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Towards multimodal HPLC separations on humic acid-bonded aminopropyl silica: RPLC and LEC behavior

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ABSTRACT

In the present study, metal binding property of humic acid (HA) was successfully adapted to the ligand-exchange concept, and metal-loaded immobilized humic acid was used as a ligand exchanger stationary phase for separation of some nucleosides. Humic-acid bonded aminopropyl silica (EC–HA–APS) was turned into ligand exchanger forms by loading aqueous solutions of Cu^{2+}, and Co^{2+} to the column (4.6 × 150; as mm) packed with EC–HA–APS. Metal ion solutions were loaded to the column in a stepwise manner where the concentration of metal ion solution being loaded to the column was increased gradually between 5 and 100 mM. The progress of metal loading process was monitored via the breakthrough curves propagated stepwise. Ligand-exchange chromatography (LEC) studies were performed on an HPLC system, and chromatographic behaviors of the studied nucleosides (i.e. uridine, Urd; thymidine, Tyd; cytidine, Cyd; adenosine, Ado; and guanosine, Guo) were investigated on Cu^{2+} and Co^{2+} loaded forms of the EC–HA–APS (*Cu*-EC–HA–APS and *Co*-EC–HA–APS). Effect of mobile phase composition, temperature, and the type of metal ion loaded to the column on the retentive behaviors of the compounds was studied, in detail. The studied solutes exhibited mixed-mode RPLC/LEC behavior on the stationary phase. Metal-loaded column (*M*-EC–HA–APS) was easily regenerated into its original form, EC–HA–APS, with 98 ± 2% metal recoveries, by using aqueous mixture of EDTA + NH_3 at pH = 7.5. Thus, the stationary phase exhibited a high flexibility between RPLC and LEC modes. This property, also, made it possible to convert the stationary phase into various ligand exchanger forms by loading different metal ions. Hence, capacity and selectivity of the stationary phase towards the studied species was manipulated easily by loading different metal ions to the stationary phase. Baseline separation for the studied species was achieved on *Cu*-EC–HA–APS and *Co*-EC–HA–APS and some differentiations were observed in capacity and selectivity, depending on the type of metal loaded. Thus, being as the first endeavor on usability of immobilized HA as a ligand exchanger stationary phase, the present study is believed to be useful to understand multifunctional character of HA-based solid/stationary phases.

1. Introduction

Despite the recent developments in detection techniques, qualitative and quantitative analyses of the components in a sample seem not to be possible, unless a supplementary separation method has been used before detection. HPLC is one of the most popular techniques being used for this target, because of its versatile application range in separation of various types of compounds. The performance of the technique is highly dependent on the properties of the stationary phase used, and so applicability of various types of materials as stationary phase has been investigated [1,2]. However, commercially available stationary phases are usually suitable for a special application (e.g. RPLC, NPLC, HILIC, etc.). Application of different modes of HPLC on the same stationary phase is an interesting area, and recent developments in design of stationary phases exhibiting multifunctional character are promising [1].

LEC has been, first, conceptualized by Helfferich [3]. The method is, classically, based on the distribution of analyte (which bear electron-donor atoms with lone electron pairs; i.e. ligand) between mobile phase (which contains molecules exhibiting ligand character) and a metal ion-immobilized stationary phase. Metal immobilized to the stationary phase contains vacant orbitals, and thus formation of (kinetically) labile metal–ligand bonds between analyte and metal ion held by the stationary phase is the main principle of the method. This is a reversible process, so that analyte bonded to the immobilized metal can be exchanged by another ligand in mobile phase (and vice versa), and this leads migration of analyte through the column. The ligands are separated according to their ability to enter into the coordination ion sphere of

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metal held by the stationary phase. With increasing ligand character of the mobile phase, analytes elute faster. Hence, selectivity of a ligand exchanger depends upon the electron-donor properties of analytes and mobile phase molecules as well as the type of metal immobilized to the stationary phase. Moreover, type and percentage of organic modifier in the mobile phase, and temperature can affect the retentive behaviors. For this reason, in LEC, there are many experimental variables that can be used to manipulate the degree of interactions in the system, and thus to improve the chromatographic separation [4].

The selectivity in LEC is, usually, higher compared to that observed in separation processes based on ion-exchange and physical adsorption. So, LEC is very popular to separate ligands as well as the compounds with very similar properties, such as geometric and positional isomers, homologues, isotopes, and optical isomers [4]. The efficiency of LEC is highly affected by the binding stability of the metal immobilized to stationary phase, and the column performance, usually, tends to diminish in course of time due to the metal leakage from the stationary phase. However, metal leakage is inevitable for most of the ligand exchangers, and this situation necessitates regenerating the ligand exchanger by loading metal ion to the stationary phase, occasionally [5].

