

Chelates of cobalt(III) and iron(II) with 2-(5-bromo-2-pyridylazo)-5-diethylaminophenol as test probes for the characterization of chromatographic effects on a reversed-phase liquid chromatography stationary phase

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Abstract

The chromatographic effects on a reversed-phase liquid chromatography (RPLC) phase were established with the use of chelates system: cobalt(III) and iron(II) with 2-(5-bromo-2-pyridylazo)-5-diethylaminophenol as the test probes. Both chelates have the same octahedral structure, M:L = 1:2, the former chelate is cationic and can be used to probe the ion-exchange phenomena on a RPLC phase, whereas the latter is not charged and can be used as reference molecule with respect to the charged one. Based on well-established LC phenomena referred in the literature, the suitability of the chelate system for examining some chromatographic effects was tested. It was concluded that the proposed test method is sensitive towards the ion-exchange phenomena on the LC phase and the hydrogen bonding between the solvent eluent and the LC phase. In addition, chromatographic effects due to the presence of ultrasonic field or due to the presence of aromatic amines in the eluent were observed with the help of the proposed test method. Based on molecular computation, the properties of chelates were compared with properties of quaternary amines, the probes most frequently used for testing LC phases, and the possible differences in an interaction of the mentioned compounds with a surface of a RPLC phase were indicated.

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1. Introduction

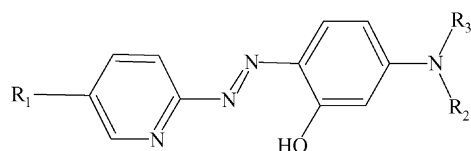
The dynamic development of chromatography has become possible thanks to a wide variety of chromatographic column packing. The silica-based liquid chromatography (LC) stationary phase is still the most used and investigated LC phase [1–3]. It was concluded that LC separation is a highly complex process where (adsorption, ion-exchange and steric exclusion) interactions between the solute and the stationary phase do take place simultaneously, and these depend on the structure of the solute and the stationary phase as well as on the composition of a mobile phase.

Organic compounds, grouped into a variety of different tests, have played the main role in the characterization of the LC

stationary phase [4]. This group of tests is used for the discrimination of LC retention mechanism and to estimate the chromatographic activity of silanol groups. Among these tests, the procedures used for characterizing silanol activities of a packing are outstandingly important (recently reviewed [5]) and are based mainly on base–acid or ion-pairing properties of amines. The new test based on the retention of the simple Li^+ cation has been published recently [6]. Although, a number of these procedures do exist, there is no universally accepted procedure to date. This is in agreement with ref. [7], where the correlation among different tests is found to be low. The reason for this is the mixed retention mechanism which involves the hydrophobic properties of the packing, the silanol properties of the packing and the degree of ionization of amines. As both, the kind as well as the content of organic phase being in eluent do affect both $\text{p}K_a$ of the silanol group and $\text{p}K_a$ of the test amine, there is a high level of uncertainty in the correct estimation of the chromatographic effect.

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In the present work, metal ion chelates are introduced as test probes for characterization of a reversed-phase liquid chromatography (RPLC) stationary phase as well as for characterization of LC retention in the case of modification of a RPLC stationary phase. So far, only a few examples of application of chelates in this regard are found in literature [8–11]. The rare use of chelates for estimating chromatographic effects is due to: (i) the existence of additional equilibria for chelate and chelate conditional stability = $f(\text{pH}, \text{kind of solvent, ionic strength})$, and (ii) low parametrization of metal ion–ligand bonding. On the other hand, chelates are regarded as a group of interesting probes for characterization of interfacial phenomena [12–14], provided that the applied measuring system is well characterized.



Ligand	R ₁	R ₂	R ₃
5-Br-PADAP	Br	C ₂ H ₅	C ₂ H ₅
5-Br-PAPS	Br	C ₃ H ₇	C ₃ H ₆ SO ₃ ⁻ Na ⁺
5-NO ₂ -PAPS	NO ₂	C ₃ H ₇	C ₃ H ₆ SO ₃ ⁻ Na ⁺

2-Pyridylazo ligands discussed in the present work.

2. Experimental

2.1. Apparatus

The chromatographic system was composed of a Hewlett-Packard HP 1100 liquid chromatograph (Waldbronn Anal. Div., Germany) consisting of a G1310A isocratic pump, G1315A diode array detection (DAD) system, a Rheodyne Model 7725i injection valve equipped with a 20 μl sample loop (Cotati, CA, USA) and analytical column. The column was connected by a polyether ether ketone (PEEK) capillary. The following columns were used: PEEK column tube PE 150/4.6 Nucleosil 100-5 C18 e.c. (150 mm \times 4.6 mm, 5 μm) (Macherey-Nagel, Düren, Germany), Zorbax, Eclipse XDB-C18 (250 mm \times 4.6 mm) and Zorbax, SB-C18 (250 mm \times 4.6 mm) (Agilent, Palo Alto, CA, USA). The columns were not new but the performed test (phenol, naphthalene and anthracene) showed that reduced plate height for columns was in the range of 7–11. Thus, the columns can be regarded as good. The chromatographic system was controlled by a Pentium MMX (200 MHz, 64 M RAM) personal computer equipped with a LC Chemstation 2D (Hewlett-Packard) program, which also stored and handled the data. The absorption spectra were recorded on a Specord spectrophotometer (Zeiss, Jena) with 10 mm glass cells controlled by a personal computer. For pH measurement an ELPO-N517 pH meter (Poland) was used. Ultrasound wave was generated in an ultrasonic bath (Sonic-6 bath, Polsonic, Poland; output power 3 \times 80 W, vibration frequency 40 kHz). The dimensions of the bath were length 30.5 cm, width 14.5 cm, height 14 cm. It was filled with water to a height of 7.5 cm. Columns were positioned at the bottom of

the ultrasonic bath. Computer programs: *Ampac 8*, (Semichem, Shawnee, USA) and *HyperChem 7* (Hypercube, Gainesville, USA) were applied for molecular computations.

2.2. Reagents

All reagents used were of analytical-reagent grade. Cobalt(II) stock solution (1 mg ml⁻¹) was prepared from cobalt(II) sulphate heptahydrate and iron(II) stock solution (1 mg ml⁻¹) from Mohr's salt (Fe(NH₄)₂(SO₄)₂·6H₂O). Salts were dissolved in water (500 ml) with addition of 1 ml of concentrated sulfuric acid. 2-(5-Bromo-2-pyridylazo)-5-diethylaminophenol (5-Br-PADAP) (Fluka, Buchs, Switzerland) solution of a concentration 5 \times 10⁻³ mol l⁻¹ in methanol was used. Water solution of 2.5 \times 10⁻² mol l⁻¹ sodium 1-dodecanesulfonate (SDeS); 2.5 \times 10⁻² mol l⁻¹ sodium salt hexane-1-sulfonic acid (SHS), 2.5 \times 10⁻² mol l⁻¹ sodium salt propane-1-sulfonic acid (SPS); 0.1 mol l⁻¹ 3-(*N*-hexadecyl-*N,N*-dimethylammonio) propane sulfonate (SB-12) (Sigma–Aldrich) were used.

Tetramethylammonium bromide (TMABr), tetraethylammonium bromide (TEABr), tetrapropylammonium bromide (TPABr), tetrabutylammonium bromide (TBABr), tetraoctylammonium bromide (TOABr) and hexadecyltrimethylammonium bromide (CTAB) (Avocado, Lancashire, England) were taken. Tetraalkylammonium bromide salts were dissolved in eluents. Acetonitrile (ACN) and methanol (MeOH) of HPLC grade (Lab-Scan, Dublin, Ireland) and water deionized and double distilled were used throughout.

