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

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ABSTRACT

The stationary phase characteristics of the material obtained through immobilization of humic acid (HA) to aminopropyl silica (APS) via amide-bond formation were investigated. The material was characterized in terms of elemental analysis, FTIR, thermogravimetric analyses, pH point of zero charge measurements, potentiometric titrations, and contact angle measurements. Amount of HA bonded to APS was determined from the elemental analysis results, and found as 170 mgHA/gAPS. Stability of the material was studied in aqueous media at different pH values, and amount of HA released at pH = 8 did not exceed 2% of the total immobilized HA. Stationary phase characteristics of the well-characterized material were investigated in an HPLC system by using some low-molecular weight polar compounds (i.e. some nucleosides and nucleobases) as test solutes. Effect of some experimental variables such as column conditioning, composition of mobile phase, and temperature on the chromatographic behavior of the studied compounds was studied. Role of ammonium solutions at different pH values on retentive properties of the species was also studied. Retention factors (k') versus volume percentage of organic modifier exhibited a U-curve, which was evaluated as an indication for RPLC/HILIC mixed-mode behavior of the stationary phase. Orthogonality between RPLC and HILIC modes was analyzed through geometric approach, and found as 48.5%. Base-line separation for the studied groups of compounds was achieved under each studied mode, and some differentiations were observed in elution order of the compounds depending on the HPLC mode applied. Chromatograms recorded under RPLC and HILIC modes were compared with those recorded on APS under similar conditions, and thus the influence/importance of HA immobilization process was evaluated in detail. In light of the obtained results, immobilized HA is represented as a useful stationary phase for HPLC separations.

1. Introduction

High performance liquid chromatography (HPLC) is one of the most popular techniques being used for qualitative and quantitative analyses of a wide range of chemical species. The performance of the technique is highly related with the properties of the stationary phase used [1,2]. So, the chromatographers attach special importance to the design of efficient stationary phases for HPLC.

Reducing the analysis time and increasing the selectivity at the same time are the main criteria in method development for HPLC [3] as well as in design of new stationary phases. In designing new stationary phases, the surface of a suitable solid support is, generally, modified by inclusion of proper functional groups and/or molecules. Such a surface modification process usually requires complicated reactions where high volume of chemicals is used both in synthesis and purification steps. As the stationary phases are usually obtained by immobilization of uni-type and/or uni-character ligands to solid supports, this type of stationary phases sometimes exhibit poor efficiency in HPLC separations, and it is, generally, difficult to increase the efficiency by applying a different mode of HPLC on the same stationary phase.

To increase the efficiency of HPLC separations, the idea "*applying more than one HPLC modes on the same stationary phase*" is deemed important, especially, by the chromatographers dealing with 2D-LC separations. The feasibility of multimodal HPLC separations is directly related to the physical and chemical properties of the stationary phase. So, the stationary phase to be used in multimodal separations must be designed carefully. Recent trends in design of new stationary phases are towards inclusion of (*i*) hydrophobic chains containing hydrophilic groups, and (*ii*) mixed phases containing various functional groups to the surface of solid support [1].

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Humic acid (HA) is the term used for definition of naturally occurring bio-macromolecules bearing hydrophobic, hydrophilic, aromatic, ionizable and electron-donor functionalities in the same structure [4]. So, HA exhibits a multifunctional character, and this special character was thought to be useful in multimodal HPLC separations of various types of compounds by immobilizing it to a suitable solid support. Through immobilization, not only HA is turned into a less-soluble form, but also mechanical properties of HA can be improved. This point of view may lead design of efficient stationary phases with multifunctional character.

Considering the experimental processes followed in immobilization of HA to solid supports, main goal of the proposed methodologies seems to be towards obtaining a stable material. So, the researchers dealing with this topic have mainly concentrated on chemical-bond formation between HA and solid support and therefore different methodologies have been proposed in the literature. In the method proposed by Bulman et al. [5], HA is immobilized via chemical bond formation between diazonium-functionalized solid support and aromatic structures present in HA. In a different method proposed by Bulman et al. [5], HA is immobilized to a glutaraldehyde-modified solid support through amide-bond formation between aldehyde groups of solid support and amine groups of HA. In the following years, Koopal et al. [6] have suggested a more efficient method that yields a stable material with low isoelectric point value. In this method, immobilization is done through amide-bond formation between carboxyl groups of HA and amine groups of aminopropyl silica. Recently, a variation of this method has been suggested by Luo et al. as well [7]. Klavins and Eglite [8] have reported immobilization of HA to different epoxy-functionalized solid supports. In the mentioned methodologies, immobilization is, generally, done in a non-aqueous media. On the other hand, there are some studies that report immobilization of HA in aqueous media, where HA macromolecules are immobilized to solid support through adsorption and/or electrostatic interactions, and in presence of a coupling-reagent [6,8–10]. However, immobilization via covalent-bond formation seems to be more efficient to obtain a stable material. According to the study of Koopal et al. [6], the method based on amide-bond formation between HA and aminopropyl silica seems to be preferable. The stability of the product obtained via this method has been improved through a supplementary process, called *end-capping*, by which residual-amino groups on the surface of the solid support are acetylated.

In the literature, the studies dealing with the usability of HA-immobilized materials have mainly concentrated on sorption behavior of various compounds, such as aminobenzene and metal ions [8], phenols [9], heavy metal ions [11–13], indigo carmin dye [14], aminobenzene, cristal violet, methylene green, flavine mononucleotide and some heavy metal ions [15], while there exists little effort on its usability as a stationary phase in HPLC.

Yu et al. [16] studied the usability of a HA-immobilized material as a hydrophilic interaction chromatography (HILIC) stationary phase for separation of some alkaloids. Kollist-Siigur et al. [17] investigated the effect of some experimental parameters on the retention of some polycyclic aromatic compounds on humic acid-(as well as fulvic acid-) bonded materials, and observed binding characteristics were tried to be related with K_{oc} (organic carbon partition coefficient) of the studied compounds. Casadei et al. [18] intensified on the separation of fullerenes by using a HA-immobilized silica material as a stationary phase. In that study, immobilization of HA was done by pumping HA solution directly to the column pre-packed with solid support. However, according to our knowledge, there is no study that comprehensively intensifies on the characteristics of HA-immobilized materials as an HPLC stationary phase.

Our recent studies have proved the applicability of three modes of HPLC (i.e. RPLC, HILIC and ligand-exchange chromatography) on HA-bonded aminopropyl silica. Moreover, owing to its special character, HA-immobilized materials are believed to be applicable in some other HPLC modes, such as NPLC, adsorption chromatography, ion-exchange chromatography, ion-pairing chromatography, etc. So, HA-immobilized material seems to be useful in multimodal HPLC separations. In the present paper, we have concentrated on RPLC and HILIC behavior of the stationary phase. In the studies, nucleosides and nucleobases, which possess hydrophobic, hydrophilic, aromatic and ionizable functionalities, are thought to be useful to reveal RPLC and HILIC behavior of HA-bonded stationary phase. Thus, the degree of various interactions, such as hydrophobic, hydrophilic, electrostatic, π–π and hydrogen-bond formation, can be evaluated reasonably. So, it is interesting to investigate the chromatographic behavior of these low-molecular weight compounds on this stationary phase.

