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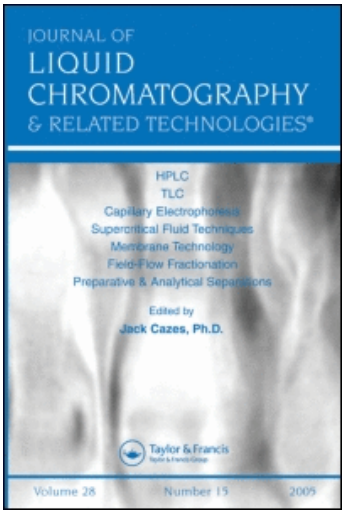

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
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Online publication date: 19 October 2000

To cite this Article Gennaro, M. C. , Marengo, E. and Gianotti, V.(2000) 'ION-INTERACTION RP-HPLC SEPARATION OF INORGANIC ANIONS ON POROUS GRAPHITIZED CARBON STATIONARY PHASE. COMPARISON WITH ODS STATIONARY PHASE', Journal of Liquid Chromatography & Related Technologies, 23: 17, 2599 — 2613
To link to this Article: DOI: 10.1081/JLC-100101821
URL: http://dx.doi.org/10.1081/JLC-100101821

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J. LIQ. CHROM. & REL. TECHNOL., 23(17), 2599–2613 (2000)

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ABSTRACT

This paper shows how a porous graphitized stationary (PGC) phase can be modified to work in RP-HPLC ion-interaction reagent (IIR) mode. Aqueous solutions of alkylammonium o-phosphate salts are used as IIRs and the analytes are the inorganic anions, iodide, iodate, bromide, bromate, nitrite, and nitrate.

Comparisons with the results obtained under the same conditions for an ODS stationary phase show that the use of the PGC column permits extension, to IIR chromatography, in the pH range of only acidic values.

INTRODUCTION

Alkylammonium o-phosphates have been extensively used in this laboratory as interaction reagents (IIRs) in the chromatographic separation, by reversed-phase ion-interaction chromatography, of cationic and anionic species.[1-2] The stationary phases were silica-based C_8 or C_{18} material packings that were dynamically modified by the ion-interaction reagent contained in the mobile phase. The modification is assumed to give rise onto the surface of the reversed-phase packing material to an electrical double-layer; the lipophilic

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alkylammonium is adsorbed onto the surface to form the first positive layer and the anion is retained through electrostatic forces.

Due to the adsorbed double layer, the original properties of the RP-stationary phase are modified and able to retain, even simultaneously, cationic and anionic species.[3] This technique offers advantages of versatility, due to the many variables that affect retention and that can be optimised to fit the problem. In particular, the pH value of the mobile phase plays a predominant effect, and its optimisation can modulate the retention of the components of a mixture and allow their resolution. When using reversed-phase material packings characterised by silica substrates, the choice of the pH value of the mobile phase is conditioned by the hydrolysis solubility of silica and ranges between pH 3 and 8. On the other hand, experiments performed to modify the surface of the stationary phases having $C_{18}H_{37}$- chains bound to polymeric substrate were unsuccessful,[4] likely due to the generally low carbon content.

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Both these limitations do not affect a carbon graphitized material packing (PGC) that consists in pure carbon micro particles and behave as a pure reversed-phase adsorbent. Carbon phases do not show the pH instability problems of silica gel and other oxide particles; they do not dissolve at high pH and do not hydrolyse under strongly acidic conditions. In addition, secondary interactions with acidic uncapped sylanol groups, often present even in ODS endcapped columns, are absent.

On the basis of these considerations, the possibility of modifying a PGC stationary phase to work in ion-interaction mode is explored in this paper. With respect to the use of ODSs, the following advantages are expected: i) inertness across the whole pH range, ii) high lipophilicity able to favour the adsorption of the IIR lipophilic moiety, iii) high homogeneity of surface, able to guarantee the absence of undesirable interactions.

