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## Comparison of the retention of organic acids on alkyl and alkylamide chemically bonded phases

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### Abstract

Retention of pyridine and indole carboxylic acids was studied on alkylamide bonded phases under reversed-phase conditions at 35°C. The alkylamide phases were synthesized by a two-step process, in which an initial amino phase was prepared under environmentally isolated conditions and subsequently reacted with alkanoyl chlorides. The retention measurements were carried out using acetonitrile–water eluent at different volume compositions and different pH values. For the comparative purposes analogous retention data were collected for conventional octyl phases bonded to various silicas. A comparison of the retention data measured on alkyl and alkylamide packings have demonstrated that the later, polymeric or monomeric, are better for separating pyridine and indole carboxylic acids. The comparative analysis of the chromatographic systems studied suggests that retention of these acidic solutes on alkylamide phases is governed by a mixed partition–displacement and ion-exchange mechanism.

### 1. Introduction

Chromatographic analysis of multifunctional compounds with acidic and/or basic groups is of great importance for many laboratories, especially those involved in agricultural and pharmaceutical research. Many of these compounds can be analyzed by high-performance liquid chromatography under reversed-phase conditions (RPLC) using alkyl-bonded phases, which currently are most popular because of their hydrophobic character and very good chemical, thermal and mechanical stabilities [1–4]. However, the performance of chromatographic columns packed with alkyl-bonded phases becomes worse

for the compounds of complex stereochemistry and functionality. Several authors [5–7] reported that these factors affect significantly the column efficiency and peak asymmetry. For instance, McCalley [5] studied the retention of pyridine and some alkyl-substituted derivatives under reversed-phase conditions using an octadecyl-bonded phase with 14% carbon loading and demonstrated that the solute stereochemistry in close vicinity of the basic group influences strongly the peak shape. According to his studies, alkyl groups in close proximity of the basic site had the most pronounced effect, apparently hindering interactions in the stationary phase. Vervoort et al. [6] and Ascah and Feibush [7] found the peak asymmetry increased with increasing  $pK_a$  of the analyte. Also, they studied the effect of the solute stereochemistry in close vicinity to the basic nitrogen atom on the peak

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shape. The same authors considered possible interactions between the protonated nitrogen atom of the solute molecule and residual surface silanols.

The molecular mechanism of retention becomes more complex when acidic groups are present in vicinity of the basic nitrogen atom, e.g., pyridine and indole carboxylic acids. These compounds, especially those with the carboxylic group in close proximity to the ring nitrogen atom, are known as the substances difficult to analyze on the currently available reversed-phase columns. The hydrogen bond, which could be formed between the ring nitrogen atom and the carboxylic group proton, facilitates ionization of the molecule and promotes interactions with the residual surface silanols. This leads to the deterioration of elution bands or to irreversible adsorption. When these acidic compounds are accompanied by analytes of another charge type or different relative hydrophobicity, addition of ion-pairing reagents is necessary in order to achieve a proper peak shape and satisfactory separation of all components of interest.

Another alternative for improving chromatographic separation of the above-mentioned analytes is application of the chemically bonded phases of specific stereochemistry and functionality. Alkylamide phases, which contain terminal alkyl chains attached to the surface via an alkylamide group, possess specific chromatographic properties. These phases have been recently studied by us [8–10] and appeared to have interesting physicochemical properties. A detailed study of alkylamide packings under reversed-phase conditions has shown that the specific interaction site, i.e., amide group, located in the hydrophobic ligand has a significant influence on the solute retention and selectivity. In addition, improved peak symmetry was observed for polar solutes such as amines which makes these phases attractive for carrying separation of base-type compounds.

In the current work we have explored the possibility of using alkylamide phase for separating complex compounds, which possess acidic groups located in close proximity to the ring nitrogen atom. Retention behavior of a series of

pyridine and indole carboxylic acids was studied on selected alkylamide phases under reversed-phase conditions using acetonitrile–water mixture as the eluent. For the comparative purposes, additional retention data were collected for these solutes on various octyl bonded phases. The retention data were measured at different compositions and pH of the mobile phase in order to get more information about retention mechanism of multifunctional acidic compounds on alkylamide phases. A long-term aim of the work is elaboration of chromatographic conditions for analyzing these compounds.

