Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1006 (2003) 137–148
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Hydrophobic interaction chromatography of homo-oligonucleotides on derivatized Sepharose CL-6B
Using and relating two different models for describing the effect of salt and temperature on retention

M.M. Diogo[a,b], D.M.F. Prazeres[b], N.G. Pinto[c], J.A. Queiroz[a,*]

[a]*Departamento de Química, Universidade da Beira Interior, R Marques d'Avila e Bolama, 6201-001 Covilhã, Portugal*
[b]*Centro de Eng[a] Biológica e Química, Instituto Superior Técnico, 1049-001 Lisbon, Portugal*
[c]*Department of Chemical and Materials Engineering, University of Cincinnati, Cincinnati, OH 45221, USA*

Abstract

The effect of salt and temperature on the interaction of homo-oligonucleotides with a derivatized Sepharose CL-6B hydrophobic support has been explained by the application of two different models: the solvophobic theory and the preferential interaction analysis. It has been shown that the two approaches give adequate descriptions of the experimental results. The models were used in order to estimate, respectively the parameter *C*, which is proportional to the reduction in hydrophobic exposed surface area upon adsorption, and the number of water and salt ions released upon adsorption. It was concluded that the magnitude of these parameters can be strongly influenced by the temperature, the hydrophobicity of the bases of the nucleotides, the molecular mass of the oligonucleotides and the presence of secondary structures. Parameter *C* was quantitatively related with the number of water molecules and salt ions released upon adsorption. These parameters were found to correlate linearly in cases where structural changes with temperature are not significant.
© 2003 Elsevier B.V. All rights reserved.

Keywords: Hydrophobic interaction chromatography; Preferential interaction analysis; Solvophobic theory; Mathematical modelling; Oligonucleotides

1. Introduction

Hydrophobic interaction chromatography (HIC) has become a very widely used bioseparation technique for the laboratory- and industrial-scale purification of biomolecules such as therapeutic proteins [1], enzymes [1] and nucleic acids [2,3]. In a typical HIC system, the molecules are bound to the hydrophobic stationary phase from an aqueous mobile phase at a relatively high concentration of a common neutral salt, such as ammonium sulfate [4]. Elution and separation are carried out by decreasing the salt concentration in order to decrease hydrophobic interactions. HIC is carried out using mildly hydrophobic stationary phases and the salt in the eluent generally has a stabilizing effect on the molecules [4]. Thus, the maintenance of the structural integrity of the biomolecules is a major advantage of this technique.

HIC was recently used with success for the downstream processing of plasmid DNA for gene therapy applications [3]. In the cited work, a weak hydrophobic interaction chromatography gel was

*Corresponding author. Tel.: +351-275-319-760; fax: +351-275-319-730.
E-mail address: jqueiroz@alpha2.ubi.pt (J.A. Queiroz).

0021-9673/03/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S0021-9673(03)00918-X

obtained by derivatization of the cross-linked agarose matrix Sepharose CL-6B with 1,4-butanediol diglycidyl ether. This chromatographic gel was able to interact with hydrophobic single stranded nucleic acid impurities from *Escherichia coli*. In order to understand the mechanism of binding of nucleic acids single strands to this support, a study at room temperature was carried out using synthetic homodeoxyoligonucleotides of adenine (polyA), thymine (polyT) and uracil (polyU) with various molecular masses and at various ammonium sulfate concentrations [5]. It was found that the oligonucleotides interacted differently with the support according to the molecular mass, the hydrophobic character of the individual bases, the secondary structure of the molecule and the concentration of the ammonium sulfate in the eluent. The results obtained were interpreted on the basis of the solvophobic theory, because of good agreement between this model and the experimental data. This theory is briefly described next.

The magnitude of hydrophobic retention is determined by the free energy change for the equilibrium of the biomolecule between the bulk mobile phase and the stationary phase domains. The equilibrium constant can be written as:

$$\log K = -(\Delta G^{0}_{\mathrm{h}\phi}/2.3RT) \quad (1)$$

where $\Delta G^{0}_{\mathrm{h}\phi}$ is the Gibbs free energy change for the retention by hydrophobic interaction, R is the universal gas constant and T is the temperature [6]. The value of K, the equilibrium constant, can be related to the retention factor measured from the chromatograms, k', according to:

$$k' = \phi K \quad (2)$$

where ϕ is the phase ratio.

In the solvophobic theory, the free energy change for retention by hydrophobic interaction chromatography can be broken into contributions from electrostatic and Van der Waals interactions, biopolymer–ligand association in the absence of surrounding solvent, cavity formation needed to accommodate the various species in the solvent, other solvent–ligand and solvent–byopolimer interactions not treated in the preceding terms, and the entropy change arising from the change in the free volume [6]. In this way, the retention of biopolymers in HIC at sufficiently high salt concentrations, where the hydrophobic interaction is the main retention mechanism, can be described by a two-parameter equation:

$$\log k' = A + Cm_{\mathrm{s}} \quad (3)$$

where m_{s} is the salt concentration.

The slope of this equation, C, is termed the hydrophobic interaction chromatography parameter and is a function of the molecular surface area change upon binding to the stationary phase surface, $\Delta A'$, which is proportional to the hydrophobic contact area, and the ability of the salt to increase the surface tension of the solution, σ_{S} [6]:

$$C = \Delta A' \sigma_{\mathrm{S}}/2.3RT \quad (4)$$

The intercept A contains information both on hydrophobic interactions and other effects such as electrostatic and Van der Waals interactions (see Refs. [6,7]). By plotting Eq. (3) for sufficiently high salt concentrations, it is possible to estimate the values of parameters C and A that can describe the absorption behaviour in hydrophobic interaction chromatography.

