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Analysis of oligonucleotides by liquid chromatography with alkylamide stationary phase

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Abstract: The main aim of the present investigation was to determine retention behavior and interactions of oligonucleotides with alkylamide stationary phase. Five oligonucleotides, differing in the sequence, were tested. Changes in the composition of the mobile phase, i.e. pH (5.0–7.0) and buffer concentration (30–75 mM) were investigated in detail. In addition, the hydrophobic and electrostatic parameters were measured for the different pH's and salt concentrations. The theoretical model of interactions between individual elements of the separation system, (e.g. solute, stationary phase, and mobile phase) based on zeta potential measurements, hydrophobic and electrostatic parameters calculation, and molecular modeling, has been considered.

Keywords: oligonucleotide, mobile phase, alkylamide stationary phase, interactions, zeta potential

1 Introduction

Reversed phase high-performance liquid chromatography (RP-HPLC) is one of the most important techniques for analysis of biomolecules, such as proteins, oligonucleotides, and so on [1-3]. Oligonucleotides are short sequences of DNA and/or RNA that have unique properties. They can be synthesized up to 160–200 bp[4]. These compounds are commonly used as starters in polymerase chain reaction, in gene delivery and as drugs for some diseases such as a virus-associated illness, AIDS, Alzheimer, cardiovascular disorder, and cancer [5-7]. Their determination is important in many biochemical analyses relevant to therapeutic applications.

High performance liquid chromatography (HPLC) is the most commonly used technique for the separation and determination of unmodified and therapeutic oligonucleotides [1-3,8]. There are many theoretical studies concerning the interaction of oligonucleotides within the surface of stationary phase [8-10]. Most of them include complex mechanisms that comprise different interactions in hydro-organic solvents [1-3,8-10]. Many different and novel approaches to the study of the retention mechanism in HPLC may be found in the literature [11-13]. One of them is the determination of zeta potential [14]. It denotes the surface charge that occurs in the presence of an aqueous solution, when functional groups dissociate on hydrophilic surface or negative ions adsorb onto hydrophobic surfaces from the solution. The changes in the mobile phase pH can cause significant changes in the equilibrium between dissociation and adsorption processes. In addition to the pH of the solution, other parameters e.g. type of salt and its concentration are also very influential when surface electric charges are considered [11-14].

Octadecyl stationary phases are most commonly used in the analysis of oligonucleotides and other compounds. Utilization of this packing material does not achieve high efficiency and selectivity in the case of the studied compounds [8-10]. Thus, another type of stationary phase was chosen for the present study, namely alkylamide. It possesses different functional groups, which cause multi-point interactions allowing for high efficiency and an ability to distinguish between oligonucleotides [15,16].

The aim of the present study was to investigate the retention behavior of the oligonucleotides on alkylamide stationary phase under different mobile phase composition and to explore the influence of eluting parameters on the retention factor. The related interactions such as hydrophobic, electrostatic, and hydrogen bonding were investigated. The results showed that the surface electrical properties of alkylamide stationary phase have a significant impact on the interaction between stationary phase and oligonucleotides.

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Table 1: Sequence and retention time of oligonucleotides in different salt concentration and mobile phase buffer pH.

Compound	Sequence	Fragment (mer)	Molecular weight (MW)	pH	k		
					30 (mM)	50 (mM)	75 (mM)
OLG **1**	5'-ATCGATCGATCGATCGATCG-3'	20	6113	7	0.182	0.500	1.546
				6	0.682	1.318	2.409
				5	5.364	4.000	2.818
OLG **2**	5'-ATCGATCGTTCGATCGATCG-3'	20	6104	7	0.227	0.727	1.864
				6	0.682	1.500	2.818
				5	9.909	6.046	3.636
OLG **3**	5'-ATCGATCGCTCGATCGATCG-3'	20	6089	7	0.227	0.636	1.773
				6	0.955	1.818	3.046
				5	23.546	12.636	6.682
OLG **4**	5'-ATCGATCGGTCGATCGATCG-3'	20	6128	7	0.227	0.546	1.273
				6	0.682	1.455	2.682
				5	10.682	5.909	3.500
OLG **5**	5'-ATCGATCGATCGATCGATCC-3'	20	6074	7	0.364	1.136	2.546
				6	1.000	1.864	3.364
				5	11.909	8.136	5.046