## 2. Experimental

### 2.1. Introductory remarks

Retention measurements were carried out on two types of alkylamide phases with an acetonitrile–water mobile phase at different pH values and different concentrations of the organic solvent. For comparative purposes, organic compounds which do not form internal hydrogen bonding, e.g., benzoic acid and 5-methylpyridinedicarboxylic acid dimethyl ester, were also studied. In addition, analogous retention measurements were performed on conventional octyl phases bonded to various silicas.

### 2.2. Reagents and materials

Two types of spherical silica used to prepare alkylamide-bonded phases were 10- $\mu\text{m}$  Li-Chrosorb Si-60 (EM Science, Cherry Hill, NJ, USA) and 5- $\mu\text{m}$  Inertsil (MetaChem Technologies, Torrance, CA, USA). The BET specific surface areas of these materials were 500 and 320  $\text{m}^2/\text{g}$ , respectively.

The surface modification reagents, 3-aminopropyltriethoxysilane and suitable alkanoyl chlorides were purchased from Aldrich (Milwaukee, WI, USA). The remaining surface modification reagents, octyldimethylchlorosilane and 3-aminopropyltrimethylmethoxysilane were purchased,

respectively, from Huls America (Bristol, PA, USA) and Pertrarch Systems (Levittown, PA, USA). The other chemicals used in chromatographic measurements and synthesis of the bonded phases, such as methanol, acetonitrile, 2-propanol, toluene, etc. were purchased from Aldrich. The chromatographic solutes (see Fig. 1) were purchased from Aldrich. The deionized water was purified in the laboratory using a Millipore (El Paso, TX, USA) Model Milli-Q water system.

### 2.3. Alkylamide-bonded phases

Mono- and polymeric alkylamide phases, i.e., AA-12 (with attached  $C_{12}$  ligand) and AA-8 (with attached octyl ligand), were prepared at Kent State University by a two-step process, in which an initial aminopropyl phase was synthesized and subsequently reacted with suitable alkanoyl chloride. The synthesis procedure was described previously [8–11]. The monomeric AA-12 phase was synthesized by modifying initially the silica surface with 3-aminopropyltrimethylmethoxysilane, however 3-aminopropyltriethoxysilane was used for preparing the polymeric AA-8 phase. The AA-12 packing was obtained using a 5- $\mu\text{m}$  Inertsil support and the AA-8 phase was synthesized using a 10- $\mu\text{m}$  LiChrosorb silica gel. The elemental analysis data for the AA-12 and AA-8 phases are as follows: (i) polymeric phase: 4.66% of C and 1.06% of N in the initial aminopropyl phase, and 14.32% of C and 1.09% of N in the final AA-8 phase, and (ii) monomeric phase: 3.37% of C and 0.39% of N in the initial aminopropyl phase, and 8.89% of C and 0.39% of N in the final AA-12 phase.

Both bonded phases were packed into 150 mm  $\times$  4.6 mm I.D. stainless-steel column blanks which were purchased from Supelco (Bellefonte, PA, USA). Prior to packing a slurry was prepared using 35 ml of 2-propanol and sonicated the mixture for 5 min. Subsequently, the columns were packed using a Haskel (Burbank, CA, USA) Model DST-52 pump at a pressure of 50 MPa and methanol as the carrier solvent.

### 2.4. Octyl-bonded phases

The commercial 150 mm 5- $\mu\text{m}$  RP  $C_8$  columns Eka Nobel Kromasil, Metachem Inertsil, Waters NovaPak, and Supelco Supelcosil DB along with a  $C_8$  column prepared at Kent State University and packed with a octyl phase bonded to 5- $\mu\text{m}$  Inertsil silica (coverage density 4.25  $\mu\text{mol}/\text{m}^2$ , carbon loading 13.27%), were used for comparative purposes.