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. Sodium form of Aldrich humic (NaA) acid was purified and converted into its protonated form (HA) before use. Aminopropyl silica (APS; 15-35 μm particle size; ~9 nm pore size) was supplied from Fluka and employed as a solid support in immobilization of HA. Immobilization of HA was done in dimethylformamide (Fluka) which included < 0.01% water and stored on molecular sieves. Aqueous mixtures of ammonia (Merck) and disodium form of EDTA (Na_2H_2Y; Merck) were used in solubility tests. Methanol (MeOH; LabScan) and acetonitrile (MeCN; LabScan) were the organic modifiers used in HPLC analyses. Aqueous ammonium solutions having different pH values were used in HPLC studies, and pH of the solutions were adjusted to a desired value by using 0.1 M HCl (Merck) and 0.1 M NaOH (Merck) solutions. The studied nucleosides (i.e. Uridine, Urd, Thymidine, Tyd, Cytidine, Cyd, Adenosine, Ado, and Guanosine, Guo) and nucleobases (i.e. Uracil, Ura, Thymine, Thy, Cytosine, Cyt, Adenine, Ade, and Guanine, Gua), were supplied from Sigma, and their test solutions were prepared in mixture of methanol and water. All the chemicals were used without further purification, and ultra-pure water (UPW; 0.059 μS cm^{-1}) was used in the experiments.

2.2. Immobilization

Solid humic acid in sodium form was purified according to the method reported in Ref. [6]. Hence, approximately 10 g of solid HA was suspended in 1.0 L of aqueous NaOH solution (pH 11) and stirred overnight. Afterwards, insoluble fractions were removed by centrifugation. Centrifugate was acidified with 1 M HCl to pH = 2 in order to re-precipitate humic macromolecules. The precipitate was separated by centrifugation and washed thoroughly with aqueous 0.05 M HCl solution. Obtained product was dried at 105 °C and stored for further use.

Purified humic acid (HA) was immobilized to APS through the route illustrated in Fig. 1. The illustrated method depends upon amide bond formation between APS and HA, and it was a slightly modified form of the method reported by Koopal et al. [6]. The process was done in DMF at 120 °C over 20 h to immobilize HA (product: HA-APS). Afterwards, residual $-NH_2$ groups on the surface of APS were end-capped in DMF medium by addition of acetyl chloride drop by drop. Obtained mixture was mixed over 5 h at ambient temperature. The product (EC-HA-APS) was rinsed successively with DMF, dichloromethane and acetone till colorless. The dried product was stored for further use.

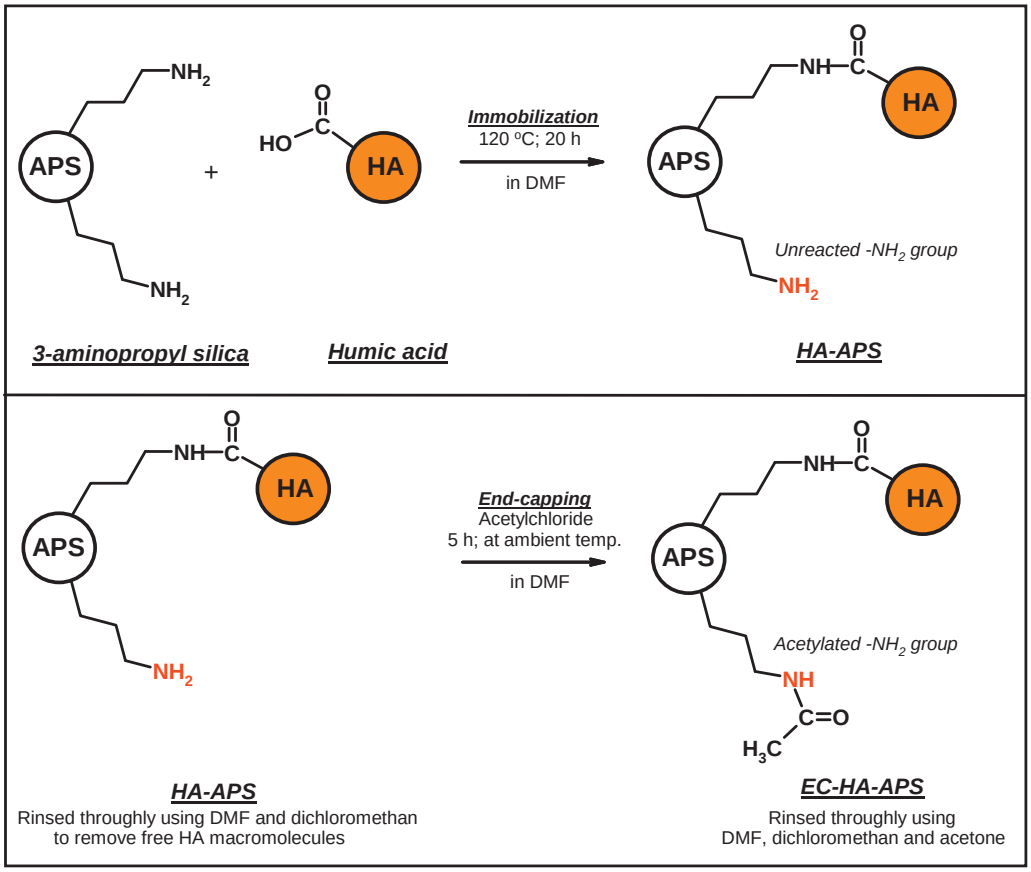

Fig. 1. Experimental route followed in immobilization of HA to APS.

2.3. Characterization

The materials used in immobilization were characterized by using various methods and techniques such as elemental analysis, FTIR, potentiometric titrations, thermogravimetric analyses, surface charge characteristics, contact angle measurements and stability tests.

Elemental analyses were performed in Middle East Technical University, 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 total mass of HA. Since Si compositions of APS, HA-APS and EC-HA-APS were not determined, O compositions of these materials were not calculated. Amount of HA bonded to APS was calculated on the basis of C/N ratio obtained for APS, HA and EC-HA-APS. So, elemental ratios on the dry material basis were used to calculate amount of HA bonded to APS.

FTIR analyses were performed for APS, HA, HA-APS and EC-HA-APS by using Perkin-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.

Number of carboxyl and phenolic hydroxyl groups in HA was determined through direct potentiometric titration of solid HA in NaCl solution of 0.05 M. So, 0.025 g of solid HA was added to 25 mL of NaCl solution and suspension was mixed till equilibration. The suspension was titrated by using aqueous solution of NaOH as titrant (~0,100 M) at ambient temperature (20 ± 2 °C). Titration was conducted by addition of 0.050 ± 0.006 mL portions of titrant, and pH of the suspension was recorded after 15–20 s for each addition. Number of functional groups were calculated using the method described in [19–21].

Thermogravimetric (TG) and differential thermogravimetric (DTG) analyses were performed for APS, HA, HA-APS and EC-HA-APS using 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}. In definition, this value corresponds to the pH value of the liquid surrounding the solid particles when the sum of surface positive charges balance the sum of surface negative charges [22]. To determine pH_{pzc}, pH-drift and mass-titration methods were followed. The experiments were performed in aqueous NaCl solutions.

In order to understand effect of HA immobilization and end-capping processes on wettability of stationary phase by water and MeOH, contact angle measurements were performed for APS, HA, HA-APS and EC-HA-APS. Measurements were conducted by using KSV (CAM 200 model) instrument, and contact angles for each material were calculated from the recorded images of water and MeOH drops on 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. Hence, wettability of APS, HA, HA-APS and EC-HA-APS was evaluated by using water and MeOH as liquid phases.

The material to be used as a stationary phase must be stable under experimental conditions studied. For this reason, the material must exhibit high stability towards most of the solvents used in liquid chromatography. Moreover, the stationary phase must be

stable within a wide range of pH in aqueous media. Hence, some experiments were performed at different pH values in aqueous media to understand the stability of HA-bonded materials. Aqueous mixtures of 0.01 M Na_2H_2Y + 0.01 M NH_3 having different pH values between 7 and 11 were used in the stability tests. The stability tests were also conducted in 0.01 M NaCl solutions having different pH values. In all cases, HA-bonded materials were 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 dissolution.