In order to guarantee that only ion-interaction mechanisms intervene in the retention process, aqueous solutions are chosen as the mobile phases and inorganic anions as the analytes, since inorganic anions can be retained as apolar PGC stationary phase only if the surface is modified and electrostatic forces intervene.

The following anions are considered: iodide, iodate, bromide, bromate, nitrite, and nitrate. Alkylammonium o-phosphate salts are used as the ion-interaction reagents, in conditions already employed with ODS stationary phases.[5]

Most of the papers reported in the literature concern the use of PGC stationary phase for separations in reversed-phase mode.[6-13] Comparisons with octadecylsilica stationary phases generally show that the hydrophobic compounds interact more strongly with PGC than with ODS material,[7,14-16] and that PGC exhibits a superior selectivity towards stereo- and geometric isomers.[7-24]

Due to the PGC planar surface, for some planar molecules (as for example benzene derivatives) retention is further increased due to π–π effects.[24] An example of the use of an aqueous mobile phase (5.0 mM heptanesulfonic acid in 5.0 mM sodium phosphate buffer at pH=9.0) can be found for the separation of alkylamines.[25] The examples of separation of inorganic anions on PGC concern: i) an ion-interaction chromatographic method with suppressed conductivity detection and tetrabutylammonium hydroxide-sodium carbonate and acetonitrile as the mobile phase,[26] and ii) the use of unmodified PGC stationary phase with evaporative light scattering detection and a mobile phase containing pyridine and carboxylic acids as electronic competitors.[27] As concerns studies of surface modification of PGC stationary phase, only a SFC[28] enantiomeric separation is reported with a chiral reagent physically anchored to the PGC surface.

In the present ion-interaction study, the effects on the retention of: i) the alkyl chain length of the IIR, ii) the IIR concentration, and iii) the pH of the mobile phase are studied and compared with the results obtained with ODS stationary phase. Also, the thermodynamic parameters of enthalpy and entropy variations for the transfer process of the analytes from the mobile to the stationary phase are compared.

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EXPERIMENTAL

Instrumentation

The chromatographic analyses have been performed by a Merck-Hitachi model L-6200 Lichrograph Chromatograph equipped with a two-channel Merck-Hitachi model D-25000 Chromato-Integrator and interfaced with a model L-4250 UV-vis detector and an L-3720 conductivity detector with temperature control.

The stationary phases used are: a Shandon Hypercarb (100 x 4.6 mm) PGC column with particles of 7 μm and a Lichrospher 100 (250 x 4 mm) RP-18 Merck 5 μm, fully end-capped with a (15.0 x 0.46 mm) Lichrospher RP-18 Merck 5 μm guard pre-column.

pH measurements have been carried out with a Metrohom pH-meter 654 equipped with a glass calomel electrode and the absorbance spectra have been recorded with an Hitachi 150-20 spectrophotometer.

Reagents and Standard Solutions

Sodium nitrate, sodium nitrite, sodium iodide, sodium iodate, sodium bromate, pentylamine, hexylamine, heptylamine, octylamine, and o-phosphoric

acid are Fluka reagents. Acetonitrile and methanol are HPLC-grade Merck reagents. For the preparation of all the solutions ultra-pure water produced by a Millipore Milli-Q system were used.

Stock solutions at concentration of 1.0 mg/L were prepared with ultra-pure water and stored at 4°C. The solutions were prepared about every 20 days, the solution of nitrite every 5 days. The solutions to be injected were prepared for dilution with ultrapure water.

Mobile Phase Preparation and Chromatographic Method

The mobile phases have been prepared by dissolving, in ultra-pure water, the required amount of the amine and by adding o-phosphoric acid to the prefixed pH value. Even if the molar ratio between the amine and o-phosphate is not exactly stoichiometric, the IIR, so prepared, will be referred to as alkylammonium o-phosphate.