### 2.5. Chromatographic equipment

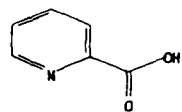
The modular liquid chromatograph, consisted of a Spectra-Physics SP8800 pump, a LKB Model 2125 column oven, a variable-wavelength UV detector (ABI 785A), and a Hewlett-Packard 1050 autosampler was used to measure the retention volumes as a function of the mobile phase composition and pH. The retention data were acquired and processed using a Hewlett-Packard 3350 laboratory data system.

### 2.6. Chromatographic measurements

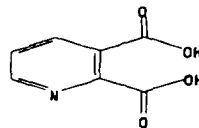
The retention data of a series of alkyl- and amino-substituted pyridine and indole carboxylic acids were measured at different acetonitrile concentrations and pH values of the mobile phase. Chromatographic measurements were collected at the pH range of 2–5.5 (50 mM sodium phosphate buffer) and the acetonitrile concentration range from 0 to 20%. The influence of pH on the solute retention was examined for the mobile phases containing 5% of acetonitrile. However, the mobile phase composition effects were studied at pH 2.0 or 2.3. All experiments for alkylamide phases were carried out at 35°C and 1 ml/min flow-rate.

## 3. Results and discussion

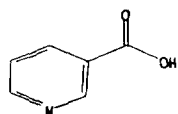
A comparative study of the alkyl- and alkylamide-bonded phases under reversed-phase conditions showed that the intercalation (competitive sorption) of solvent molecules into the stationary phase depends strongly on the specific



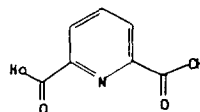
Picolinic Acid



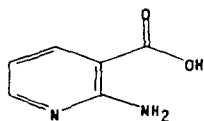
2,3-Pyridine Dicarboxylic Acid (2,3-PDC)



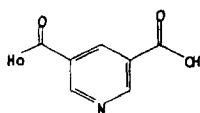
Nicotinic Acid



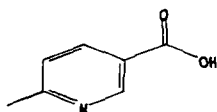
2,6-Pyridine Dicarboxylic Acid (2,6-PDC)



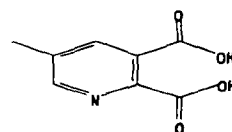
2-Amino Nicotinic Acid



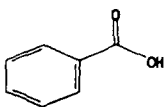
3,5-Pyridine Dicarboxylic Acid (3,5-PDC)



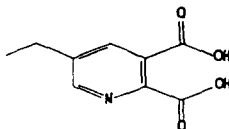
6-Methyl Nicotinic Acid



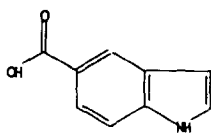
5-Methyl-Pyridine Dicarboxylic Acid (MPDC)



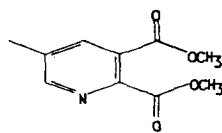
Benzoic Acid



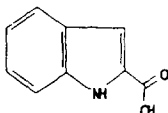
5-Ethyl-Pyridine Dicarboxylic Acid (EPDC)



Indole-5-Carboxylic Acid (5-Indole COOH)



5-Methyl Pyridine Dicarboxylic Acid Dimethyl Ester (MPDC.DME)



Indole-2-Carboxylic Acid (2-Indole COOH)

Fig. 1. Chemical structures of the pyridine- and indolecarboxylic acids studied.

interaction groups localized in hydrophobic ligands [8]. These specific groups, e.g., amide group in the alkylamide phases, decrease hydrophobic character of the bonded phase. This effect is stronger for the alkylamide phases with shorter terminal alkyl chains. Thus, for the alkylamide phases, especially those with short terminal alkyl chains, the preferential sorption of organic solvent into the stationary phase is smaller in comparison to the corresponding alkyl-bonded phases. While the residual surface silanols are the only polar groups in the monomeric alkyl-bonded phases, the alkylamide-bonded phases in addition to the specific alkylamide ligands contain also the residual silanols and aminopropyl groups. All specific groups present in the alkylamide phases are able to form a special structure of the stationary phase, the physicochemical properties of which differ significantly from that for a conventional alkyl phase [8,9]. This structure plays an essential role in separating multifunctional solutes under reversed-phase conditions.

