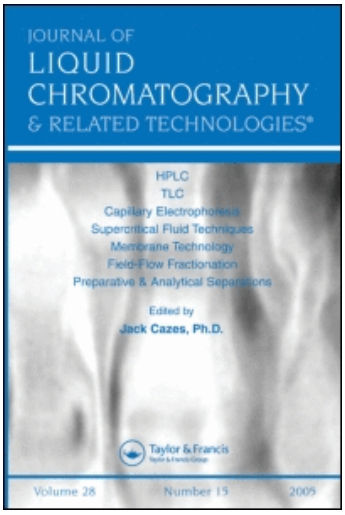

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Investigations into the Use of Poly (3-Methylpyrrole-4-Carboxylic Acid) Coated Silica as a Chromatographic Stationary Phase

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INVESTIGATIONS INTO THE USE OF POLY(3-METHYLPYRROLE-4-CARBOXYLIC ACID) COATED SILICA AS A CHROMATOGRAPHIC STATIONARY PHASE

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ABSTRACT

Poly(3-methylpyrrole-4-carboxylic acid) (PMPC) was coated on to a silica support for use as an HPLC stationary phase. Reversed phase anion exclusion and cation exchange chromatographic behaviour have been characterised using a group of test compounds. The predominant mode of interaction was found to be dependent on the eluent conditions. Separation of various compounds was carried out and the applicability of this stationary phase for protein separations was investigated.

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INTRODUCTION

The use of conducting polymers as chromatographic stationary phases has been investigated previously (1-6). In some of this work (3, 5) the ability to alter retention by application of electrical potentials has been utilised, and this aspect of the work has been reviewed recently (6). In all previous work polypyrrole coated on to various substrates was employed as the chromatographic packing material. Both anion exchange and reversed phase interactions were observed using these phases. Other workers have shown that the availability of a mixed mode mechanism of retention can be of use in attaining separation in complicated samples such as those containing protein aqueous material (7). However, peak distortion and a decrease in column efficiency may sometimes be the result of mode separations.

It has been found by other workers that polypyrrole undergoes an oxidation degradative process via unblocked 3,4 positions of the pyrrole ring with a subsequent loss of anion exchange capacity (8, 9). Recently pyrroles derivatised in either the 3 or 4 position have been used to form new conducting polymers. These polymers not only had novel chemical selectivity but also improved chemical stability (10-15). This derivatisation approach has been used to introduce hydrophobicity (10), optical activity (11) or further ion exchange capabilities (15) to the resultant polymers.

Poly (3-methylpyrrole-4-carboxylic acid) (PMPC) has previously been synthesized using electrochemical methods (15-17) and was found to exhibit cation exchange capabilities (15). In this work, attempts to use PMPC as an HPLC stationary phase has been investigated with a view to use as a multimode chromatographic system. A chemical polymerization process was investigated. The stationary phase has been characterised using a variety of test compounds. Protein separations have been studied. The stationary phase has been used for separation of a

range of compounds and the separation of proteins was investigated.

EXPERIMENTAL

Reagents, Materials and Standard Procedures

3-Methylpyrrole-4-carboxylic acid (MPC) was synthesized in the laboratory as described previously (17). Tetra-n-butylammonium perchlorate (TBAP) (Fluka) and p-toluene sulfonic acid (PTS) (Aldrich) were used as obtained. Acetonitrile (AR, Millinckrodt) was used as the solvent for electrochemical polyermization while 50% methanol and 50% deionized water was used as the solvent for chemical polymerization. 1.0mol L^{-1} Ferric chloride (BDH) solution was prepared in water. Silica (Ultrasphere, 10μm, 220m^2/g) was a gift from Beckman Instruments, USA. Methanol (AR) was obtained from Marlinckrodt. Acetate buffers were prepared by mixing certain amount of 2mol L^{-1} acidic acid and 2mol L^{-1} sodium acetate solutions. The pH of the buffers was monitored by a pH meter. Test compounds were prepared as 1% (w/v) stock solutions in methanol or water and diluted in the eluent before use. Proteins were from Sigma used without further purification. The dead time of the chromatographic system was measured using water.