2 Experimental

2.1 Reagents and chemicals

Standards of oligonucleotides were purchased from GeneSys (Wrocław, Poland), and purified by desalting. Their sequences and molecular weights are in Table 1. Since the studied substances (0.1 mM) were provided in lyophilized form, the standard solutions were prepared by dissolution in deionized water. Methanol of gradient grade purity (J.T. Baker, Deventer, Netherland), and deionized water from Milli-Q system (Millipore, El Paso, TX, USA) are commonly used to prepare mobile phases. The salts and acids were also used for buffer preparation, namely ammonium acetate (CH_3COONH_4), potassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4), and acetic acid (CH_3COOH). These reagents were purchased from Sigma-Aldrich (Gillingham, Dorset, UK).

2.2 Chromatographic analysis of oligonucleotides

Chromatographic studies were performed on an Agilent 1100 series liquid chromatograph (Santa Clara, California, United States) consisting of a quaternary pump, an automatic injector, a column oven, and a DAD detector. The ChemStation software was used to collect and process the data. The packing materials were home-made and functionalized with alkylamide molecule [16-18]. The phase was packed into (125 × 4.6 mm ID, 5.0 μm) stainless-steel columns by using a home-made apparatus equipped with Haskel pump (Burbank, CA, USA) under constant pressure.

The magnitude of 1 and 3 μL of the various oligonucleotide samples were loaded onto the column and isocratic elution was carried out with the three different mobile phase pH levels (5.0, 6.0, and 7.0), in various mobile phase buffer salt concentration (30, 50, and 75 mM). The CH_3COONH_4 solution was used for pH 5.0, while in the case of pH 6.0 and 7.0, KH_2PO_4/K_2HPO_4 buffer was studied. The pH of CH_3COONH_4 solution was adjusted with 1.0 M acetic acid. Phosphate buffers were made by mixing KH_2PO_4 and K_2HPO_4 solutions to prepare pH 6.0 and 7.0. The organic solvent content in the mobile phase was constant and equalled 10% v/v of methanol. The autosampler and column temperatures were constant at 30°C. The flow rate was equal to 0.7 mL min^{-1}. The absorbance of the eluate was continuously measured at $\lambda = 254$ nm.

2.3 Molecular modeling of alkylamide stationary phase

The structure and electrostatic potential of alkylamide stationary phase was modeled with the use of Gaussian 03 package program [19,20]. Molecular structures were visualized with GaussView. The geometry of the molecule was optimized by the use of the molecular mechanics method, MM3, until the root mean square gradient

became smaller than 0.01 kcal mol^{-1} Ĺ$^{-1}$. To produce a 3D plot of the MEP, the electrostatic potential cube file was calculated from total SCF density. The contour maps of the electrostatic potential were then drawn using a distance between grid points of 0.02 Ĺ and the isovalues 0.0004.

2.4 Zeta potential measurements for alkylamide stationary phase

The zeta potential of alkylamide stationary phase was performed using Zetasizer Nano ZS instrument (Malvern Instruments Ltd., United Kingdom). The charge on the surface of particles is characterized by measuring the zeta potential of a suspension at 30°C, as a function of pH (5.0–7.0) and different buffer salt concentration (30–75 mM). 1 μg of packing materials was suspended on 1.5 mL of solution and next sonicated for 20 min. Then samples were injected directly into the capillary cell electrophoresis measurement and a measurement of the particle electrophoretic mobility resulted in the calculated zeta potential. The obtained results were processed using Dispersion Technology Software (Malvern Instruments).

3 Results and Discussion

3.1 Alkylamide stationary phase

Alkylamide stationary phase possesses different functional groups bonded to the silica surface: alkylamide, aminopropyl and residual silanols. Results obtained from elemental analysis permit determination of carbon and nitrogen content in the packing material surface. Alkylamide stationary phase constitutes 5.17% of carbon on the silica support and five-fold lower nitrogen content (which equals 1.01%). This has an impact on the charge distribution on the stationary phase surface. The twelve carbon alkyl chain will interact by hydrophobic interactions during the chromatographic process. Contrarily, aminopropyl and amide groups will bind oligonucleotides because of the polar interactions i.e. hydrogen bonding or electrostatic interactions [15-18].

The alkylamide stationary phase was used for four months with a flow rate of 1 mL min^{-1} and mobile phases containing organic or inorganic buffers of various concentrations. It was stable during this period of time, results were repeatable, and the column was efficient (the number of theoretical plates).

