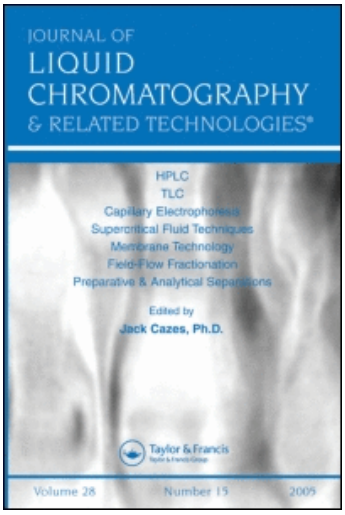

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THE ROLE OF pH OF THE MOBILE-PHASE IN ION-INTERACTION RP-HPLC

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ABSTRACT

The dependence of retention on pH of the mobile phase is investigated in reversed-phase ion interaction chromatography, being the mobile phase an aqueous solution of 5.0 mM octylammonium and ortho-phosphoric acid at different pH values.

The analytes considered were amines, diamines, amides, and species characterized by different functionalities. The behaviour of retention as a function of pH is discussed with comparison with literature data and correlation with pk_a values is shown.

The optimization of separation and resolution in a mixture of acids and amines through pH variations is also presented.

INTRODUCTION

The dependence of retention on *pH* of the mobile phase has been studied in reversed-phase chromatography (1-8) and models able to

predict retention as a function of *pH* have been described (2-8). Some examples are also found in ion-pair chromatography, in which the mobile phase is an hydroorganic mixture which contains a ion-pairing agent (9-14). At our knowledge, only one study from this laboratory concerns the dependence of anions in ion-interaction chromatography, which makes use, as the mobile phase, of an aqueous solution of the interaction reagent (15). In reversed-phase ion-interaction chromatography retention greatly depends on the *pH* of the mobile-phase, since *pH* variations can affect the reaction equilibria of both the analytes and the components of the ion-interaction reagent. Dissociation equilibria as well as of ion-pair formation must be considered.

According to the interaction model which better fit our experimental data, the interaction reagent flowing in the mobile-phase is dynamically adsorbed as ion-pair onto the surface of the reversed-phase material packing, whose originary interaction properties are therefore modified (15-19). In these conditions, the *pH* of the mobile phase not only can influence the capability of the analytes to be retained but can also affect the amount and the interaction properties of the moiety adsorbed onto the surface of the stationary phase. As a function of the mobile-phase *pH*, the properties and the capacity of the modified stationary phase can therefore vary.

As mentioned, a previous study from this laboratory dealt with the retention of anions as a function of the *pH* of the mobile phase, when the interaction reagent was octylammonium phosphate (15). Different behaviours were observed for anions of strong and weak acids and the results obtained were explained by considering different

effects. In particular, a different capacity of the stationary-phase for different *pH* values was suggested, on the basis of the stability of the different ion-pair species which can be formed between octylammonium and o-phosphate.

This paper studies the *pH*-dependence of amines, diamines, amides and of species, like *4*-aminobenzoic, nicotinic and orotic acids, which contain in their molecule two different functionalities.

It is also shown how separation and resolution of components of a mixtures can be optimized as a function of mobile-phase *pH*.

MATERIALS

Apparatus

Analyses were carried out with a Merck-Hitachi Lichrograph chromatograph Model L-6200, equipped with a two-channel Merck-Hitachi model D-2500 Chromato-integrator, interfaced with a UV-vis detector model L-4200 and a L-3720 conductivity detector with temperature control, of the same firm.

A Metrohom 654 *pH*-meter equipped with a combined glass-calomel electrode was employed for *pH* measurements and a Hitachi mod.150-20 spectrophotometer for absorbance measurements.

Chemicals and Reagents

Ultrapure water from Millipore Milli-Q was used for the preparation of all solutions. Sodium iodide, nicotinic acid, benzylamine,

nicotinamide were Merck analytical grade reagents. Octylamine, sodium azide, sodium bromate, sodium nitrate, *1,2*-phenylenediamine, *1,3*-phenylenediamine, phenethylamine, orotic acid, creatinine, *4*-aminobenzoic acid, aniline, ortho-phosphoric acid were Fluka analytical grade chemicals. Potassium thiocyanate and potassium chromate were C.Erba analytical grade chemicals.