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The PGC column, before being able to work in IIR mode, required a wetting treatment of an addition of 5% acetonitrile to the aqueous mobile phase. If washed and left in acetonitrile after use, the column does not require any additional acetonitrile to the aqueous mobile phases. Between the use of different mobile phases the column was washed with ultra-pure water, with a water-acetonitrile mixture (50/50 v/v) and with 100% acetonitrile. When not in use the column is left in acetonitrile.

Every second month of intensive use and when anomalous high pressures are observed, the washing treatment suggested by the producer is performed. The column flow-rate is inverted and an acidic washing (flow-rate 1.0 mL/min) is performed with 50 mL of a mixture of tetrahydrofuran and water (50/50 v/v) containing 0.1% of trifluoroacetic acid; then a basic washing (flow-rate 1.0 mL/min) is performed with 50 mL of a mixture of tetrahydrofuran and water (50/50 v/v) containing 0.1% of NaOH. The acidic washing is repeated, the stationary phase is equilibrated with 50 mL of a mixture of methanol/water (95/5 v/v) and again inverted.

RESULTS

Modification of the PGC Surface

As mentioned, the aim of this investigation is: i) to study the possibility of using PGC columns in ion-interaction mode, ii) to evaluate the effect on retention of the different variables involved, and iii) to compare the performances of PGC and ODS stationary phases in RP-HPLC-IIR chromatography.

On the basis of the results previously obtained in the separation of inorganic anions on ODS stationary phases,[5] experiments were performed by using, as the mobile phases, aqueous solutions of alkylammonium o-phosphates. The analytes considered are: iodide, iodate, bromide, bromate, nitrite, and nitrate. The variables studied are: i) the alkyl chain length of the IIR that was varied between 5 (pentylamine) and 8 (octylamine), ii) the concentration of IIR varied between 3.00 mM and 15.00 mM, and iii) the pH of the mobile phase that, as already mentioned, can be varied in a large range (0-14), and that is expected to give relevant advantages with respect to the use of ODS stationary phase.

A study was also performed as a function of temperature, with the aim to collect information about the transfer process of the analytes between the mobile and the stationary phases.

The first separation of the mixture of the anions was tried under the experimental conditions already successfully used with ODS column.[5] While the first modification process of the surface of GPC required a previous wetting of the column for addition of some acetonitrile (5%) to the aqueous mobile phase, this addition was not required in the following elutions, where totally aqueous mobile phases were always employed. In the conditions of 5.00 mM octylamine o-phosphate as the IIR at pH = 6.40, UV detection at 230 nm, all the inorganic anions considered were retained at reasonable times (within 15 min) and a good resolution was obtained. This result indicates that PGC stationary phase surface can be modified to work in IIR mode.