Preparation of an HPLC Column

Chemical synthesis of PMPC coated silica is described as follows according to previous method (18): 2.0g MPC and 3.2g PTS were mixed in a 50ml measuring cylinder. The mixture was dissolved in 25ml of methanol. Then 5.0g silica was added into the solution and 25ml of 1.0mol L^{-1} $FeCl_3$ (aqueous) was mixed with the above suspension. The suspension was allowed to stir

for 48 hours with nitrogen and some methanol was added in during the reaction to compensate the evaporated solvent. The resultant particles were filtered with Buchner filter and washed with copious water and then acetone. The small polymer particles were classified by flotation to obtain an evenly sized packing material. The size of the particles was monitored using a microscope. The polymer-coated silica particles were packed into a 1.6mm ID x 3.2mm OD x 65mm Teflon tubing (ACTIVON) using a previously described method (19) and a 4.6mm ID x100mm stainless steel column by a slurry-packing method using acetone under 400atm pressure.

Chromatography

A Beckman 114M Solvent Delivery System, an Altex 210 valve with a 20µl sample loop, and a Beckman 165 variable wavelength detector consisted of an HPLC system for isocratic chromatography. Chromatograms were recorded on an ICI DP-600 chart recorder. For gradient elution a Dionex Series 4000i gradient pump and injector, an ICI SD-2100 UV-VIS variable wavelength detector and a Kipp and Zonen BD41 chart recorder were used.

Eluents used in isocratic chromatography were a mixture of water and methanol with acetate buffers if required while eluents for gradient elution of proteins were (A) 0.1% TFA in H_2O with or without 0.05mol L^{-1} NaCl and (B) 0.1% TFA in 20% H_2O and 80% CH_3CN with or without 0.05mol L^{-1} NaCl.

RESULTS AND DISCUSSION

The carbon loading of the polymer coated HPLC packing material was 2.76%. The specific surface area is 204m^2/g, which is smaller than pure silica (220m^2/g). This is probably due

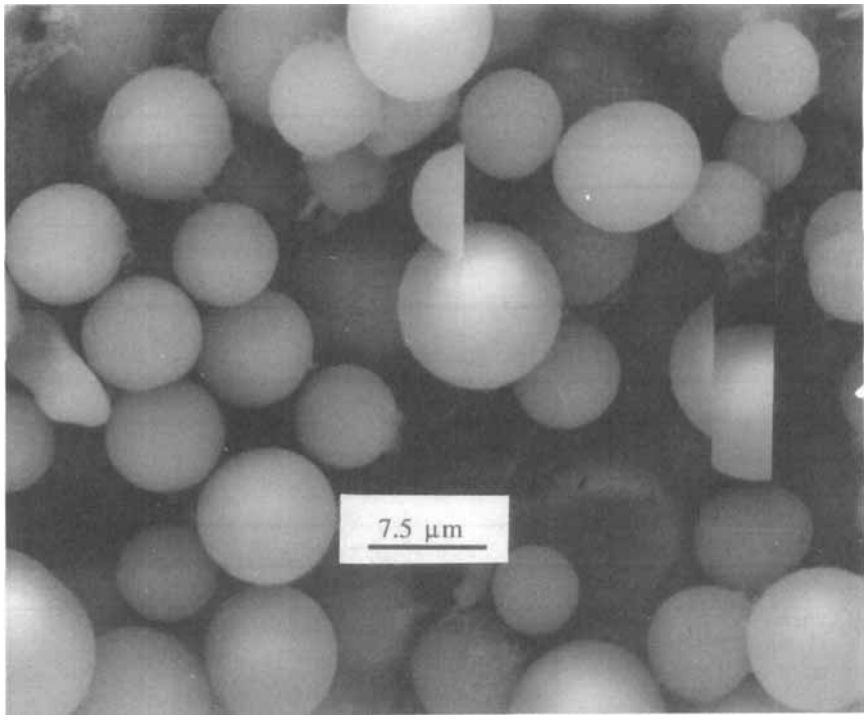


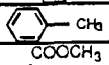
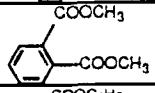
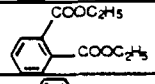
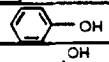
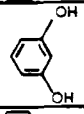
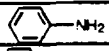
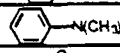
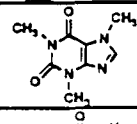
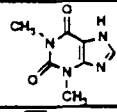
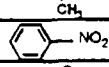
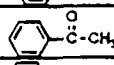
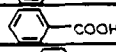
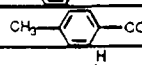
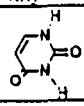
Figure 1 Scanning electron micrographs of PMPC modified silica.

to the fact that the polymer coating reduced the surface area. A scanning electron micrograph of the packing material is depicted in Figure 1.