Humic acid (HA) binds heavy metal ions through various mechanisms, such as adsorption, ion-exchange and complex formation, depending on the conditions. Comparing with some common complexing agents (such as EDTA and ammonia), HA interacts with heavy metal ions moderately [6]. So, metal bindings to HA can be evaluated as (*i*) sufficiently tight in aqueous media, and (*ii*) sufficiently weak to strip the metal ion in presence of a complexing agent. Owing to this property, HA-immobilized materials may act as flexible ligand-exchange stationary phases.

Despite the extensive knowledge on HA-metal ion interactions, there appears no study that deals with applicability of HA-based materials as ligand-exchange stationary phase in the literature. Carboxyl groups are highly preferred functionalities in the stationary phases used in LEC [7], and it is well known that these groups present within the structure of HA, abundantly. The studies dealing with HA-immobilized materials mainly concentrate on RPLC and HILIC behavior [8–10]. We have, recently, intensified on the stationary phase characteristics of humic acid (HA)-bonded aminopropyl silica (EC–HA–APS), and both RPLC and HILIC behaviors of this material towards some nucleosides and nucleobases have been proven [11]. Further studies showed that EC–HA–APS could also be used as a ligand-exchange chromatography (LEC) stationary phase by loading a suitable metal ion. In this sense, the present study has been devoted to ligand exchanger behavior of the metal-loaded EC–HA–APS (*M*-EC–HA–APS) in order to represent the applicability of the third mode of HPLC on EC–HA–APS. EC–HA–APS was turned into Cu^{2+} or Co^{2+} loaded forms (*Cu*-EC–HA–APS and *Co*-EC–HA–APS), and ligand exchanger behavior was investigated on two different *M*-EC–HA–APS forms. Some nucleosides were the test solutes used throughout the study.

2. Experimental

2.1. Chemicals

All the chemicals used were of analytical reagent grade or HPLC grade, and supplied from Merck, Fluka, Sigma and LabScan. Aldrich humic acid in sodium form (NaA) was purified and converted into its protonated form (HA) before use. Aminopropyl silica (APS; 15–35 μm particle size; ~9 nm pore size; Fluka) was used as a solid support in immobilization of HA. Immobilization and end-capping processes were performed in dimethylformamide (DMF; included <0.01% water and stored on molecular sieves; Fluka). Aqueous solutions of Cu^{2+} and Co^{2+} were prepared from their respective nitrate and chloride salts, respectively. Those solutions were used to turn EC–HA–APS to *M*-EC–HA–APS. The studied nucleosides (i.e. uridine, Urd, thymidine, Tyd, cytidine, Cyd, adenosine, Ado, and guanosine, Guo) were supplied from Sigma, and their test solutions were prepared in methanol (MeOH)-Water mixture. MeOH (LabScan) and acetonitrile (MeCN; LabScan) were the organic modifiers used in HPLC analyses. In some experiments, aqueous solutions of ammonia (Merck), sodium acetate (NaAc; Merck), and sodium chloride were used in the mobile phase. All the chemicals were used without further purification, and ultra-pure water (0.059 μS cm^{-1}) was used throughout the experiments.

2.2. Immobilization of HA, and characterization

Purified HA was immobilized to APS through a slightly modified form of the method proposed in Ref. [12]. In this method, HA is thought to be immobilized via amide-bond formation mechanism, yielding HA–APS. Residual $-NH_2$ groups remained on APS were acetylated (*end-capping*) in DMF medium by addition of acetyl chloride drop by drop. The product (EC–HA–APS) was rinsed successively with DMF, dichloromethane (Merck) and acetone (Merck) till colorless. The product was dried at 105–120 °C, and afterwards stored for further use [11].

HA, APS, HA–APS and EC–HA–APS were characterized in terms of elemental analysis, FTIR, potentiometric titrations, thermogravimetric analyses, surface charge characteristics, contact angle measurements and stability tests.

Elemental analyses were performed in Middle East Technical Universtiy, and C, N, H, and S compositions of APS, HA, HA–APS and EC–HA–APS were determined directly using LECO, CHNS-932 equipment. Composition of O element in HA was calculated by subtracting C, N, H, and S elemental compositions, and ash content determined by thermogravimetry from the total mass of HA. Amount of HA bonded to APS was calculated on the basis of C/N ratio obtained for APS, HA and EC–HA–APS.