An alternative representation to the solvophobic theory is the preferential interaction (PI) analysis that focuses on the interactions of the salt with the biomolecules. The effect that a biomolecule has on the distribution of the solvent and the solute molecules is characterised in the PI approach by the preferential interaction coefficient:

$$\Gamma^{\mathrm{m}}_{32} \equiv (\delta m_3/\delta m_2)_{\mathrm{T},\mu_1,\mu_2} = \upsilon_3 - (m_3/m_1)\upsilon_1 \quad (5)$$

where m is the molal concentration, and the subscripts 1, 2 and 3 refer to the solvent, the biomolecule and the solute, and υ_i is the moles of species i in the vicinity of the biomolecule per mol of biomolecule [8].

Considering a hydrophobic interaction adsorption process using an aqueous salt solution as the mobile phase:

$$b\mathrm{B} + s\mathrm{S} \rightleftarrows c\mathrm{C} \quad (6)$$

where B is the biomolecule, S is the surface site and C is the biomolecule–surface complex, it has been shown that the variation of the observed equilibrium constant [8] with the mean ionic activity of the salt

$a_{\pm}$, at a given temperature, T, and pressure, P, is given by the stoichiometrically weighted sum of the preferential interaction coefficients, Γ^{m}:

$$[\delta(\ln K_{obs})/\delta(\ln a_{\pm})]_{T,P,EQ} = c(\Gamma^{m}_{+,c} + \Gamma^{m}_{-,c}) - p(\Gamma^{m}_{+,p} + \Gamma^{m}_{-,p}) - s(\Gamma^{m}_{+,s} + \Gamma^{m}_{-,s}) = (\Delta\upsilon_{+} + \Delta\upsilon_{-}) - [nm_3\Delta\upsilon_1/m_1] \quad (7)$$

where $\Delta\upsilon_1$, $\Delta\upsilon_{-}$ and $\Delta\upsilon_{+}$ are the stoichiometrically weighted change in the number of mol of water, salt anions and salt cations, respectively, in the local regions of the products and reactants of the process, and n is the total number of anions (−) and cations (+) per formula unit of the salt [8]. For a biomolecule in an aqueous solution of ammonium sulfate, m_3 is the molal concentration of ammonium sulfate and m_1 is the molal concentration of water.

Rewriting the last equation in terms of the capacity factor and the molal salt concentration, gives:

$$[\delta(\ln k')/\delta(\ln m_3)]_{T,P,EQ} = [(\Delta\upsilon_{+} + \Delta\upsilon_{-})/g] - [(nm_3\Delta\upsilon_1)/(m_1 g)] \quad (8)$$

where:

$$g = [\delta(\ln m_3)/\delta(\ln a_{\pm})]_{T,P} \quad (9)$$

The values for g can be calculated from tabulated data for activity coefficients as a function of salt concentration, or by applying appropriate thermodynamic models.

If ion and water stoichiometries are independent of the salt concentration, integration of the last equation yields:

$$\ln k' = c + [(\Delta\upsilon_{+} + \Delta\upsilon_{-})/g]\ln m_3 - [(nm_3\Delta\upsilon_1)/(m_1 g)] \quad (10)$$

At low salt concentrations, where adsorption is governed by electrostatic interactions, the second term in the right hand side of the last equation is dominant and the process is controlled by the displacement of counter-ions $-(\Delta\upsilon_{+} + \Delta\upsilon_{-})$ [8]. At the higher salt concentrations, used in hydrophobic interaction chromatography, the third term in the right hand side of Eq. (10) is dominant and the increase in retention times with salt concentration is controlled by the displacement of water molecules, $-(\Delta\upsilon_1)$ [8]. By plotting the last equation, these two parameters can be estimated. The results obtained by Perkins et al. for a number of HIC systems reported that the release of water molecules (100–200) was significantly more than the release of salt ions (0.5–3) [8]. This is consistent with the commonly accepted idea that the adsorption in HIC is an entropically driven process [8].

As was mentioned earlier, the solvophobic theory has been shown to be a good model for explaining the interaction of homo-deoxyoligonucleotides with a hydrophobic derivatized Sepharose gel using different salt concentrations at room temperature. In this work, new experimental data were used in order to verify the applicability of both the PI analysis and the solvophobic theory for describing the behaviour of the same class of molecules on the same hydrophobic support at different temperatures. This is motivated by an inadequate understanding of the effects of temperature on hydrophobic interactions. The experimental data were also used to relate the parameters calculated from the two different theories. In fact, the value of parameter C from the solvophobic theory is proportional to the value of the surface area change upon binding to the stationary phase surface ($\Delta A'$), according to Eq. (4), which is proportional to the hydrophobic contact area. Thus, the value of C should, in principle be related to the number of water molecules and salt ions released upon adsorption, as determined by the PI analysis.

2. Experimental

2.1. Materials

Lyophilised homo-deoxyoligonucleotides with different molecular masses were purchased from Interactiva (Ulm, Germany), ressuspended in 10 m*M* Tris, pH 8.0 and used without further purification. The HIC support was a Sepharose derivative synthesised by covalent immobilization of the ligand 1,4-butanediol diglycidyl ether from Aldrich (Steinheim, Germany) on Sepharose CL-6B [9] purchased from Pharmacia (Uppsala, Sweden). The amount of epoxy groups immobilized on the support, determined after treatment of the activated matrix with sodium thiosulfate was around 500 μmol/g dry gel [9]. For the modulation of the chromatography

experiments, analytical-grade ammonium sulfate and tris(hydroxymethyl)aminoethane (Tris) from Merck (Darmstadt, Germany) were used.

2.2. Chromatography of the oligonucleotides

Chromatography was performed in a Pharmacia fast protein liquid chromatography (FPLC) system (P500 pump, LCC 500 chromatography controller). An XK 16/20 (20 cm×1.6 cm I.D.) column was packed with 28 ml of the HIC gel and the temperature in the thermostatic jacket was controlled using a Thermomix BU thermostat from B Braun (Melsungen, Germany). The column was equilibrated at 15, 25, 30 and 35 °C with various concentrations of ammonium sulfate in 10 m*M* Tris–Cl (pH 8.0) at a flow-rate of 1 ml min^{-1}. A 500-μl volume of the various oligonucleotides samples was loaded onto the column, and isocratic elution of each oligonucleotide was carried out at identical concentrations of ammonium sulfate. The absorbance of the eluate was continuously measured at 254 nm. After the chromatographic runs, the FPLC system was cleaned with 2 column volumes of 1 *M* NaOH.