2.3. Eluent

Eluents were prepared in a 250 ml calibration flask by mixing solvents in appropriate proportions with or without the presence of an appropriate additive. The composition of each eluent is given as a description in a figure, which illustrates an appropriate effect. Such prepared eluents were degassed in an ultrasonic bath (mixture of solvents) or by filtering, using 0.45 μm PTFE filter under suction (solvent with a salt component).

2.4. General procedure for separation of Co(III)/Fe(II) chelates with 5-Br-PADAP

In a 10 ml calibrated flask 0.3 ml of 5-Br-PADAP solution, 10 μg of Co(II), 1 μg of Fe(II) and 5 ml of methanol were added and the solution was filled with water up to the mark. A 20 μl portion of the solution was injected onto the column. The flow rate of the eluent was 1 ml min⁻¹ and the eluate was simultaneously monitored at 440 nm (5-Br-PADAP), 590 nm (Co(III) chelate) and at 555 and 740 nm (Fe(II) chelate) using DAD detector. Columns were equilibrated with eluent not less than 40 min before the sample injection. The value of k was measured by double injection (triple when difference in retentions was higher than 5%). Note that the concentration of iron(II) chelate in the pH range below 3.5 of an eluent should be increased for visualizing the peak of the chelate, as the efficiency of formation of iron(II) chelate is found to be lower below pH 3.5. Alternatively, the peak of ligand (5-Br-PADAP) can serve as the reference peak

as retention of both iron(II) chelate and ligand (5-Br-PADAP) is found to be close for the whole pH range of the eluent.

3. Results and discussion

3.1. Criteria for choice of metal ion chelates as LC probe

Chelates of metal ions with azo ligands are used most frequently in metal ion determination, due to high chelate stability in wide range of pH and high optical molar absorption coefficient ensuring low level of detection. These include chelates of PAR, PAN and 5-Br-PADAP, which have been reviewed recently [15,16]. In the present work, the chelates of Co(III)L_2^+ and Fe(II)L_2^0 ($\text{L}^0 = 5\text{-Br-PADAP}$) were applied in order to examine some LC effects. Both chelates have the same structure, metal ion:ligand = 1:2, cobalt chelate is positively charged (Co(III)L_2^+), whereas iron chelate is neutral (Fe(II)L_2^0). The Co(III)L_2^+ chelate is interesting. It has been reported that the chelate once formed is stable in a wide pH range, from fairly concentrated acid (e.g., 3 mol l^{-1} hydrochloric acid) to pH at least 10 [17]. The same was reported for other pyridylazo chelates of Co(III) [16]. Further, the outstanding ion-pairing properties of the cationic Co(III) chelates with 2-pyridylazo ligands toward negatively charged species have been reported as follows (anion determined/chelate used): $\text{BF}_4^-/\text{Co(III)(5-Cl-PADAP)}$ [18], anionic surfactants/ $\text{Co(III)(5-CF}_3\text{-PADAP)}$ [19]/ Co(III)(PADAP) [20]/ $\text{Co(III)(5-Cl-PADAP)}$ [21] and albumin/ $\text{Co(III)(5-Br-PADAP)}$ [22]. The formation and partition constants of ion associates formed between the cationic cobalt(III) complexes and counter anions (ClO_4^- , SCN^- , Br^- and Cl^-) [23] were established and these factors mostly depended on the bulkiness and the polarity of the pyridylazo compounds. Next, both voltammetric and electrophoretic techniques were applied for the characterization of Co(III)L_2^+ chelate ($\text{L}^0 = 5\text{-Br-PADAP}$) [24]. In the present work, the basic properties of Co(III)L_2^+ chelate were established by the spectrophotometric method and these are presented in Table 1.

Studies on RPLC retention of metal ions (Cu, Co, Fe, Ni, V and Pd) as 5-Br-PADAP chelates by RPLC showed that only the retention of Co(III) chelate is affected by varying the concen-

tration of a surfactant in the eluent [25]. The deviation in the retention of cobalt chelates with azo ligands in RPLC has been reported [26,27].

The second chelate used in this work is Fe(II)L_2^0 , which has already been used in many applications dealing with determination of iron ion by RPLC method. The chelate has been described in refs. [28,29].

In the present work, measurement system (application of two chelates $\text{Co(III)L}_2^+/\text{Fe(II)L}_2^0$) was used for the characterization of some LC effects. It was proposed that Co(III)L_2^+ can be used as a molecular probe sensitive toward cation exchange/ion-pairing properties of LC phase, whereas the Fe(II)L_2^0 was regarded as reference molecule. Testing a RPLC phase by a probe consisting of two molecules is accepted and frequently used in chromatographic practice (e.g., Tanaka test (benzylamine/phenol) or Waters test (amitriptyline/acenaphthalene)). However, it was claimed that for structurally different molecules used as test probes the non-specific interactions may be different depending on an applied separation condition. The present system ($\text{Co(III)L}_2^+/\text{Fe(II)L}_2^0$) is different from those applied so far. As both chelates have the same structure (octahedral, M:L = 1:2), the non-specific interactions (hydrophobic or shape dependent) between the LC phase and chelate should be similar. Both chelates have their λ_{max} in the VIS spectral region and this allows investigation of the retention using a RP phase, which is modified by various compounds, usually having their λ_{max} in the UV range.

3.2. Choice of eluent solvents and LC phases

Based on the description in the previous section assumptions about the character of interaction of $\text{Co(III)L}_2^+/\text{Fe(II)L}_2^0$ chelates with RPLC stationary phase were examined using silica-based RP phases: (i) with diisobutyl-*n*-octadecylsilane ligand (Zorbax SB-C18) and (ii) dimethyl-*n*-octadecylsilane ligand (Nucleosil 100-5 C18 e.c. and Zorbax Eclipse XDB-C18). The mentioned phases have been recently characterized in ref. [30], in terms of seven basic RP variables and in ref. [1] in terms of selectivity parameters. It should be noted that the determined variables in ref. [30]: hydrophobicity (α_{CH_2}), total ion-exchange capacity, $\alpha_{\text{B/P}}$ pH 7.6 and acidic ion-exchange capacity, $\alpha_{\text{B/P}}$, pH 2.7, for phases applied in the present work (Nucleosil C18: 1.44, 2.18, 0.13; Zorbax, Eclipse XDB-C18: 1.5, 0.35, 0.09; Zorbax, SB-C18: 1.49, 1.46, 0.13, respectively) show that all these phases have similar hydrophobicity but different ion-exchange properties. The concentration of silanol group (internal, surface and shielded) for Zorbax phase (Rx-C18 and SB-C18) has already been reported in ref. [31].

In the present work, these three C18 phases were chosen according to their diversity in the retention of CoL_2^+ chelate with the use of pure solvents as eluent (Table 2). Thus, no elution of CoL_2^+ was observed on Nucleosil C18 phase using both ACN and MeOH eluents. Using Zorbax SB-C18 phase the CoL_2^+ chelate was not eluted by ACN but eluted by MeOH, and on Zorbax Eclipse XDB-C18 the chelate was eluted by both ACN and MeOH solvents. This behavior indicates that the content of silanol groups available for interaction with CoL_2^+ decreased

Table 1

The basic properties of $\text{Co(III)(5-Br-PADAP)}$ chelate determined by spectrophotometric measurement

Parameter	$\text{Co(III)(5-Br-PADAP)}$
Optimum pH range ^{a,b}	2–10
λ_{max} ^c (nm)	590
Molar absorptivity (ϵ) ($1 \text{ mol}^{-1} \text{ cm}^{-1}$)	1.1×10^5
Metal to ligand ratio ^d	1:2
Stability constant ($\log \beta$) ^d	11.8

^a Chelate once formed is then stable in 3 mol l^{-1} HCl [17], 3 mol l^{-1} H_2SO_4 or 1.8 mol l^{-1} HNO_3 [16].