Chromatographic Characterisation

Capacity factors as a function of methanol concentration were determined for the 16 test compounds listed in Table 1. Results are summarised in Figure 2. For most compounds, the capacity factors increased with decreased methanol

TABLE 1 - Test compounds *

Name	Structure	pKa*
Benzene		-
Toluene		-
Dimethyl-phthalate		-
Diethyl-phthalate		-
Phenol		10
Resorcin		-
Aniline		4.60 (+1)
N,N-Dimethyl-aniline		5.15 (+1)
Caffeine		14
Theophylline		<1(+1)
Nitrobenzene		-
Acetophenone		-
Benzoic acid		4.204
p-Toluic acid		4.362
Cytosine		4.58 (+1), 12.16 (0)
Uracil		-

* pk_a values are from References 26 and 27. Numbers in parentheses indicate ionic states.

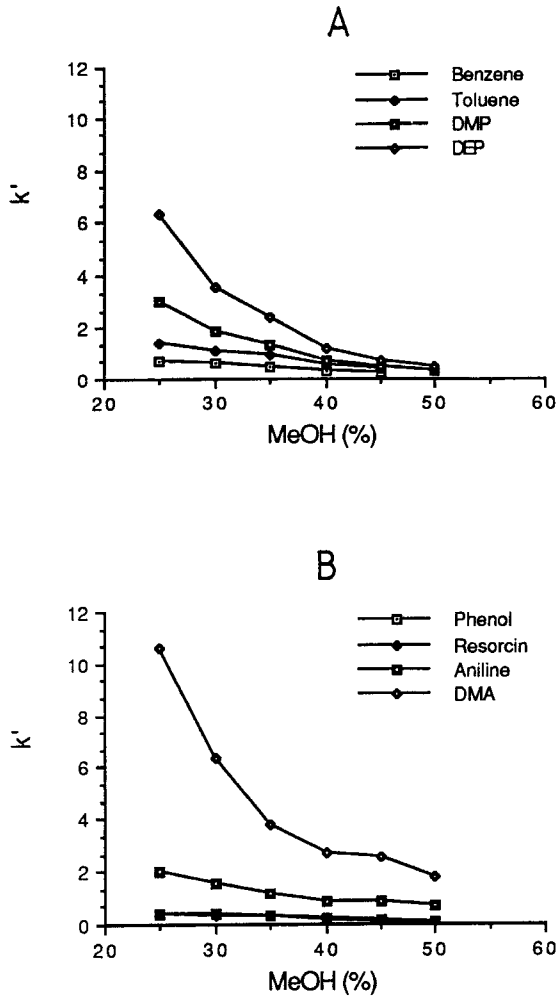


Figure 2 Plots of capacity factor vs methanol concentration in the eluents.
Column: PMPC coated silica, 10μm in a 3.2 mm ID x 65mm Teflon column.
Eluent: Mixture of methanol and water at 0.5ml/min.
Detector: UV; λ = 254nm

(continued)

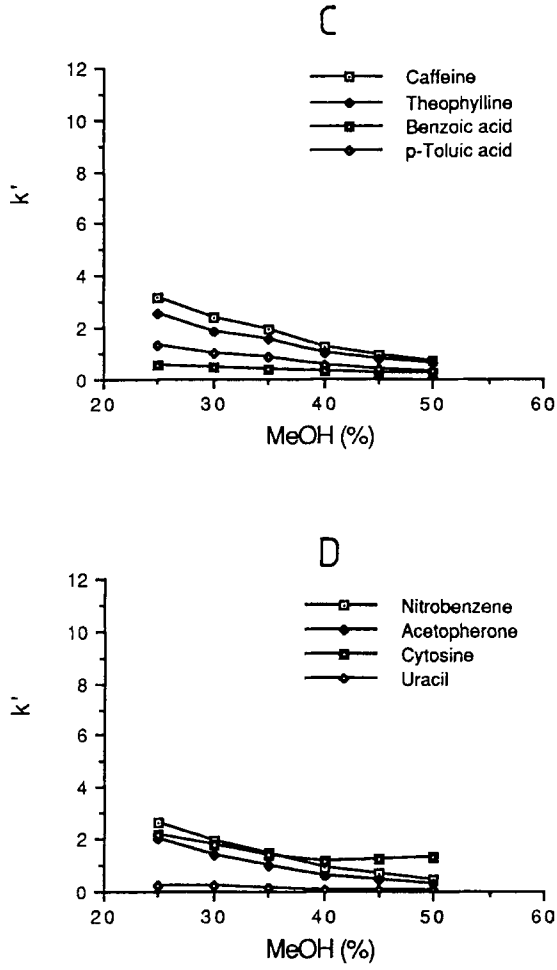


Figure 2 Continued

TABLE 2 - Comparison of polypyrrole and poly(3-methyl-4-carboxylic acid) modified silica *

Test Compound	Relative retention to benzene on PP**	Relative retention to benzene on PMCP
Benzene	1.0	1.0
Toluene	1.6	1.2
Dimethylphtalate	2.0	1.3
Diethylphthalate	2.1	1.7
Phenol	0.9	0.4
Aniline	8.0	2.5
N,N-Dimethylaniline	2.2	6.7
Benzoic acid	∞	0.7

* Eluent = 50% MeOH and 50% H_2O.

