



The influence of a carbon layer deposited on a zirconia surface on the retention of polar analytes in an organic rich mobile phase

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ABSTRACT

Hydrophilic interaction liquid chromatography (HILIC) represents a modern MS-friendly approach to the analysis of polar compounds. To date especially silica-based HILIC stationary phases are utilized. Recently the papers concerning retention of polar analytes on unmodified titania and zirconia as well as a polybutadiene modified zirconia stationary phase under HILIC conditions have been published. In this work the investigation of a highly hydrophobic carbon-coated zirconia column under HILIC conditions was reported. The influence of buffer concentration, buffer type, pH and temperature on the elution and chromatographic efficiency were studied. The processes participating on the retention of polar compounds showed a multimodal character. The retention was governed rather by surface adsorption than phase partition in high ACN mobile phases. The ligand exchange remained an important retention force although the access of the analytes to the active surface was significantly hindered by the carbon coating. Besides ligand exchange and hydrophilic interactions also the hydrophobic carbon layer was involved in the retention of carboxylic acids and especially xanthines in the organic rich mobile phase.

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1. Introduction

The separation of polar analytes on conventional reversed-phase (RP) columns is often limited by a poor retention of these compounds although a water rich mobile phase is used. Another problem is represented by the limited stability of some RP stationary phases in an aqueous environment [1]. The use of polar stationary phases and high concentration of a water miscible organic solvent (mostly acetonitrile) was found to be an effective tool for the analysis of polar organic compounds [2]. The term hydrophilic interaction liquid chromatography (HILIC) was proposed by Alpert for this chromatographic modus. He suggested that the strongly eluting solvent is water and the retention mechanism is partitioning [3].

Nowadays it is believed that the retention mechanism is more complex and the resulting retention is dependent on miscellaneous interactions between the analyte and the stationary phase. Besides partitioning also ion interactions, hydrogen bonding, adsorption, ligand exchange etc. are involved in separation [2,4–6]. However, the retention mechanism has not been fully elucidated yet. The term aqueous normal phase chromatography (ANP) was

introduced when other retention forces than partitioning take part in the retention process [7].

Similarly to RP-HPLC the most HILIC separations are performed on bare silica or modified silica stationary phases [2,8]. Despite the number of advantages, silica suffers from limited pH and thermal stability. This drawback led to search for alternative materials usable in chromatography. Metal oxides, especially zirconia and titania, seem to be the most promising. These materials are more stable in extremes of pH and are also able to withstand high temperature. Hence, the metal-based phases enable the analysis of compounds in their non-ionized form even if they are strong acids or bases. In addition the advantages of high temperature chromatography can be utilized [9].

Since water molecules play an important role in interactions between analytes and metal oxides [10] as well as in HILIC [3], titania and zirconia have been recently tested as potential stationary phases for the separation of polar compounds under HILIC conditions.

Zhou et al. studied the HILIC behavior of nucleotides [11] and carboxylic acids [12] on bare titania. The Randon's group utilized a capillary titania monolith for extraction of organo-phosphorous compounds [13] as well as a particle titania column for investigation of the HILIC behavior of N-methylated xanthines [14] and beta blockers [15]. Generally, it was found that except partitioning also ligand exchange and ion interactions influenced the retention

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in dependence on the mobile phase composition. Titania was exploited under ANP conditions also in practice [16–18].

Relatively lower attention was paid to zirconia. The separation ability of zirconia monolith and zirconia-coated silica monolith was studied by Randon et al. using several xanthine derivatives [19].