3. Results and discussion

3.1. Chromatographic experiments

A 50-μg amount of each homo-deoxyoligonucleotide (polyA and polyT with 6, 15 and 30 bases) was injected into the HIC column and eluted with different concentrations of ammonium sulfate and at different temperatures. The maximum temperature was 35 °C since, according to the manufacturers, Sepharose melts on heating above 40 °C. As observed earlier [5], the retention of the oligonucleotides increased with the salt concentration of the eluent. For the same concentration of salt, the retention times also increased with the temperature. If a sufficiently high salt concentration or temperature is used, the oligonucleotide is retained in the column and can only be eluted by decreasing the salt concentration. The magnitude of the salt concentration necessary to promote this type of interaction is a function of the hydrophobicity of the oligonucleotide.

The retention factors were determined from the chromatograms by calculating the quotient between the net retention volume and the void volume: $k' = V_N/V_O$ [10]. The net retention value is calculated by subtracting the void volume from the total retention volume ($V_N = V_R - V_O$). The value of the void volume was determined by injecting and eluting (with water at 1 ml min^{-1}) 500 μl of a 8.5 kilobase pair (Kbp) plasmid solution. Due to its high molecular mass, and to the fact that it is a highly hydrophilic molecule with the hydrophobic bases packed and shielded inside the double helix, this plasmid elutes with a retention volume equal to V_O. A value of 12.5 ml was thus estimated and used in all calculations. A list of the retention factors obtained for the different oligonucleotides at various molar concentrations of ammonium sulfate and at various temperatures is shown in Table 1. In the case of the polyT 6, the retention times at a specific salt concentration were the same for all temperatures. In the case of the polyA 6, the retention times at 35 °C were the same as at 30 °C.

3.2. Analysis with the solvophobic theory

In accordance with Eq. (3) from the solvophobic theory, plots of the logarithm of the retention factor versus the concentration of ammonium sulfate for the temperatures of 15, 25, 25 and 30 °C were constructed (Fig. 1) for each of the oligonucleotides studied. Using the linear least-squares fitting procedure, it was established that, for all the molecules and temperatures studied, Eq. (3) is satisfactorily obeyed only at the three highest salt concentrations. In fact, according to Ref. [6], the dependence of the logarithm of the retention factor on the salt concentration is influenced both by electrostatic and hydrophobic interactions. Although neglecting the influence of electrostatic interactions, Eq. (3) is a very good description of the chromatographic behaviour for high salt concentrations because the hydrophobic interaction is the main mechanism. However, for low salt concentrations electrostatic interactions cannot be neglected and Eq. (3) is not obeyed. The *C* coefficient of the solvophobic theory was thus estimated using only these high salt concentration data and the results are presented in Table 2. The molecular surface area change upon binding

Table 1
Retention factors of the oligonucleotides at different concentrations of ammonium sulfate

Oligonucleotide	T (°C)	Retention factors									
		[Ammonium sulfate] (M)									
		1	1.1	1.2	1.3	1.4	1.5	1.6	1.7	1.8	2.0
PolyT 6	15, 25, 30, 35	1.72		2.08			3.12	3.68		5.68	8.60
PolyT 15	15	1.72		2.44	3.24	4.52	6.36				
	25	1.84		2.88	3.96	5.64	8.00				
	30	1.90		3.00	4.20	6.20	8.60				
	35	1.98		3.20	4.40	6.36	9.16				
PolyT 30	15	1.98	2.84	4.56	7.00						
	25	2.40	3.78	5.80	10.68						
	30	2.62	4.12	6.60	12.20						
	35	2.80	4.60	7.40	13.96						
PolyA 6	15	2.04		2.52		3.28	4.12	5.18	6.52		
	25	2.12		2.68		3.52	4.20	5.40	6.86		
	30, 35	2.12		2.76		3.68	4.36	5.64	7.24		
PolyA 15	15	2.04	2.44	3.08	4.20	6.04					
	25	2.40	3.04	4.08	5.72	8.44					
	30	2.68	3.28	4.62	6.60	10.12					
	35	2.92	3.76	5.24	7.64	11.60					
PolyA 30	15	1.24	1.52	2.00	2.70						
	25	1.66	2.16	3.00	4.54						
	30	1.90	2.56	3.80	5.80						
	35	2.12	3.08	4.68	7.80						

to the stationary phase, $\Delta A'$, which is proportional to the hydrophobic contact surface area [6], was then calculated using Eq. (4) and the best-fit C values.

Fig. 2a shows a plot of C versus the number of bases for all the temperatures studied. For polyT, C was found to increase with the number of hydrophobic bases at all the temperatures. As is seen in Eq. (4), the value of C depends on $\Delta A'$, σ_s (specific for each salt), R and T. Thus, at a constant temperature and for a fixed salt, in the absence of special effects (such as the presence of secondary structures), the value of C varies only with $\Delta A'$ [6], which is proportional to the number of bases of the oligonucleotide [5,11]. In the case of polyA, the value of C also increases with the number of hydrophobic bases for the temperatures 25, 30 and 35 °C. However, the rate of increase from polyA 15 to polyA 30 is lower when compared with polyT. In fact, parameter C determined at these temperatures for polyA 30 is very similar to the one determined for polyA 15. Furthermore, at the temperature of 15 °C the value of C decreases from polyA 15 to polyA 30. Once parameter C is proportional to the hydrophobic contact area of the molecule ($\Delta A'$) [6], as explained in the introduction chapter, these results mean that the hydrophobicity of the polyA 30 at 25, 30 and 35 °C is similar to that of the polyA15. The results also prove that, for the temperature of 15 °C, the hydrophobicity of polyA30 is lower than that of polyA15. These results are probably a consequence of the existence of secondary structures for the case of polyA30. In fact, secondary structures can shield the interaction sites of the molecule giving $\Delta A'$ values that are lower than expected. As seen in Fig. 2a, this effect is less pronounced at high temperatures because the increase in temperature tends to disrupt the interactions that stabilise the secondary structures [12].