The graph presented in Fig. 1 visualizes the distribution of charge on the surface of stationary phase. Regions of the negative electron density are marked with red color, while fragments of positive density (or less negative) are mapped using blue color. Negative potentials correspond to the attraction of the probe unit charge, whereas, positive ones are related to repulsive interactions of the probe charge. Colors ranging from green to yellow account for the hydrophobic properties of packing material. As it was assumed, alkyl ligands remain non-polar. In the region of amide group, as well as in the case of aminopropyl, one or two contrary regions may be observed – a positive (aminopropyl) one and a negative one (amide). The presence of hydrogen bonded to nitrogen atom provides positive potential. However, it has to be pointed out that this modeling was done in vacuum conditions, without taking into account the influence of the mobile phase in liquid chromatography. Therefore, present investigation was extended to study the influence of solvent on the surface properties of packing material and its interaction with oligonucleotides.

3.2 The selection of oligonucleotides

The synthetic and unmodified oligonucleotides differing in sequence were chosen for present investigations. All tested compounds had the length of 20 mers and differed in the position of the nucleobase or base substitution (Table 1). Four oligonucleotides (OL1, OL2, OL3, OL4) possess various nitrogen bases in the 9th position of oligomer (Table 1). In the case of the fifth compound (OL5), the guanidine in the 20th position of the sequence was replaced by cytidine (Table 1). Oligonucleotides used during the study may form hairpin loops. The most probable secondary structure of these biomolecules was presented in Fig. 2 A and B. The studies were carried out under non-denaturating conditions, therefore, oligonucleotides were not single stranded. Sequences of the studied compounds are very similar, and structural differences are not significantly different. There are various functional groups in their structures: hydrophobic and hydrophilic ones. Thus, these compounds may interact with the stationary phase surface by many different types of interactions, such as hydrophobic, electrostatic, and/or hydrogen bonding.

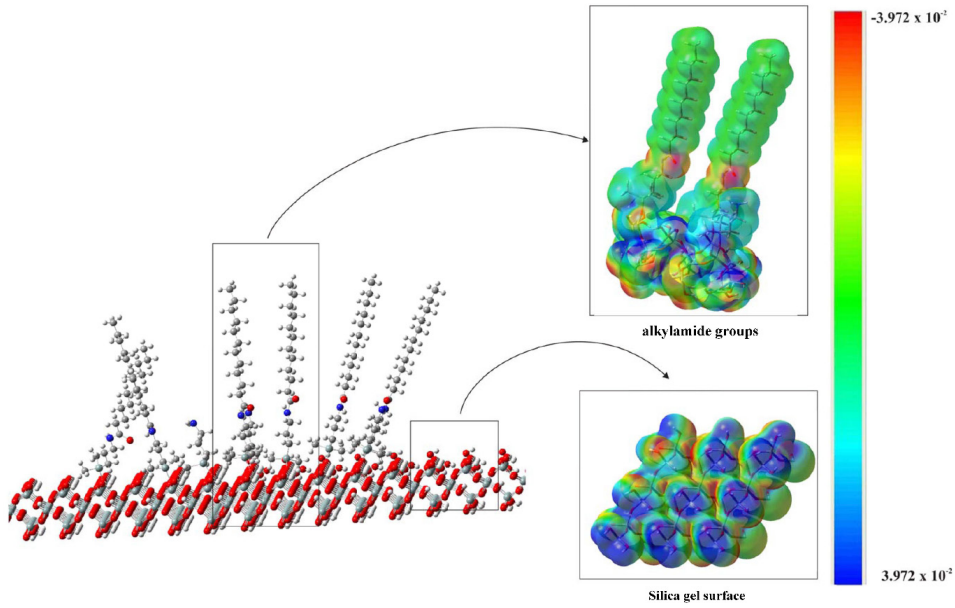

Figure 1: Structure of synthesized alkylamide stationary phase.

