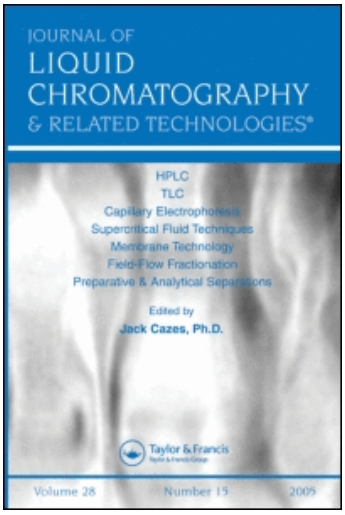

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Ge, H. , Gelmore, K. G. and Wallace, G. G.(1994) 'Studies on Poly (3-Octadecyl Pyrrole) Modified Silica as a Reversed Phase HPLC Packing Material', Journal of Liquid Chromatography & Related Technologies, 17: 6, 1301 — 1316
To link to this Article: DOI: 10.1080/10826079408013764
URL: http://dx.doi.org/10.1080/10826079408013764

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(6), 1301-1316 (1994)

STUDIES ON POLY (3-OCTADECYL PYRROLE) MODIFIED SILICA AS A REVERSED PHASE HPLC PACKING MATERIAL

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ABSTRACT

In this work poly(3-octadecyl pyrrole) (PODP) modified silica has been employed as a reversed phase material. The stationary phase has been characterized using a selection of test compounds and compared with polypyrrole and ODS phases. The stability of this stationary phase in acidic and basic media has been tested. Protein separations have been carried out on this phase. The results obtained indicate that this stationary phase is stable in acidic media and that the performance, with respect to stability, in basic media is similar to commercial ODS columns. Protein separations and basic compound separations have been achieved using this stationary phase.

INTRODUCTION

Separation of proteins and peptides using high-performance liquid chromatography (HPLC) has been extensively studied. A number of different modes of

chromatography have proven useful for such separations. These include size-exclusion, ion-exchange, affinity, hydrophobic interaction and reversed phase (1-3). Among them, reversed phase chromatography using alkyl (4-8) or phenyl (9) bonded silica has been widely used. Often aqueous-organic solvent systems with acids such as trifluoroacetic acid (TFA) (6-9), phosphoric acid (4-5) and perchloric acid (6) have been used. These eluents have a pH value of 2-3 and such conditions are known to promote the cleavage of the carbon chain from the silica in bonded phases (10-12). Separation of basic compounds has also proven difficult on silica-based reversed phase columns due to the solubility of silica under such conditions. Therefore, the development of a stationary phase which is more resistant to low and high pH eluents or alternatively more selective under moderate conditions is desirable.

The use of polymer materials may go some way towards providing such a stationary phase (12,13). In our laboratories conducting polymer based stationary phases have been developed (14-18). These phases possess several unique characteristics which make may make their use as chromatographic stationary phases attractive. Polymers, such as polypyrrole, are electrically conductive, chemically stable, electrochemically active and they can be easily coated on to silica to produce mechanically stable stationary phases. In addition they are capable of a diverse array of chromatographic interactions.

In other laboratories a derivative of pyrrole with a C-18 hydrophobic chain, 3-octadecyl pyrrole (3ODP), has been polymerised either electrochemically (19, 20) or chemically (21).

In this work, 3ODP has been absorbed onto silica and then polymerised. Synthesis of the polymer and characterisation of the stationary phase has been investigated. Stability in acidic and basic media has been examined and the suitability of the stationary phase for separation of proteins or basic compounds has been studied.

EXPERIMENTAL

Reagents and Materials

All reagents were Analytical Reagent (AR) grade unless otherwise stated. 3-octadecyl pyrrole monomer was synthesized using previously reported methods (19).

The chemical polymerization reagent employed was 0.2M $FeCl_3$. Chromatographic test compounds were benzene, toluene, dimethylphthalate (DMP), diethylphthalate (DEP), phenol, resorcin, aniline, N,N-dimethylaniline (DMA), caffeine, theophylline, nitrobenzene, acetophenone, benzoic acid, p-toluic acid, cytosine, uracil, procainamide and N-acetylprocainamide.

Protein samples included α-lactalbumin (LA), ovalbumin (OVA), bovine serum albumin (BSA), human serum albumin (HSA), myoglobin (MYO) and transferrin (TRAN). The proteins were dissolved in purified water and 0.05% (W/W) solutions were stored in a refrigerator.

Preparation of Packing Materials

1.0g of 3ODP was added to 20 ml CH_2Cl_2 in a 50ml beaker. Then, 10g of silica (Beckman, 10μm Ultrasphere, 80Å average pore size, 220 m^2/g specific surface area) were mixed with the solution in the beaker. A nitrogen stream was used to evaporate the CH_2Cl_2. 40 ml of 0.2 mol l^{-1} $FeCl_3$ solution was added to the slurry containing the 3ODP coated silica. Oxidation was carried out with vigorous stirring of the solution using nitrogen for 30 minutes. The particles were transferred to a Buchner filter and washed sequentially with purified water, acetone, dichloromethane, acetone and a mixture of water and acetone (50/50). Fine powder was removed by flotation in water. Finally, 5cm and 15cm x 4.6mm I.D. stainless steel columns were slurry-packed using acetonitrile as the solvent at 300 atm and 400atm respectively. A

Beckman ODS column (5μm, 80A pore size, 15cm x 4.6mm I.D) was used for comparative studies.