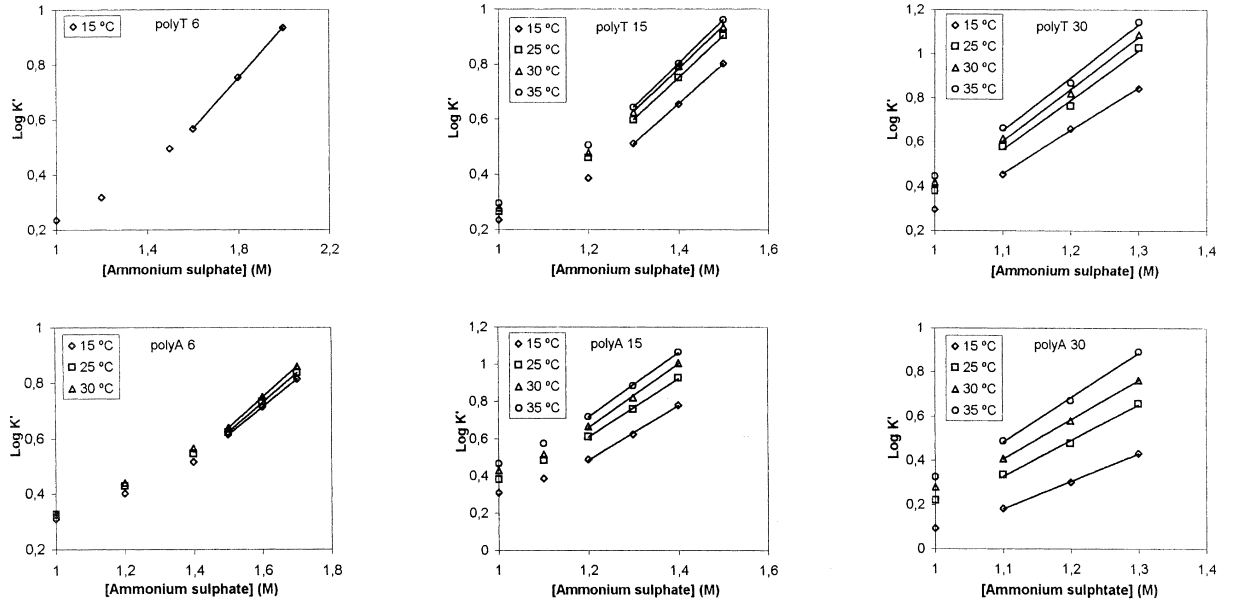

Fig. 1. Plots of the decimal logarithm of the retention factors of the oligonucleotides on derivatized Sepharose CL-6B against the ammonium sulfate concentration and respective trendlines of the linear zones.

With the exception of the molecules with 30 nucleotides, for the same temperature, C is always higher for the case of oligo A when compared with oligo T. In fact, previous studies using reversed-phase liquid chromatography (RPLC) of nucleotides reported that the hydrophobicity of adenine is higher than that of thymine [12,13]. Our experiments showed that, in HIC, this rule can be extrapolated for the case of poly6 and poly15: $H_{nA} > H_{nT}$. This order of elution, however, is reversed for the case of the poly 30 molecules, probably because of the presence of secondary structures in the case of polyA 30. The influence of the presence of secondary structures is also an adequate explanation for the reported lower retention of relatively high-molecular-mass polyA molecules in RPLC when compared with poly T [14].

For the majority of the molecules studied, the value of C (proportional to $\Delta A'$) increases with the temperature (Fig. 2b). An increase in temperature decreases the degree of local base stacking, which increases the hydrophobic contact area of the oligonucleotides [10]. As shown in Fig. 2b, the rate of increase of C is higher for the case of polyA 30. This is because the polyA 30 molecules manifest a much higher degree of local base stacking at low temperatures in order to stabilise the very rigid conformations that occur at these temperatures. Only for the polyT 6 is the value of C approximately constant with the temperature; this is due to the low hydrophobicity of this molecule.

3.3. Analysis with the PI model

Fig. 3 shows plots of the natural logarithm of the retention factor against the natural logarithm of the salt concentration for the temperatures of 15, 25, 30 and 35 °C for each of the oligonucleotides studied. These data were fitted to the PI model (Eq. (10)) using a non-linear least squares fitting procedure [15], and the parameters C, $(\Delta\upsilon_+ + \Delta\upsilon_-)/g$ and $(-n\Delta\upsilon_1)/(m_1 g)$ were calculated. The values used for n, m_1 and g in Eq. (10) are 3, 55.15 and 1.6, respectively. Good agreement between the model and the data was generally observed.

Table 3 presents the number of water molecules $-(\Delta\upsilon_1)$ and ions $-(\Delta\upsilon_+ + \Delta\upsilon_-)$ released on adsorption. For all cases, adsorption leads to the release of a much larger number of water molecules (119–418) than salt ions (1–18). This situation is consistent

Table 2
Estimates of the parameters determined by the model of solvophobic theory describing the variation of the interaction of the oligonucleotides with the ammonium sulfate concentration and with the temperature

Oligonucleotide	T (°C)	Parameter C
PolyT 6	15, 25, 30, 35	0.9216
PolyT 15	15	1.4645
	25	1.5270
	30	1.5562
	35	1.5922
PolyT 30	15	1.9589
	25	2.2554
	30	2.3573
	35	2.4106
PolyA 6	15	0.9968
	25	1.0654
	30, 35	1.1013
PolyA 15	15	1.4624
	25	1.5784
	30	1.7027
	35	1.7256
PolyA 30	15	1.2476
	25	1.6130
	30	1.7759
	35	2.0177

with the fact that hydrophobic interactions are driven by an increase in entropy resultant from the release of water molecules from the surface of the stationary phase upon adsorption, as has been described for the hydrophobic interaction of several proteins [8,16,17].