^b The cobalt(III) ion in chelate is not prone to a reduction in the presence of reducing agents up to pH 9 [12].

^c In 50:50 MeOH/water solution. The value of λ_{max} was stable in different solvents: ACN/water, from 20% to 100% of ACN or in MeOH/water mixture, in the same proportions.

^d The Bent-French method.

Table 2

Comparison of k of CoL_2^+ , FeL_2^0 and L species on different RP-LC columns using pure solvents (ACN or MeOH) as eluent^a

ACN			MeOH		
CoL_2^+	L	FeL_2^0	CoL_2^+	L	FeL_2^0
Nucleosil 100-5 C18 e.c.					
No elution	1.53	1.73	No elution	0.86	1.24
Zorbax, SB-C18					
No elution	0.81	1.01	0.92	0.40	0.51
Zorbax, Eclipse XDB-C18					
3.22	0.77	0.98	0.17	0.41	0.45

^a (Column cleaning) column was purged by $0.05 \text{ mol l}^{-1} \text{ H}_2\text{SO}_4$ for 30 min and next it was left stand in 50:50 MeOH:water for 1 day, otherwise the column behaves as if the separation was performed with the use of acidic eluent.

in the same order and this is in agreement with the $\alpha_{B/P}$ order for these phases, mentioned above [30]. The differences in elution of CoL_2^+ by ACN and MeOH are discussed in detail in Section 3.9. To overcome the lack of retention of CoL_2^+ on Nucleosil or Zorbax SB-C18 columns, the ion-pairing reagent (NaClO_4) in concentration of $1 \times 10^{-3} \text{ mol l}^{-1}$ (Nucleosil) or $5 \times 10^{-4} \text{ mol l}^{-1}$ (Zorbax SB-C18) was added in order to visualize the peak of CoL_2^+ (with a higher concentration of NaClO_4 no further influence on the value of k of the chelate).

In the present work (ACN:water) and (MeOH:water) mixtures as eluent were examined. First, these solvents are most often applied to a RPLC separation and the second, we would like to compare the present results with those obtained in ref. [8], where metal chelates with 5-Br-PAPS and 5-NO₂-PAPS (structures are presented in Section 1) have been applied for examining the LC retention phenomena. In ref. [8], the correlation of $\log k$ with solubility parameters (δ) was found to be linear for MeOH:water eluent, whereas for ACN:water eluent the correlation was curved for (5-Br-PAPS, 5-NO₂-PAPS) chelates. Fig. 1a shows the present results obtained for CoL_2^+ and FeL_2^0 ($\text{L}^0 = 5\text{-Br-PADAP}$) chelates with the use of eluents (ACN, MeOH) containing NaClO_4 ($5 \times 10^{-4} \text{ mol l}^{-1}$) as ion-pairing (IP) reagent. It was found that for FeL_2^0 using both (ACN, MeOH) eluents, the correlation $\log k = f(\delta)$ is linear and the slope is found to be independent from the column applied (for MeOH eluents the slope is 0.20–0.22 and for ACN eluents it is 0.58–0.61). For CoL_2^+ , the relation $\log k = f(\delta)$ is curved, especially for the region with high content of the organic solvent in the eluent (>80%), whereas for content of organic solvent <80%, the observed relations are linear. In the second experiment (Fig. 1b) eluents without IP reagent were examined. Only Zorbax Eclipse column was permitting for observation of the retention of CoL_2^+ and FeL_2^0 in the absence of IP reagent in the eluent (Table 2). Comparing Fig. 1a and b, the greatest difference is observed for $\log k = f(\delta)$ relation for CoL_2^+ chelate in ACN eluent. Thus, ion-pairing ($\text{CoL}_2^+ \cdots \text{ClO}_4^-$) seems to be effective in an eluent which contains high content of ACN (> 80%), whereas for the lower ACN content, such association is replaced by the other ($\text{CoL}_2^+ \cdots \text{SiO}^-$) as the value of $\log k$ of CoL_2^+ in ACN/water eluent containing IP reagent (Fig. 1a) reaches the level of $\log k$ of CoL_2^+ in ACN/water eluent with-

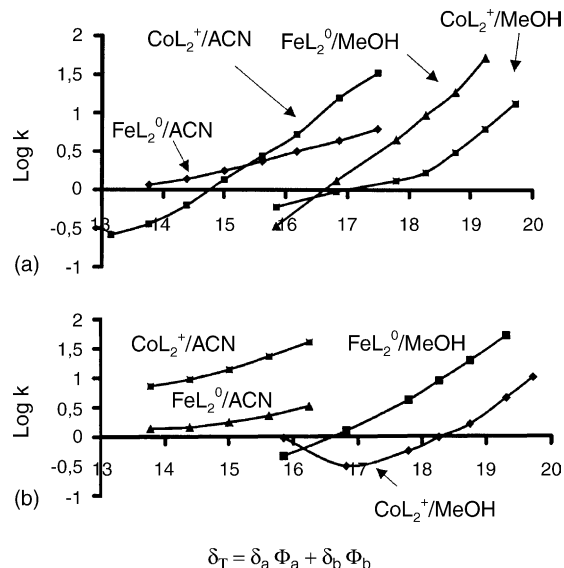


Fig. 1. Retention of CoL_2^+ , FeL_2^0 and L^0 species as a function of mobile phase composition. The correlation of $\log k$ of these species with the solubility parameter (δ_T); (a) Zorbax, SB-C18 column, eluent: organic phase (ACN, MeOH):water containing $5 \times 10^{-4} \text{ mol l}^{-1} \text{ NaClO}_4$. (b) Zorbax, Eclipse XDB-C18, eluent: organic phase (ACN, MeOH):water, without IP reagent. Solubility parameters for MeOH, ACN and water: 15.85, 13.15 and 25.52 $\text{cal}^{1/2} \text{ cm}^{-3/2}$ were taken from ref. [8].

out IP reagent (Fig. 1b). For FeL_2^0 , ion-pairing has no effect on $\log k = f(\delta)$ and slopes for FeL_2^0 in the presence or absence of ion-pairing reagent (NaClO_4) were similar to each other and this confirms the choice of the FeL_2^0 chelate as reference molecule in such LC conditions.

3.3. Influence of pH of the eluent

It is well recognized that in the silica-based LC phase different silanol groups are present [32]. It was found that two different pK_a values of silanol were found on a bare silica as well as on a RPLC phase [6] and a particular pK_a of silanol at the bare silica is different from appropriate pK_a found on a RP bonded silica [2]. In the present work, the influence of pH of an eluent on k of both ($\text{CoL}_2^+/\text{FeL}_2^0$) chelates using the RPLC columns were examined (Fig. 2). The results obtained here indicates that in

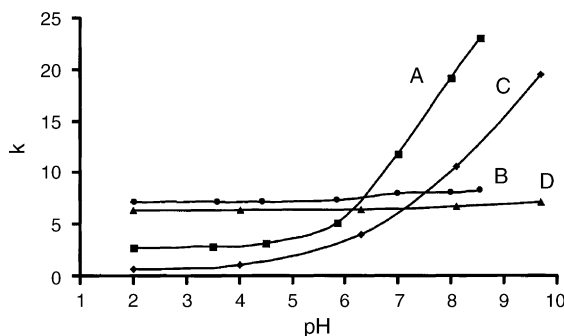


Fig. 2. Influence of pH on retention of CoL_2^+ and FeL_2^0 chelates—two different columns with the same eluent (MeOH:water, 80/20, v/v, containing $5 \times 10^{-4} \text{ mol l}^{-1} \text{ NaClO}_4$). Labels, (A and C) denote CoL_2^+ , (B and D) FeL_2^0 ; columns: Nucleosil, 100-5 C18 e.c. (A and B), Zorbax, SB-C18 (C and D).