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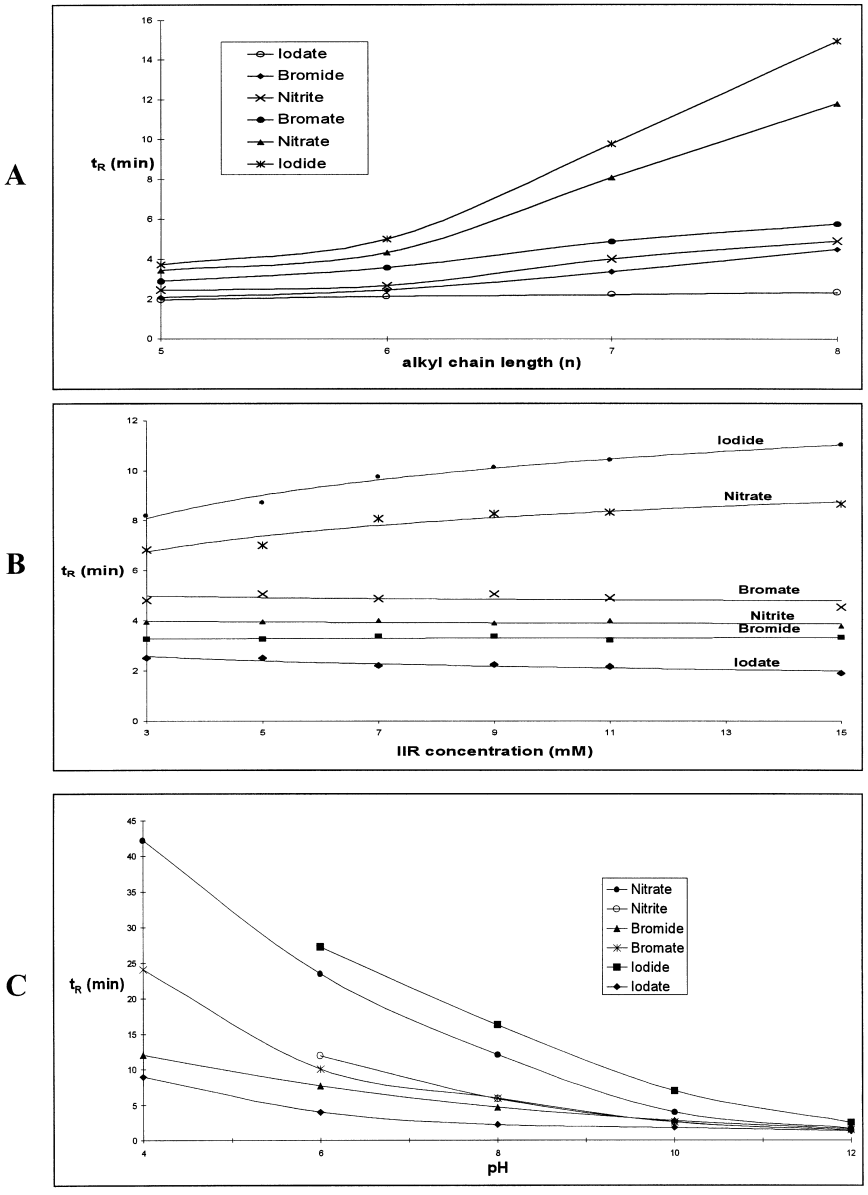
Effect of the Alkyl Chain

In order to optimise the separation and to study the effect on the retention of the different variables as well, experiments were performed for different IIR alkyl-chain lengths, ranging from n = 5 (pentylamine) to 8 (octylamine); o-phosphate was the anion, 5.00 mM the IIR concentration, and 6.4 the mobile phase pH. The results show that the IIR alkyl chain length exerts the same effect already observed with the ODS column,[29-30] since retention increases as the alkyl chain length increases, in agreement with the increased lipophilicity of the chain (Figure 1A). The use of heptylamine was chosen since it offers the advantages of resolution.

Effect of the Ion-Interaction Reagent Concentration

Figure 1B shows that retention, in the concentration range of octylamine o-phosphate between 3.00 and 15.00 mM, is practically less affected by IIR concentration for the anions of the weaker acids than for iodide and nitrate, that elute later. Retention still increases to reach a maximum at concentrations around 12 mM, after the maximum retention is essentially constant, (as can be



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expected in the hypothesis that increasing concentration leads to a progressive increase of the number of the active sites onto the surface of the stationary phase) to the surface capacity (the maximum amount of modifiable sites). This predictable behaviour, not observed with ODS surface,[31] is very likely due to the homogeneity of the PGC column surface and, in particular to, the absence of other contributions in the retention mechanism, such as those due to metal impurities and to residual silanol groups.

Effect of the Mobile Phase pH

For all the anions investigated, retention decreases as the pH of the mobile phase increases, up to become for pH values around 10, very close to the dead time. On the other hand, at lower pH values, the retention dependence can only be studied for the anions of strong acids, since the anions of the weaker acids are too strongly retained (Figure 1C): at lower pH, predominantly the undissociated neutral forms exist, which do not participate in ion-interaction mechanisms.