The values of $-(\Delta\upsilon_1)$ for polyA and polyT were plotted against the number of nucleotides for all the temperatures studied (Fig. 4a). It was found that $-(\Delta\upsilon_1)$ increased with the number of hydrophobic bases for polyT for all the temperatures studied, and for polyA for the temperatures of 25, 30 and 35 °C. This increase of $-(\Delta\upsilon_1)$ with the hydrophobic character is consistent with results for several protein/hydrophobic ligand systems in the literature [8]. As was mentioned earlier, this trend is expected for adsorption processes dominated by hydrophobic interactions, because the number of water molecules released should be proportional to the hydrophobic contact area between the molecule and the ligand [8].

For the case of the polyA, the number of water molecules released upon interaction of polyA 30 with the support was found to be similar to that of polyA 15. This result is consistent with the generally similar values of the parameter C obtained for polyA 30 and polyA 15. As explained for Fig. 2a, this means that the hydrophobicity of polyA 30 is similar to that of polyA 15. Again, this behaviour is also probably due to the existence of secondary structures for the case of polyA 30.

In comparing the values of $-(\Delta\upsilon_1)$ for homo-oligonucleotides composed of adenines with those composed of thymines, the trend is consistent with expectations. For the low-molecular-mass oligonucleotides (without secondary structures) the interaction of molecules containing adenines is accom-

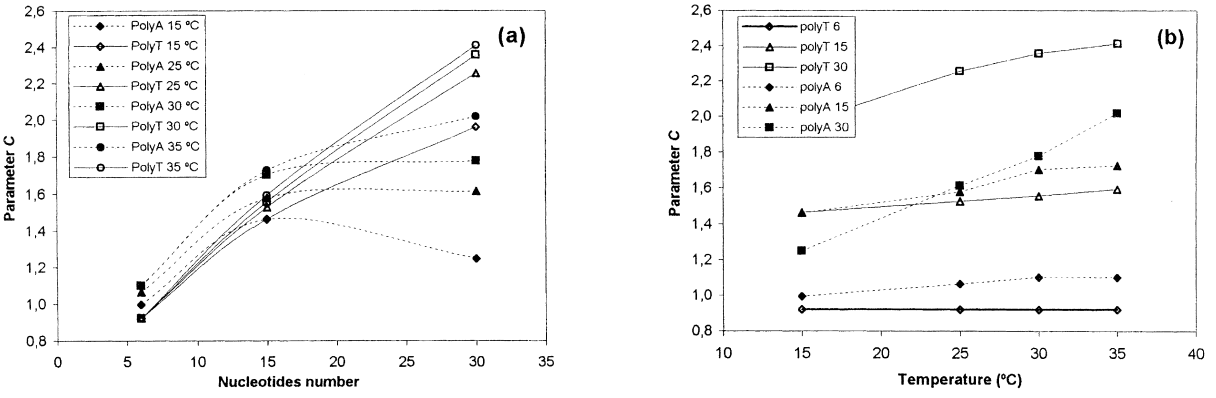

Fig. 2. Variation of parameter C with the number of bases of the oligonucleotides (a) and with the experimental temperature (b).

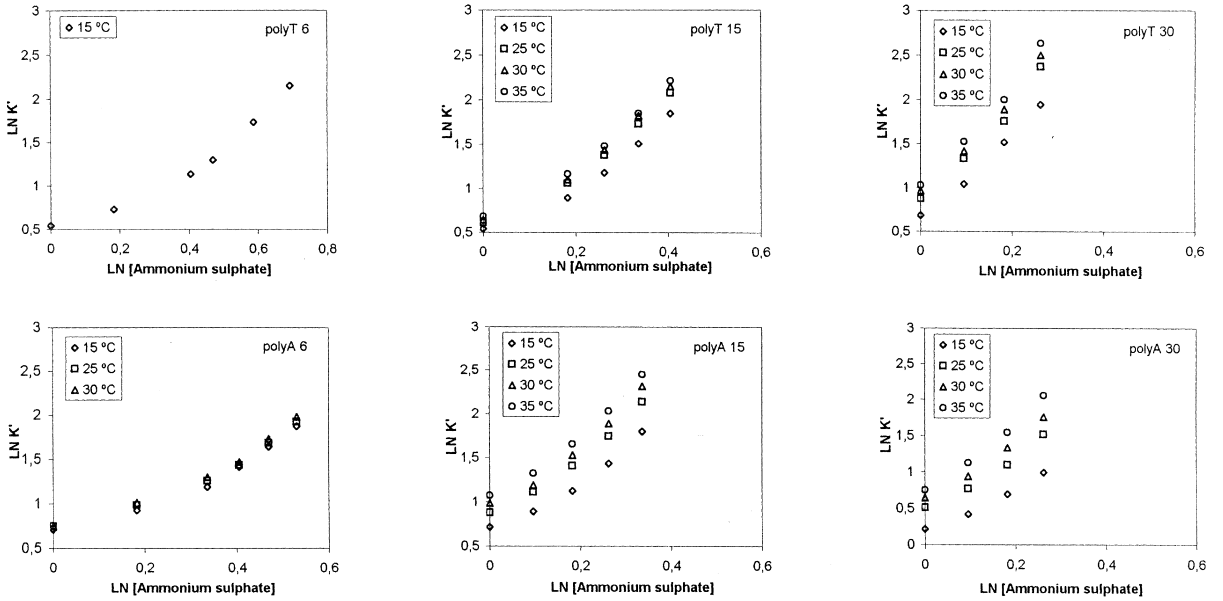

Fig. 3. Plots of the natural logarithm of the retention factors of the oligonucleotides on derivatized Sepharose CL-6B against the natural logarithm of the ammonium sulfate concentration.