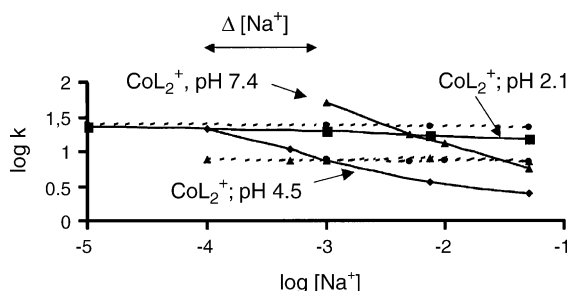


Fig. 3. The relationship between $\log k$ of (CoL_2^+ , FeL_2^0) and $\log[\text{NaCl}]$ being in the eluent. The solid lines denote retention of CoL_2^+ at different pH, whereas dashed lines denote retention of FeL_2^0 at pH 7.4 and 4.5 (bottom) or L^0 at pH 2.1 (upper). The label $\Delta[\text{Na}^+]$ denotes the concentration of NaCl in eluent necessary to start the ion-exchange process, and this reflects the column ion-exchange capacity at different pH.

the pH range 2–4.5, the hydrophobic interaction between both chelates and the RP phase is found to be the main retention mechanism as the slope of k versus pH for both Fe(II)L_2^0 and Co(III)L_2^+ is the same. Above pH 4.5, the hydrophobic interaction remains the main factor governing the retention of Fe(II)L_2^0 chelate, whereas k of Co(III)L_2^+ is the superposition of both hydrophobic and the continuously increasing the ion-pairing ($\text{SiO}^- \cdots \text{CoL}_2^+$) interactions between the CoL_2^+ and the LC phase. Thus, the k of Fe(II)L_2^0 reflects the non-specified interaction common for both chelates, whereas the increasing dissimilarity between k (CoL_2^+) and k (FeL_2^0) reflects an increase in the specific chromatographic effect ($\text{SiO}^- \cdots \text{CoL}_2^+$) due to an increase in the pH of the eluent.

3.4. Analysis of $\log k$ versus $\log [C^+]_m$ plot

Analysis of k of a test solute in function of the counterion concentration in eluent ($[C^+]_m$) is accepted for the discrimination of LC retention mechanism [3]. In the previous Section it was postulated that k of CoL_2^+ versus pH of the eluent can be divided onto two parts. In the pH range of the eluent up to 4.5 the hydrophobic separation mechanism dominates, whereas for pH above 4.5 superposition of hydrophobic and ion-exchange mechanisms is governed the CoL_2^+ retention. In this section, curves $k = f[C^+]_m$ were registered in the first pH range at the pH (2.1 and 2.9) as well as in the second pH range (pH 4.5 and 7). As the counterion $[C^+]_m$, the Na^+ (as NaCl) was chosen as the Cl^- anion that has the lowest ion-pairing properties among (ClO_4^- , SCN^- , Br^- and Cl^-) anions [23]. Careful study of Fig. 3 shows (the lowest, middle and highest pH were shown in the figure for clarity) that the obtained regressions and slopes for the curves $\log k = f(\log[\text{Na}^+])$ for CoL_2^+ were as follows: $\log k = -0.048 \log[\text{Na}^+] + 1.13$, $R^2 = 0.9518$ (pH 2.1); $\log k = -0.07 \log[\text{Na}^+] + 1.15$, $R^2 = 0.971$ (pH 2.9), $k = -0.353 \log[\text{Na}^+] - 0.132$, $R^2 = 0.9789$ (pH 4.5); $\log k = -0.558 \log[\text{Na}^+] + 0.007$, $R^2 = 0.9946$ (pH 7). Thus, taking the relation $\log k = f[C^+]_m$ into account, an increase in the slope for CoL_2^+ chelate due to an increase in pH of the eluent was observed and no-influence of pH of the eluent on the slope for FeL_2^0 or L^0 was noticed. In the present work the slope obtained for $\log k = f[C^+]_m$ for CoL_2^+ is higher than the slope reported for

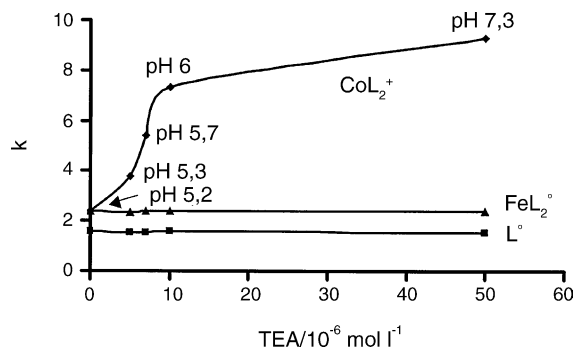


Fig. 4. Influence of triethylamine (TEA) on k of CoL_2^+ , FeL_2^0 and L^0 species examined in the present work. Column: Zorbax SB-C18; eluent: ACN:H₂O, 80:20, v/v containing NaClO_4 ($5 \times 10^{-4} \text{ mol l}^{-1}$) and TEA. The pH of the eluent is shown in the figure.

Ace or Alltima columns (with the help of quaternary amines) [3], thus the content of silanol group is expected to be higher at the Nucleosil phase than on these (Ace, Alltima) columns. Another possible explanation is about a different interaction of cobalt chelate than the test quaternary amine with a stationary phase and this is discussed in Section 3.12. The analysis of another relationship ($k = f(1/[\text{Na}^+])$) revealed that a decrease in an intercept with an increase in pH of the eluent was observed. Taking into account the criteria presented in ref. [3], such behavior points to the increasing contribution of ion-exchange process between CoL_2^+ and LC phase in the total retention. Fig. 3 shows some additional features of the experiment. It was found that for eluent (pH 2.1), the eluent with the lowest organic solvent content (MeOH, 65%) was sufficient for elution of both chelates (only RP mechanism), whereas eluent (pH 7.4) needs greater content of organic phase (MeOH, 80%) due to (RP + IC) mechanisms. Also, an increase in pH of eluent leads to the situation where the counterion $[C^+]_m$ concentration in eluent has to be higher to start the ion-exchange process (denotes as $\Delta[\text{Na}^+]$, Fig. 3), which points to the growing cation exchange capacity of the column with an increase in the pH of the eluent.