Shown in Fig. 2 are the experimental dependencies of the capacity ratio  $k'$  on pH for EPDC on all columns studied. Analysis of these dependencies indicates that in the case of alkylamide phases the ionic type of interactions plays a

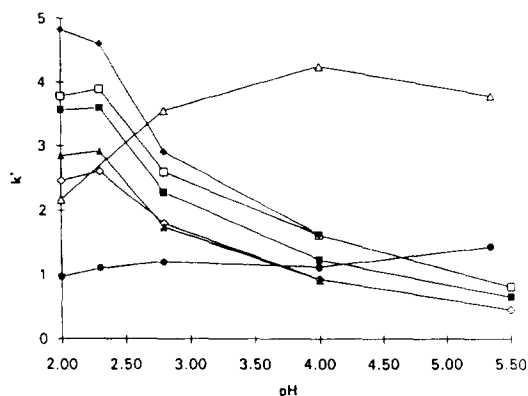


Fig. 2. A comparison of the  $k'$  vs. pH dependencies for 5-ethylpyridinedicarboxylic acid (EPDC) on the alkyl- (45°C) and alkylamide- (35°C) bonded phases. The hydro-organic mobile phase contained 5% of acetonitrile. Columns: ■ = Kent Inertsil  $C_8$ ; □ = MetaChem Inertsil; ◆ = Kromasil  $C_8$ ; ◇ = Supelcosil  $C_8$ -DB; ▲ = NovaPak  $C_8$ ; △ = Kent AA-8; ● = Kent AA-12.

substantial role in the retention mechanism. For all alkyl columns the retention of EPDC decreases with the degree of acid ionization, while on both alkylamide columns the acidic solutes are retained longer at the higher pH values. The obtained pH-dependent plots are analogous to those observed in ion chromatography [12]. However, the retention of EPDC dimethyl ester, which does not form ionic species, remains independent of pH. Similar behavior is observed for all other acids studied as shown in Figs. 3–5. To explain the difference in retention on the AA-8 and AA-12 phases one should consider the following factors:

(i) Structural difference between two phases: AA-8 is a polymeric phase with  $C_8$  terminal chain, whereas AA-12 is a monomeric phase with  $C_{12}$  terminal chain.

(ii) Different nature of silicas used in the synthesis of both phases: AA-8 was prepared on LiChrosorb, whereas AA-12 was synthesized on Inertsil.

(iii) Different concentrations of surface ligands: for the AA-8 phase the coverage density of alkylamide ligands and residual amino groups

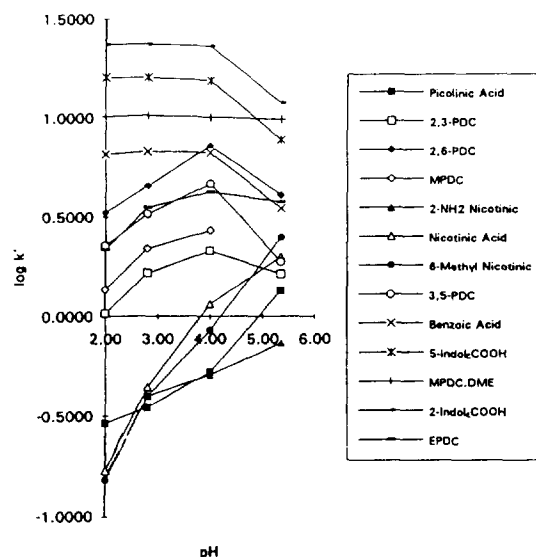


Fig. 3. A comparison of the  $\log k'$  vs. pH dependencies for all solutes listed in Fig. 1 on the polymeric AA-8 phase. Chromatographic conditions as in Fig. 2.