Table 3
Estimates of the parameters determined by the model of preferential interaction analysis describing the variation of the interaction of the oligonucleotides with the ammonium sulfate concentration and with the temperature

Oligonucleotide	T (°C)	$(-n\Delta\upsilon_1)/(m_1 g)$	$(-\Delta\upsilon_1)$	$(\Delta\upsilon_+ + \Delta\upsilon_-)/g$	$-(\Delta\upsilon_+ + \Delta\upsilon_-)$
Poly T 6	15, 25, 30, 35	4.06	119.4	−3.54	5.7
PolyT 15	15	9.30	273.5	−8.21	13.1
	25	8.18	240.6	−6.44	10.3
	30	8.29	243.9	−6.55	10.5
	35	8.25	242.7	−6.38	10.2
PolyT 30	15	14.21	418.0	−11.51	18.4
	25	13.63	400.9	−9.98	16.0
	30	14.20	417.7	−10.43	16.7
	35	13.02	383.0	−8.84	14.1
PolyA 6	15	5.38	158.2	−4.92	7.9
	25	5.10	150.0	−4.56	7.3
	30, 35	4.90	144.1	−4.21	6.7
PolyA 15	15	10.27	302.1	−9.00	14.4
	25	9.07	266.8	−7.05	11.3
	30	10.32	303.5	−8.22	13.2
	35	9.87	290.3	−7.62	12.2
PolyA 30	15	8.17	240.3	−6.36	10.2
	25	9.23	271.5	−6.56	10.5
	30	10.94	321.8	−8.23	13.2
	35	12.13	356.8	−8.93	14.3

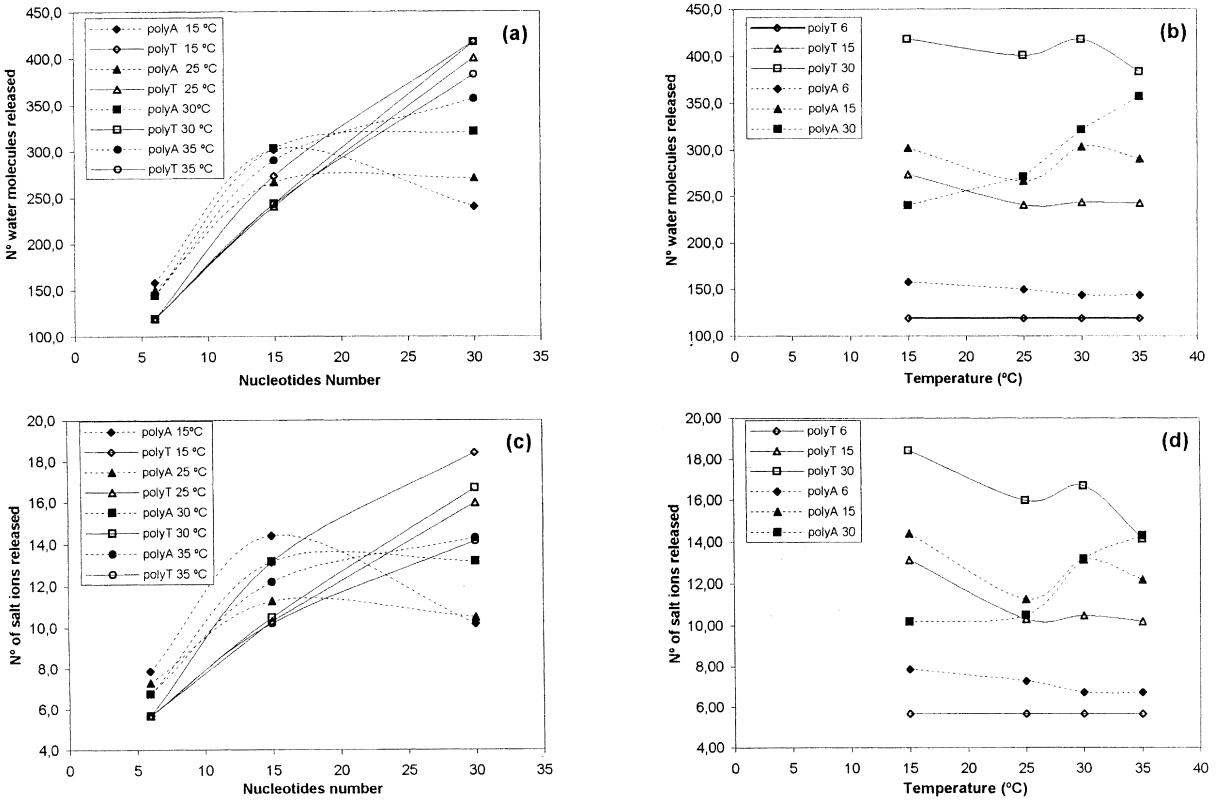

Fig. 4. Variation of the number of water molecules released upon adsorption with the number of bases of the oligonucleotides (a) and with the experimental temperature (b); and variation of the number of salt ions released on adsorption with the number of bases of the oligonucleotides (c) and with the experimental temperature (d).

panied by the release of a larger number of water molecules. As discussed earlier, this is due to the larger decrease in the exposed hydrophobic area, due to the higher hydrophobicity of adenine relative to thymine [12,13].