3.5. Modification of RPLC stationary phase by aliphatic R_3N amines

The tertiary aliphatic amines are used in chromatographic practice for screening of free silanol groups [32]. The proposed mechanism is the hydrogen bonding between free silanol group and R_3N amine ($\text{Si}-\text{O} \cdots \text{H} \cdots \text{NR}_3$) [32] and the amine concentration proposed for it is in the range of $10^{-3} \text{ mol l}^{-1}$. In the present experiment, the eluent based on MeOH or ACN was modified by addition of aliphatic R_3N amines. For this, R_3N amines, where $\text{R} = \text{CH}_3$, C_2H_5 , C_4H_9 , $\text{C}_{10}\text{H}_{21}$ were applied in the concentration range, up to $1 \times 10^{-4} \text{ mol l}^{-1}$ in the eluent. This experiment allows checking whether suppression of silanol groups has an effect on the retention of $\text{CoL}_2^+/\text{FeL}_2^0$ chelates. The obtained results (Fig. 4) shows that the fast increase in k of Co(III)L_2^+ due to the presence of R_3N in eluent ($>1 \times 10^{-5} \text{ mol l}^{-1}$) was observed. This is evidently an effect of the high pK_a of R_3N (e.g., pK_a 9.9, $\text{R} = \text{CH}_3$; pK_a 10.5, $\text{R} = \text{C}_2\text{H}_5$), which leads to the effective ionization of silanol

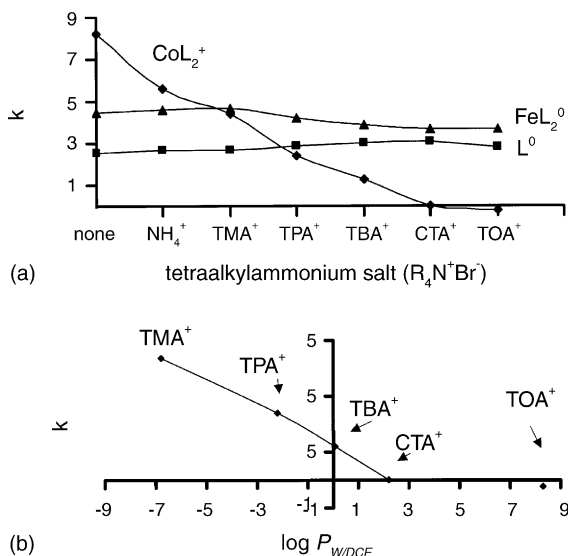


Fig. 5. (a) Influence of $R_4N^+Br^-$ salt on the retention of CoL_2^+ and FeL_2^0 chelates and L^0 ligand. (b) Relationship between k of CoL_2^+ and $\log P_{W/DCE}$ of $R_4N^+Br^-$ salts ($P_{W/DCE}$ = partition coefficient: water-1,2-dichloroethane). Concentration of $R_4N^+Br^-$ in the eluent $1 \times 10^{-3} \text{ mol l}^{-1}$. Column: Zorbax SB-C18, eluent: ACN/water 70/30, v/v (in the absence of R_4N^+ salt, 70/30, v/v ACN/water with $NaClO_4$, $5 \times 10^{-4} \text{ mol l}^{-1}$ was used).

groups, because the small amount of R_3N added to the eluent leads to drastic increase in the pH of the eluent (Fig. 4). This means that the pH effect described in Section 3.3 is dominating the $Co(III)L_2^+$ retention in the present experiment. However, taking into account that k of (FeL_2^0 or L^0) was unchanged due to an increase in the concentration of R_3N in the eluent it can be concluded that the chromatographic effect related to an interaction of free silanol groups with chelate ($SiOH \cdots ML_2$) has no influence on the retention of these species.

3.6. Modification of RPLC stationary phase by tetraalkylammonium salts ($R_4N^+Br^-$)

The tetraalkylammonium salts are able to screen the ionized silanols groups due to ion-pairing effect ($Si-O^- \cdots N^+R_4$) as well as free silanol groups due to hydrogen bonding ($Si-HO \cdots H-R_4N^+$) [32]. The amount of adsorbed ammonium compound on the RP phase depends on an alkyl chain length of R_4N^+ and can reach a maximum surface concentration of $2.5\text{--}3 \mu\text{mol m}^{-2}$ [33]. In the present work, the examined $R_4N^+Br^-$ salts were: TMA⁺, TPA⁺, TBA⁺, TOA⁺, CTA⁺, all bromide salts. The eluent based on MeOH or ACN solvents, containing $R_4N^+Br^-$ at constant concentration $1 \times 10^{-3} \text{ mol l}^{-1}$ was used. The eluent pH for all R_4N^+ was 6.3–6.5. It was found that a decrease in the retention of $Co(III)L_2^+$ was observed irrespective of the kind of R_4N^+ salt applied, whereas for the not charged species ($Fe(II)L_2^0$ and L^0) the retention was unchanged in this respect (Fig. 5). This is in agreement with the effect observed for $Co(III)L_2^+$ and $Fe(II)L_2^0$ chelates with azo ligands using several RP columns [27]. Using R_4N^+ salt in modification of RPLC phase two parameters are found to be important: (i) charge of nitrogen atom of R_4N^+ , and (ii) hydrophobicity of R_4N^+ . Charges of R_4N^+ used in the present

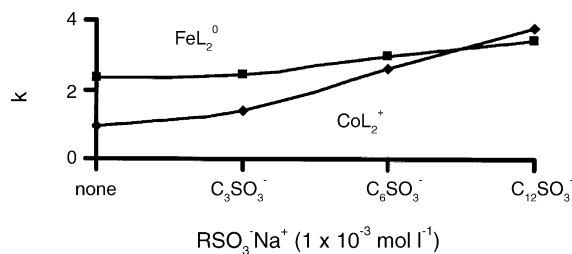


Fig. 6. Influence of sulphonate salts on retention of CoL_2^+ and FeL_2^0 . Column: Nucleosil 100-5 C18 e.c., eluent: ACN:water 80:20, v/v and $RSO_3^-Na^+$ ($1 \times 10^{-3} \text{ mol l}^{-1}$), (in absence of $RSO_3^-Na^+$ salt, 80:20, v/v ACN:water with $NaClO_4$, $1 \times 10^{-3} \text{ mol l}^{-1}$ was used).

work were calculated using the PM3 wavefunction ($q=0.62$, TMA⁺; $q=0.57$, TPA⁺; $q=0.58$, TBA⁺) and partition coefficients P of transfer of bromide quaternary ammonium salt from water to 1,2-dichloroethane, used as the representation of R_4N^+ hydrophobicity, were taken from ref. [34] ($10^3 P_{W/DCE}$, 1.6×10^{-7} TMA; 6.2×10^{-3} TPA; 1.16 TBA, 158 CTA). It can be concluded that a decrease in k of $Co(III)L_2^+$ is related to hydrophobicity of R_4N^+ , as the charges of all R_4N^+ are similar and even slightly lower for higher R. The correlation between k of CoL_2^+ and $\log P_{W/DCE}$ of R_4N^+ salts is found to be excellent ($k = -0.4778 \log P_{W/DCE} + 1.21$ ($R^2 = 0.9938$)) and is presented in Fig. 5b. There is a huge disparity between $\log P_{W/DCE}$ for TMA⁺ and TOA⁺ and the LC effect due to the presence of TOA⁺ is not expressed properly. In addition, the effect of the type of a LC phase on a change of k of CoL_2^+ (Nucleosil > Zorbax) and effect of the eluent (Δk , ACN > Δk , MeOH) were also observed.