2.4. Equipment used in HPLC analyses

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 × 100 in mm; *internal diameter* × *length*) as its aqueous suspension by using a slurry packer. The column was used after rinsing thoroughly with water and MeOH. A laboratory-made 2 μL injection loop was used in experiments. A valve adapted to the system made it possible to perform both HPLC and flow injection analyses (FIA) on the same instrument.

2.5. HPLC analyses

At first, the column packed with EC-HA-APS was conditioned by pumping portions of mobile phase comprised water and methanol (MeOH). In order to evaluate the degree of conditioning, some experiments were repeated periodically, and thus reproducibility of k' values was monitored in course of time. When standard deviation in k' values became ≤0.03, the stationary phase was accepted to be reached a suitable conformation and/or geometric structure. Then, the effect of some experimental variables on chromatographic behavior of some nucleosides and nucleobases was studied on the column conditioned enough time.

The relation between flow rate and HETP (*height equivalent to a theoretical plate*) was studied. In the experiments, Urd and Cyd were used as the probe molecules, and mean value of HETP was calculated from the results obtained for each molecule at different flow rates ranging between 0.15 and 1.50 mL/min.

Effects of type and percentage of organic modifier, ammonium solution, and temperature on k' values of nucleosides and nucleobases were studied at the defined flow rate of 0.50 mL/min by using single-solute samples. Test solutions of nucleosides and nucleobases were prepared in MeOH–water mixture, and few drops of 1.0 M NaOH solution were added to increase the solubility of species. All the samples were filtered through 0.20 μm Nylon filters before use.

Effect of organic modifier was studied by using MeOH and MeCN as the organic solvents. So, k' values of the studied compounds were derived by using MeOH–water and MeCN–water mobile phase systems having different percentages of organic modifiers (1–90%; v/v). During these experiments, flow rate and temperature were fixed at 0.50 mL/min and 25 °C, respectively.

Since nucleosides and nucleobases includes nitrogen atom in their molecular structure, studying with ammonium solutions having different pH values were thought to be interesting and useful in chromatographic separations. So, pH and temperature studies were performed by using mobile phases consisted of 0.1 F NH_4^+ at various pH values (3.0–7.0) and organic modifiers. pH of NH_4^+ solutions were adjusted to a desired value by using 0.10 M HCl and 0.10 M NaOH solutions. The studies under RPLC and HILIC modes were performed with NH_4^+ (0.1 F)-MeOH (99:1; v/v) and NH_4^+ (0.1 F)-MeCN (10:90; v/v) mobile phase systems, respectively. Effect of temperature was studied between 25 and 45 °C, and van't Hoff plots were derived for each studied pH values of NH_4^+ solutions. pH measurements were performed on a combined pH-measurement system (Jenway).

On the basis of the results obtained from single-solute experiments, the conditions suitable for base-line separation of the studied compounds were sought for. Some recorded chromatograms were compared with those recorded on a column filled with APS to understand role of HA immobilization on chromatographic behavior.

Signals were acquired at UV 254 nm wavelength, 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

3.1.1. Elemental analysis and amount of HA bonded to APS

Results of the elemental analyses are tabulated in Table 1. As can be seen from the table, there is an obvious change in C element content of APS after immobilization of HA. The increment observed in C% can be attributed to organic backbone of HA immobilized to APS. This high C loading reveals immobilization of HA macromolecules to APS. Amount of HA immobilized to APS was calculated on the basis of differentiation in C/N ratio, and amount of HA bonded to APS was calculated as 170 mgHA/gAPS.

3.1.2. FTIR analysis

Elemental analyses revealed immobilization of HA to APS, but further information was necessary to evaluate the role of chemical bond formation. So, FTIR analyses were performed for HA, APS, HA-APS and EC-HA-APS. The spectra recorded for each material are shown in Fig. 2.

Actually, it is difficult to acquire compact information from the FTIR spectra, because most of the significant bands of HA are shielded by Si–O and H-bonded Si–OH vibrations around 1250–900 cm^{-1} (broad) and 1600 cm^{-1} [6]. The most significant change in the spectra after immobilization of HA is the band appeared in the spectra of HA-APS and EC-HA-APS, which is around 1655 cm^{-1}. This band is attributed to vibrations arising from C=O groups of amide structures. As can be seen from the spectra, this band is not present both in HA and APS spectra, and so the appearance of this band confirms immobilization of HA to APS via amide-bond formation. Comparing the spectra of HA-APS and EC-HA-APS, the band arising from C=O vibrations of carboxyl groups (around 1705 cm^{-1}) is more evident in the spectrum of EC-HA-APS. This situation can be attributed to effect of end-capping process, by which some of the immobilized HA macromolecules are removed from APS surface and residual $-NH_2$ groups on the surface are acetylated.

3.1.3. Potentiometric titrations

Acidity of HA is mainly attributed to –COOH and phenolic –OH groups, and determination of the amount of these functional groups

Table 1
Elemental compositions for HA, APS and EC-HA-APS.

	C (%)	H (%)	N (%)	S (%)	O (%)
HA[a]	64.57	5.18	1.73	0.59	28.56
APS[b]	5.80	1.89	2.06	–	–
EC-HA-APS[b]	11.65	1.86	1.79	–	–

[a] On the dry and ash-free material basis.
[b] On the dry material basis.

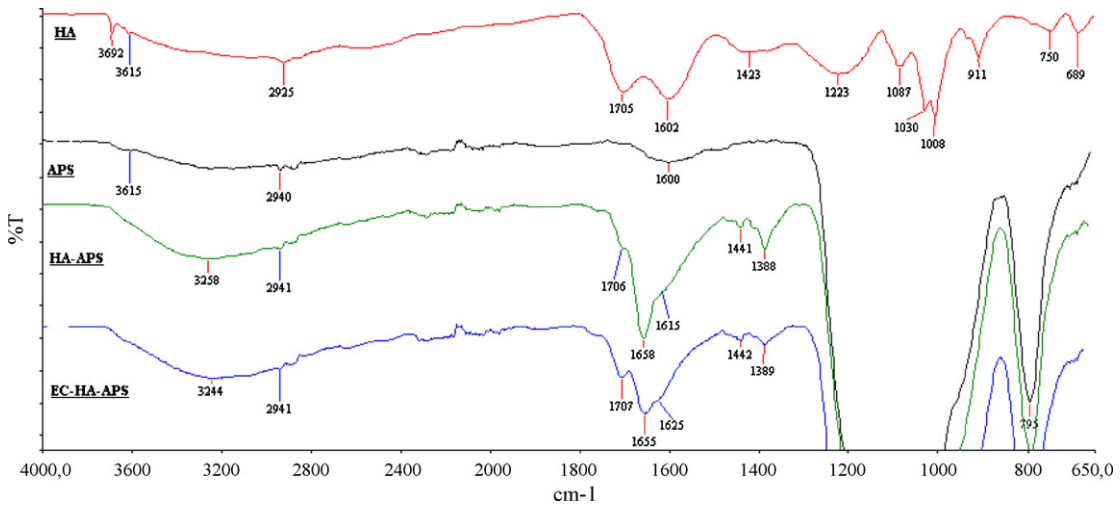

Fig. 2. FTIR spectra for HA, APS, HA-APS, and EC-HA-APS.

is deemed important in studies dealing with HA. Direct titration methods are very useful for this purpose, and are very popular. Direct titration methods not only give some information about amount of acidic groups, but also give some insight into distribution of dissociation constants of acidic groups present in the structure [21].