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This trend, as a function of pH, is similar to that observed with ODS packing material,[32] but the increase of retention observed at lower pHs is greater for PGC, most likely because of the greater lipophilicity of PGC material packing that favours the formation of a greater number of modified active sites.

The decrease of retention for higher pH values is of relevant interest because it confirms the ion-interaction mechanism already hypothesised for ODS packing. Retention decrease can, in fact, be ascribed to a lower number of modified sites onto the surface, due to the lower molar fraction of the protonated amine that constitutes the positive electrical layer and, in addition, to competitive equilibria from hydroxyl ions.

Therefore, the results obtained with PGC while, on one hand give a confirm of the mechanism proposed for ODs, on the other hand do not allow us to enlarge the possibility of application of the IIR technique at higher pH values.

Figure 1. A) The effect of the alkyl chain length (*n*) on retention time. Stationary Phase: Shandon Hypercarb (100 x 4.6 mm); Mobile Phase : 5.0 mM aqueous solution of respectively pentylamine, hexylamine, heptylamine and octylamine at pH= 6.4 (for o-phosphoric acid); flow rate 1.0 mL/min; UV detection at 230 nm. B) Retention time as a function of IIR concentration. Stationary Phase: Shandon Hypercarb (100 x 4.6 mm); Mobile Phase : aqueous solution of heptylamine at pH= 6.40 (for o-phosphoric acid) at concentrations ranging between 3.00 and 15.00 mM; flow rate 1.0 mL/min; UV detection at 230 nm. C) Retention time as a function of pH of the mobile phase Stationary Phase: Shandon Hypercarb (100 x 4.6 mm) Mobile Phase : 7.0 mM aqueous solution of heptylamine at different pH value; flow rate 1.0 mL/min; UV detection at 230 nm.

Separation of the Mixture on PGC Column and Validation of the Method

As shown in Figure 2A, is the separation obtained (spectrophotometric detection at λ=230 nm) for the mixture containing iodate (0.25 mg/L), bromide (0.50 mg/L), nitrite (0.05 mg/L), bromate (0.05 mg/L), nitrate (0.05 mg/L), and iodide (0.05 mg/L) in the optimised conditions of the mobile phase: 7.00 mM heptylamine o-phosphate at pH=7.0, flow-rate =1.0 mL/min.

Plots of peak area vs. standard concentrations showed a good linearity. From sensitivity (peak area for 1.00 mg/L concentration) and for a signal to noise ratio =3, detection limits below 2.00 μg/L were obtained for nitrite, nitrate, and iodide; around 10.00 μg/L for bromate and 50.00 μg/L for iodate and bromide.

The method was also tested for its ruggedness by robustness tests performed[33,34] by imposing variations of ± 10% to the optimised pH value and to the IIR concentration.

As expected, retention is affected differently for different analytes by these variations; ranges are within 15%. In any case, the resolution and the sequence elution order of the analytes in the mixture are always preserved to indicate the robustness of the method.

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Separation of the Mixture on ODS Column and Comparison with PGC Column

For a comparison, the same mixture was separated under the same conditions of mobile phase but with an ODS stationary phase (Figure 2B). The comparison between the separation of the mixture performed with PGC column (Figure 2A) and the ODS column (Figure 2B) shows that: i) sensitivity is, on average, comparable (being even better for PGC column), ii) the elution sequence order is the same, and iii) retention times are comparable considering that the ODS column is 25 cm and PGC is 10 cm long. Obviously, the total analysis time required for the separation is much lower with PGC. Furthermore, peaks are sharper and efficiency higher, due to the different linear velocity. In turn, baseline noise is much lower for the ODS column.