In our previous paper we described the retention behavior of acidic polar analytes (4-aminobenzenesulfonic acid, 4-aminobenzoic acid, 4-hydroxybenzoic acid, 3,4-diaminobenzoic acid) on a polybutadiene modified zirconia phase (Zr-PBD) under HILIC conditions [20]. The results were compared with those obtained on bare zirconia (b-ZrO₂) and a silica HILIC phase. The retention on both zirconia phases seemed to be primarily governed by surface adsorption, whereas partition appeared as the main retention mechanism on the silica material. Differences in the retention of 4-aminobenzenesulfonic acid and carboxylates on zirconia were observed thanks to a distinct ability of these species to interact via ligand exchange. Nevertheless, the similar retention profile and selectivity were observed on both zirconia columns in the HILIC region. The stronger retention on b-ZrO₂ than on Zr-PBD suggested that the polybutadiene layer affected only the strength of retention without substantial influence on the selectivity.

Based on these interesting results we decided to continue in mapping of the behavior of RP zirconia stationary phases under HILIC conditions. The aim of this work was to study a differently modified zirconia phase, namely carbon-coated zirconia (Zr-C). This material was chosen thanks to its distinct properties contrary to polymer modified zirconia phases, i.e. enhanced selectivity towards polar and non-polar geometric isomers, its ability to electronic (π – π) interactions etc. [21]. We focused on the influence of a very hydrophobic carbon layer on the retention of selected polar analytes and on comparison with our previous observations.

2. Experimental

2.1. Chemicals

HPLC gradient grade acetonitrile (ACN) was purchased from Merck (Darmstadt, Germany). Acetic acid, acetone, ammonium hydroxide and all analyzed compounds (i.e. 4-aminobenzoic acid, 4-hydroxybenzoic acid, 4-aminobenzenesulfonic acid, 4-toluenesulfonic acid, theophylline, theobromine, caffeine, guaifenesin and resorcinol) were obtained from Sigma Aldrich (Schnellendorf, Germany). Milli-Q water was produced by a Millipore purification system (Schwalbach, Germany).

2.2. Preparation of stock and working solutions

The stock solutions (0.5 mg/mL) were prepared by dissolving the appropriate amount of each substance in either acetonitrile (most of the analytes) or 1% (v/v) acetic acid solution in acetonitrile (theobromine). These solutions were further diluted to get the working solutions (50 μ g/mL) with ACN–water mixture of either 50:50 or 10:90 (v/v) ratios for analyses using mobile phases with ACN content below or above 80%, respectively.

2.3. HPLC instrument

HPLC-PDA analyses were performed on a chromatographic system LC 20A Prominence (Shimadzu, Duisburg, Germany) which consisted of a DGU-20A3 degasser, two LC-20AD pumps, a SIL-20AC autosampler, a CTO-20AC column oven, a SPD-M20A photodiode array detector and a CBM-20AC communication module. The data were processed using LC solution software, version 1.21 SP1 (Shimadzu, Duisburg, Germany).

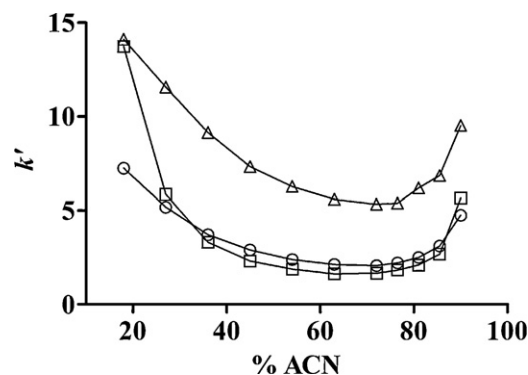


Fig. 1. Typical retention behavior of carboxylic acid as a function of acetonitrile concentration on tested zirconia stationary phases: Zr-C (□), Zr-PBD (○), b-ZrO₂ (△). 4-Hydroxybenzoic acid was used as an example.

2.4. Stationary phases and chromatographic conditions

A zirconia modified with a carbon layer, a polybutadiene layer and a bare zirconia column (150 mm × 4.6 mm; 5 μ m particle diameter, ZirChrom, Anoka, MN, USA) were used in our study. These stationary phases are stable within the pH range of 1–14 and up to 200 °C, Zr-PBD up to 150 °C. During this work the pH value of aqueous part in all mobile phases was adjusted prior mixing with ACN.