** Data from Reference 4.

concentration, indicating that a reversed phase retention mechanism was involved. Benzene and toluene were not strongly retained on the polymeric stationary phase indicating that the PMPC phase had limited interaction with non-polar compounds. On the other hand, longer retention was observed for the polar test compounds, DMP, DEP, nitrobenzene and acetophenone. Compared with the behaviour previously observed on polypyrrole phases (4) this stationary phase showed lower relative retention to benzene for the selected test compounds (Table 2). Like polypyrrole phases, phenol as well as resorcin had short retention on the PMPC phase indicating that the ability to undergo H-bonding interactions was minimal. Organic acids, benzoic and p-toluic acids were also unretained using reversed phase conditions. Their capacity factors were similar to benzene and toluene respectively. This indicated that the anion exchange capability previously observed on polypyrrole phase (4) was not

found on PMPC. This could be that the polymer was self-doped as indicated previously (18).

Basic compounds, aniline, N,N-dimethyl aniline, caffeine, theophylline and cytosine had relatively higher retention. This behaviour was due to the expected acid-base interactions between the -COOH group on the stationary phase or uncoveredSiOH group on silica and the amine groups on the test compounds. Uracil had almost no interaction with the stationary phase, presumably because its amide group is really basic.

Capacity factors as a function of pH for the 16 test compounds were determined (Figure 3). The polymer was deprotonating from pH=3 and completely deprotonated over pH=6 (15). For nonpolar test compounds, benzene and toluene, the effect of pH on retention was negligible. A slight increase in capacity factors with increased pH was found for the polar compounds DMP, DEP, nitrobenzene and acetophenone. This could be that deprotonation of the -COOH group on the polymer stationary phase increased the polarity of the stationary phase. Caffeine, theophylline and uracil were not cationic in this pH range so that the retention behaviour was more like other polar compounds. Aniline, N,N-dimethylaniline, and cytosine were expected to undergo cation exchange. However, deprotonation of -COOH and protonation of amino groups occurred in a reversed way so that no apparant cation exchange was observed when changing pH. Significant changes of capacity factors for benzoic and toluic acids with eluent pH were observed. The higher the pH, the lower the capacity factors. This indicated that at low pHs both the stationary phase and the acids were protonated enabling interactions to occur, while at high pHs the deprotonated stationary phase excluded the deprotonated acids.

Capacity factors as a function of ionic strength were determined for the 16 test compounds (Figure 4). For most test compounds retention was not affected by the ionic strength of