Effect of the Temperature on Retention and Evaluation of ΔH° and ΔS° for the Transfer Process of the Analyte from the Mobile to the Stationary Phase

Experiments have been performed in the optimised conditions as a function of temperature, in the range between 25°C and 60°C, with steps of 5°C. The aim is: i) to evaluate how the IIR retention mechanism is affected by the

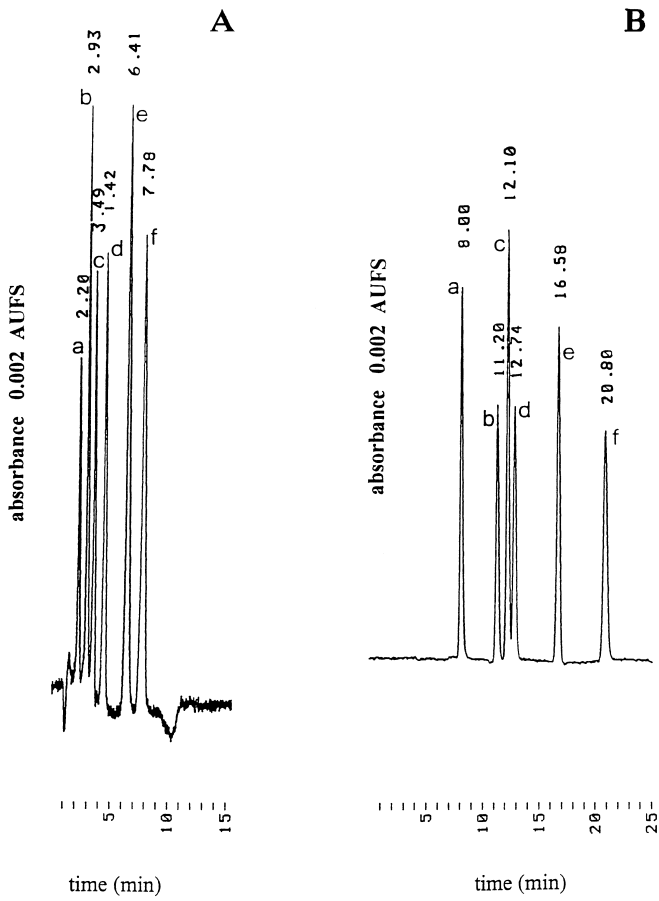

Figure 2. A) Separation of the mixture of the inorganic anions on PGC column. Conditions: Stationary phase: Shandon Hypercarb (100 x 4.6 mm). Mobile phase: 100% H_2O, heptylamine 7.0 mM, pH 7.0. Mobile phase flow rate 1.0 mL/min. Peak identification: a) iodate (0.25 mg/L), b) bromide (0.50mg/L), c) nitrite (0.05 mg/L), d) bromate (0.05mg/L), e) nitrate (0.05mg/L), f) iodide (0.05 mg/L). Volume injected: 100 µL; UV detection at 230 nm. B) Separation of the mixture of the inorganic anions on ODS column. Conditions: Stationary phase: Merck Lichrospher 100 (250 x 4 mm) RP-18 5 µm fully endcapped with a Lichrospher RP-18 (5 µm) guard precolumn. Other conditions and peak identification as in Figure 2A.

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temperature, ii) to evaluate the thermodynamic parameters of enthalpy $\Delta H°$ and entropy $\Delta S°$ for the transfer process from the mobile to the stationary phase, and iii) to compare the behaviour to temperature variations of PGC and ODS stationary phases.

As expected, retention times decrease with temperature increase, likely due to increased solubility of the analytes in the mobile phase and in general to an increased mass transfer rate.

From the retention times, the capacity factor $k' = (t_R - t_0)/t_0$ (being t_R the retention time and t_0 the dead time) in the van't Hoff equation can be calculated:

$$\ln k' = -\frac{\Delta H°}{RT} + \frac{\Delta S°}{R} + \ln \phi$$

ϕ is the phase ratio defined as the ratio between the volumes of the stationary phase V_S and of the mobile phase V_M.