The effect of ACN content was investigated in the range 18–90% (v/v). The mobile phases were prepared from two parts. The part A was composed of 50 mM acetic acid, pH adjusted to 4.5 with ammonium hydroxide. The part B was prepared by mixing 0.5 M acetic acid, pH adjusted to 4.5 with ammonium hydroxide and ACN in the ratio 10:90 (v/v).

The effect of acetate concentration was evaluated at following levels: 5, 10, 20, 35 and 50 mM. The mobile phase was composed of adequate concentration of acetic acid (pH adjusted with ammonium hydroxide to 4.5) in 85.5% ACN.

The effect of pH was evaluated at 3.8, 4.5, 5.6, 6.7, 8.5 and 9.5, respectively. The mixture of 35 mM acetic acid (adjusted with ammonium hydroxide to desired pH) in 85.5% ACN was used in these experiments.

The effect of the Lewis base type was evaluated using acetate and formate. Both mobile phases were composed of corresponding acid (50 mM, pH adjusted with ammonium hydroxide to 4.5) in 85.5% ACN.

The effect of column temperature was studied at 20, 25, 30, 35, 40, 45, 55, 65, 75 and 83 °C. The mobile phase was composed of acetic acid (50 mM, pH adjusted with ammonium hydroxide to 4.5) in 85.5% ACN.

The following chromatographic conditions remained constant throughout the study: a flow rate of 1 mL/min, an injection volume of 20 μ L and UV detection set at 254 nm. The void volume was determined using acetone dissolved in ACN–water mixture (50:50, v/v). The estimation of theoretical plate counts, tailing factors and plate heights was accomplished according to the USP [22].

3. Results and discussion

We previously studied the influence of polybutadiene coating on zirconia surface on the retention of polar acidic analytes under HILIC conditions [20]. It was shown that the unmodified areas were responsible for the retention under HILIC conditions. The obtained retention factors on Zr-PBD were lower in contrast to native zirconia (see Fig. 1) owing to the polymer barrier, which hindered the full access of the analytes to the zirconia surface. However, the selectivity for studied compounds was not affected.

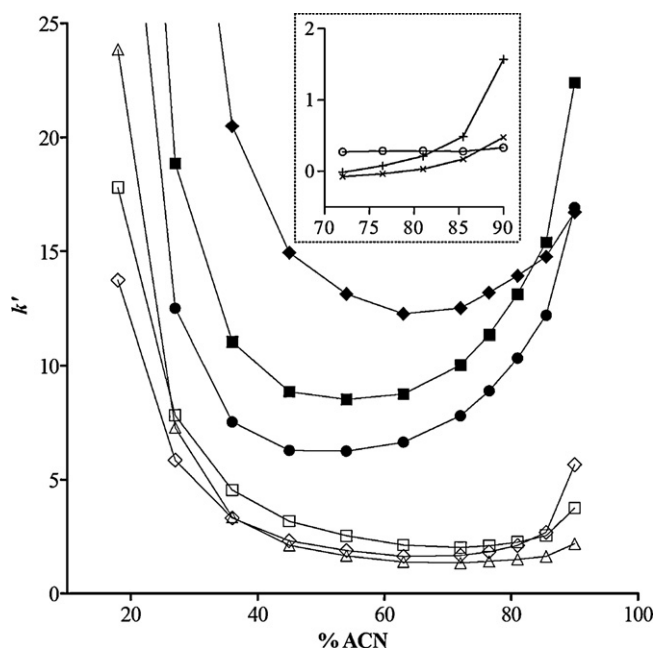


Fig. 2. The effect of acetonitrile concentration on the retention of model compounds on Zr-C. The inset shows the retention curves for weakly retained compounds in HILIC region. Compounds: 4-aminobenzoic acid (\square), 4-hydroxybenzoic acid (\diamond), theophylline (\blacksquare), theobromine (\bullet), caffeine (\blacklozenge), guaifenesin (\triangle), 4-aminobenzenesulfonic acid ($+$), 4-toluenesulfonic acid (\times), resorcinol (\circ).