FTIR analyses were performed for APS, HA, HA–APS and EC–HA–APS by using Pelkin-Elmer (Spectrum 100 model) instrument, and the spectra were recorded between 4000 and 400 cm^{-1} range. The spectra were recorded directly using solid materials, and thus KBr pellets were not prepared. FTIR spectra were also recorded for Cu^{2+} and Co^{2+} loaded EC–HA–APS; *Cu*-EC–HA–APS and *Co*-EC–HA–APS, respectively. So, EC–HA–APS was brought into interaction with 0.1 M metal ion solutions for 1 h, and then the solid phase was rinsed thoroughly with water to remove unbounded and weakly bonded metal ions from the surface of EC–HA–APS. After drying the products at 105–120 °C, FTIR spectra were recorded on the same instrument.

Number of carboxyl and phenolic hydroxyl groups in HA was determined through direct potentiometric titration of solid HA in NaCl solutions having different concentrations (0.01; 0.05 and 0.10 M). In titrations, aqueous solution of NaOH (~0.100 M) was used as titrant. Number of –COOH and phenolic –OH groups was calculated from the titration curves according to the method proposed in Refs. [11,13–15].

Thermogravimetric (TG) and differential thermogravimetric (DTG) analyses were performed for APS, HA, HA–APS and EC–HA–APS on a TA instruments Q500 model in 10 °C increments per minute from 40 °C to 1100 °C, using Al_2O_3 pans under dynamic air atmosphere of 60 mL/min. Differentiation in ash content of APS and EC–HA–APS was related with amount of HA bonded to APS.

Surface charge characteristics of APS, HA–APS and EC–HA–APS were evaluated in terms of pH point of zero charge, pH_{pzc}, by using pH-drift and mass-titration methods [11,16]. The measurements were done in 0.01 M NaCl solution, using a combination pH measurement system (Jenway).

Contact angle measurements were performed for APS, HA, HA–APS and EC–HA–APS by using KSV (CAM 200 model) instrument. Contact angles for each material were calculated from the recorded images of water and MeOH drops on the pellets of the solids. Contact angles were automatically calculated by the instrument software on the basis of Young–Laplace equation. Mean values of the contact angles calculated from left and right sides of the drop images were used in evaluations.

0.01 M Na_2H_2Y + 0.01 M NH_3 (Na_2H_2Y, dissodium form of EDTA) and 0.01 M NaCl solutions having different pH values between 7 and 11 were used in the stability tests of EC–HA–APS. In all cases, EC–HA–APS was introduced to mentioned aqueous media, and shaken intermittently over 24 h at ambient temperature. Afterwards, the supernatant was analyzed by a UV–vis spectrophotometer (Shimadzu 1700) at 410 nm wavelength to evaluate amount of HA released from EC–HA–APS.

2.3. HPLC system

HPLC analyses were performed on an Agilent 1100 series system consisted of quaternary pump with degasser, thermostatted column compartment, variable wavelength detector, and a manual injection port. EC–HA–APS was packed into a commercially available stainless steel HPLC column (4.6 × 150 in mm; internal diameter × length) as its aqueous suspension by using a slurry packer. The column was used after being rinsed thoroughly with water and MeOH. A laboratory-made 2 μL injection loop was used throughout the experiments.

2.4. HPLC studies

2.4.1. Converting EC–HA–APS to M-EC–HA–APS

At first, EC–HA–APS was conditioned enough time by a mobile phase system consisted of water and MeOH. Afterwards, aqueous solutions of metal ions (Cu^{2+} and Co^{2+}) were loaded to the column in a stepwise mode where the influent concentration was increased gradually in the range 5–100 mM. The progress of the metal loading was monitored via the breakthrough curves propagated in course of time, at wavelengths 595 and 507 nm for Cu^{2+} and Co^{2+}, respectively. After having been reached the equilibrium conditions for the highest influent concentration (that corresponds to the last plateau), water was loaded to the column to remove unbounded and/or weakly bonded metal ions from the column. In this way, EC–HA–APS was turned into *M*-EC–HA–APS. To test the binding stability of the metal ions to EC–HA–APS, approximately 1.5 L of water was passed through the column and the effluent was collected for subsequent atomic absorption spectrophotometry (AAS) analyses. Also, stripping efficiency of the studied metal ions by Na_2H_2Y + NH_3 (pH = 7.5) was studied through AAS analyses.