METHODS

A 5 μm ODS-2 Spherisorb Phase Separation column fully end-capped 250.0 x 4.6 mm with a carbon load of 12% (0.5 mM/g), together with a 15.0 x 4.6 mm Lichrospher RP-18, 5 μm guard pre-column.

The solutions to be used as mobile phase were prepared by adding to the amount of octylamine weighed to prepare a 5.0 mM solution the required amount of ortho-phosphoric acid up to obtain the desired *pH* value. The solutions prepared at the different *pH* values contained therefore the same analytical concentration of octylamine (5.0 mM) and different analytical concentrations of the acid. With this procedure the presence in the mobile phase of any other component different from octylamine and o-phosphoric acid was avoided. A *pH* range within 2.5 and 8.0 was explored.

The chromatographic system was conditioned by passing the eluent through the column until a stable baseline signal was obtained; a minimum of 1 hour was necessary. This procedure was always followed when a new mobile phase was used. After use, the column

was washed and regenerated by flowing a 50/50 v/v water/methanol mixture (0.5 mL/min for 1 hour).

No particular degradation of the column was observed with *pH* variations.

The dead time was evaluated through injection of $NaNO_3$ (20 ppm) and conductometric detection of the unretained Na^+ ion. It was shown that the dead time does not significantly depend on the *pH* of the mobile phase and, at the operating conditions of flow-rate (0.7 mL/min), the average measured was 3.56 min.

RESULTS AND DISCUSSION

Table 1 lists the retention times (as the average of at least three experiments) obtained as a function of *pH* for the analytes studied. Analytes containing aminic functionality as well as aminic- carboxylic functionalities were chosen: the structures are reported in Figure 1 (A, B). 5.0 mM octylammonium phosphate was the interaction reagent and spectrophotometric detection at 230 nm was employed. The range of *pH* investigated (between 2.5 and 8.0) was imposed by the use the of silica-based stationary phase, since it was not possible (15) to obtain a reproducible and good extent of surface modification for a reversed-phase C-18 polymer-based material packing, that would have allowed to investigate a larger *pH* range.

Previous results, based on the calculation of the distribution, as a function of *pH*, of all the species formed between octylammonium and o-phosphate, suggested that the greater capacity of the stationary phase

TABLE 1

Retention times t_R (minutes) at different pH values for the Analytes investigated. The values are the average of at least three experiments. Stationary phase: Spherisorb ODS-2, 5 μm, fully endcapped, 250 x 4.6 mm; Ion Interaction Reagent: Octylammonium 5.0 mM and ortho-phosphoric acid; Flow Rate=0.7 mL/min; Spectrophotometric Detection at 230nm.

	pk_a (25°C, 0)	t_R (min) pH 2.5	t_R (min) pH 3.0	t_R (min) pH 4.5	t_R (min) pH 5.0	t_R (min) pH 6.4	t_R (min) pH 7.0	t_R (min) pH 8.0
aniline	4.63	3.76	4.50	22.05	27.96	29.00	29.79	21.30
1,2-phenylenediamine	4.63*	n.r.	4.02	8.00	15.20	17.40	15.20	13.40
1,3-phenylenediamine	2.50° 5.11°	n.r.	n.r.	6.93	8.37	9.57	8.70	8.20
creatinine	3.55	n.r.	n.r.	4.93	5.04	6.12	5.77	5.89
nicotinamide	3.35	7.19	14.20	25.17	25.11	17.27	18.13	12.40
benzylamine	9.35	n.r.	n.r.	4.00	4.11	6.30	8.36	15.80
phenethylamine	9.97*	4.21	4.16	4.97	5.15	8.60	11.16	19.50
4-aminobenzoic acid	2.08 4.96	14.89	22.80	41.45	41.93	22.50	21.50	15.00
nicotinic acid	2.05 4.81	7.95	13.69	94.68	105.43	56.68	52.00	39.38
orotic acid	4.05 8.78	42.80	52.47	47.65	44.00	20.40	20.50	17.20