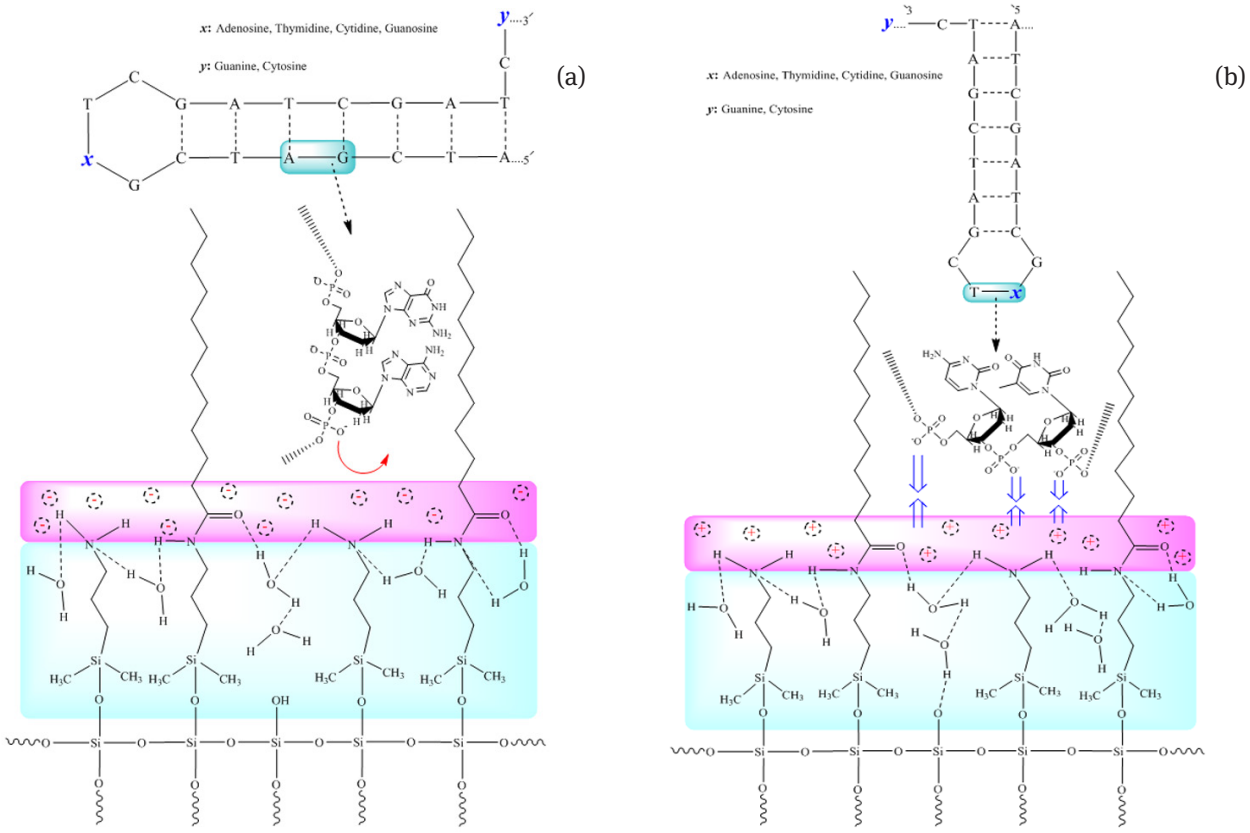

Figure 2: (a) Interaction schemes of secondary structure of oligonucleotides with alkylamide stationary phase at pH ≥ 6.0 in low salt concentration. (b) Interaction schemes of secondary structure of oligonucleotides with alkylamide stationary phase at pH ≤ 5.0 in low salt concentration.

3.3 The influence of pH and concentration of salt on oligonucleotide retention

Retention studies of oligonucleotides on the alkylamide stationary phase were done during the present investigation. The *k* values for each oligonucleotide were determined for different concentrations of mobile phase buffer and its pH. All experiments were performed with the use of alkylamide stationary phase and results were collected in Table 1. It was proven that with the decrease in pH of the mobile phase buffer, the *k* value was increasing for all of analyzed oligonucleotides (Table 1). Moreover, two opposite effects related to the concentration of buffer were also observed. In the case of pH 7.0 and 6.0, the retention of studied compounds was greater for higher concentrations of salts. An opposite tendency was noticed for pH 5.0, where *k* values were decreasing with the increase in buffer concentration.

The binding strength of oligonucleotides to the stationary phase will increase by increasing their hydrophobic surface area, but also charge, size and biospecific recognition must be considered. In the backbone of oligonucleotides, the phosphate groups carry a negative charge that will be attracted to the stationary phase, while the positively charged groups will present at surface of the support. Alkylamide packing material possess aminopropyl groups, which are able to undergo protonation at low pH of mobile phase buffer (pH 5.0). The zeta potential was investigated to determine the charge distribution on alkylamide packing.