2.4.2. HPLC analyses of nucleosides

In HPLC analyses, some nucleosides (i.e. Urd, Tyd, Cyd, Ado and Guo) were used as the test solutes. In the experiments performed by using single-solute-samples, relation between *retention factor* (k') and experimental parameters studied was evaluated. Effect of mobile phase composition, temperature and the type of metal loaded to EC–HA–APS was investigated. Thus, retentive behaviors were evaluated on two ligand exchanger forms of EC–HA–APS (i.e. *Cu*-EC–HA–APS, and *Co*-EC–HA–APS). Temperature studies were performed for 25, 35, and 45 °C. The results obtained from single-solute experiments led determining the conditions suitable for separation of the studied compounds from their mixtures. Chromatograms recorded on EC–HA–APS and *M*-EC–HA–APS were compared with each other to understand effect of metal loading. Signals were acquired at UV 254 nm wavelength for the studied nucleosides, and processed by ChemStation data processor. MS Excell and OriginPro 7.5 were used for calculations and graphical demonstrations.

3. Results and discussion

3.1. Characterization

After immobilization of HA, a significant change was observed in C-element content, and it was attributed to organic backbone of HA, which is rich in C. From the elemental analyses results, amount of HA bonded to APS was calculated as 170 mgHA/gAPS. Immobilization of HA to APS via chemical bond formation was confirmed by the band appeared around 1655 cm^{-1} after immobilization. This band is attributed to the vibrations arising from C=O groups of amide structures, and implies the formation of amide-bond between HA and APS. Intensity of the bands around 1707 and 1655 cm^{-1} was found to be decreased in the case of metal loaded EC–HA–APS (figure not shown). These are the bands related with carbonyl groups, and such a decrement in the intensity of the bands has been attributed to the interaction of metal ions via carbonyl groups [17].

Number of –COOH and phenolic –OH groups was determined through direct titration method [15]. In 0.05 M NaCl solution, number of carboxyl and phenolic hydroxyl groups was calculated as 5.2 and 2.1 meq/gHA, respectively. Derivative titration curves revealed the presence of at least three types of carboxyl groups having different pK_a values distributed between 3 and 7, and two types of phenolic hydroxyl groups having pK_a values between 7 and 10.

Another important finding about the immobilization of HA was acquired from thermogravimetric analyses. Mass-loss in the case of EC–HA–APS was found to be higher, and it was attributed to the organic backbone of HA immobilized to APS. Also, thermal stability was found to be increased after end-capping process.

Surface charge characteristics of APS, HA–APS and EC–HA–APS were evaluated in terms of pH_{pzc} measurements. An obvious increment was observed in surface acidity after immobilization of HA to APS, so that pH_{pzc} value of APS, 9.8, diminished to 6.6 (HA–APS). After end-capping process that value diminished to 2.7 (EC–HA–APS).

Stability tests were performed for EC–HA–APS in 0.01 M Na_2H_2Y + 0.01 M NH_3 (pH = 7–11) and 0.01 M NaCl (pH = 7–11) media revealed high stability of EC–HA–APS. Amount of HA released into solution never exceeded 2% at pH = 7 and 8. All the results confirmed high stability of EC–HA–APS, and accordingly immobilization of HA via chemical bond formation.

3.2. M-EC–HA–APS

EC–HA–APS was turned into ligand exchanger forms through a practical methodology where metal ion solution was loaded to the column packed with EC–HA–APS using the HPLC system. So, 100 mM aqueous solution of the studied metal ion was diluted to a desired concentration in the HPLC system, and the influent concentration was increased stepwise. When the column became equilibrated with the solution having the highest concentration (100 mM), the column was rinsed with water till base-line (Fig. 1). Afterwards, aliquots of water were passed through the column for a complete conditioning. Through the mentioned stepwise manner, *M*-EC–HA–APS is thought to gain a stable conformation and/or geometric structure, gradually. Amount of the metal held by EC–HA–APS for the *i*th step was calculated via the following formula, which is used in *stepwise frontal analysis* [18]:

$$q_i = q_{i-1} + \frac{(V_{R,i} - V_0) \times (C_i - C_{i-1})}{W_S}; \quad C_i > C_{i-1} \geq 0$$

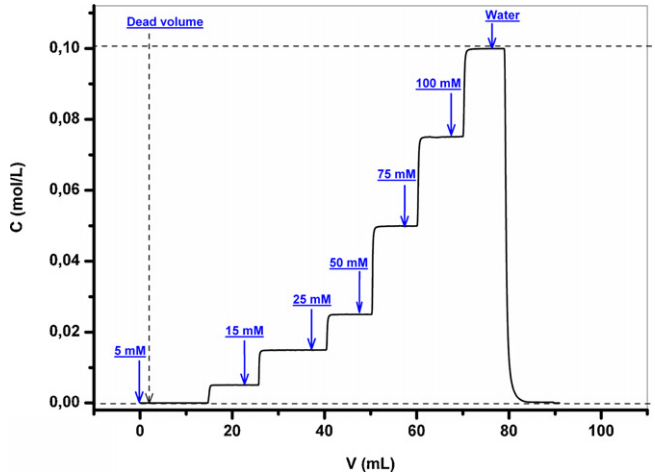

Fig. 1. Breakthrough curves recorded during the stepwise manner followed in metal loading process. Loading solution: $CoCl_2$; flow rate: 0.50 mL/min; temperature: 25 °C; detection wavelength: 507 nm; column: 4.6 × 150 (i.d. × length; as mm).