* 25°C, 0.1 ° 20°C, 0 n.r.= not retained

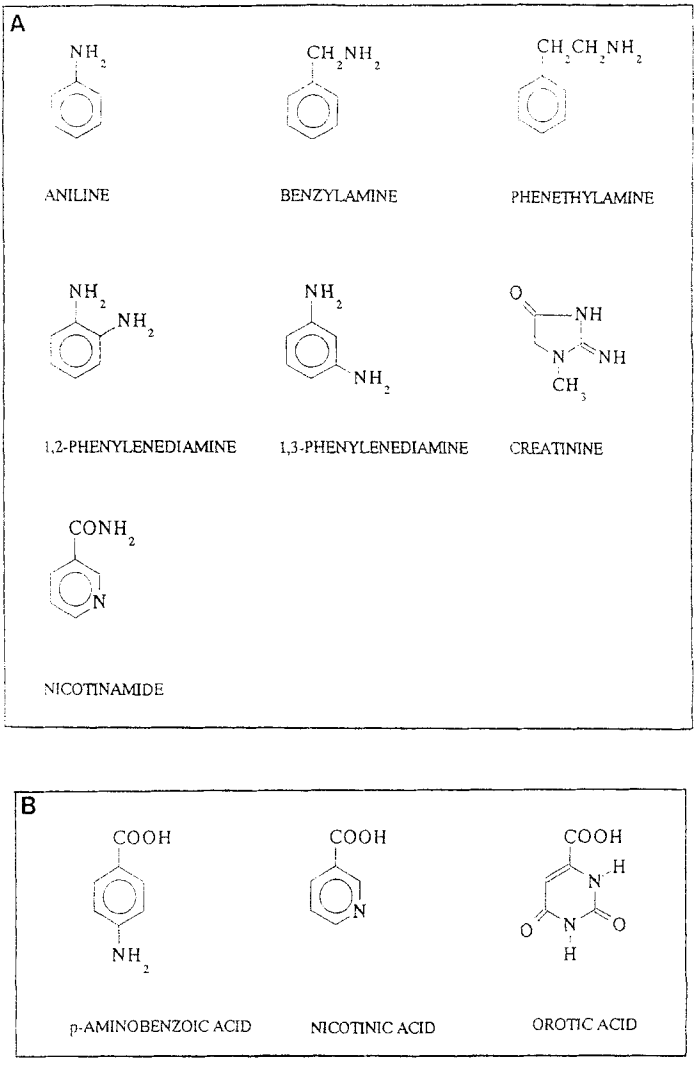

FIGURE 1 (A, B).
Analyte structures.

(expressed as the maximum number of active sites) can be hypothized at lower *pH* values. In agreement, anions of strong acids showed at lower *pH* values higher retention values. For the anions of weak acids, retention depends on the dissociation constant k_a and a maximum of retention was shown for *pH* values close to pk_a values.

Figures 2 and 3 show the behaviour of capacity factor k' ($k' = \frac{t_R - t_o}{t_o}$, where t_R is the retention time and t_o the dead time) for the amines, diamines and amides studied. Two kind of behaviours can be envisaged. Aniline, creatinine, nicotinamide, *1,2-* and *1,3-* phenylenediamine (Figure 2) show a maximum of retention in the *pH* range of about 4-6, while benzylamine and phenylethylamine (Figure 3) show a progressive increase of retention for the whole *pH* range investigated. It can be observed that the amines characterized by a maximum of retention are characterized by pk_a values around 4-5 (see Table 1) while benzylamine and phenethylamine show pk_a values around 9.5 (see Table 1).These results are in agreement with literature results (12) obtained for adenine (pk_a = 4.12) in ion-pair chromatography (sodium octylsulphonate as the ion-pairing and 10% v/v of methanol in the mobile phase).

The behaviours observed in Figure 2 and 3 can be explained as follows. The results previously obtained for the same chromatographic system (15) indicated a greater capacity of the stationary phase for lower *pH* values. In addition, at lower *pH*, amines are preferentially present in their protonated form, under which the formation of ion-pairs with o-phosphate should be favoured. But, as lower is the *pH* and more protonated the amines, as stronger are the repulsion electrostatic forces