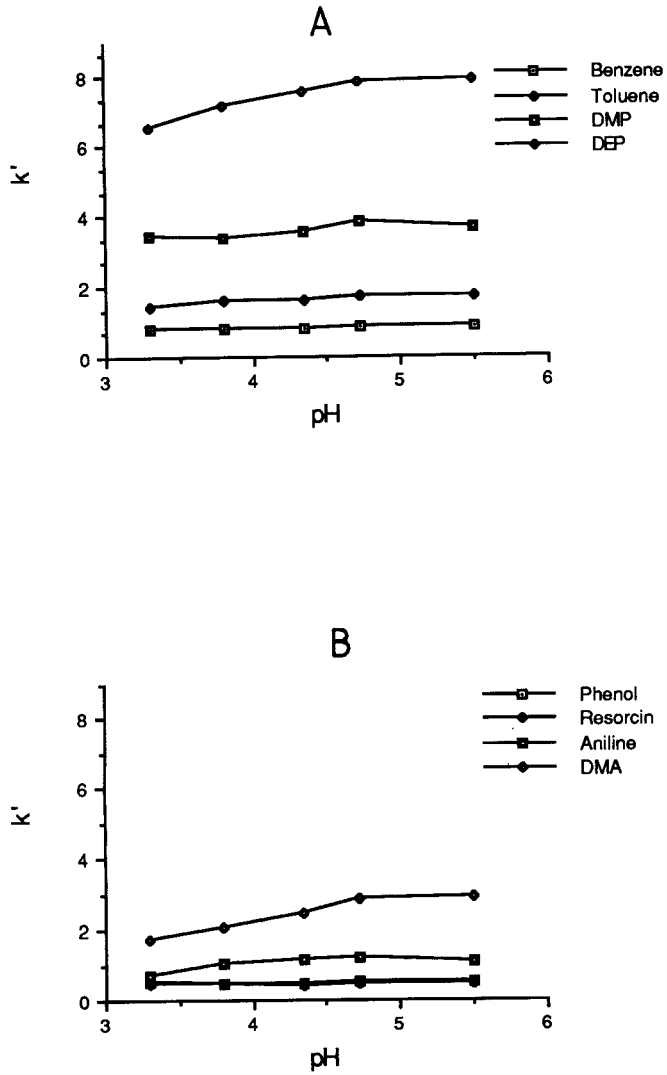


Figure 3 Plots of capacity factor vs eluent pH
Column: As in Figure 2
Eluent: 25% Methanol and 75% H_2O containing 0.05mol L^{-1} acetate buffer at various pH
Flow rate: 0.5ml/min.
Detector: UV; λ = 254nm

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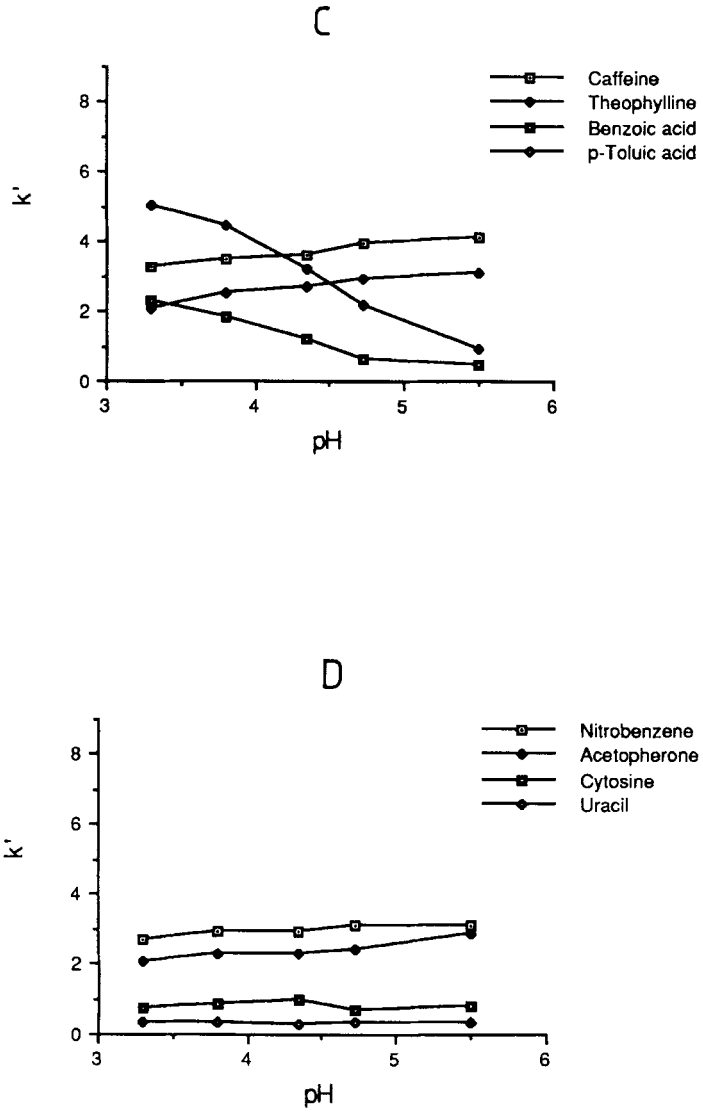

