitrile (Figure 16a) is associated with imperfect separation of B3 and B2 peaks and pronounced B1 peak tailing, the application of methanol eliminates these disadvantages (Figure 16b), resulting in successful separation

below 2.5 min, which is an outcome comparable to or better than that presented in works by Karatapanis et al. [38–40] (the detailed comparison is difficult due to differences in the applied eluent flow rates).

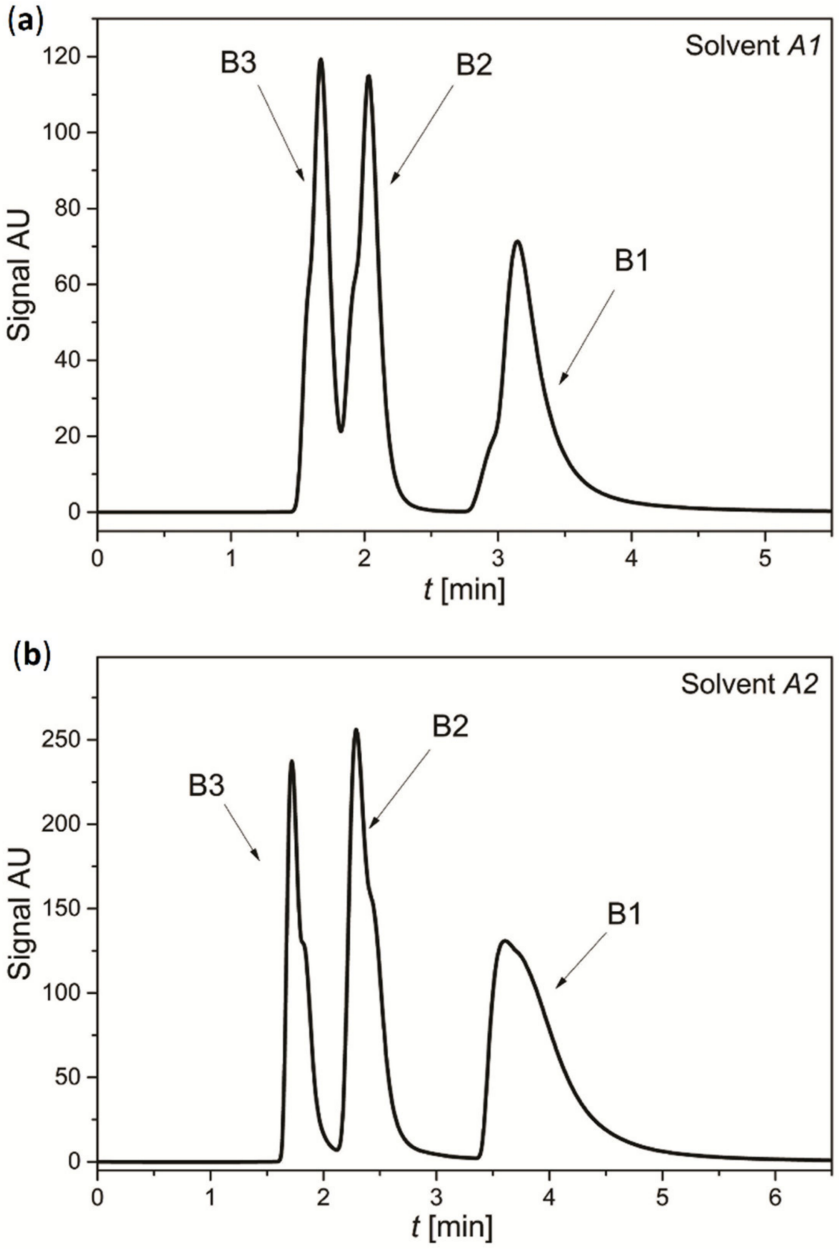

Figure 14. Separation of vitamin mixture obtained using column *A* and solvent: (**a**) *A*1 and (**b**) *A*2 as mobile phase.