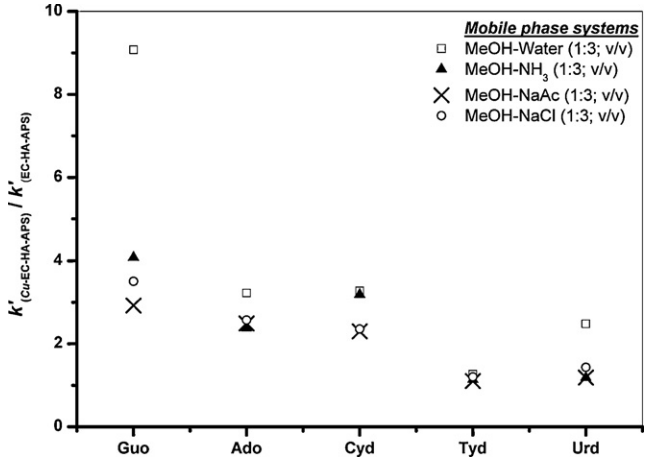

Fig. 2. Relative k' values calculated for nucleosides when mobile phase had different compositions. Flow rate: 1.00 mL/min; temperature: 25 °C; detection wavelength: 254 nm; injection volume: 2 μL. ($k'_{(EC-HA-APS)}$ and $k'_{(Cu-EC-HA-APS)}$ represent the k' values recorded on EC–HA–APS and *Cu*-EC–HA–APS, respectively).

where $V_{R,i}$ and V_0 represent retention volume and dead volume (mL), respectively. Equilibrium concentration of metal ion in mobile phase and stationary phase is represented by C_i (mol/L) and q_i (mmol/g), respectively. Mass of the stationary phase packed to the column is given by W_S (g). Observable capacities for the conditions correspond to the equilibrium concentration of 100 mM were found as 0.278 and 0.297 mmol *M*/gEC–HA–APS for Cu^{2+} and Co^{2+}, respectively.

In order to evaluate the metal binding stability, approximately 1.5 L of water was passed through the column and effluent was subjected to AAS analyses. Results showed that ~75 ± 2% of the metal held by the stationary phase was stable in the column at 25 °C. 95 ± 2% of the total metal held by the stationary phase was found to be stripped by the mixture of 0.1 M Na_2H_2Y + 0.1 M NH_3 (pH = 7.5) as stripping agent. This property made it possible to switch between RPLC and (different) LEC conditions, easily.

3.3. HPLC analyses of nucleosides

HA has a macromolecular structure that bears different functionalities. For this reason, nucleosides are expected to interact with *M*-EC–HA–APS through hydrophilic, hydrophobic, π–π, hydrogen-bonding, and ligand-exchange mechanisms at varying degrees, depending on the conditions. So, retentive behaviors are thought to resulting from a combination effect of the mentioned mechanisms.

To express the role of metal loading to the stationary phase, in the first set of the experiments, relative k' values were calculated for different mobile phase compositions. So, k' values recorded on EC–HA–APS and *Cu*-EC–HA–APS were used to calculate relative k' values (Fig. 2). As can be seen from figure, k' values recorded on *Cu*-EC–HA–APS are higher than those recorded on EC–HA–APS. This situation is clearly seen in the case of Cyd, Ado and Guo rather than that in the case of Urd and Tyd. Longer retention of Cyd, Ado and Guo was related with the presence of $–NH_2$ group within their molecular structure. Presence of this group was thought to increase the ligand character of species, and thus they might exhibit higher affinity towards the metal held by the stationary phase in comparison to Urd and Tyd. Also, as shown in Fig. 2, with increasing ligand character of the mobile phase, k' values of Cyd, Ado and Guo were found to decrease, while the retention of Urd and Tyd was little affected by the changes in mobile phase composition.