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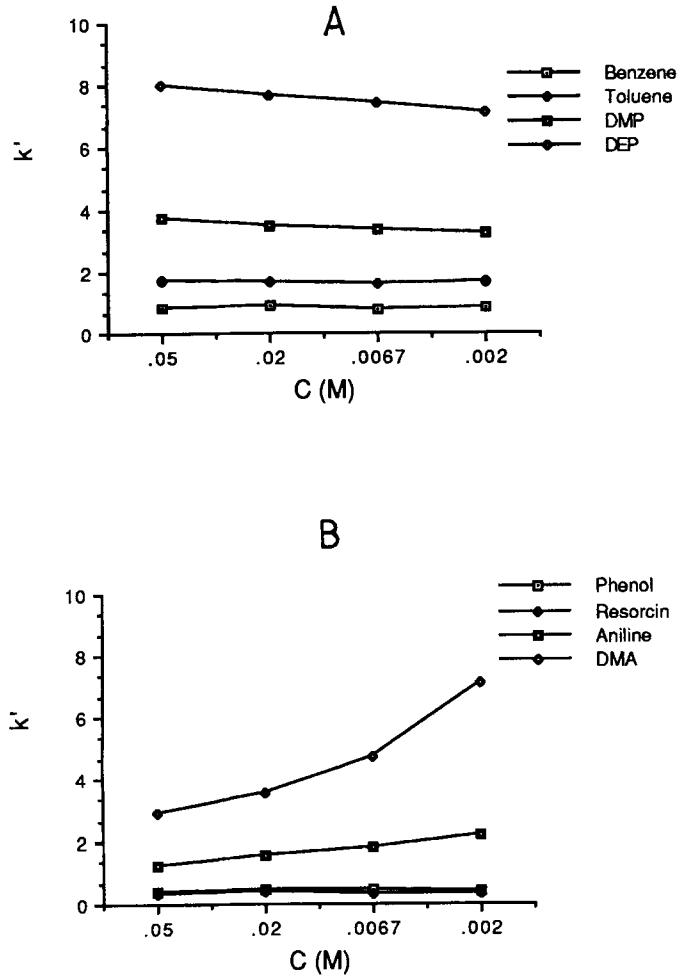

Figure 4 Plots of capacity factor vs ionic strength of the eluent
Column: As in Figure 2
Eluent: 25% Methanol and 75% H_2O, pH=5.5 acetate buffer, Ionic strength varied.
Flow rate: 0.5ml/min.
Detector: UV; $\lambda = 254nm$

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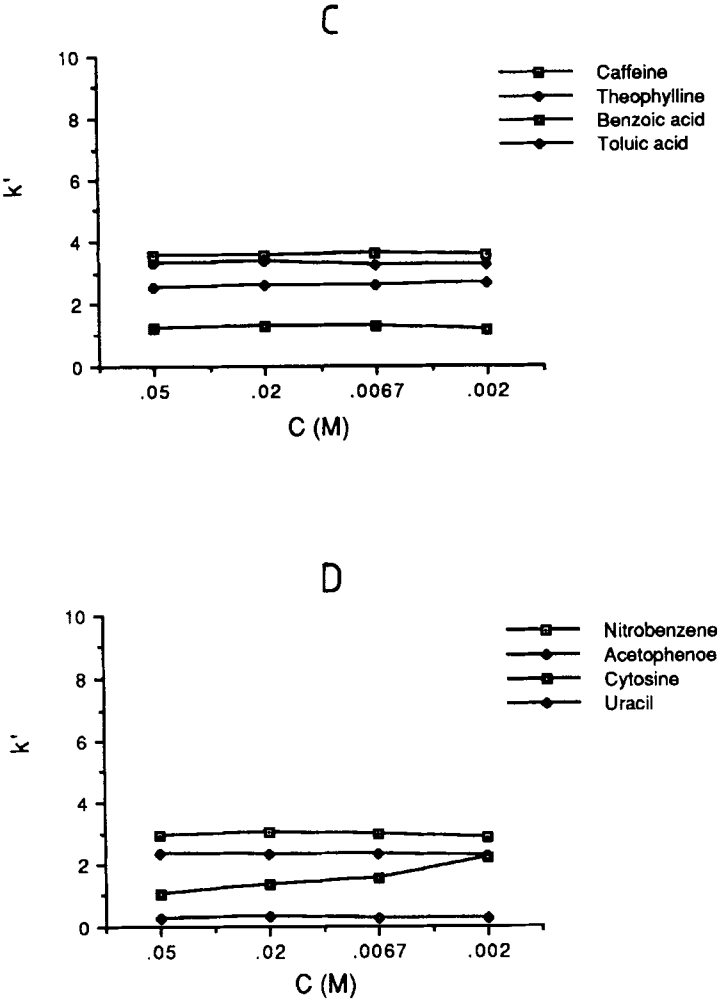

Figure 4 Continued

the eluent. However, capacity factors for aniline, N,N-dimethylaniline and cytosine decreased with increased ionic strength. This is typical of the behaviour expected for cation exchange processes. At this pH -COOH of the stationary phase was mostly deprotonated while $-NH_2$ of the compounds was partly protonated. The protonation pk_a for DMA was high so that significant cation exchange interaction was observed. Capacity factors of DMP and DEP increased slightly with increased ionic strength. This may be due to increased hydrophobic interactions induced by the increased ionic strength (20, 21).