The van't Hoff plot (Figure 3) reporting *ln k'* as a function of *1/T* shows good linearity (R^2 always > 0.98) for all the analytes. This suggests that, in the investigated temperature range, the retention process proceeds through only one kind of mechanism.

From the slope and for R=1.987 cal/mole, the $\Delta H°$ values reported in Table 1 are calculated. From the intercept the $\Delta S°$ values can be calculated, if the value of $\phi = V_s/V_M$ is known. The evaluation of ϕ is not so easy, mainly due to the difficulty in the evaluation of the volume of the stationary phase V_s. While ϕ has been evaluated as ranging between 0.351 and 0.819 for a series of different commercial C_{18} columns,[35] no data is available, to our knowledge, for PGC columns. Anyway, a good accepted approximation is the calculation of V_s as the difference between the total volume of the stationary phase and the volume of the mobile phase:[36] the ϕ value can, thus, be estimated as ranging between 0.5 and 1.0. The $\Delta S°$ values are given, herein, with this approximation (Table 1).

The negative values obtained for $\Delta H°$ indicate that the process of transfer from the mobile to the modified stationary phase is enthalpically favoured, while the negative $\Delta S°$ values suggest an increased order of the chromatographic system as the solute is transferred to the stationary phase.

For every solute, when comparing $\Delta H°$ to $T\Delta S°$ over the temperature range studied, the magnitude of $\Delta H°$ is greater (with iodate the only exception) than that of $T\Delta S°$, to indicate that enthalpy plays a greater role than entropy in the

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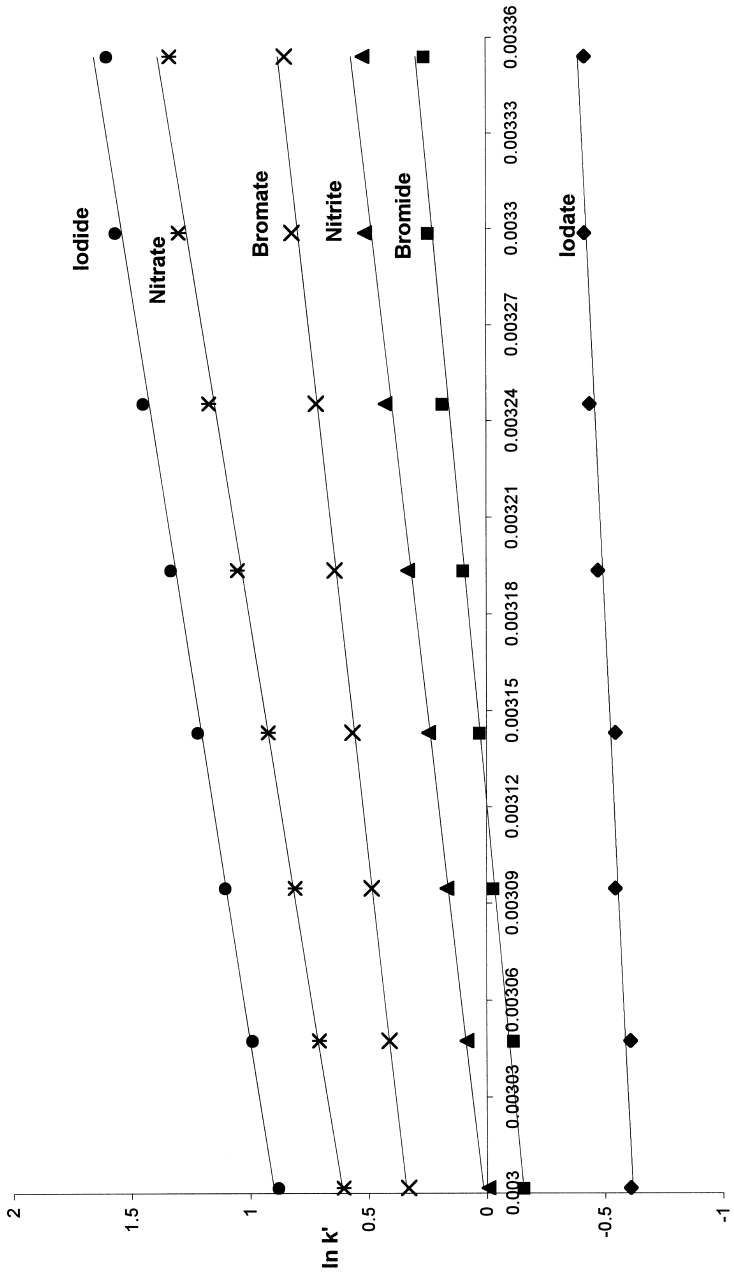