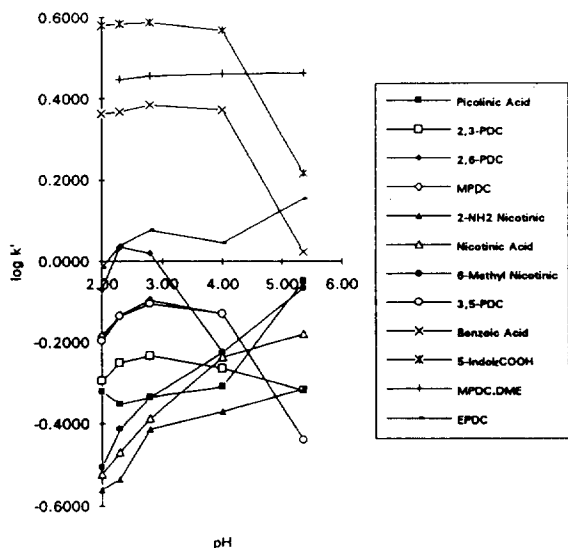


Fig. 4. A comparison of the  $\log k'$  vs. pH dependencies for all solutes listed in Fig. 1 on the monomeric AA-12 phase. Chromatographic conditions as in Fig. 2.

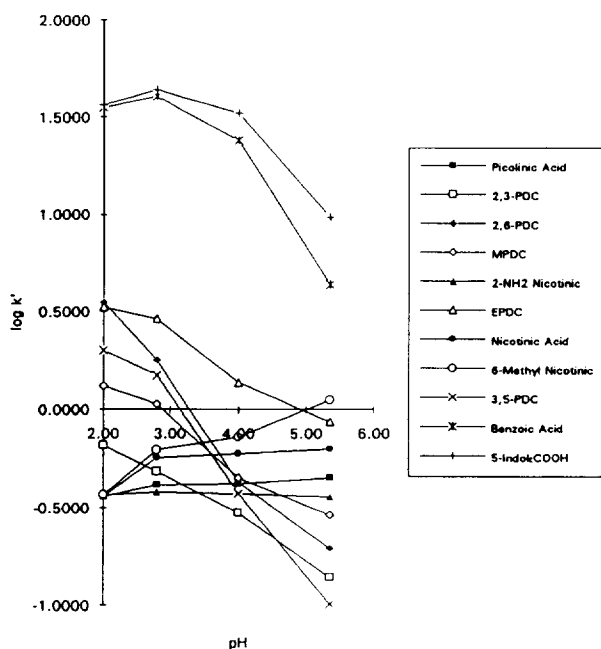


Fig. 5. A comparison of the  $\log k'$  vs. pH dependencies for all solutes listed in Fig. 1 on the Kent monomeric  $C_8$  phase. Chromatographic conditions as in Fig. 2.

are 1.36 and  $0.4 \mu\text{mol}/\text{m}^2$ , respectively; however, for the AA-12 phase these concentrations are 1.4 and  $0.4 \mu\text{mol}/\text{m}^2$ .

The basic properties of the nitrogen atom in the amide group are very weak. So, when the amide group accepts a proton, it does through the oxygen atom. In comparison to the amide nitrogen the basicity of the nitrogen atom in the amine group is much stronger. Therefore, the aminoalkyl phases retain much longer organic acids than the corresponding alkylamide phases. Since both, monomeric and polymeric, alkylamide phases contain ligands with terminal amino groups (residual amino groups) and internal amide groups, the overall retention is strongly affected by the concentration of the unreacted amino groups. Although the coverage densities of the AA-8 and AA-12 packings studied were similar, residual amino groups and silanols were screened more effectively for the later material. In addition, an internal masking of the amino groups by the residual surface silanols is also possible. Thus, the AA-8 phase adsorbs more water than the AA-12 phase and its structure and physicochemical properties depend much more on pH of the mobile phase. Due to the possibility of mutual interactions between residual silanols and amino groups the amide groups seem to play an important role in the retention mechanism on the AA phases. Additional spectroscopic studies are in progress in order to identify molecular interactions that control retention on the AA phases.