Direct titration curves and first order derivative curves were analyzed (figures not shown). Amount of the functional groups were calculated as 5.2 and 2.1 meq/g for carboxyl and phenolic hydroxyl groups, respectively, on the dry and ash-free basis. From the derivative curves it was concluded that there were at least three types of carboxyl groups having different p*K*a values distributed between 3 and 7, and two types of phenolic hydroxyl groups having p*K*a values between 7 and 10. Also, most of the acidic groups were understood to be dissociable under neutral and slightly acidic conditions.

3.1.4. Thermogravimetric analysis

Thermal stability of HA, APS, HA-APS and EC-HA-APS was studied by thermogravimetric analyses. Recorded thermograms (TG) and differential thermograms (DTG) were analyzed (figures not shown), and results showed that mass loss was higher after immobilization of HA. Also, thermal stability of EC-HA-APS was found to be higher than that of HA-APS, which implied positive effect of end-capping process on the stability of HA-bonded material. Hence, immobilization of HA to APS was confirmed by TG and DTG analyses, too, and increased thermal stability was related with chemical bond formation.

3.1.5. Surface charge characteristics

Surface charge characteristics of APS, HA-APS and EC-HA-APS were evaluated in terms of pH_{pzc}. Comparing pH_{pzc} values of each material, there is an obvious increment in surface acidity after immobilization of HA to APS, so that pH_{pzc} value of APS, 9.8, diminishes to 6.6 (HA-APS). More importantly, pH_{pzc} diminishes to 2.7 after end-capping process, and obtained value is in accordance with iso-electric point value (2.6) reported for EC-HA-APS in Ref. [6]. Further decrement in pH_{pzc} after end-capping process was related with acetylation of residual $–NH_2$ groups which pose a basic character on the surface. Acetylation turns these groups into amide structures, and thus basic character becomes diminished. So, pH_{pzc} studies revealed perfect acetylation of un-reacted $–NH_2$ groups on the surface, and thus decreased effect of these groups.

3.1.6. Contact angle measurements

Differentiation in wettability of APS after immobilization was evaluated in terms of contact angle (θ) measurements. Analyses were performed with water and MeOH, and calculated θ values are given in Table 2. Comparing the results, wettability of all the materials is better in the case of MeOH. Among the θ values calculated for APS, HA-APS and EC-HA-APS, the lowest values were obtained for EC-HA-APS. This reveals effect of HA immobilization and end-capping processes on the wettability. Since θ values of EC-HA-APS is lower than 90°, it can be said that this material get wet by water and MeOH, perfectly. So, EC-HA-APS was thought to be useful as a stationary phase to be used in RPLC and HILIC modes of HPLC.

3.1.7. Stability tests

Results showed that amount of HA released from the surface was lower than 5% of total immobilized HA to APS at pH = 9. On the other hand; approximately 10% and 22% HA was found to be released at pH = 10 and 11, respectively. Amount of HA released into solution never exceeded 2% at pH = 7 and 8. So, in HPLC studies to be performed on EC-HA-APS, aqueous solutions having pH values above 7.5 were avoided to study with. Finally, in all cases, amount of HA released into solution was higher in the case of 0.01 M Na_2H_2Y + 0.01 M NH_3 mixture, compared to NaCl solution. Results confirmed high stability of EC-HA-APS, and accordingly immobilization of HA via chemical bond formation.

3.2. HPLC analyses

Molecular structure and p*K*a values of the studied nucleosides and nucleobases are given in Fig. 3 [23]. As can be seen from the figure, unique difference between the molecular structures of the nucleosides and their respective nucleobases is the presence of a ribosile (or deoxyribosile) structure in nucleosides. Analyzing the molecular structures, it can be seen that some of the nucleosides and nucleobases involve $–NH_2$ group which can be protonated depending on medium pH. Another property of this

Table 2
Contact angles (θ) measured for HA, APS, HA-APS and EC-HA-APS using water and MeOH as liquid phases.

	HA	APS	HA-APS	EC-HA-APS
θ (using water)	62.0°	35.0°	33.8°	14.6°
θ (using MeOH)	8.46°	14.9°	15.5°	11.7°

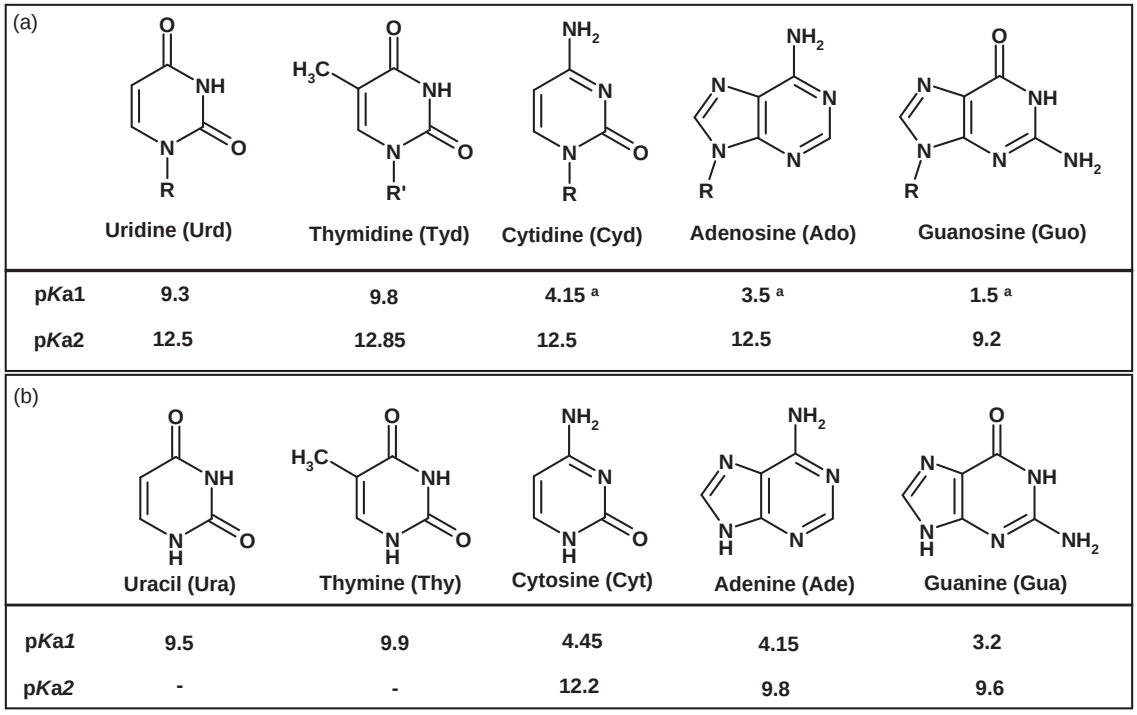

Fig. 3. Molecular structure and p*K*a values of the studied nucleosides and nucleobases (R: Ribosile; R′: Deoxyribosile) [a] i.e. $-NH_3^+$.

type of nucleosides and nucleobases is their association in aqueous media through enol forms. This effect is known as "*vertical stacking*" [23], and its degree depends upon the medium (i.e. percentage of organic modifier and pH) in which the nucleosides and nucleobases have been dissolved as well as the temperature. So, chromatographic behavior of these compounds should be studied carefully with respect to mobile phase composition, pH and temperature to understand role of mechanisms took role. For this reason, effect of composition of mobile phase, pH of ammonium solution, and temperature on retention factors (k') of the studied nucleosides and nucleobases were investigated using single-solute samples. Differentiation in k' values was carefully analyzed and related with possible mechanisms. Analyses were performed on the column conditioned enough time.