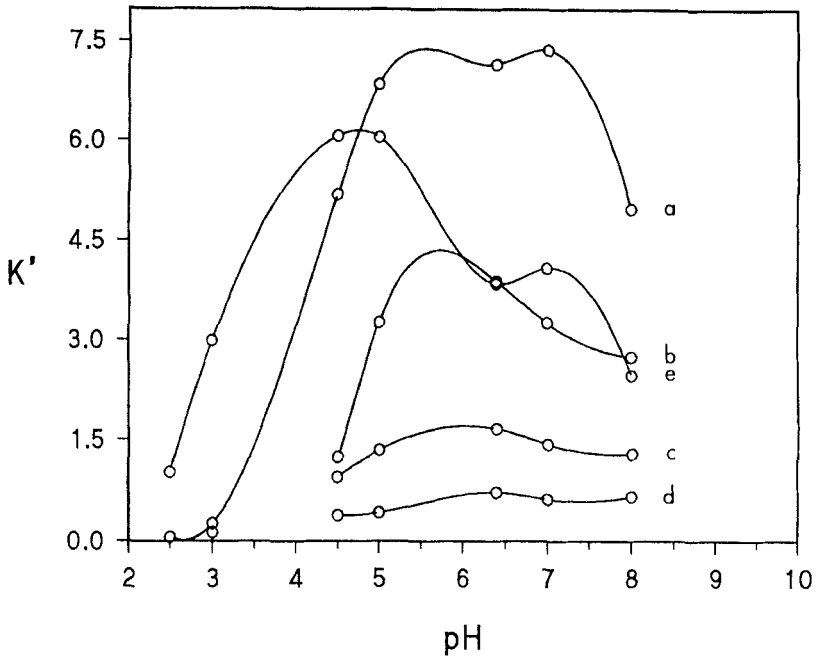

FIGURE 2.
Capacity factor k' as a function of pH. Operating conditions: Stationary phase: Phase Separation Spherisorb ODS-2, 5 μm, fully endcapped, 250 x 4.6 mm; Ion interaction reagent: 5.0 mM octylammonium and ortho phosphoric acid; Flow rate: 0.70 mL/min; Injection volume: 100 μL; Spectrophotomectric detection at 230 nm.
Analytes: a= aniline, b= 1,2-phenylenediamine, c= 1,3-phenylenediamine, d= creatinine e= nicotinamide.

aging between the adsorbed octylammonium and the analytes and the final result is a lower retention.

The retention decrease observed (Figure 2) for higher *pH* values can be ascribed to the combined effect of the lower retention capacity of the stationary phase together with the always lower molar fraction of the analyte which is present in the protonated form.

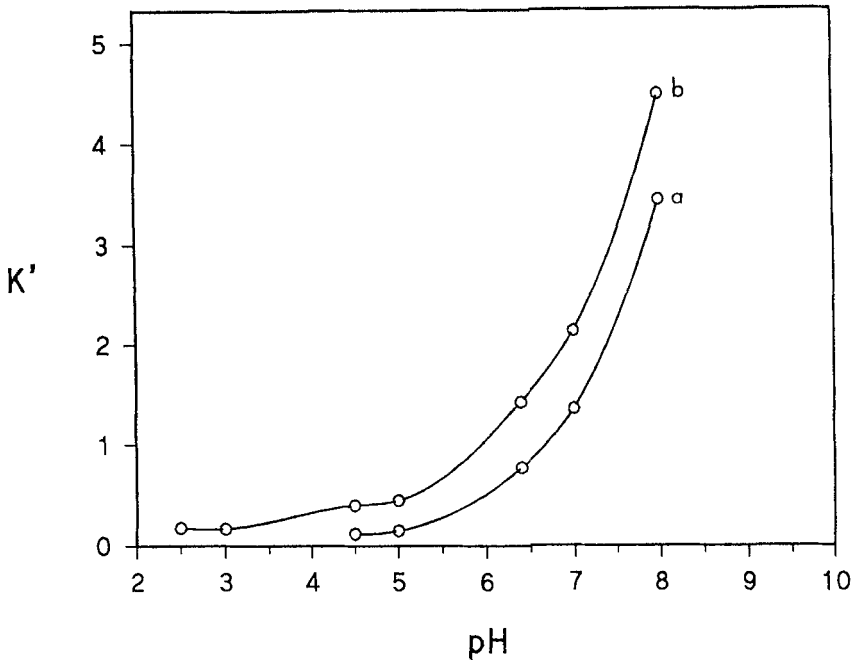

FIGURE 3.
Capacity factor k' as a function of pH. Experimental conditions as in Figure 2.
Analytes: a= benzylamine, b= phenethylamine.