In order to prove higher interaction character of $–NH_2$-bearing species with metal ion, some supplementary experiments were carried out. In that set of experiments, Cu^{2+} solutions having different concentrations were used as mobile phase, and change in k' values of Urd, Tyd and Cyd was evaluated. The results are graphically shown in Fig. 3, and reveal that the retention of Cyd is very dependent on the concentration of metal ion in the mobile phase. Decrement observed in retention of Cyd with increasing concentration of Cu^{2+} in mobile phase might be due to the formation of mixed Cu–Cyd complexes in mobile phase. Because of this effect, the solubility of Cyd in mobile phase might be increased in presence of Cu^{2+} in the mobile phase. This situation may explain why Cyd, Ado and Guo exhibited higher retention on *Cu*-EC–HA–APS compared to Urd and Tyd. As expected, k' values of Urd and Tyd was found to be almost independent of the Cu^{2+} concentration. The results acquired from the experiments proved that ligand-exchange behavior of the stationary phase could be better observed in the case of Cyd, Ado and Guo which bear $–NH_2$ group. As be seen from the chromatogram recorded on *M*-EC–HA–APS (Fig. 4), retention times of the studied compounds decrease in the order Urd < Tyd < Cyd < Ado < Guo. The chromatogram recorded on EC–HA–APS under same conditions was also given in figure for comparison purposes. In all cases, though Urd and Tyd were little affected, retention times of the compounds were higher

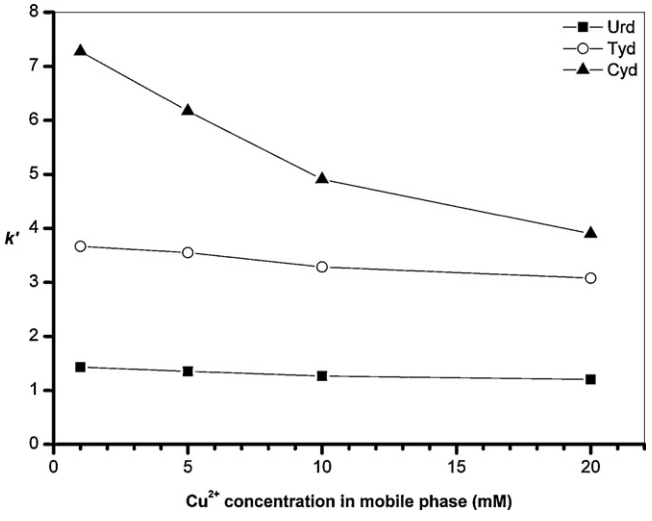

Fig. 3. Relation between k' values of some nucleosides and Cu^{2+} concentration of mobile phase. Mobile phase: $Cu(NO_3)_2$; flow rate: 1.00 mL/min; temperature: 25 °C; detection wavelength: 254 nm; injection volume: 2 μL.

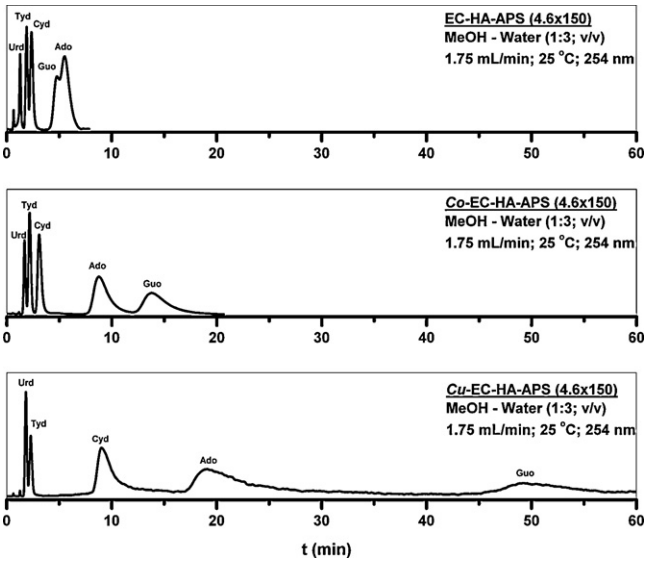

Fig. 4. Some chromatograms recorded on EC–HA–APS, *Co*-EC–HA–APS and *Cu*-EC–HA–APS by isocratic elution (effect of metal loading process as well as type of metal loaded on chromatographic separation). Experimental conditions are annotated on the chromatograms.

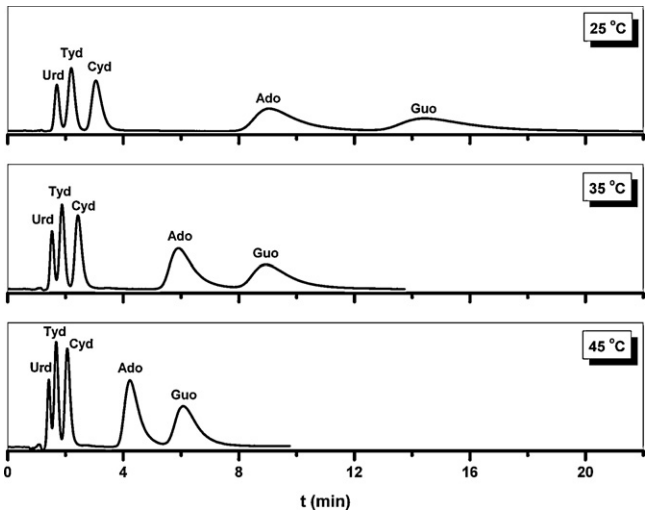

Fig. 5. Chromatograms recorded on *Co*-EC–HA–APS at different temperatures. Mobile phase: MeOH–water (25:75, v/v); flow rate: 1.75 mL/min; detection wavelength: 254 nm; injection volume: 2 μL; temperature: (a) 25 °C, (b) 35 °C, (c) 45 °C.