3.7. Modification of RPLC stationary phase by alkylsulphonate salts ($RSO_3^-Na^+$)

It was expected that, the modification of RPLC stationary phase by an addition of $RSO_3^-Na^+$ salt to the eluent leads to a reverse effect to the effect which was observed in previous section. This is consistent with ref. [35], where the RPLC phase modified by alkylsulphonate salts is described as cation-exchange LC phase. The examined $RSO_3^-Na^+$ salts were $R = C_3H_7$, C_6H_{13} and $C_{12}H_{25}$ and these were dissolved in the eluent ($c_{RSO_3Na} = 1 \times 10^{-3} \text{ mol l}^{-1}$). Fig. 6 shows the typical effect due to the modification RPLC phase by eluent containing $RSO_3^-Na^+$ salt. As in the previous section, the change in retention is observed for $Co(III)L_2^+$ chelate but to a much lesser extent. Also the increase of k of FeL_2^0 due to the presence of $RSO_3^-Na^+$ salts in the eluent was observed, especially for higher R (C_6 , C_{12}) salts. This means that the coverage of the phase by $RSO_3^-Na^+$ salt can be different than that by the R_4N^+ salt as the former clearly leads to an increase in hydrophobicity of the RPLC phase. It should be pointed out that, like in previous the section, the k of $Co(III)L_2^+$ does rely on R of $RSO_3^-Na^+$ salts as the negative charge of $RSO_3^-Na^+$ is found to be similar for all $RSO_3^-Na^+$. In addition, the degree of an increase in retention of $Co(III)L_2^+$, thus the degree of RP phase modification, does depend on the kind of RP phase examined here (Δk of $Co(III)L_2^+$ on Zorbax > Nucleosil).

3.8. Modification of RPLC stationary phase by zwitterionic salt (SB-12)

Zwitterionic salts ($R_1N^+-R_2-SO_3^-$) are an interesting group of compounds used in modification of the RPLC phase. The dynamic coating of the RP phase allows for simultaneous retention of cationic and anionic species. The process has already been characterized in the ref. [36]. In the present work eluents (MeOH/water, 80/20; ACN/water, 80/20) containing $5 \times 10^{-4} \text{ mol l}^{-1}$ NaClO_4 and SB-12 in the range of $0-6 \times 10^{-3} \text{ mol l}^{-1}$ were applied. It was established that retention of both Co(III)L_2^+ and Fe(II)L_2^0 were not influenced by SB-12 concentration in the eluent, which indicates that such modification did not lead to formation of ion-exchange properties of RPLC phase. Such observation is in agreement with ref. [37], where MeOH/water eluent containing SB-12 was applied to modify the Spherisorb ODS phase and no ion-exchange properties of such prepared phase was detected (one of the explanations is presented in ref. [37]).

To summarize the results presented in Sections 3.1–3.8, which were based on modifications of RPLC phase, it can be concluded that the $\text{Co(III)L}_2^+/\text{Fe(II)L}_2^0$ system can be an interesting test probe for testing the LC chromatographic effects and the obtained results were in agreement with many publications presented in literature. In the following sections the proposed test method was applied for establishing chromatographic effects on the RP phase, which have not been reported in the literature so far.

3.9. Hydrogen bond formation between stationary phase and methanol

The influence of a solvent (ACN versus MeOH) on retention of chelates has already been observed in the previous work [14], where retention of Fe(NN)_3^{2+} chelates ($\text{NN} = 1,10\text{-phenanthroline}$ and its derivatives) was examined using C_{18} RPLC phase with ACN:water or MeOH:water eluents. It was observed that for the former eluent the order was $k_{\text{chelates}} < k_{\text{ligands}}$, whereas for the latest one, the reversed order, $k_{\text{chelates}} > k_{\text{ligands}}$ has been observed. Also, in the presence of SDS ($\text{C}_{12}\text{SO}_4^-\text{Na}^+$) in an eluent, mixed retention mechanism for these chelates (RP+SEC) has been observed [14].

The difference in interaction of ACN or MeOH with the RPLC stationary phase has already been discussed [32]. Generally, it was pointed out that MeOH is prone to the formation of hydrogen bonding with silica-based LC phase, whereas ACN does not possess such an ability. Both solvents possess similar permittivity ($\epsilon_{\text{MeOH}} = 32.7$, $\epsilon_{\text{ACN}} = 37.5$) but different values of Abraham's hydrogen bond acidity ($\alpha_{\text{MeOH}} = 0.43$, $\alpha_{\text{ACN}} = 0.07$) and Abraham's hydrogen bond basicity ($\beta_{\text{MeOH}} = 0.47$, $\beta_{\text{ACN}} = 0.32$) [38]. In addition ion-pairing or ion-exchange depends on solvents properties, e.g., dielectric constant. An example is the observation of values of association constant (K_{assn}) of TBA^+ with Br^- ($K_{\text{assn}}/\text{solvent dielectric constant}$): 103/19 and 0.6/78 [39], thus the ion association is favorable in low polarity solvents.

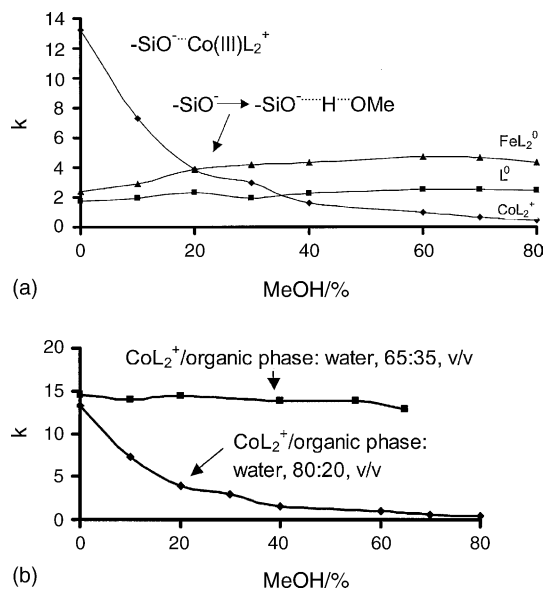


Fig. 7. The application of CoL_2^+ chelate to the determination of degree of interaction of methanol with RPLC stationary phase (Zorbax, Eclipse XDB-C18). Upper, (a) the strong interaction of CoL_2^+ with stationary phase is observed using the eluent 20:80, v/v, water:organic phase (ACN + MeOH) with the content of MeOH up to 20% (v/v). At the MeOH concentration (>20%) the interaction of CoL_2^+ with stationary phase is replaced by the interaction of MeOH with stationary phase. It should be noted that k of FeL_2^0 and L^0 were insensitive about changes in eluent composition, which indicates that CoL_2^+ is a specific test probe. (b) Comparison of retention of CoL_2^+ using two different eluents, which indicates that the specific interaction between organic solvent and stationary phase (20:80, v/v water:organic phase eluent) is replaced by water–stationary phase interaction when eluent 35:65, v/v water:organic phase (ACN + MeOH) was applied.

In the present work the retention of both chelates using pure solvents (MeOH and ACN) was investigated (Section 3.2, Table 2). Two conclusions can be drawn from the experiment.

The first conclusion is given in Section 3.2 (relative content of silanol groups on a particular phase). The second conclusion is that the retentivity of Co(III)L_2^+ using both pure solvents were: $k(\text{ACN eluent}) > k(\text{MeOH eluent})$ (Table 2). As both solvents (MeOH versus ACN) possess similar values of dielectric constant (ϵ), values of association constant ($\text{SiO}^- \cdots \text{Co(III)L}_2^+$) in both solvents should be similar in respect of ϵ . Therefore, the reduced retention of Co(III)L_2^+ in MeOH is due to influence of the hydrogen bonding between the MeOH and the LC phase ($\text{SiO}^- \cdots \text{H} \cdots \text{OMe}$) on ion-pairing ($\text{SiO}^- \cdots \text{Co(III)L}_2^+$), because of higher α value for MeOH (Abraham's hydrogen bond acidity). The following experiments were performed with the aim of confirming the observation. In the experiment (Fig. 7a), the retention of all species (Co(III)L_2^+ , Fe(II)L_2^0 and L^0) was observed using the eluent: 20% water and 80% mixture of organic solvents (ACN + MeOH, in various proportions). At the beginning the ACN/water (80:20, v/v) as eluent was applied following by gradual increase in the MeOH content in the eluent. It can be concluded that the initial good conditions for ion-pairing ($\text{SiO}^- \cdots \text{Co(III)L}_2^+$) in ACN gradually diminish owing to the presence of hydrogen bonding ($\text{SiO}^- \cdots \text{HO} \cdots \text{Me}$) caused by an increased concentration of MeOH in the eluent. This fact is confirmed by next experiment, where the eluent 35:65 (v/v)