3.2.1. *Effect of organic modifier in mobile phase*

Effect of organic modifier on k' values of the studied compounds was investigated by using MeOH–water and MeCN–water mobile phase systems, where percentage of organic modifier was changed between 1 and 90% (v/v). Obtained results are graphically shown in Fig. 4. As can be seen from the figure, two trends are sensible in distribution of k' values depending on the increments in percentage of organic modifier: (*i*) k' values tend to diminish in the range 1–50% (v/v), and (*ii*) k' values tend to increase in the range 70–90% (v/v). So, k' values exhibited a "U-shaped" curve versus percentage of organic modifier, and this situation is more clear when MeCN–water mobile phase system was used. This trend was related with mixed-mode RPLC/HILIC behavior of the stationary phase towards the studied compounds. So, chromatographic separation of the studied groups of compounds was thought to be achieved under two modes of HPLC when the percentage of organic modifier was (*i*) 1–50% (RPLC), and (*ii*) 70–90% (HILIC). However, MeOH–water mobile phase system was found not to be suitable for HILIC separations, because k' values of the compounds were very close to each other when percentage of MeOH was higher than 70% (v/v).

For the conditions corresponding to RPLC mode (MeOH and MeCN = 1–50%; v/v), k' values of the nucleosides increase in the order Urd < Tyd < Cyd < Guo < Ado. Among the studied nucleosides, k' values of Urd and Tyd were less affected by the changes in percentage of organic modifier. This implies that the degree of hydrophobic and/or other interactions is minimal for the two species. Both elution order and the degree of k' values reveal higher affinity of the stationary phase towards nucleosides with $-NH_2$ group (i.e. Cyd, Guo and Ado). This situation was attributed to vertical-stacking and accordingly increased degree of hydrophobic interactions for these compounds. Comparing the k' values of Cyd, Guo and Ado, it can be seen that k' values diminish in the order Cyd < Guo < Ado. This order of elution may be related with the combined effect of hydrophobic interactions and ionization degree of the compounds on chromatographic behavior. However, under the studied conditions (pH ~ 6–7), the three nucleosides are present mainly in their molecular form, and thus the effect of ionization can be omitted. So, the main effect seems to be hydrophobic interactions and vertical stacking. Since Ado and Guo have higher molecular weight, the degree of hydrophobic interactions resulting from London forces might be higher for these compounds. This might be the reason of why they eluted later than Cyd.

Because nucleobases exhibited similar trends to nucleosides, above explanations are valid for nucleobases, too. As a general trend, nucleobases exhibited higher k' values compared to their respective nucleosides, except for Ura and Thy. This situation was related with the presence of ribosile (or deoxyribosile) in the structure of nucleosides. Presence of the sugar ring, which has high hydrophilic character, might increase the solubility of nucleosides in the mobile phase. Under RPLC conditions, Ura exhibited k' values very close to the unity in most cases. As known, in RPLC studies, it is very common to use Ura as a probe molecule for determination of dead volume because of its low degree of interactions with RPLC stationary phases. So, the chromatographic behavior of Ura on EC-HA-APS was found to be in accordance with that observed on common RPLC stationary phases.

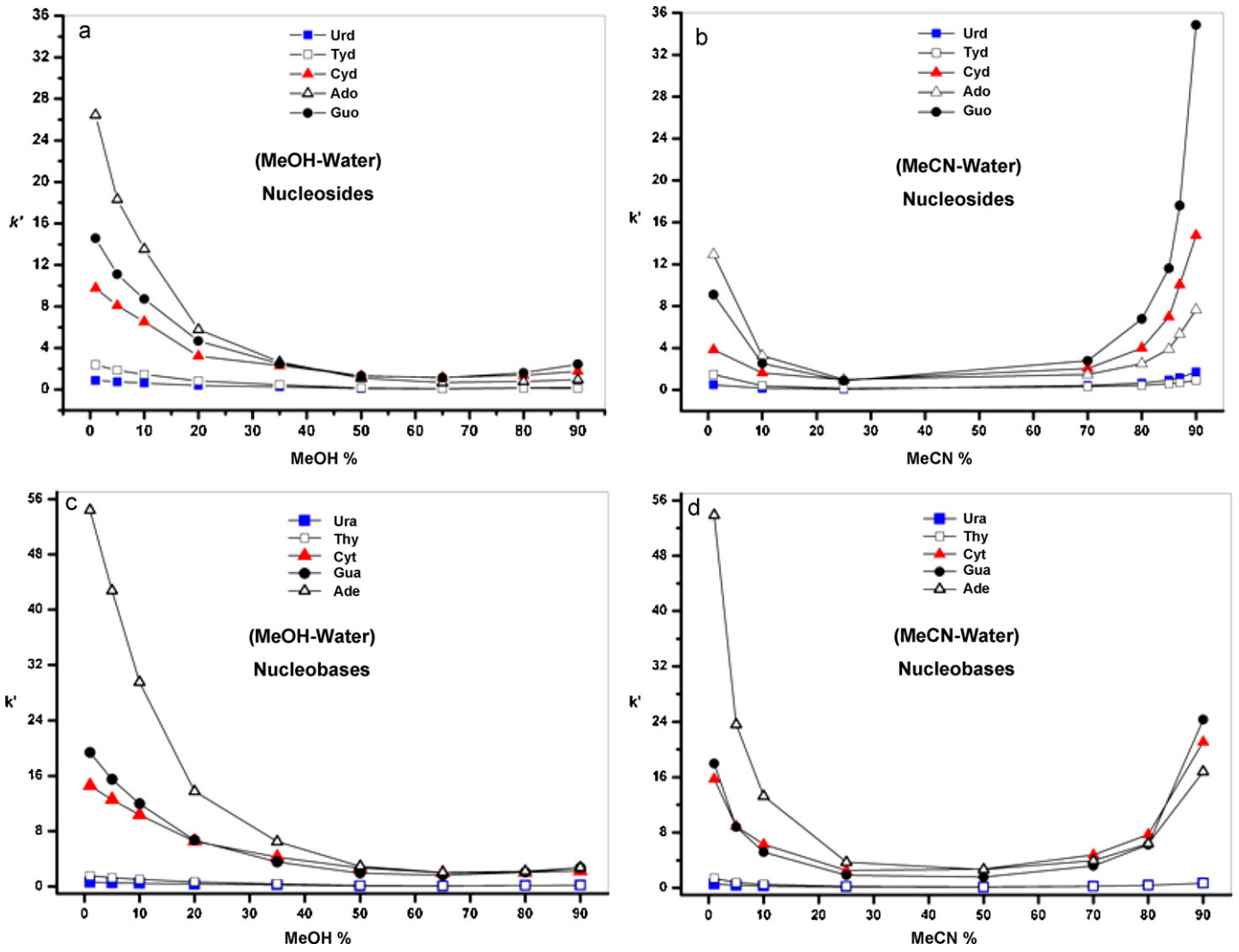

Fig. 4. Differentiations in k' values of nucleosides (a) and (b), and nucleobases (c) and (d) depending on the type and percentage of organic modifier used in mobile phase. Mobile phase: MeOH–water (a) and (c), MeCN–water (b) and (d); flow rate: 0.50 mL/min; detection wavelength: 254 nm; temperature: 25 °C; Injection volume: 2 μL (*Note*: Each data point on the graphs is the mean of at least two replicates where standard deviation in k' values does not exceed 0.03).