These considerations do not hold for benzylamine and phenethylamine, which are characterized by very higher pK_a, so that at *pH* 8 the molar fraction a of the protonated form is still sufficiently high ($\alpha > 0.95$) to give easily rise to the formation of ion-pairs.

Figure 4 shows the behaviour of retention as a function of *pH* for the following acids: *4*-aminobenzoic, nicotinic and orotic acids which (see Figure 1 B) show in their molecule both carboxylic and nitrogen containing functionalities. The dependence shows in every case a

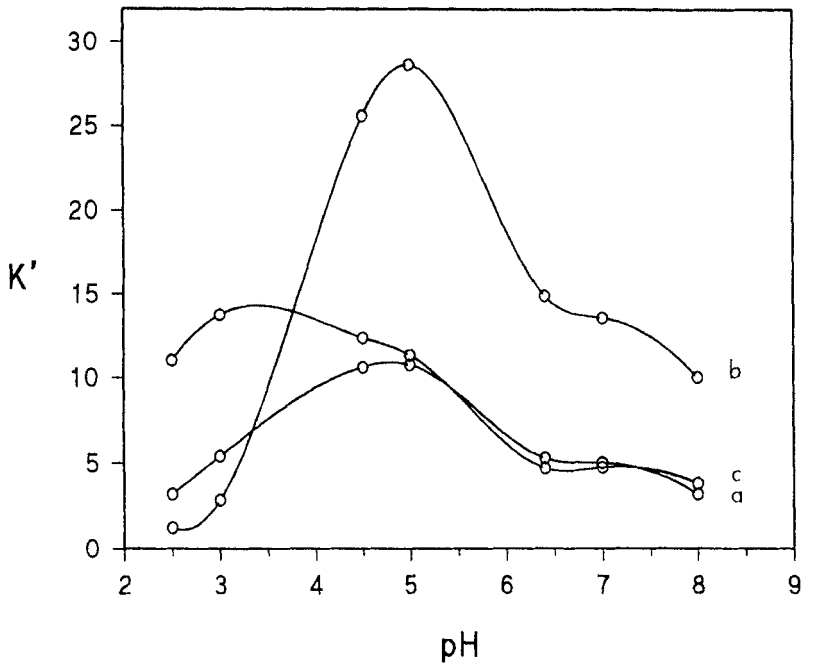

FIGURE 4.
Capacity factor k' as a function of pH. Experimental conditions as in Figure 2.
Analytes: a= 4-aminobenzoic acid, b= nicotinic acid, c= orotic acid.

maximum, around *pH* 5 for nicotinic and 4-aminobenzoic acids and around 3.0 - 3.5 for orotic acid.

It would be of interest to understand which of the two functional groups present in the molecule is participating in the retention.

On the basis of the results collected up to now for anions (15) and amines, it was always shown that the maximum of retention nearly corresponds to the pK_a value of the analyte. We can therefore propose that the carboxylic group is always responsible for retention for the

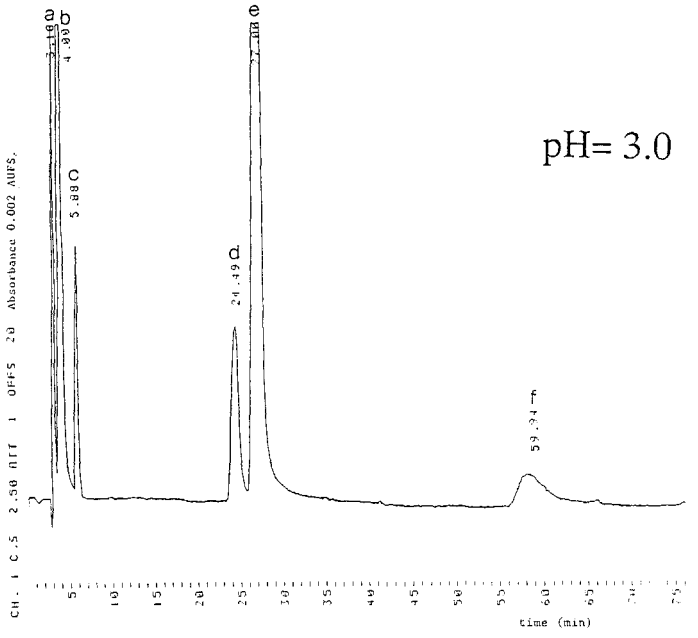

FIGURE 5.
Separations at pH=3.0 (A), pH=6.4 (B), pH=8.0 (C) of a mixture containing: a= 1,3-phenylenediamine (0.50 ppm), b=1,2-phenylenediamine (0.50 ppm), c= sodium azide (0.50 ppm), d= nitrate (0.50 ppm) e= iodide (0.50 ppm), f= thiocyanate (0.50 ppm) and g= chromate (1.00 ppm). Operating conditions as in Figure 2.

three compounds investigated, with respect to the nitrogen-containing group.