3.4 Zeta potential for alkylamide packing material

Determination of zeta potential is useful when the characterization of surface charge is needed. Therefore, it was applied during present investigation, to study the real charge distribution as a function of salt pH and concentration. The magnitude of the zeta potential was estimated for various compositions of mobile phases (pH 4.0–7.0 and salt concentration 30–75 mM).

The obtained zeta potential values ζ (in mV), standard deviation STD (in mV) for the three repeated measurements of each sample in different pH and salt concentration are shown in Fig. 3. At pH 4.0 and 5.0, zeta potential decreased with increasing salt concentration because of the increasing ionic strength. In the case of pH = 4.0, the highest potential (ζ = +1.35 mV) is measured in comparison to other samples at a salt concentration of 30 mM. This is possibly caused by the positive surface charges. This indicates the protonation of aminopropyl groups at pH 4.0 and 5.0. This phenomenon can be related to the diminution of the repulsive potential. Additionally, at pH 7.0 and 6.0, opposite effects were observed. As shown in Fig. 3, in the case of salt concentration of 30 mM, the lowest surface charge potentials (ζ = -19.88 mV) were detected at pH = 7.0. The results showed that salt concentration has an influence on zeta potential, but alkylamide surface charges, significantly depend on pH values. Moreover, at pH lower than 6, aminopropyl groups are protonated, while at pH higher than 6 residual silanols possess partial negative charges.

3.5 The discussion on retention mechanism of oligonucleotides on alkylamide packing material

The influence of the pH appears to have great impact on the analysis of oligonucleotides in the case of alkylamide packing material. This is a consequence of the change in magnitude of protonic charge on the oligonucleotides and stationary phase. The modifications made in the pH of mobile phase buffer cause the change in the retention mechanism of oligonucleotides. Consequently, the hydrophobic interactions are the most influential for mobile phases with buffers of pH 6.0 or 7.0. Contrary oligonucleotides interact with alkylamide stationary phase mainly by electrostatic interactions at pH 5.0 (Table 1). Therefore, it is possible to increase the strength of binding of oligonucleotides with stationary phase on the basis of electrostatic interactions. At pH 6.0 and 7.0, the negative charge of the stationary phase is not sufficiently shielded at low salt concentrations. Consequently, negatively charged phosphate backbones of oligonucleotides are electrostatically repulsed from the surface.

The *k* values are increasing with the buffer concentration when hydrophobic interactions take place during the retention mechanism. By increasing the salt concentration at pH 7.0 and 6.0, the viscosity of the mobile phase will increase, whereas diffusivity of the oligonucleotides decreases.

The opposite situation may be observed for pH 5.0, when electrostatic attraction between oligonucleotides and stationary phase appears (Table 1). The *k* values are lower for 75 mM than for 30 mM of mobile phase buffer. These tendencies are characteristic of an ion-exchange mode. The phosphate groups in oligonucleotides are attracted to the stationary phase surface. The alkylamide packing material becomes a weak anion exchanger at pH 5.0. Consequently, increasing the salt concentration at this value of pH, causes greater ionic strength of the mobile phase. The salt concentration can also affect the dipole-

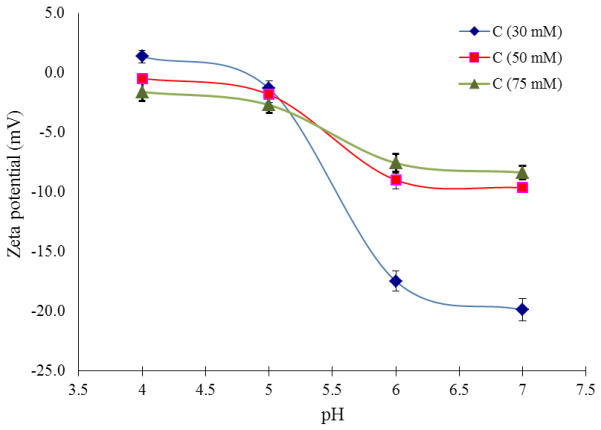

Figure 3: Plots of the zeta potential values obtained for different salt concentration prepared at different pH values.