Separation Examples

Reversed phase separations of some test compounds were considered (Figure 5). Separation of basic compounds with and without a buffer in the eluent was tested. There was no significant difference observed (Figure 6). Usually silica-based packing materials cause undesirable effects for HPLC of basic samples, which may be reduced by addition of buffers (22, 23). Separation of benzoic and toluic acids under various conditions was investigated. Better resolution was achieved at a low pH since both the stationary phase and the acids were protonated so that a reversed phase separation was achieved. (Figure 7). These separations were carried out on a test column (3.2mm ID x 65 mm) and hence overall column efficiency was low. This will be improved using longer columns in future work.

Protein Separation

The proteins used are shown in Table 3. Separations initially were carried out using CH_3CN gradient with 0.1% TFA. However, ghost peaks were observed after protein elution which were probably due to irreversible adsorption from previous injections. 0.05mol L^{-1} NaCl was added in the eluents to produce

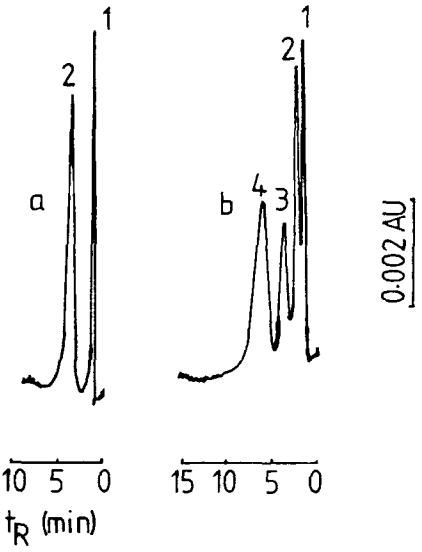

Figure 5 *Examples of reversed-phase separation on a PMPC column*
Column: As in Figure 2
Eluent: 25% Methanol and 75% H_2O
Flow rate: 0.2ml/min.
Samples:
(a)1. 0.15ppm uracil; 2. 0.5ppm cytosine
(b)1. 10ppm benzene; 2. 10ppm toluene
3. 2ppm DMP 4. 5ppm DEP
Detector: UV; λ = 254nm

TABLE 3 - Protein Properties

Protein	Molecular Weight (x 1000)	pI
α-Lactalbumin (LA)	14	5.1
Ovalbumin (OVA)	44	4.6
Bovine Serum Albumin (BSA)	66	5.3
Human Serum Albumin (HSA)	66	4.7
Myoglobin (MYO)	18	7.0
Transferrin (TRAN)	77	5.9-6.0

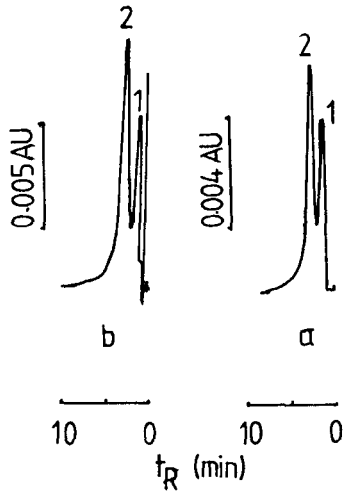

Figure 6 Separation of basic compounds
Column: As in Figure 2
Eluent: (a) 25% Methanol and 75% H_2O, 0.02moi L^{-1} acetate buffer, pH=4.4, 0.5ml/min;
(b) 25% Methanol and 75% H_2O at 0.5ml/min.
Samples:
1. 20ppm aniline 2. 5ppm N,N-dimethylanile
Detector: UV; λ = 254nm

a higher ionic strength. However, such effects were still observed. Protein gradient elution under these conditions is summarised in Table 4 and separation examples are given in Figure 8. The recovery was estimated from comparison of peak areas with and without the column. High recovery for transferrin without salt in the eluent and for α-lactalbumin with salt in the eluent was found. The recovery was low for most proteins, probably due to the nature of proteins under reversed phase conditions as reported (24). Retention sequence of proteins was different from other reversed phases. For example, on a C_{18} phase (24, 25) retention sequence was OVA>BSA>MYO while it was MYO>BSA>OVA on the PMPC phase. This is probably due to effect of cation exchange capability or polar nature of the stationary phase.