In this study we continued in the same topic with the carbon-coated zirconia stationary phase. In order to obtain the retention data for various chemical compounds, 4-toluenesulfonic acid, resorcinol, guaifenesin and xanthines (caffeine, theobromine, theophylline) were added to the previous set of analytes (4-aminobenzoic acid, 4-hydroxybenzoic acid, 4-aminobenzenesulfonic acid). The effect of acetonitrile amount, acetate concentration, pH of the aqueous phase and type of the Lewis base in the mobile phase as well as temperature on the retention of model analytes were studied. The compounds that were practically not influenced by a particular condition were excluded from the discussion in the text.

3.1. Effect of acetonitrile concentration

The retention behavior of model compounds on Zr-C was evaluated under similar conditions as on Zr-PBD and b-ZrO₂ and is shown in Fig. 2. Although the deposition of the carbon layer on the zirconia surface is more effective than in case of polybutadiene (up to 97% of the surface could be modified [23]), the population of accessible Lewis acid sites on zirconia was sufficient to interact with analytes under HILIC conditions. The dependence of retention factors on ACN concentration for 4-aminobenzoic acid (PAB) and

Table 1

The regression analysis of log–log and lin–log plots of model compounds on Zr-C in HILIC region.

Compound	r^2	
	log–log	lin–log
4-Aminobenzoic acid	0.912	0.813
4-Hydroxybenzoic acid	0.922	0.828
4-Aminobenzenesulfonic acid	0.993	0.996
4-Toluenesulfonic acid	0.950	0.982
Theophylline	0.988	0.940
Theobromine	0.996	0.960
Caffeine	0.994	0.960
Guaifenesin	0.937	0.852
Resorcinol	N/A ^a	N/A ^a

^a Not applicable.

4-hydroxybenzoic acid (PHB) provided characteristic U-shape curves similar to those obtained on b-ZrO₂ and Zr-PBD [20] (Fig. 1). As apparent the retention on Zr-C was comparable with results on b-ZrO₂ and approximately two times higher than on Zr-PBD in mobile phase containing 18% ACN. However, thanks to the surface modification the retention in 90% ACN was significantly stronger than on Zr-PBD (by 30% and 20% for PAB and PHB, respectively) and lower than on b-ZrO₂. Taking into account that the retention of carboxylates is governed by the ligand exchange on bare zirconia [20,24] and that the retention of carboxylates was lowered in presence of the polybutadiene layer, it seems that the carbon moiety affected their retention also in the organic rich mobile phase.

The U-shape retention profile of xanthines appeared to be mediated in a different way than in case of carboxylates. The excessive retention in low ACN area can be ascribed to a strong interaction with the graphitized carbon layer (elution order theobromine, theophylline and caffeine) because only negligible interactions of xanthines with bare zirconia and Zr-PBD were found. Similarly only slight retention was found on these phases in an acetonitrile rich mobile phase, whereas a marked retention was observed on Zr-C. This result indicated that the carbon layer was involved in the retention process of xanthines. Moreover the retention order was adjusted according to increasing polarity. On the other hand 4-aminobenzenesulfonic acid (SUL) and 4-toluenesulfonic acid did not behave differently in comparison with our previous results.

The log–log and lin–log plots of retention factors and the content of the aqueous part of the mobile phase were compared within the range 72%–90% ACN. Apart from benzenesulfonic acids the higher correlations were found in log–log plots (see Table 1), which should favor rather adsorption than partitioning on Zr-C.

3.2. Effect of acetate concentration

The influence of acetate concentration on the retention under HILIC conditions is demonstrated in Table 2. Slightly suppressed retention of carboxylic acids as a consequence of raising acetate

Table 2

The effect of acetate concentration on the retention, plate numbers [1/m] and tailing factors of model compounds under HILIC conditions (85.5% ACN).