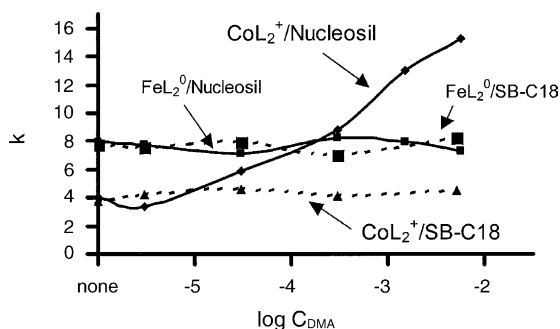


Fig. 8. Modification of RPLC phase by aromatic amine (DMA—dimethylaniline). Solid lines, Nucleosil 100-5 C18 e.c.; dashed lines, Zorbax SB-C18. Eluent: MeOH/water = 80/20, v/v; NaClO₄, 4×10^{-3} mol l⁻¹.

water:organic phase (ACN + MeOH, in various proportions) was applied (Fig. 7b). It was found that in this case the k of CoL_2^+ was at the same level, in spite of the relatively large concentration of MeOH in the eluent. Thus, the influence of MeOH on interaction ($\text{SiO}^- \cdots \text{Co(III)L}_2^+$) (Fig. 7a) was hidden due to the strong interaction between water and the stationary phase.

To summarize the present experiment, it can be proposed that CoL_2^+ is sensitive toward hydrogen bonding between a solvent and a stationary phase and the influence of the parameter (α Abraham's hydrogen bond acidity) can be detected in real chromatographic conditions.

The second conclusion from the experiment is that the interaction of MeOH with RPLC phase is stronger than ACN with RPLC phase. Beginning with the methanol-based eluent and increasing contents of the ACN in the eluent, all the time the retention was similar to that obtained at the beginning, despite the high content of ACN in the eluent. On the other hand, starting from ACN-based eluent and increasing the content of MeOH in the eluent the retention was fast adapting to current conditions.

3.10. Modification of RPLC stationary phase by aromatic amines tested by present method

Aromatic amines are useful in detection of silanol activity in RPLC phase [32]. Aromatic amines have pK_a lower than aliphatic ones, thus the pH effect due to the presence of amine in the eluent, reported in the Section 3.5, could not be observed. The application of aromatic amines (pyridine, aniline and dimethylaniline), as eluent additive, in modification of the RP phase leads to the formation of two chromatographic effects (Fig. 8). First, an increase in k of Co(III)L_2^+ in relation to k of Fe(II)L_2^0 was observed due to an increase in the concentration of amine in the eluent (for all concentrations of amine the pH of the eluent was similar (pH 5.2–5.5)). Second, the effect was observed on the RPLC phase with the highest content of silanol group (Nucleosil), whereas on the phase (Zorbax) with the lowest content of silanol group the effect was not observed. The observed effect, is not an ion-pairing (amine/ CoL_2^+ ; charge of nitrogen atom in amines is low, e.g., pyridine, $q = -0.08$, PM3), because such effect should be independent from the phase used. Two mechanisms can be proposed: (i) the partition of an aromatic amine to C_{18} phase can induce the formation of areas with the negative

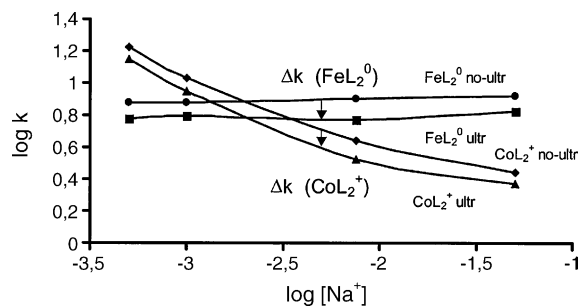


Fig. 9. Influence of ultrasonic field on retention of $\text{CoL}_2^+/\text{FeL}_2^0$ chelates reflected by $\log k = \log[\text{Na}^+]$ curve (see Section 3.4). Column: Nucleosil 100-5 C18 e.c., eluent: MeOH:water, 80/20 containing NaCl, pH 4.5. Descriptions: “no-ultr” denotes k of chelate in the absence of ultrasonic field and description ‘ultr’ in the presence of the field.

charge due to the fact that amines are basic compounds and differences in structure of these RP phases may induce such effect, and (ii) it is possible to assume that aromatic amines bound to RP phase are protonated by the most active silanol groups, which leads to formation of ionized silanol group ready for interaction with a cationic chelate. Although the effect is well established, it seems to be that additional experiments are needed for adequate explanation of the effect.

3.11. Influence of ultrasonic field on retention of $\text{CoL}_2^+/\text{FeL}_2^0$ chelates

The ultrasound field has been recently introduced into the chromatographic practice [40,41]. Also the field was applied in order to differentiate interactions of the metal ion with surfactants [28]. The aim of the present experiment is applying the ultrasonic field in order to differentiate separation mechanisms with the help of $\text{CoL}_2^+/\text{FeL}_2^0$ chelates as molecular probes. In the experiment the relationship $\log k = f(\log[\text{Na}^+])$ was applied (Section 3.4) as the relation is allowing estimation of the degree of (hydrophobic versus ion-exchange) interaction of a solute with a LC phase. Fig. 9 shows the relationship between $\log k = f(\log[\text{Na}^+])$ for CoL_2^+ and FeL_2^0 on Nucleosil phase, in the presence or absence of the ultrasonic field. As was stated previously, the former chelate is sensitive to ion-exchange phenomena, whereas the latest one is sensitive to non-specific interaction on the RPLC phase. It was observed (Fig. 9) that k for both FeL_2^0 and CoL_2^+ is shifted to the same extent (denoted as Δk), which indicates that for both chelates a common factor influences the retention of both probes to the same extent. The next observation is that the efficiency of the field on k of chelates is related to a decrease in back pressure (pressure of the eluent in the absence of the field minus pressure in the presence of the field) noticed for a particular eluent. Taking the eluent (80:20, organic phase:water) into account, in ACN:water eluent, where the decrease in back pressure is much lower than in MeOH:water eluent, the effect Δk , due to the presence of ultrasonic field is significantly smaller. However, taking into account the value of Δk (Fig. 9) it can be concluded that ultrasound effect in affordable LC conditions is related with hydrophobic interaction between the probe and the LC phase and not with ion-exchange, as the

slope for $k = f[\text{Na}^+]$ for CoL_2^+ was left the same and Δk is related with a decrease in k of FeL_2^0 .