Elution of nucleosides was found to be highly affected by the type of organic modifier used, so that MeCN–water mobile phase system eluted nucleosides more easily than MeOH–water, when the percentage of organic modifier was 1–50% (v/v). Under similar conditions, k' values of nucleobases were found not to be affected by the type of organic modifier, significantly. Under RPLC conditions, as in the case of MeOH–water mobile phase system, Urd, Tyd, Ura and Thy exhibited relatively low k' values in MeCN–water system, too. Finally, depending on the type of organic modifier used in mobile phase, no change was observed in elution order of all the studied compounds, while significant changes were observed in selectivity for nucleosides.

All the studied compounds were successfully separated under so-called RPLC conditions (Fig. 5), and RPLC behavior of EC-HA-APS was observed. RPLC behavior of EC-HA-APS was related mainly with hydrophobic backbone of HA, and also hydrophilic and ionizable groups were thought to contribute in selectivity. Under RPLC conditions, elution order of the studied compounds was compared with that previously obtained on C18 stationary phase [23], and some important differences were observed for elution of some compounds. This might be resulted from cooperation of different separation mechanisms in the case of EC-HA-APS, as HA bears both hydrophobic and hydrophilic structures. Owing to this nature, it is actually difficult to distinguish RPLC and "*per* aqueous liquid chromatography (PALC)" behaviors which are likely to be observed on EC-HA-APS. Both behaviors may cooperate under highly aqueous conditions at varying degrees, and further studies are needed to understand this issue well. So, in the present study, we used the term RPLC (or highly aqueous RPLC) in order to not to complicate the discussions.

As known, separation mechanism in HILIC mode is based on the distribution of analytes between a water-enriched layer stagnant to the stationary phase and mobile phase [24]. Depending on the properties of stationary phase, different interactions may cooperate in chromatographic separation at varying degrees. Owing to the hydrophilic groups (e.g. –COOH, –OH, etc.) present in HA, EC-HA-APS was thought to exhibit HILIC behavior, and this behavior was clearly observed in MeCN–water system when percentage of MeCN was between 70 and 90% (v/v).

Comparing the trends in k' values of nucleosides under RPLC and HILIC conditions, the most significant change was which observed in elution order. So, retention times for the studied nucleosides increased in the order Urd < Tyd < Cyd < Guo < Ado and Tyd < Urd < Ado < Cyd < Guo, respectively, under RPLC and HILIC conditions.

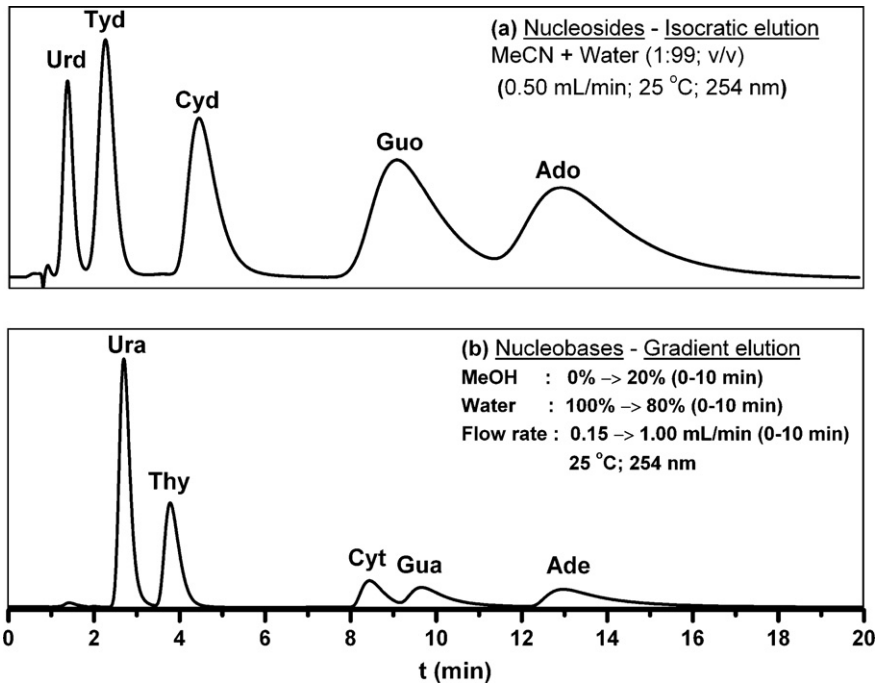

Fig. 5. Chromatograms recorded for (a) nucleosides, and (b) nucleobases on EC-HA-APS using isocratic and gradient elution, respectively (RPLC conditions). Experimental conditions are annotated on the figure.

Under HILIC conditions, elution order of nucleobases was found to be similar to that in the case of nucleosides: Thy∼Ura < Ade < Cyt < Gua. Comparing the k' values obtained under RPLC and HILIC conditions, the most striking change was that observed in k' values of Ade: Under RPLC conditions, it was difficult to elute Ade, whereas it was eluted easily under HILIC conditions.

Through isocratic elution, the studied nucleosides were successfully separated under HILIC conditions, too (chromatogram was shown in the following sections). However, as retention factors of Ura and Thy were too close to each other, all the five nucleobases could not be separated in this mode. Elution order of some of the studied compounds was found to be very similar to those obtained on various HILIC stationary phases [25]. As a conclusion of this section, HILIC behavior of the stationary phase was confirmed, too. The selectivity observed for nucleosides under HILIC conditions was found to be higher in comparison to nucleobases. This was attributed to the structural differences between the two groups of compounds.

3.2.2. Effect of ammonium solution and temperature

Another important finding was the efficiency of ammonium solution in elution of the studied compounds on EC-HA-APS both in RPLC and HILIC modes. Both nucleosides and nucleobases were found to be eluting easily by using aqueous mixtures of MeOH–$NH_4^+{}_{(aq)}$ as mobile phase, comparing to MeOH–water. When NH_4^+ solution was used in the mobile phase (instead of Water), k' values of the compounds, especially which bear $-NH_2$ group (Cyt, Cyd, Gua, Guo, Ade, and Ado), were found to decrease, significantly, both in RPLC and HILIC modes. Also, selectivity of the stationary phase was found to be changed depending on the pH of ammonium solution between 3.0 and 7.0. Another advantage of NH_4^+ solution may appear in LC–MS analyses, because it does not exhibit any difficulty in ionization when it is introduced to MS. So, studying with ammonium solution was thought to be interesting and useful in HPLC studies of nucleosides and nucleobases to be performed on EC-HA-APS.

Temperature was found not to have an effect on elution order both under RPLC and HILIC conditions. However, significant decrements were observed in k' values under RPLC mode with increasing temperature. On the other hand, temperature was found to have little effect on k' values under HILIC conditions. The temperature dependence of the retention mechanism was evaluated through van't Hoff plots. So, $\ln k'$ values were graphed versus $1/T$, and the trends in data points were evaluated (figures not shown). As a general trend, the data points exhibited a linear distribution ($0.92 \leq r^2 \leq 1.00$), and thus there was no evidence that could be attributed to the changes in retention mechanism depending on temperature under RPLC and HILIC conditions. Based on van't Hoff plots, exothermic character of retention enthalpies was concluded almost for all the studied species under RPLC and HILIC modes. Thus, retention mechanisms under RPLC and HILIC conditions were understood not to be changed depending on the temperature. Under both RPLC and HILIC conditions, it is difficult to explain the retentive behaviors on the basis of a uni-type mechanism. So, the observed behaviors were thought to be resulting from a combination effect of various mechanisms taking role at varying degrees. On the other hand, depending on the experimental conditions, one of the mechanisms is believed to predominate, and this may be the reason of the linearity observed in van't Hoff plots. Hence, it was concluded that (*i*) hydrophobic interactions between the solutes and stationary phase, and (*ii*) distribution of the solutes between water-enriched layer stagnant to stationary phase and mobile phase might be the mechanisms predominating under RPLC and HILIC conditions, respectively. So, RPLC/HILIC mixed-mode behavior of EC-HA-APS should be analyzed, carefully.