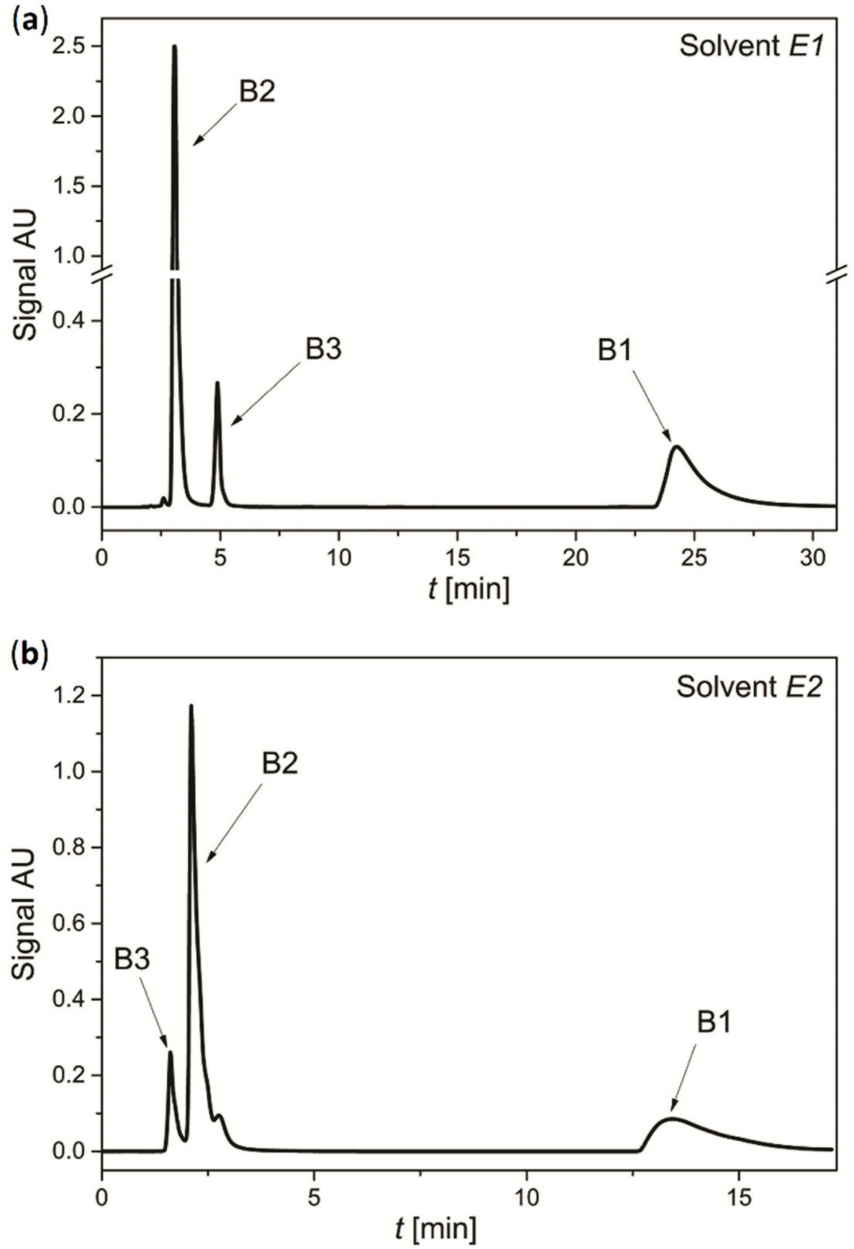

Figure 15. Separation of vitamin mixture obtained using column *E* and solvent: (**a**) *E*1 and (**b**) *E*2 as mobile phase.