Compound	k'		N		T_f	
	5 mM	50 mM	5 mM	50 mM	5 mM	50 mM
4-Aminobenzoic acid	5.1	2.9	1000	2300	2.9	2.5
4-Hydroxybenzoic acid	5.0	3.1	900	2600	3.0	2.5
4-Aminobenzenesulfonic acid	0.0	0.5	13,300	28,200	1.3	1.7
4-Toluenesulfonic acid	−0.2	0.2	15,100	26,100	1.2	1.3
Theophylline	16.6	15.8	17,800	19,100	4.5	4.3
Theobromine	13.6	12.4	33,400	43,700	3.5	2.9
Caffeine	16.0	14.8	37,300	36,700	3.0	3.0
Guaifenesin	1.8	1.7	9400	26,900	3.2	2.2
Resorcinol	0.3	0.3	24,800	24,900	1.4	1.4

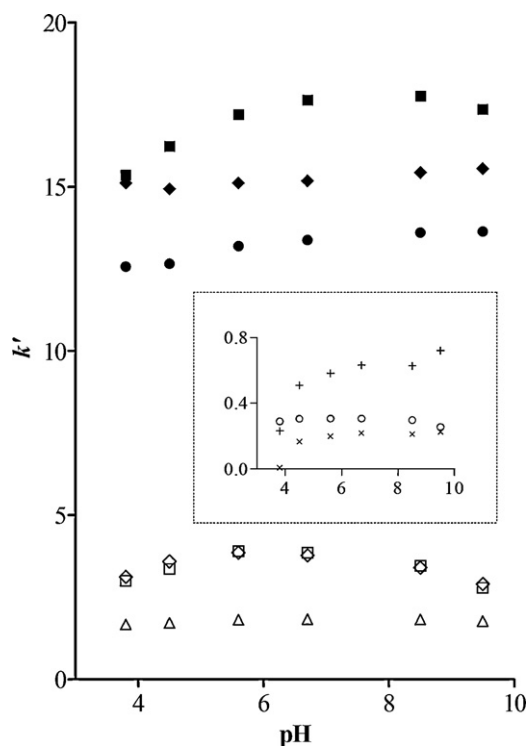


Fig. 3. The effect of pH on the retention of model compounds under HILIC conditions (85.5% ACN). The inset shows the retention behavior of weakly retained compounds. Compounds: 4-aminobenzoic acid (□), 4-hydroxybenzoic acid (◇), theophylline (■), theobromine (●), caffeine (◆), guaifenesin (△), 4-aminobenzenesulfonic acid (+), 4-toluenesulfonic acid (×), resorcinol (○).

amount can be interpreted by a mutual Lewis bases competition for the ligand exchange sites on zirconia. The more acetate was available in the eluent, the higher saturation of zirconia Lewis acid sites and thus the reduced retention, higher plate count and better peak symmetry were observed. The role of ligand exchange was further confirmed by replacing acetate with water. No elution of carboxylic acids was found on b-ZrO₂ in 85.5% ACN–water whereas both compounds were eluted on Zr-C phase thanks to the shielding of the zirconia surface by the carbon layer. However, the retention factors increased more than twice for PHB and more than three times for PAB in comparison to ACN–acetate (5 mM, pH 4.5).

Xanthines showed also a mild decrease in retention and this trend was found for slightly acidic theophylline and theobromine as well as neutral caffeine. Therefore the ion exchange is unlikely to be the main reason. The explanation might lie in a presence of ligand exchange as these heterocycles possess electron pairs on nitrogen as it has been discussed by Abi Jaoudé and Randon in case of a titania phase [14]. Considering that the retention of benzenesulfonic acids seemed to be governed by partitioning the retention increase