when *M*-EC–HA–APS was used instead of EC–HA–APS. Also, elution orders of Ado and Guo switched after metal loading. Longer retention of Ado and Guo was related with their higher molecular weight, and accordingly increased degree of London forces between the stationary phase and the mentioned compounds. So, *M*-EC–HA–APS was understood to have a mixed-mode RPLC/LEC character towards the studied compounds, and both capacity and selectivity of EC–HA–APS were found to be improved through the metal loading process. Finally, type of the metal loaded to the stationary phase was observed to have effect on capacity and selectivity, so that the retention of Cyd, Ado and Guo was found to be higher in the case *Cu*-EC–HA–APS compared to *Co*-EC–HA–APS. This might be due to higher affinity of copper towards $-NH_2$ bearing nucleosides, compared to cobalt. Thus, separation efficiency was found to be improved by loading a metal ion, which is suitable for special application, to the stationary phase.

The effect of temperature on chromatographic behaviors was also studied within the range 25–45 °C. The experiments were performed by using MeOH–water (25:75, v/v) mobile phase system, and the chromatograms recorded on *Co*-EC–HA–APS were given in Fig. 5. Despite the fact that the elution of Urd and Tyd was little affected by the changes in temperature, Cyd, Ado and Guo eluted more easily with increasing the temperature from 25 °C to

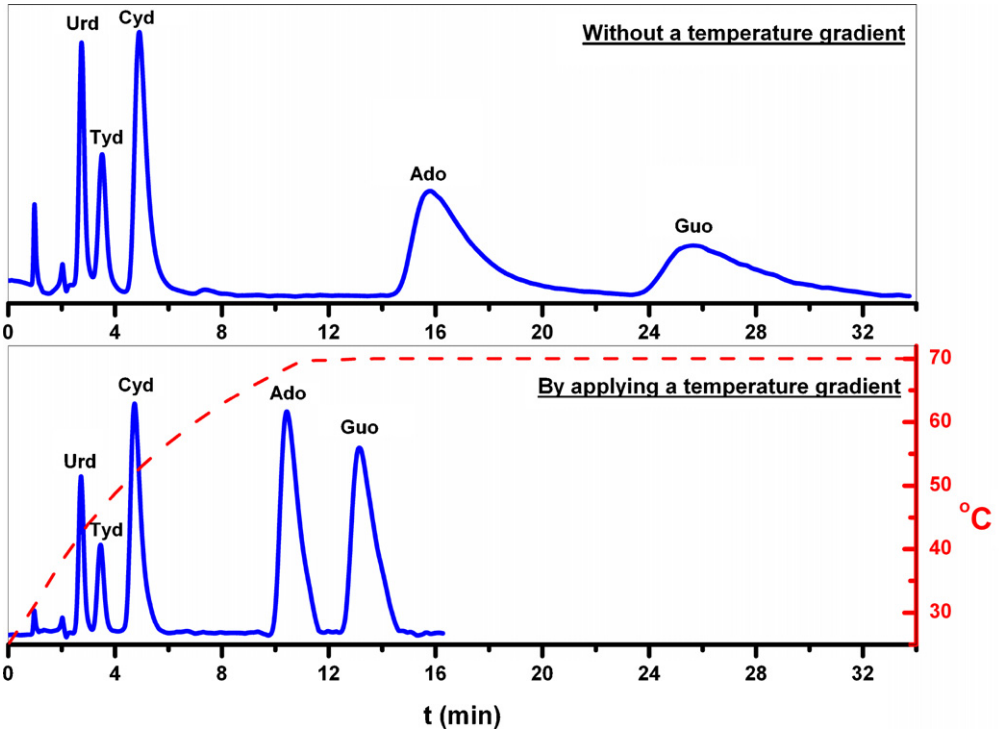

Fig. 6. Comparative chromatograms to express the influence of temperature gradient on separation efficiency. Form of the stationary phase: *Co*-EC–HA–APS; mobile phase: MeCN–water (10:90; v/v); flow rate: 1.00 mL/min; detection wavelength: 254 nm; injection volume: 2 μL; temperature gradient is shown on the chromatogram.