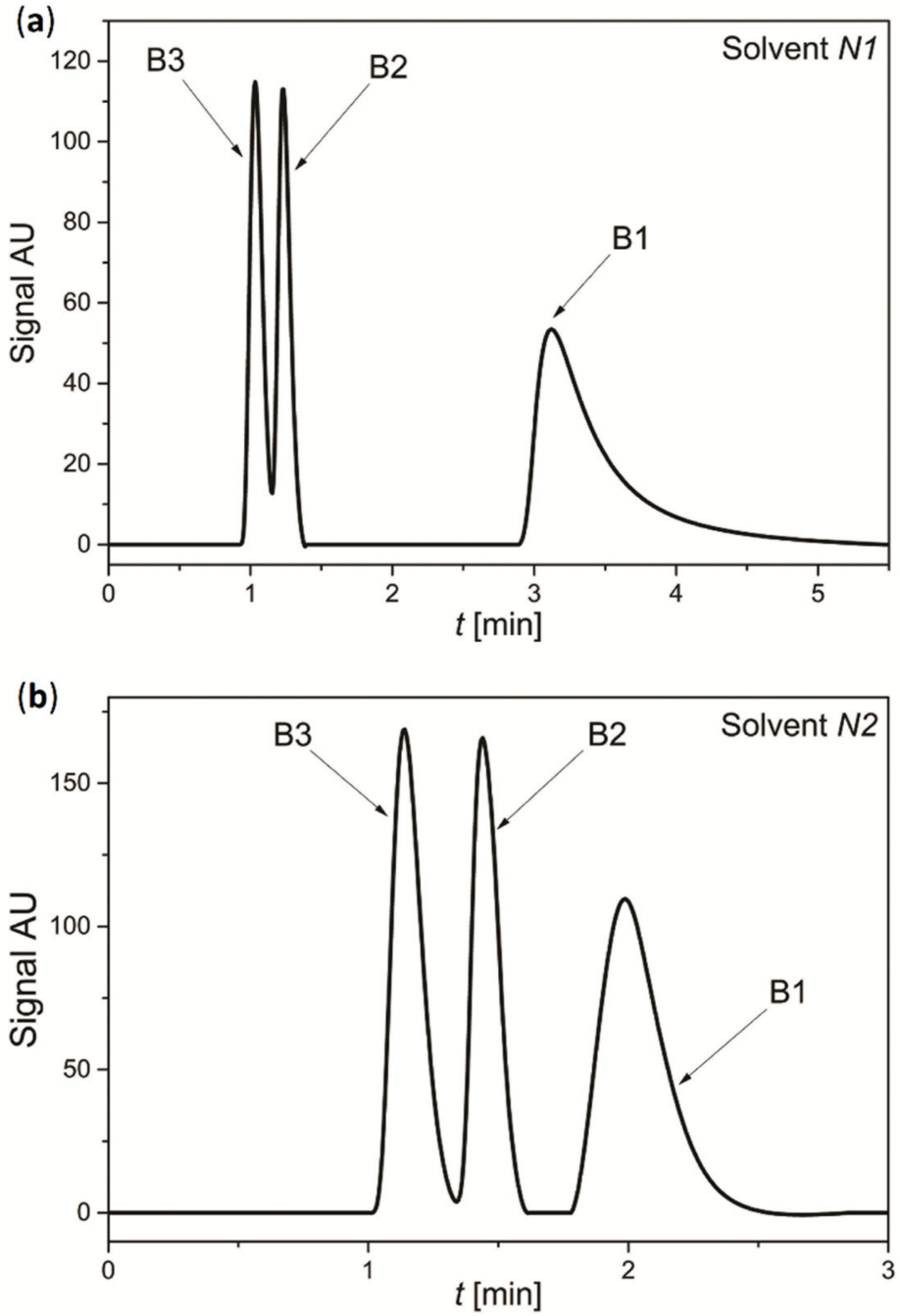

Figure 16. Separation of vitamin mixture obtained using column *N* and solvent: (**a**) *N*1 and (**b**) *N*2 as mobile phase.

4. Conclusions

Liquid chromatography has long been used in various fields of science, everyday life and also in modern agriculture engineering. There are many research stations in the world that take advantage of chromatographic quantitative and qualitative analysis for several chemical substances in various biological materials. Nowadays, chromatographic methods provide the most accurate results of such analysis using a single operation/apparatus. Of course, the proper use of this technique requires a series of basic research processes necessary to optimize the conditions of the analytical method (practical aspect) and understanding the mechanisms governing this process (theoretical aspect). The results of such investigations connected with the HILIC technique have been presented in this work. From

the practical point of view, the results included in this work can be used for the quantitative and/or qualitative analysis of B-complex vitamins during the monitoring of various types of biological systems within the framework of broadly understood agricultural engineering. The results presented in this paper also contribute to the investigations on the separation mechanism in the HILIC technique, which (as mentioned in the introduction part) is not yet fully understood. In the present study, the effects of stationary phase, temperature as well as pH and the concentration of the buffering salt in eluent on the retention of vitamins B1, B2 and B3 have been investigated in order to find optimal conditions for separation of the vitamin mixture. In view of the applied conditions of chromatography, the possible retention mechanisms have been discussed that led to the selection of the most effective separation method of the vitamin mixture in the two-step optimization process. The specific conclusions are as follows:

- Since B1 and B3 vitamins are present in ionic form in the test conditions, the dominant trend along with the growth of the mobile phase pH was the increase in vitamin B1 retention and the decrease in vitamin B3 retention due to the opposite electrostatic interactions of the analyzed substances with modifying ligands and free silanol groups of adsorbents. The effects of pH for neutral vitamin B2 retention were insignificant and, for most systems, it was associated with a moderate increase in retention;
- The unequivocal effect of buffering salt concentration on the retention of analyzed vitamins was dependent on the properties of the given analyte, the stationary phase type and the organic solvent used for eluent. Depending on the particular chromatographic system, an increase or decrease in retention with an increase in the concentration of ammonium acetate in the eluent was observed;
- The obtained results of temperature effects on retention of the vitamins in selected systems indicate the dominant exothermic nature of the sorption processes with increasing temperature. There were also few observed systems for which the energy processes were endothermic, resulting from the contribution of adsorption interactions in the global retention mechanism. However, the general conclusion from the temperature studies is that application of higher process temperatures is not justified since the energy expenditure would not be balanced by possible improvement in the separation quality;
- As a result of two-step optimization of the process conditions, a HILIC chromatographic system has been proposed involving Nucleodur® C18 Gravity-SB column—although it is not an HILIC-dedicated one—and the eluent consists of 90% methanol, pH 6 and buffering salt (ammonium acetate) concentration C_{buff} = 20 mmol/dm^3. The system enables the separation of a mixture of vitamins B1, B2 and B3 in a shorter time than in the systems described in literature, in the isocratic conditions and using methanol, which is a more environmentally friendly organic solvent compared to commonly used acetonitrile.

Author Contributions: Conceptualization, M.C. and W.Z.; methodology, P.Z.; software, M.C.; validation, P.Z., W.Z. and J.K.; formal analysis, W.Z.; investigation, P.Z. and J.K.; resources, P.Z. and J.K; data curation, W.Z.; writing—original draft preparation, W.Z.; writing—review and editing, M.P.; visualization, M.C., M.P. and P.Z.; supervision, W.Z.; project administration, W.Z. All authors have read and agreed to the published version of the manuscript.

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