From a practical point of view, the different *pH* dependence shown by the retention of different analytes can helpfully assist in solving problems of identification and resolution between the components of a mixture. In Figure 5 typical chromatograms recorded at three different *pH* values (*pH* = 3.0, 6.4 and 8.0, 5.0 mM

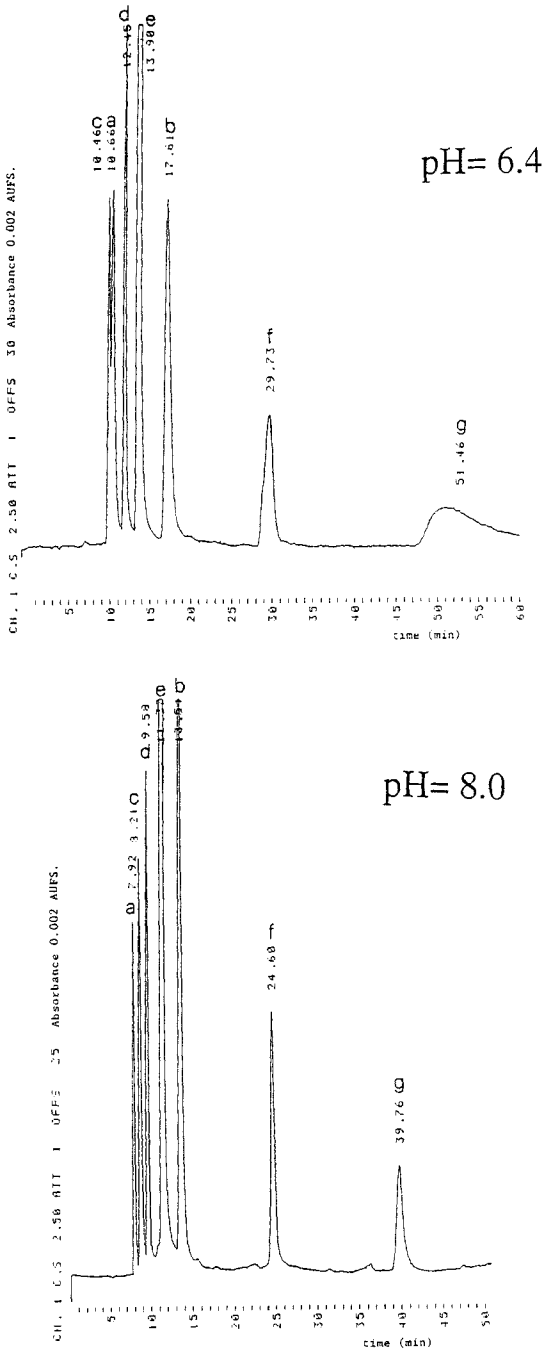

FIGURE 5 (continued)

octylammonium o-phosphate as the ion-interaction reagent and spectrophotometric detection at 230 nm) are reported for a mixture which contains anions and amines, namely: *1,3*-phenylenediamine (0.50 ppm), *1,2*-phenylenediamine (0.50 ppm), hydrazoic acid (1.00 ppm), nitrate (0.50 ppm), iodide (0.50 ppm), chromate (1.00 ppm) and thiocyanate (0.50 ppm).

It can be observed that not only sensitivity and resolution can vary with pH but also the elution sequence order as it can be for example observed for hydrazoic acid, nitrate and iodide and *1,2*-phenylenediamine at *pH* 3.0 and 6.4 and for *1,3*-phenylenediamine and hydrazoic acid at *pH* 6.4 and 8.0.

Figure 5 also shows the remarkable improvement which can be obtained in sensitivity and resolution when working with a *pH* of the mobile phase equal to 8.0.

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