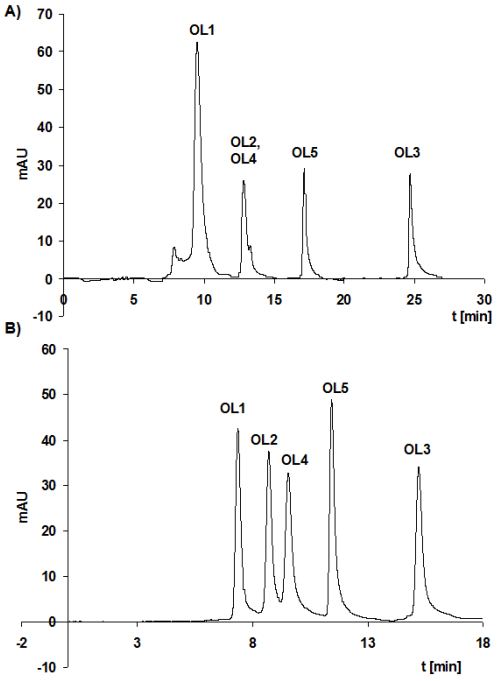

Figure 4: Results of oligonucleotides separation with the use of alkylamide stationary phase. Chromatographic conditions: A) mobile phase: 90% *v/v* 50 mM CH_3COONH_4 (pH 5), 10% *v/v* ACN; B) mobile phase: 90% *v/v* 75 mM CH_3COONH_4 (pH 5), 10% *v/v* ACN; flow rate 1.0 mL min^{-1}. The autosampler and column temperature 30°C. UV-Vis detection λ = 254 nm.

dipole interactions with the dipolar oligonucleotide molecules.

It may be concluded that in the case of pH 6 and 7, a salting-out effect may be observed, but if the hydrophobic interactions are neglected, salting-in takes place. Weakly hydrophobic interactions cause a small change in the retention factor of oligonucleotides at pH 7.0 and 6.0. The opposite effect was observed when electrostatic interactions were dominant during retention mechanism.

3.6 Separation of oligonucleotides

The attempt of oligonucleotide mixture separation was also done during the study. An acidic pH of mobile phase was selected, since retention factor for pH 6 and 7 was low (Table 1). Consequently, complete separation was not possible. Fig. 4 presents the best results obtained for isocratic elution mode. The board peak shape may be observed; however, *k* values were high and separation of four oligonucleotides was possible (Fig. 4 A). The utilization of higher buffer concentration caused a decrease of *k* values, reduction of analysis time and peak asymmetry (Fig. 4B). Therefore, the separation of all five biomolecules was obtained. Application of alkylamide packing may be successfully used for the separation of oligonucleotides; however, the mix mode elution conditions should be used, when retention depends on both ionic and hydrophobic conditions.

3.7 The effect of oligonucleotide structure on the elution order

The data summarized in Table 1 allows for the estimation of the impact of oligonucleotide sequence on their retention for alkylamide stationary phase. The differences between the retention factor with respect to the elution order are as follows: adenine < thymine ≤ guanine < cytosine. The greatest differences in the retention factor between various oligonucleotides were observed for acidic pH. It is a consequence of the change in retention mechanism and the influence of electrostatic and hydrogen bonding interactions. The reason also lies in the orientations of the bases in the oligonucleotides through interactions with alkylamide stationary phase.

Elution of oligonucleotides depends on the hydrogen atom positions involved in the interactions. It can be suggested that nucleotide bases can be oriented and form hydrogen bonding interactions between sequences and stationary phase by electrostatic adsorption of phosphodiester backbone with alkylamide packing material. Also, by changing adenine (OL 1) to thymine (OL 2) or guanine (OL 4), the retention factor is nearly doubled, but replacement of adenine by cytosine (OL 3) causes about 4-fold greater *k* values (Table 1), which corresponds to greater hydrogen bonding interactions with studied packing material (Fig. 2).

4 Conclusions

The results in this work show a relationship between the retention of the oligonucleotides and mobile phase composition for alkylamide stationary phase. It was shown that retention of these compounds depends on several interaction types. Retention factor of the studied compounds can be described by change of stationary phase charge. The retention factor increases with the decrease of mobile phase pH, regardless of salt concentration. Consequently, the retention and separation of oligonucleotides may be changed by changing the mobile phase composition and pH. Moreover, simple reversed-phase mode may be succesfully used for the determinetion of these biomolecules, instead of commonly used ion pair chromatography. Despite that, the peak asymmetry mixture of oligonucleotides may be separated with the mobile phase of acidic pH containing high concentration of buffer.

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