3.2.3. RPLC/HILIC mixed-mode behavior

RPLC/HILIC mixed-mode behavior of EC-HA-APS was evaluated in terms of orthogonality. Gilar et al. [26] proposed a useful approach called "geometric approach" for quantitative description of orthogonality. In this approach, retention times, $t_{R,i}$, recorded

under each chromatographic mode was converted to normalized retention times, $t_{R,i(\mathrm{norm})}$, according to the following relation:

$$t_{R,i(\mathrm{norm})} = \frac{t_{R,i} - t_{R,\mathrm{min}}}{t_{R,\mathrm{max}} - t_{R,\mathrm{min}}}$$

where $t_{R,\mathrm{min}}$ and $t_{R,\mathrm{max}}$ were the retention times for the first and last eluted peaks, respectively. In this way, $t_{R,i(\mathrm{norm})}$ values are calculated for each chromatographic mode, and are graphed on a 2D surface containing $N \times N$ bins, number of bins being equal to the number of peaks recorded. Surface coverage by the data points is related with the orthogonality between the two chromatographic modes applied, and percent orthogonality ($O\%$) is estimated according to the following relation:

$$O\% = \frac{\sum \mathrm{bins} - \sqrt{P_{\mathrm{max}}}}{0.63 \times P_{\mathrm{max}}}$$

where $\sum$bins and $\sqrt{P_{\mathrm{max}}}$ represent the number of bins covered by the data points and sum of bins in 2D surface, respectively.

In this approach, number of analytes is desired to be as high as possible for a good estimation of the degree of orthogonality. However, in the present study, the number of analytes was 10, and so we could study with 9 analytes to obtain a 3×3 bins system. Since the number of analytes was too low, we followed a different approach for a reasonable estimation of $O\%$. For this purpose, $t_{R,i(\mathrm{norm})}$ values calculated for HILIC mode were graphed versus $t_{R,i(\mathrm{norm})}$ values obtained under different RPLC conditions where different types and percentages of organic modifiers had been used (Fig. 6). The $O\%$ values calculated from each graph were used to derive a mean value for $O\%$. As can be seen from the figure, calculated $O\%$ values are ranging between 17.6 and 70.6, distributing within a wide range. So, $O\%$ value seems to be highly dependent on the type and percentage of organic modifier used in RPLC mode. The approach we proposed in the present study is thought to be reasonable and useful to predict $O\%$ value. So, the mean value for $O\%$ was calculated as 48.5%, representing a moderate orthogonality between the two modes of HPLC. However, by studying with analytes having higher molecular weight (e.g. proteins), the degree of $O\%$ is expected to be higher. So, RPLC/HILIC mixed-mode behavior of EC-HA-APS has been proven by the orthogonality analysis.

Chromatographic separation of nucleosides was optimized through a two-level full-factorial design where *steepness of MeOH gradient* and *pH of ammonium solution* were the experimental parameters studied. The results obtained through factorial design

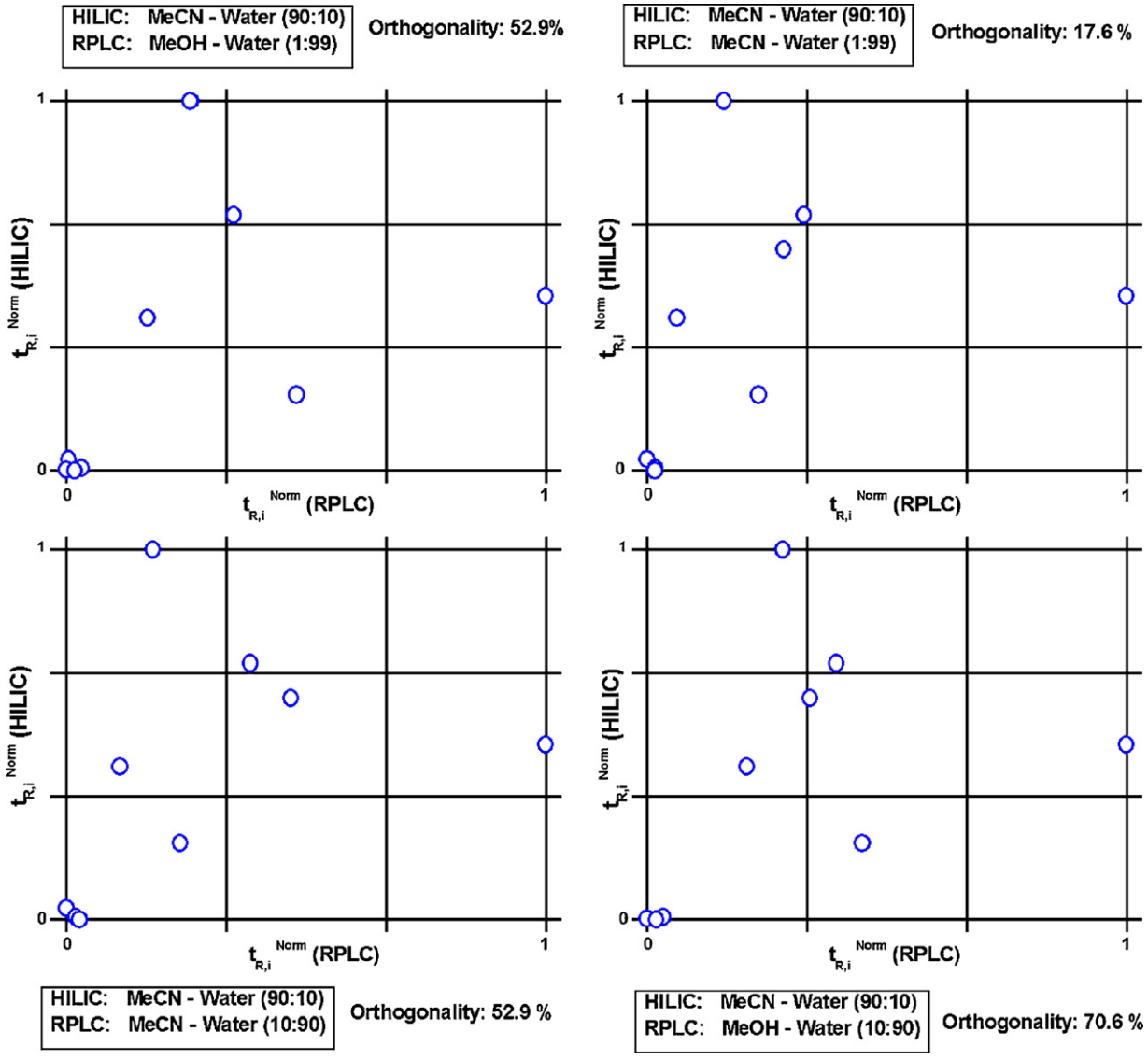

Fig. 6. Graphical demonstration for normalized retention times of nine selected compounds to evaluate $O\%$ between RPLC and HILIC modes. Experimental conditions are annotated on the figure.