As shown in Figs. 6-9 the acetonitrile concentration influences differently the retention of multifunctional acids on the alkyl- and alkylamide-bonded phases. For the conventional  $C_8$  column (Figs. 6 and 7) the retention volumes of all acids decrease in a similar manner as the concentration of acetonitrile increases from 0 to 20%. In this case the experimental dependencies of  $k'$  versus the acetonitrile concentration for acids are similar to that for diester on the AA-8 and AA-12 columns (Figs. 8 and 9). For the later columns, the retention volumes of the acids studied decrease much more slowly with increasing acetonitrile concentrations than for the corresponding RP  $C_8$  columns. The dependencies of

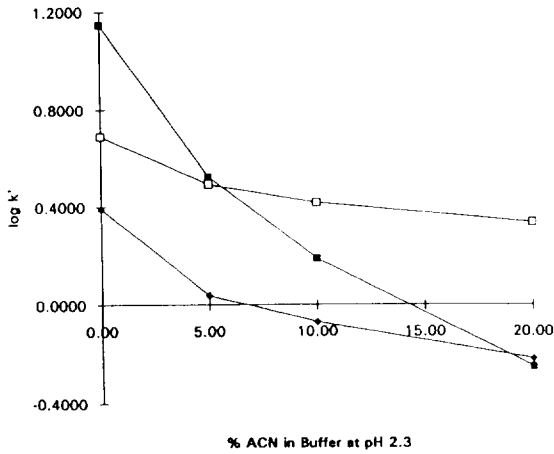


Fig. 6. A comparison of the  $\log k'$  vs. % acetonitrile (ACN) dependencies for 5-ethylpyridinedicarboxylic acid (EPDC) on the Kent monomeric  $C_8$  (■), polymeric AA-8 (□) and monomeric AA-12 (◆) packings at 35°C and pH 2.3.

$\log k'$  on the acetonitrile concentration in the mobile phase are almost flat in the range between 10 and 20% due to the lower methylene selectivities on the AA phases. It was shown in

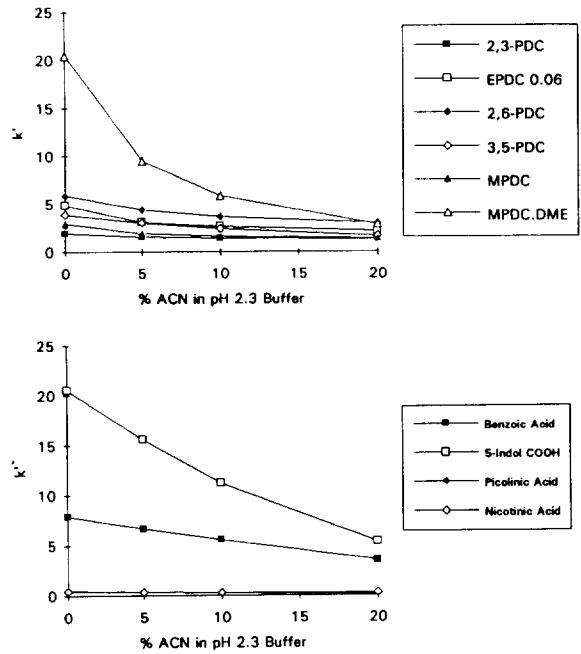


Fig. 8. A comparison of the  $k'$  vs. % ACN dependencies for diacids (top panel) and monoacids (bottom panel) on the polymeric AA-8 packing at 35°C and pH 2.3.