3.12. Rationalizing the observed effects with the help of molecular computation—comparison of cationic probes

In this section we would like to compare cationic probes used for characterization of ion-exchange properties of a RPLC phase by means of the molecular computational methods. First, charge of metal ion in the chelate ($q = +0.45$, Co(III)L_2^+ by DFT method (B88-LYP functional with DZVP basic set) was established and this is an additional argument for treating the Co(III)L_2^+ chelate as the cationic probe. Also, structures (octahedral) and molar volumes (Van der Waals volume: $\text{vdW}_{\text{CoL}_2} = 1646 \text{ \AA}^3$ and $\text{vdW}_{\text{FeL}_2} = 1648 \text{ \AA}^3$) of both chelates obtained by *HyperChem* are found to be similar, which indicates that the structurally dependent interactions between these chelates and the LC phase should be similar. In this section the quaternary amines used for testing the LC stationary phases: (i) bretylium [2], (ii) recently introduced berberine [42], along with (iii) tetramethylammonium (TMA^+) cation as an example of the simplest quaternary amine, were analysed and compared with the cationic CoL_2^+ . The obtained charges of these amines are higher than the charge of CoL_2^+ chelate (nitrogen atom, PM3 method: TMA^+ (0.62), bretylium (0.60), berberine (0.50)). Thus, taking into account the Coulombic interaction between the probe and the anionic site, the cationic quaternary amines are advantage probes. However, additional factors are important for the RPLC separation of these probes. Both, the change in a charge of a probe, due to the presence of various solvents and an influence of one interaction (between probe and a hydrophobic site on a RP phase) on the second interaction (between probe and a charged site on a phase), should be considered. The former was detected by Ampac program, which has built-in the SM [38] package allowing modeling in various solvents. It was established that the solvents have no influence on the charged quaternary amine, having the symmetry (point group T_d , TMA^+) and this is due to the lack of dipole moment of the molecule. For amines without point symmetry (e.g., bretylium, dipole moment 11.4 D) the decrease in charge of nitrogen atom of amine is evident (5%, SM 5.2 or 10%, COSMO methods; from water to butanol). The second factor, the influence of the non-specified interaction on the specified one is explained by taking TMA^+ as an example. The combined effect (ion-exchange + hydrophobic interactions in RPLC) has recently been postulated [3] and the effect (hydrophobically assisted ion-exchange process) is claimed to be the dominating process in RPLC separation for charged solutes. In the present theoretical experiment, the energy effects being a result of interaction of TMA^+ with anionic site were determined (Table 3) and compared with energy gain due to interaction of TMA^+ with hydrophobic surface. In this experiment the molecular mechanic (mm+/*HyperChem*) was applied. The energy effect due to TMA^+ interaction with anionic site (e.g., Cl^- (*HyperChem*)) is presented in Table 3. A similar problem (the interaction of NH_4^+ with BF_4^-) has been reported [43]. From Table 3 it can be concluded that on bringing TMA^+ closer to the anionic site the bigger distortion of TMA^+ geometry was observed, which

Table 3

The influence of TMA^+ interaction with Cl^- on the TMA^+ parameters

$\text{N}^+ \cdots \text{Cl}^-$ distance (Å)	Angle C–N–C (°) ^a	Charge of N atom ^b	Energy (kcal/mol) ^c
3.2 (optimal)	109.5	0.71	–52.1
2.5 ^d	114.8	0.76	–26.9 ($\Delta E = +25.2$)
2.1 ^d	118.4	0.81	–19.2 ($\Delta E = +32.9$)

^a (mm+, *HyperChem*), the angle 108.5° was determined for the free TMA^+ cation.

^b The charge of N atom was determined (PM3) at the appropriate TMA^+ geometry. Both AM1 and PM3 (*HyperChem*) were indicated an increase in N charge due to dipper distortion of TMA^+ geometry; values obtained by PM3/*HyperChem* are close to these obtained by AM1/*Ampac*.

^c Energy of molecule at the appropriate geometry (mm+, *HyperChem*). The (ΔE) denotes energy of the molecule at its distorted geometry minus energy of molecule at its optimal geometry.

^d Restrained distance.

results in an increase in the charge of the nitrogen atom ($\sim +14\%$, at the reduced $\text{N}^+ \cdots \text{Cl}^-$ distance of about 1.1 Å). The second effect (Table 3), an increase in TMA^+ energy due to distortion of TMA^+ geometry was observed, which shows that distortion of TMA^+ geometry is an energy consuming process. In such a situation it is necessary to compare the energy gain for TMA^+ due to hydrophobic interaction with the energy required for distortion of TMA^+ geometry, due to binding of TMA^+ to negative site. In other words, if energy gain due to hydrophobic interaction is small in relation to the energy of TMA^+ at a given geometry (due to binding of TMA^+ to negative site), the ion-pairing process should dominate, whereas reversed energy proportion indicates that the hydrophobic interaction dominates over the electrostatic one with some consequences. The interaction of TMA^+ with the flat surface containing carbon atoms, designed by multiplying the anthracene skeleton (rectangle, $7 \times$ skeleton), was assumed as the equivalent of hydrophobic interaction for TMA^+ . The obtained binding energy, $\Delta E = E_{\text{complex}} - (E_{\text{TMA}} + E_{\text{flat surface}})$ for TMA^+ was negative (-38 kcal/mol) and the energy gain is higher than the energy required for distortion of TMA^+ geometry due to its binding to the negative site as close as 2.1 Å ($+33 \text{ kcal/mol}$, Table 3). Thus, the binding of TMA^+ to the hydrophobic site can reduce the distance ($\text{N}^+ \cdots$ charged site) to be much shorter than the optimal distance. This situation denotes the distortion of the TMA^+ geometry and change in TMA^+ charge ($\sim +14\%$, at the reduced $\text{N}^+ \cdots \text{Cl}^-$ distance), factor which is important in ion-exchange process. Taking into consideration the facts presented above the rest of probes were analysed. Shortly, these results are presented in the following manner: $\text{N}^+ \cdots \text{Cl}^-$ optimal distance/energy of the molecule at this geometry, followed by the restrained $\text{N}^+ \cdots \text{Cl}^-$ distance/ $(\Delta E = \text{energy of a molecule at its distorted geometry minus the energy of a molecule obtained at its geometry, where } \text{N}^+ \cdots \text{Cl}^- \text{ distance is optimal})$ and finally, the value of the energy due to hydrophobic binding of the molecule to the flat carbon surface (all energies in kcal/mol): (i) bretylium, 3.4 Å/ -159 , 2.6 Å/ $(\Delta E = +17)$, 2.1 Å/ $(\Delta E = +49)$, binding energy = -124 ; (ii) berberine, 2.6 Å/ -55 , 2.1 Å/ $(\Delta E = +15)$, 1.7 Å/ $(\Delta E = +40)$, binding energy = -24 ; (iii) CoL_2^+ , ($\text{Co}^+ \cdots \text{Cl}^-$) 2.97 Å/ -78 , 2.5 Å/ $(\Delta E = +2.5)$, 2.1 Å/ $(\Delta E = +22)$, binding energy is positive

(+19). Taking these data into account, the distortion of a molecule parameters (geometry, charge), due to molecule interaction with a negative site, which is enforced by hydrophobic binding, is more probable for bretylium than for berberine or CoL_2^+ chelate. This leads to another important conclusion that parameters of a quaternary amine can be conditional during RPLC separation, and this can be an additional argument that concerns the problem with the low correlation between results obtained by various testes, based on protonated (quaternary) amines [7]. Regarding the CoL_2^+ chelate, three facts are important. First, the Co(III) configuration $t_{2g}^6 e_g^0$ indicates the well-separated areas of low and high charge densities around the metal ion and such a charge distribution is achieved even when the chelate geometry is distorted (e.g., John-Taller effect). Second, for CoL_2^+ chelate, no change in λ_{max} of CoL_2^+ was observed in the present work, in solvents (20–100% of ACN or MeOH; in solvent/water mixtures) (lack of solvatochromism), which points to stable CoL_2^+ geometry, in spite of the presence of media possessing various properties, e.g., dielectric constant. Finally, CoL_2^+ is not an acid–base probe, so the problems with pH, mentioned for amines (e.g., benzylamine [7]) do not refer to the chelate as the LC probe.

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