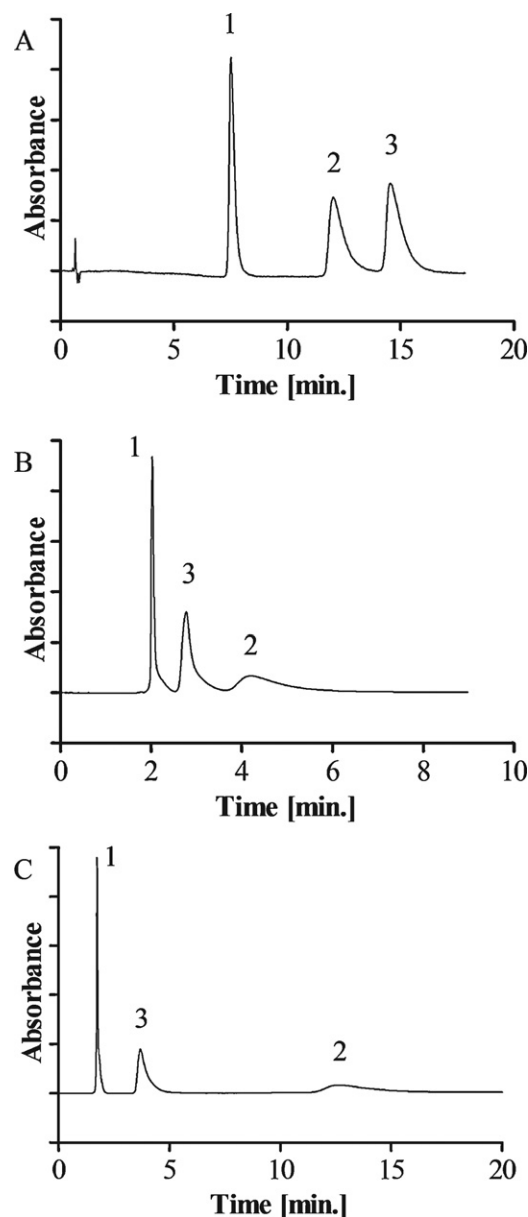


Fig. 4. The separation of xanthines on zirconia stationary phases in mobile phase ACN–water (95:5, v/v), detection at 254 nm. (A) Zr-C, flow rate 3 mL/min, column temperature 55 °C; (B) b-ZrO₂, flow rate 1 mL/min, column temperature 15 °C; (C) b-ZrO₂, flow rate 1 mL/min, column temperature 80 °C; 1 = caffeine; 2 = theobromine; 3 = theophylline.

Table 3

The effect of Lewis base type on the retention, plate numbers [1/m] and tailing factors of model compounds under HILIC conditions (85.5% ACN).

Compound	Acetate			Formate		
	k'	N	T_f	k'	N	T_f
4-Aminobenzoic acid	2.6	2600	2.5	3.3	2100	2.5
4-Hydroxybenzoic acid	2.7	2800	2.7	3.5	2100	2.9
4-Aminobenzenesulfonic acid	0.5	27,400	1.7	0.8	29,200	1.7
4-Toluenesulfonic acid	0.2	27,100	1.3	0.3	25,300	1.2
Theophylline	15.5	23,100	3.3	18.1	18,600	3.6
Theobromine	12.2	42,800	2.6	15.8	32,900	2.8
Caffeine	14.8	41,900	2.5	16.9	31,900	2.7
Guaifenesin	1.6	28,700	2.0	1.8	15,800	2.7
Resorcinol	0.3	26,400	1.4	0.3	21,600	1.2

Table 4The effect of temperature on the retention, plate numbers [1/m], tailing factors and ΔH° values [kJ/mol] of model compounds under HILIC conditions (85.5% ACN).

Compound	k'		N		T_f		ΔH°	r^2
	20 °C	83 °C	20 °C	83 °C	20 °C	83 °C		
4-Aminobenzoic acid	2.8	3.4	2000	7600	2.8	2.4	2.6	0.973
4-Hydroxybenzoic acid	3.0	4.0	2000	7800	3.0	2.4	3.7	0.976
4-Aminobenzenesulfonic acid	0.5	0.4	28,000	28,100	1.6	1.5	-2.5	0.927
4-Toluenesulfonic acid	0.2	0.1	21,700	22,900	1.2	1.2	-9.3	0.842
Theophylline	17.9	4.4	16,100	44,000	4.5	2.5	-19.3	1.000
Theobromine	14.4	3.6	35,800	60,400	3.1	1.7	-18.9	1.000
Caffeine	17.1	4.3	31,700	59,400	2.9	1.8	-19.0	1.000
Guaifenesin	1.8	0.7	26,300	8200	2.1	3.4	-13.1	1.000
Resorcinol	0.3	0.2	24,200	21,200	1.3	1.4	-7.3	0.978