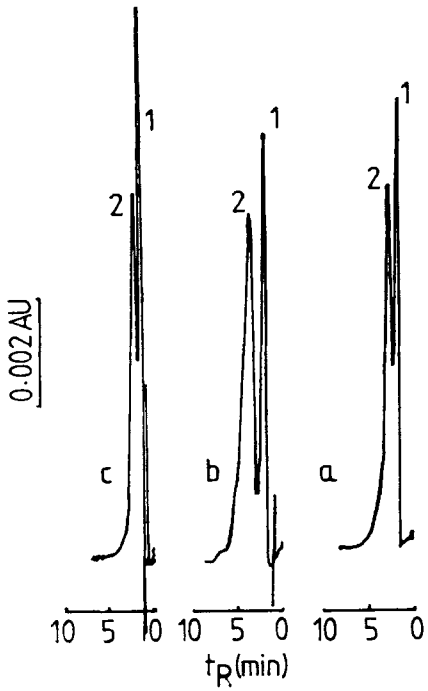

Figure 7 Separation of acidic compounds
Column: As in Figure 2
Eluent: (a) 25% MeCH and 75% H_2O
(b) 0.05mol L^{-1} acetate buffer (pH = 4.2) in (a)
(c) 0.05mol L^{-1} acetate buffer (pH = 5.2) in (a)
Flow rate: 0.2 ml/min.
Samples: 1. 4ppm benzoic acid
2. 4ppm p-toluic acid
Detector: UV; λ = 254nm

TABLE 4 - Protein retention and recovery on PMPC column

Gradient elution from A to B in 20 minutes	A. 0.1% TFA in H_2O B. 0.1% TFA in 80%CH_3CN + 20% H_2O		A. 0.1% TFA and 0.05M NaCl in H_2O B. 0.1% TFA and 0.05M NaCl in 80% CH_3CN + 20% H_2O	
	Retention (min.)	Recovery (%)	Retention (min.)	Recovery (%)
HSA	4.05	35	4.56	76
BSA	4.91	77	5.27	60
Ovalbumin	0.81, 5.00	78	0.81, 4.24	61
Transferrin	5.75	97	5.75	45
Myoglobin	6.37	31	8.75	41
α-Lactalbumin	5.37	55	8.28	100

CONCLUSIONS

Poly (3-Methylpyrrole-4-carboxylic acid) was coated on silica as a stationary phase. Reversed phase ion exclusion and cation exchange type interactions were observed. The predominant mode of interaction is influenced by solution conditions such as organic solvent content, pH and ionic strength. Anion exclusion occurred at low pH's when both the stationary phase and analytes were deprotonated. Cation exchange effects were observed when the stationary phase was deprotonated and the analyte was protonated. Protein separations on PMPC were investigated. The elation order was different from other

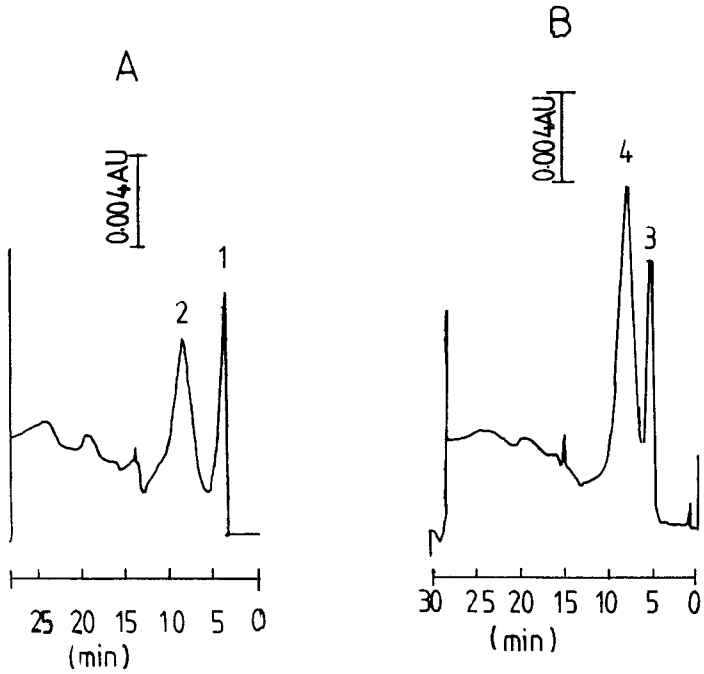

Figure 8 Separation of proteins on the PMPC column
Column: PMPC coated silica, 10μm, in a 4.6mm IDx100mm stainless steel column.
Eluent: (A) 0.1% TFA and 0.05M NaCl in H_2O and
(B) 0.1% TFA and 0.05mol L^{-1} NaCl in 80% CH_3CN and 20% H_2O; gradient from A to B in 20 minutes at 1.0ml/min.
(a) Separation of 50μg HSA (1) and 50μg MYO (2)
(b) Separation of 50μg BSA (3) amd 25μg LA

reversed phase columns. Separations of various compounds were carried out.

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