The number of water molecules released is plotted in Fig. 4b against the temperature for all the molecules studied. The variations observed cannot be easily interpreted. In contrast to the trend observed for parameter *C*, the number of water molecules released is not proportional to the temperature. A study carried out on the effects of temperature on the HIC of dansyl derivatives of amino acids [10] does provide help in interpreting this result. It showed that the variation of the entropy associated with retention (ΔS^0) is strongly dependent on the temperature. This value was found to be positive at low temperatures and to decrease with increasing temperature [10], which is typical for processes governed by the hydrophobic effect. It is known that a positive entropy change results from the release of water molecules upon adsorption of the molecules to the stationary phase [8]. This implies that for the dansyl amino acids $-(\Delta\upsilon_1)$ will decrease with an increase in temperature. However, these molecules are very simple and they undergo no significant conformational changes at the temperatures used [10]. In contrast, it has been shown (Fig. 2b) that the values of $\Delta A'$ for the oligonucleotides increase with increasing temperature, which implies the values of $-(\Delta\upsilon_1)$ should increase with increasing temperature. Thus, in combination, these two independent phenomena can be expected to give a complex variation of $-(\Delta\upsilon_1)$ with temperature, as has been observed.

In contrast to the other oligonucleotides, for polyA 30, the number of water molecules released always

increases with an increase in temperature. This is because the increase of the hydrophobic exposed area with the temperature is very high, due to the disruption of the secondary structures (Fig. 2b). In this particular case, the conformational changes induced by the increase in temperature seems to be the predominant phenomenon that governs the release of water molecules.

The complexity of the adsorption process can be more fully appreciated from a Van 't Hoff analysis of the retention characteristics. It was found that the standard-state entropy determined at 1.3 *M* ammonium sulfate (results not shown) decreases with the temperature for the case of the polyT 15, polyT 30 and also for polyA 15. This behaviour is expected since that, for processes governed by HIC, the entropy changes are large and positive at low temperatures and become negative at higher temperatures [10]. For the case of polyA 30, however, the standard-state entropy was found to increase with the temperature. This variation is in agreement with the variation of $-(\Delta\upsilon)$ determined by the preferential interaction analysis. In fact, the values of ΔS^0 are higher for higher temperatures because more water molecules are released due to the increase of the exposed hydrophobic surface area due to the thermal denaturation of the polyA 30.

The number of salt ions released on adsorption was also plotted against the number of nucleotides and against the temperature (Fig. 4c and d, respectively). The trends observed are very similar to the ones in Fig. 4a and b. This is probably because each monomer in the oligonucleotides is constituted by a hydrophobic base and a negatively charged phosphate. Thus, the hydrophobic and electrostatic interactions between the molecule and the stationary phase are proportional, as observed.

3.4. Relationships between model parameters

In order to examine the relationship between the number of water molecules released, as calculated from the PI model, and parameter *C* in the solvophobic theory, the values of *C* were plotted versus the corresponding $-\Delta\upsilon_1$ for each temperature (Fig. 5a and b). For both polyA and polyT a linear dependence was observed at all temperatures with relatively good correlation coefficients. This was expected because both models characterised the experimental data effectively.

Another interesting observation can be made by plotting the number of salt ions released on adsorption (from PI analysis) versus parameter *C* (Fig. 5c and d). For polyT, linear relationships with relatively good correlation coefficients were observed between the two parameters. As mentioned earlier, the electrostatic interactions should be proportional to the hydrophobic interactions because each nucleotide has simultaneously a hydrophobic base and a negatively charged phosphate group. In the case of polyA, however, the two parameters (*C* and ions released) are not in general linearly related. This is due to the presence of secondary structures in polyA; the hydrophobic and electrostatic interactions between the molecule and the stationary phase may not be proportional, as occurs with totally denatured molecules. The polyA data with a correlation between *C* and ions released closest to linear is at 35 °C, because at this temperature the molecule is more denatured than at lower temperatures.

4. Conclusions

The application of the solvophobic theory and the PI analysis to HIC of homo-oligonucleotides on derivatized Sepharose CL-6B has shown that both models characterise the retention behaviour effectively. Parameter *C* from the solvophobic theory, which is proportional to the hydrophobic surface area of the molecules, was found to increase with the number of nucleotides, the hydrophobicity of the individual bases and with the temperature. Exceptions to these general trends were due to the presence of secondary structures (polyA 30) that decrease the exposure of the hydrophobic bases. The number of water molecules $-(\Delta\upsilon_1)$ and salt ions $-(\Delta\upsilon_+ + \Delta\upsilon_-)$ released on adsorption were estimated with the PI model. The magnitude of $-(\Delta\upsilon_1)$ was found to be much larger than $-(\Delta\upsilon_+ + \Delta\upsilon_-)$, which is typical for hydrophobic interactions. In general, the number of water molecules and salt ions released increases with the number of nucleotides and the hydrophobicity of the individual bases. This is because $-(\Delta\upsilon_1)$ should be proportional to the reduction in the exposed hydrophobic surface area upon adsorption. Similarly,