in 50 mM acetate may be elucidated as follows. Higher salt concentration might drive the more solvated salt anions into the water enriched layer yielding an increase in the volume of stagnant water layer [12] and thus the benzenesulfonates remained longer in the aqueous phase.

Guaifenesin seemed to interact with zirconia via ligand exchange thanks to vicinal hydroxyls [18]. This hypothesis was supported by better peak shape in 50 mM than in 5 mM acetate on Zr-C and by no elution on b-ZrO₂ in 85.5% ACN–water.

3.3. Effect of acetate pH

In spite of considerably lower retention of PAB and PHB on Zr-C in comparison with Zr-PBD and b-ZrO₂ [20], the results confirmed the principal role of the ligand exchange process. It was reported by Blackwell that the strongest contribution of ligand exchange to carboxylic acid retention was found around pH 6, but then a steep decrease in retention to the zero value was observed due to prevailing ion repulsion [25]. Nearly the equal retention of PHB (pKa 4.57) and PAB (pKa 4.86) [26] at pH 3.8 and at pH 9.5 (Fig. 3) indicated the weakening of repulsion between analyte and stationary phase in the mobile phases with higher pH. According to Alpert, the electrostatic repulsion was attenuated in presence of high concentration of organic solvent [27]. Consequently a noteworthy retention was obtained, however three times higher plate count at alkaline pH reflected considerably minor contribution of the slow ligand exchange process to the retention.

Assuming the ability of xanthenes to ligand exchange the dependence of their retention factors on pH may be explained similarly. However, caffeine seemed to be practically unaffected by the chosen pH in contrast to dimethylxanthenes. Similar results were observed by Abi Jaoudé et al. on titania [14]. The retention behavior of guaifenesin showed the different ability to ligand exchange than in case of xanthenes. Whereas the retention seemed not to be affected by the eluent pH, the plate count dropped from 23,500/m in acidic mobile phase to 12,900/m in alkaline environment due to the increased peak tailing.

3.4. Effect of Lewis base type

Substituting acetate for formate led to longer retention times of acids and xanthenes (by approx. 15%–25%), see Table 3. Generally, lower plate counts and also the adverse effect on peak symmetry were detected in formate. The elucidation of these results may lie in a weaker Lewis base character of formate. Hence, the analytes that underwent a ligand exchange could strongly interact with the zirconia surface resulting in higher retention and lower chromatographic efficiency.

3.5. Effect of temperature

The results including the plate counts, tailing factors as well as ΔH° values calculated from van't Hoff plots are summarized in Table 4. The temperature effect on the retention of acidic analytes on Zr-C was not as strong as on b-ZrO₂ and Zr-PBD [20], probably due to higher coverage of zirconia surface by the carbon layer. The increasing retention of carboxylic acids is in conformity with the previous results on b-ZrO₂ and Zr-PBD [20]. Linear segments (in both $r^2 > 0.998$) separated by an obvious curvature at 45 °C were confirmed. Such observation could be related to a change of interaction mechanism and/or a change in the thickness of water layer [28,29]. The slopes of van't Hoff plots of benzenesulfonic acids were positive, however, the non-linearity around 60 °C was found as well.

Surprisingly the superior linearity of van't Hoff plots (r^2 surpassing 0.9999 in the whole temperature range) were obtained for xanthenes and guaifenesin. As can be seen in Table 4, the efficiency was markedly increased for xanthenes and simultaneously the peak symmetry was improved. Nevertheless, completely opposite behavior was observed for guaifenesin. This diverse temperature effect needs a further investigation.