Table 1
Ratio of peak area recorded by HPLC and FIA techniques.[a]

	Ratio of peak area (HPLC/FIA)				
	Urd	Tyd	Cyd	Ado	Guo
EC–HA–APS	0.99	1.02	1.10	1.03	1.01
Cu-EC–HA–APS	1.10	1.05	1.05	0.99	0.98
Co-EC–HA–APS	1.15	1.10	1.12	0.96	0.95

[a] Experimental conditions: mobile phase, MeOH–water (60:40; v/v); flow-rate: 1.00 mL/min; temperature: 25 °C; detection: 254 nm; column: 4.6 × 150 (*i.d.* (mm) × length (mm)).

45 °C. So, temperature was thought to be useful to improve the chromatographic separation of the studied compounds. Since Ado and Guo had high retention on *Cu*-EC–HA–APS, chromatographic separation on this ligand exchanger form could be improved only after applying suitable gradients both in mobile phase composition and temperature (chromatogram not shown). On the other hand, *Co*-EC–HA–APS offered mild conditions for elution of all the studied compounds. Base-line separation on *Co*-EC–HA–APS was achieved by applying a gradient program in temperature and by using MeCN instead of MeOH as organic modifier (Fig. 6). In this way, the importance of temperature-gradient, which is not a common experimental parameter in HPLC separations, has been shown, as well.

Metal binding stability and capacity of the column was found to be better when the stepwise manner was followed instead of one-step metal loading manner. Standard deviation in k' values of the studied compounds did not exceed 0.03 even after multiple metal loading and stripping steps performed at different times. So, stepwise metal loading process is proposed as a useful methodology to convert EC–HA–APS to *M*-EC–HA–APS under column conditions.

Supplementary experiments have been performed in order to understand elution efficiency of nucleosides on EC–HA–APS and *M*-EC–HA–APS. So, peak areas obtained by flow injection analysis (FIA) were compared with those obtained by single solute HPLC studies. Obtained results are tabulated in Table 1, revealing good elution efficiency of the studied nucleosides on EC–HA–APS and *M*-EC–HA–APS. Therefore, there found no evidences that could be attributed to irreversible sorption of the studied nucleosides under the studied conditions.

Together with its efficiency in HPLC separations, *M*-EC–HA–APS made it possible to study at high flow rates, under relatively low system pressures. The system pressure did not exceed 120 bars at the flow rate of 4.00 mL/min, when Water and MeOH–water (25:75, v/v) mobile phase systems were used. This superior property seems promising in separations to be performed under high pressure. So, immobilization of HA to smaller particular-size APS is thought to be useful as improving the separation efficiency.

Comparing the results we obtained on *M*-EC–HA–APS under so-called mixed-mode RPLC/LEC conditions with those previously obtained on different stationary phases under RPLC (C18) [19] and ion-pairing RPLC (ZORBAX SB-Aq) [20] conditions, it can be said that the order of elution is quite different. This situation implies differentiation in the mechanism of separation when *M*-EC–HA–APS has been used as stationary phase. Analysis time for the separation of the studied nucleosides was found to be shorter than those recorded on the mentioned stationary phases, and as a general trend, *M*-EC–HA–APS exhibited good separation efficiency. Moreover, it was possible to manipulate the retentive behaviors easily by loading a different type of metal to EC–HA–APS. Hence, EC–HA–APS made it possible to switch between RPLC/HILIC [11] and RPLC/LEC mixed-mode conditions, easily. So, *M*-EC–HA–APS was found to be advantageous because of the mentioned reasons.

4. Conclusion

Metal-loaded immobilized HA was found to be efficient for chromatographic separation of nucleosides as an HPLC stationary phase. The observed retentive behaviors were thought to be resulting from a mixed-mode RPLC/LEC behavior of the stationary phase. Both capacity and selectivity of the column were easily manipulated by loading different metal ions to the column. This property is believed to be useful for chromatographic separation of various chemical species having ligand character. Furthermore, a good flexibility was observed to switch between different forms of the stationary phase, by using suitable complexing agents for metal stripping purposes. So, different modes of HPLC were found to be applicable on the same column. The studied compounds were easily separated by using MeOH–water and MeCN–water mobile phase systems. *M*-EC–HA–APS made it possible to study at high flow rates, and therefore separation efficiency could be improved by studying at high flow rates with low system pressure. Finally, it is understood that EC–HA–APS can exhibit high flexibility between different modes of HPLC, and so it seems promising as a stationary phase to build new concepts based on chromatographic separations.

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