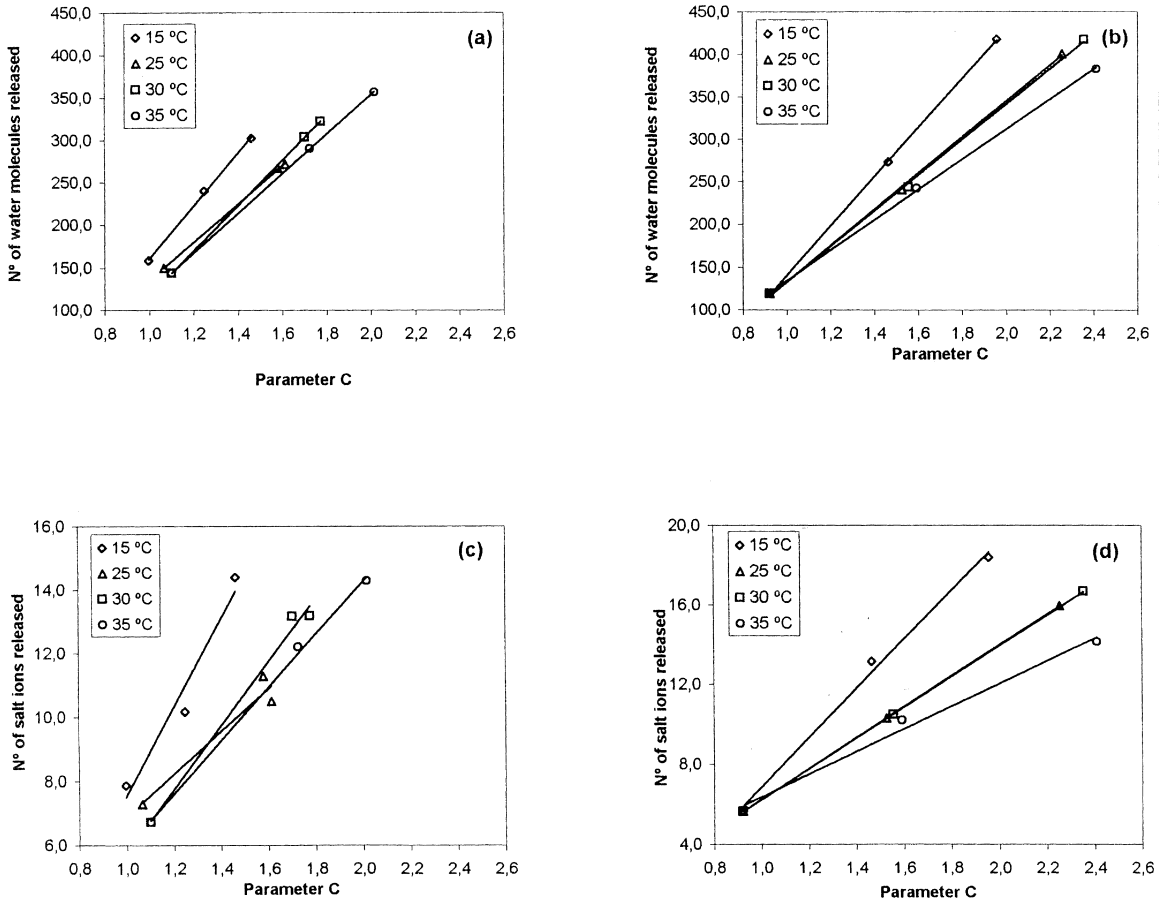

Fig. 5. Linear trendlines of the plots of the number of water molecules released against parameter *C* on adsorption of polyA (a) and polyT (b) and linear trendlines of the plots of the number of salt ions released against parameter *C* on adsorption of polyA (c) and polyT (d).

$-(\Delta\upsilon_+ + \Delta\upsilon_-)$ should also be proportional to the reduction in the exposed hydrophobic area upon adsorption, due to the simultaneous polyanionic and hydrophobic nature of the nucleotides. However, exceptions to the expected trend were noted (polyA 30), and these were due to the presence of secondary structures. It was also observed that the amount of water and salt released upon adsorption was not in general proportional to the temperature, probably due to the dual influences of thermodynamic and conformational phenomena.

In correlating the solvophobic parameter *C* versus the water and salt release values from the PI analysis, linear trendlines were obtained for the majority of the plots, except for the plot of $-(\Delta\upsilon_+ + \Delta\upsilon_-)$ versus *C* for polyA. It was concluded that the presence of secondary structures modifies the proportion of hydrophobic and ionic residues exposed in this molecule.

Acknowledgements

This work was partially supported by the Program Praxis XXI—Portugal in the form of a fellowship to M.M.D. (BD/21241/99) and also by project POCTI/43620/BIO/2000 from FCT.

References

[1] J.A. Queiroz, C.T. Tomaz, J.M.S. Cabral, J. Biotechnol. 87 (2001) 143.
[2] M.M. Diogo, S.C. Ribeiro, J.A. Queiroz, G.A. Monteiro, N. Tordo, P. Perrin, D.M.F. Prazeres, J. Gene Med. 3 (2001) 577.
[3] M.M. Diogo, J.A. Queiroz, G.A. Monteiro, G.N.M. Ferreira, S.A.M. Martins, D.M.F. Prazeres, Biotechnol. Bioeng. 68 (2000) 576.
[4] D. Haidacher, Cs. Horváth, in: F. Dondi, G. Guichon (Eds.), Physicochemical Basis of Hydrophobic Interaction Chromatography, Kluwer, Dordrecht, 1992.
[5] M.M. Diogo, J.A. Queiroz, D.M.F. Prazeres, J. Chromatogr. A 944 (2002) 119.
[6] W.R. Melander, Z. El Rassi, Cs. Horváth, J. Chromatogr. 469 (1989) 3.
[7] Cs. Horváth, W. Melander, I. Molnár, J. Chromatogr. 125 (1976) 129.
[8] T.W. Perkins, D.S. Mak, T.W. Root, E.N. Lightfoot, J. Chromatogr. A 766 (1997) 1.
[9] L. Sundberg, J. Porath, J. Chromatogr. 90 (1974) 87.
[10] D. Haidacher, A. Vailaya, Cs. Horváth, Proc. Natl. Acad. Sci. USA 93 (1996) 2290.
[11] D.P. Lee, J.H. Kindsvater, Anal. Chem. 52 (1980) 2428.
[12] C.R. Cantor, P.R. Schimmel, Biophysical Chemistry, Part III, W.H. Freeman, New York, 1980.
[13] S. Ikuta, R. Chattopadhyaya, R.E. Dickerson, Anal. Chem. 56 (1984) 2256.
[14] J.P. Liautard, J. Chromatogr. 584 (1992) 135.
[15] Table Curve 2D version 2.0, AISN Software, Jandel Scientific, 1989–1994.
[16] M.A. Esquibel-King, A.C. Dias-Cabral, J.A. Queiroz, N.G. Pinto, J. Chromatogr. A 865 (1999) 111.
[17] A.C. Dias-Cabral, N.G. Pinto, J.A. Queiroz, Sep. Sci. Technol. 37 (2002) 1505.