Figure 3. van't Hoff plots.

Table 1

Thermodynamic Parameters Calculated from the van't Hoff Plot*

	m	b	R^2	$\Delta H°$ (kcal mol^{-1})	$\Delta S°$ (cal mol^{-1} K^{-1})	$-\Delta S^0$	β
Nitrate	2190.80	-5.962	0.9910	-4.35	-10.47 ÷ -11.83	-11.15	390.41
Nitrate	2190.80	-5.962	0.9910	-4.35	-10.47 ÷ -11.83	-11.15	390.41
Nitrite	1577.20	-4.719	0.9824	-3.13	-8.00 ÷ -9.38	-8.69	360.59
Bromide	1279.30	-3.994	0.9840	-2.54	-6.56 ÷ -7.94	-7.25	350.61
Bromate	1526.50	-4.240	0.9932	-3.03	-7.05 ÷ -8.43	-7.74	391.87
Iodide	2135.30	-5.507	0.9884	-4.24	-9.56 ÷ -10.94	-10.25	413.93
Iodate	640.01	-2.537	0.9410	-1.27	-3.66 ÷ -5.04	-4.35	292.34

* $y = b + mx$.

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transfer of a solute from the mobile to the stationary phase and, therefore, in the retention process.

To understand if the difference in the thermodynamics parameters that seem to characterise the different analytes (Table 1) is significant, the compensation factor $\beta = \Delta\mathbf{H}°/\Delta\overline{\mathbf{S}}°$ was calculated. Again, the values obtained show some differences that can hardly be correlated to the analyte structure.

As concerns the comparison with data obtained for other classes of compounds, not many examples are available in literature. Most of them concern reversed phase retention[35,37-39] on ODS columns and some examples concern PGC stationary phases;[40-41] only one example concerns ion-interaction chromatography.[42]

The comparative analysis of all these data show as all the $\Delta H°$ and $\Delta S°$ values reported are similar and seem to be independent on the analyte, on the stationary phase packing material, and on the chromatographic technique used.

DISCUSSION

In conclusion, this study shows that it is possible to modify a PGC column to work in ion interaction mode and that the mechanisms of modification and retention are very similar for PGC and ODS stationary phases. Also, the ther-

modynamic parameters of $\Delta H°$ and $\Delta S°$ for the transfer process of the solute from the mobile to the stationary phase are comparable.

The use of PGC offers some disadvantages of cost but advantages of a general, greater efficiency which allows the mixture resolution in a lower total analysis time.

As concerns the experimental range of pH, this can be advantageously extended, (with respect to ODS stationary phase) to more acidic values. The higher values are precluded by the mechanism itself that governs the IIR technique.

ACKNOWLEDGMENTS

The authors gratefully acknowledge financial support by CNR (Consiglio Nazionale delle Ricerche, Roma) and MURST (Ministero dell'Università e della Ricerca Scientifica e Tecnologica, Roma).

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Received March 3, 2000 Author's Revision June 16, 2000
Accepted March 31, 2000 Manuscript 5269

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