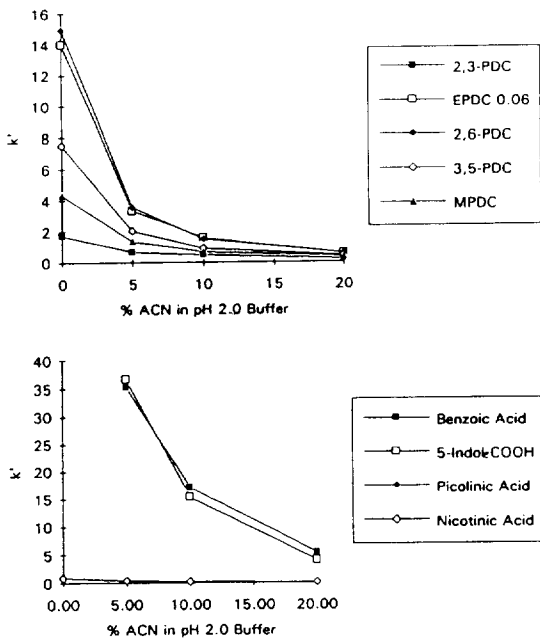


Fig. 7. A comparison of the  $k'$  vs. % ACN dependencies for diacids (top panel) and monoacids (bottom panel) on the Kent monomeric  $C_8$  packing at 35°C and pH 2.0.

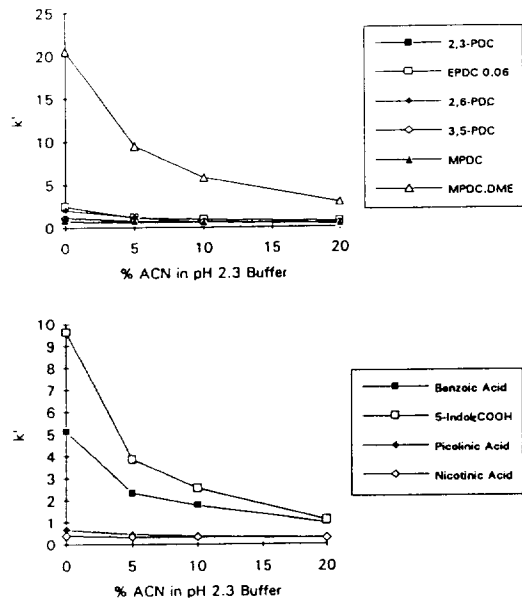


Fig. 9. A comparison of the  $k'$  vs. % ACN dependencies for diacids (top panel) and monoacids (bottom panel) on the monomeric AA-12 packing at 35°C and pH 2.3.

the previous work [8] that the methylene selectivities on the AA phases can be about 25–50% lower in comparison to those on the corresponding alkyl phases. For instance, at 20% of acetonitrile in the mobile phase ester elutes before 2,6-pyridinedicarboxylic acid. A comparison of all types of the columns studied is shown in Fig. 6. Similarly, as in the case of pH-dependent retention studies, the concentration effects are more pronounced for the polymeric AA-8 phase. As shown in Fig. 8 the concentration-dependent plots for acidic solutes are quite well separated. However, for the AA-12 packing these plots overlap. This means that the AA-8 phase is better for separating the acidic solutes studied.

The dependencies of  $\log k'$  on pH or acetonitrile concentration for mono- and diacids falls into two different categories. Experimental points for monoacids seem to fit a linear pH dependence of retention, while points for diacids seem to lie on a parabolic curve. This different behavior can be probably attributed to the greater charge effects of the latter compounds as well as to their ability to form intermolecular hydrogen bonding.

#### 4. Conclusions

The current work suggest that the retention mechanism of multifunctional organic acids on the conventional alkyl phases, including those with high coverage density (e.g., Kent C<sub>8</sub>) occurs according to the partition–displacement mechanism, which is characteristic for typical RPLC systems [13]. However, the retention mechanism of these solutes on alkylamide phases is governed by a mixed ion-exchange and partition–displacement mechanism. It has been shown that the contribution of ion-type interaction to the overall retention is greater for polymeric alkylamide phases than for monomeric ones.

A comparison of the retention measurements for alkyl and alkylamide phases have demonstrated that the later phases, polymeric or mono-

meric, are better for separating the pyridine and indole carboxylic acids than conventional alkyl packings. They allow separation of these acidic solutes from hydrophobic compounds over a wide range of the mobile phase compositions (including highly aqueous conditions) without adding ion-pair reagents and/or applying the mobile phase gradient. Currently chromatographic and spectrometric studies are in progress in order to characterize further the structural features of the alkylamide bonded phases and to enrich our understanding about the molecular mechanism of the solute retention on these packing materials.

#### Acknowledgement

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