3.6. Separation of model samples

It was demonstrated that Zr-C column provides number of possibilities how to control the retention of compounds and thus the resulting separation. Besides the common mixed-mode behavior at lower ACN, a new application area in higher ACN region opens up. The separation ability of Zr-C in organic rich eluent is presented on two model samples: acids (SUL, PAB, PHB) and xanthenes (theophylline, theobromine, caffeine).

3.6.1. Acids

This sample was analyzed according to the HPLC conditions used in our previous paper, i.e. ACN–acetate (50 mM, pH 4.5) (90:10, v/v), temperature 60 °C and flow rate 1 mL/min [20]. It allowed the comparison with the results obtained on b-ZrO₂ and Zr-PBD (3,4-diaminobenzoic acid was omitted due to strong retention on Zr-C). A satisfactory separation but similar selectivity and efficacy to that on b-ZrO₂ and Zr-PBD were obtained. The tailing factors 1.95, 2.01 and 2.01 and plate heights 32, 211 and 175 μm for SUL, PAB and PHB respectively, showed slightly less efficiency of Zr-C than on previously studied columns.

3.6.2. Xanthenes

The co-elution of theobromine and caffeine was found under HPLC conditions used for the separation of acids. Several solutions were possible to solve this problem, e.g. the reduction of ACN amount to ca. 75%. However, good discrimination between caffeine and dimethylxanthenes was also achieved in a simple mobile

phase ACN–water (95:5, v/v). The elevated column temperature and higher flow rate were utilized with respect to excessive retention ($k' > 25$) and low column backpressure. All three compounds were separated in approx. 15 min at 55 °C and flow rate 3 mL/min, see Fig. 4A. The co-elution of theobromine and theophylline was observed at higher temperature than 60 °C.

The ability of Zr-C to retain xanthenes was in stark contrast to b-ZrO₂ and Zr-PBD, where practically a null retention of caffeine and very low retention ($k' < 1.3$) of dimethylxanthenes were observed even in 95% ACN–water. This observation implied the crucial role of the carbon layer during the separation of these species.

In addition interesting temperature effect on xanthine retention was found on b-ZrO₂ in 95% ACN–water. Whereas the “maximal” retention of caffeine ($k' = 0.3$) was reached at 15 °C (Fig. 4B), the temperature increase led to the elution of caffeine in the void volume. Nevertheless, the enhanced retention of dimethylxanthenes, especially theobromine was found (Fig. 4C) at higher temperature. The switch in order of dimethylxanthine peaks as well as their significant band broadening in comparison with Zr-C are also worth to be mentioned.

4. Conclusion

The assessment of carbon-coated zirconia stationary phase for the analysis of selected polar compounds under HILIC conditions was reported. The results clearly demonstrated the ability of this material to retain polar compounds under HILIC conditions despite the presence of hydrophobic barrier. Different mechanisms especially ligand exchange, hydrophilic and most interestingly hydrophobic interactions were participated on the final retention. Their relative importance was strongly dependent on the chemical structure and properties of the analyte and experimental conditions.

Based on the comparison with reference zirconia phases, the carbon layer influenced the overall retention of carboxylates and xanthine derivatives differently than polybutadiene. In contrast to Zr-PBD the carbon layer was more involved in the retention process in water rich and acetonitrile rich mobile phases as well. Especially xanthenes showed strong interactions with the carbon layer under HILIC conditions while preferably ligand exchange took place on native zirconia.

Generally, these results signified that modified RP phases might be successfully used in separations under HILIC conditions. The ability to ligand exchange seems to be important for the interaction with zirconia surface. In some cases also the hydrophobic

layer could be involved in the retention even though the organic rich mobile phase is used. However, with respect to the multimodal retention character further experiments are necessary to elucidate in detail all aspects influencing separation in the organic rich environment, e.g. the role of ligand exchange, temperature, organic modifier, Lewis base additive, etc.

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