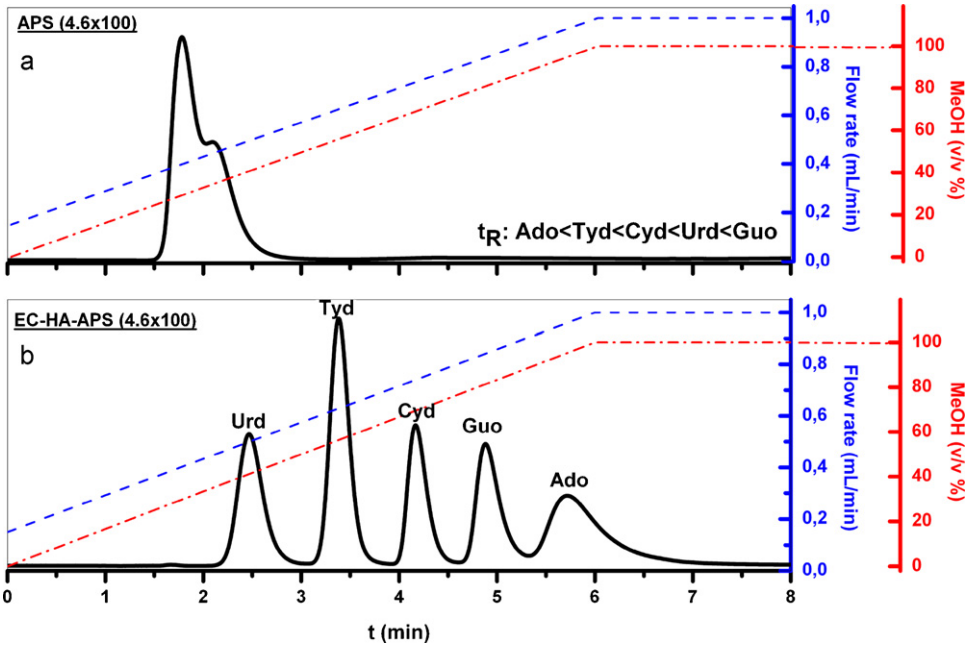

Fig. 7. Comparison of the chromatograms recorded for nucleosides on (a) APS and (b) EC-HA-APS using gradient elution (RPLC/HILIC mixed-mode conditions). Mobile phase: MeOH–$NH_{4(aq)}^{+}$; detection wavelength: 254 nm; temperature: 25 °C; injection volume: 2 μL); pH of ammonium solution was adjusted to 5.0 using 0.10 M HCl and 0.10 M NaOH solutions; gradients applied in flow rate and MeOH percent are annotated on the graph. Column was conditioned with MeOH–water (50:50, v/v%) and 100% water before run.

manner led application of steep MeOH gradients when pH of NH_4^+ solution was 5.0. The chromatogram recorded under determined optimal conditions (Fig. 7) clearly revealed mixed-mode RPLC/HILIC behavior of the stationary phase. As be seen from the chromatogram, the applied MeOH gradient scans mobile phase compositions corresponding to RPLC and HILIC modes thoroughly in one run. Thus, combination effect of RPLC and HILIC behaviors led to a chromatographic separation where the peak resolutions were around 1.5. So, the recorded chromatogram visualizes the mixed-mode RPLC/HILIC behavior of the stationary phase towards the studied compounds.

3.2.4. Column performance

On the column conditioned enough time, within-day and between-day reproducibility was evaluated in terms of standard deviation in k' values. Over 12 months, approximately 900 analyses were performed on the column packed with EC-HA-APS, and standard deviation in k' values never exceeded 0.03.

The chromatograms recorded by using EC-HA-APS and APS columns are comparatively given in Figs. 7 and 8. The effect of HA immobilization to APS is seen clearly from the chromatograms, so that both capacity and selectivity seem to be improved after HA immobilization to APS. Besides, there are some changes in elution order when RPLC/HILIC mixed-mode conditions are applied (Fig. 7). However, elution orders are almost similar when HILIC conditions are applied (Fig. 8). This may imply that under HILIC conditions the retention mechanism is virtually independent of the type of stationary phase used. On the other hand, there seem significant differences in capacity and selectivity after HA immobilization to APS under the two modes of HPLC applied.

Another superior property of EC-HA-APS was observed when studying with highly aqueous mobile phases under RPLC conditions. As known, most of the RPLC stationary phases collapse when they are interacted with highly aqueous mobile phases, and thus exhibit low capacity and selectivity towards low-molecular weight polar compounds. Nevertheless, some of the studied species were separated easily on EC-HA-APS by using highly aqueous mobile phases (percentage of organic modifier was less than 5%) as shown in Fig. 5. This implies that EC-HA-APS has an open structure even under highly aqueous conditions, too. This behavior must be due to the presence of hydrophilic groups covalently bonded to the organic backbone of HA, which may restrict the collapse of hydrophobic backbone of immobilized HA macromolecules due to water molecules.

The peak area recorded through (*i*) HPLC analyses and (*ii*) FIA were compared with each other. The results showed that peak area recorded via HPLC and FIA modes fitted over 95% with each other, and so there was no evidence for irreversible bindings of the studied solutes to EC-HA-APS. This implies the suitability of EC-HA-APS as a stationary phase.

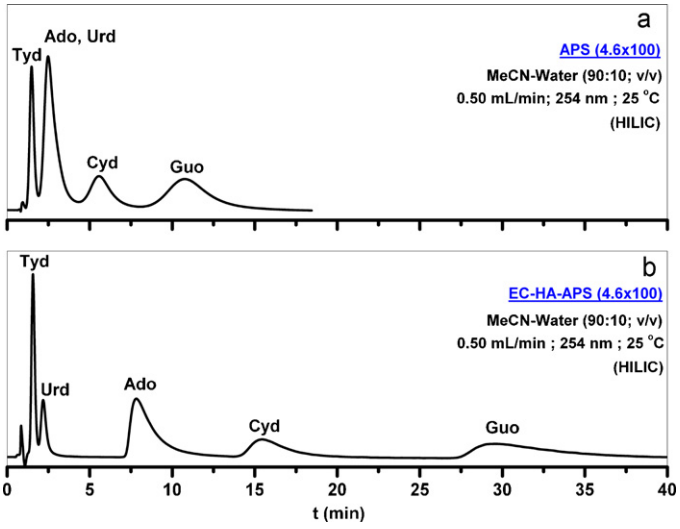

Fig. 8. Comparison of the chromatograms recorded for nucleosides on APS and EC-HA-APS using isocratic elution (HILIC conditions). Experimental conditions are annotated on the figure.

4. Conclusion

As a conclusion, the stationary phase, which was obtained by immobilization of HA to APS via chemical bond formation and subsequent end-capping process, exhibited mixed-mode RPLC/HILIC behavior towards some nucleosides and nucleobases. The orthogonality between the two modes of HPLC was calculated as 48.5%, revealing the applicability of EC-HA-APS in mixed-mode RPLC/HILIC separations. Chromatographic separation of the studied species was achieved under RPLC and HILIC modes. Depending on the HPLC mode applied, some differences were observed in retentive behaviors of the compounds, and this situation was related with the changes in mechanism of separation. Chromatograms recorded on EC-HA-APS were compared with those recorded on APS under similar conditions. The results showed that there were significant changes both in capacity and selectivity of APS after HA immobilization. Also, some differentiations were observed in elution order of the compounds, by comparing the separations on APS and EC-HA-APS. Chromatographic behaviors could easily be related with chemistry of the studied species and stationary phase. Thus, the column packed with EC-HA-APS is understood not to be a "*black-box*" where it is difficult to understand the mechanism of separation, and accordingly to interpret the retentive behaviors, due to the unknown structure of HA. It should be, however, noted that further studies are needed to understand its behavior and efficiency well.

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