Chromatography

All isocratic chromatographic experiments on the PODP column were carried out using a Beckman 114M solvent delivery system, an Altex 210 valve (20μl sample loop), a Beckman 165 variable wavelength detector and an ICI DP-600 chart recorder. Gradient chromatographic experiments on the PODP column and stability tests in basic media were performed using a Dionex Series 4000i gradient pump and injector (50 μl), an ICI SD-2100 UV-VIS variable wavelength detector and a Kipp and Zonen BD41 chart recorder.

Eluents used for protein separations were,
(A) 0.1% TFA in water, and,
(B) 0.1% TFA in acetonitrile.
Stability tests in acid media were carried out by pumping 0.1% TFA in 50% water and 50% acetonitrile, flow rate = 1ml/min, through the column at 50°C. The temperature was controlled by a Waters column temperature controller.

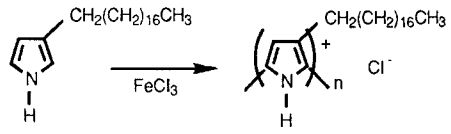
Stability tests in basic media were carried out by pumping 10mM phosphate buffer (pH=9) solution, containing 40% MeOH, at 1ml/min through the column at 80°C. Deterioration of column performance was tested using 20mM phosphate buffers (pH=3 and 7) containing 20% MeOH. All other chromatographic eluents were a mixture of methanol and water. The pH and ionic strength were adjusted using acetate buffers.

RESULTS AND DISCUSSION

Using the method described in the experimental section, poly(3-octadecyl pyrrole) (PODP) was deposited on the silica surface making the particles black in appearance.

Elemental analysis showed that the loadings of C, H, N and Cl were 3.54%, 0.56%, 0.31% and 1.13% by weight.

The presence of Cl indicated that chloride was incorporated as a counterion as follows:



The mole ratio of Cl to N (i.e. n) was approximately 1:4. This is lower than expected and may be due to the presence of $FeCl_4^-$ as a counterion in the polymer material as has been reported previously[18]. The surface area of the coated stationary phase was 172 m^2/g compared to 220m^2/g for the non-coated phase indicating that the polymer coating reduced the silica surface area by about 20%. Presumably some of the pores on the silica are blocked by the polymer coating.

A scanning electron micrograph (SEM) of the coated packing material was obtained (Figure 1). No significant changes were observed after coating even though a black deposit was visually obvious. This indicated that the coating was very thin.

Chromatographic Characterisation

Capacity factors as a function of methanol concentration were determined for 16 test compounds. Results are summarized in Figure 2. A distinct reversed phase interaction was observed with the retention of all species decreasing as the percentage of organic modifier was increased. Non-polar compounds, benzene and toluene have higher capacity factors than those obtained on PP/Cl previously (Table 1) which indicated that the PODP conducting polymer phase undergoes stronger hydrophobic interactions. Phenol and resorcin interacted weakly with the PODP

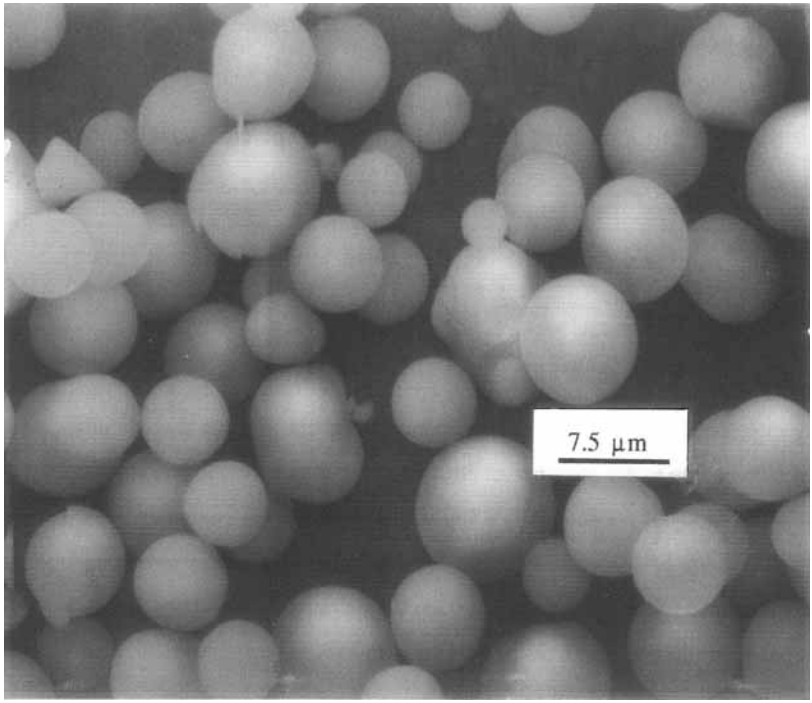


Figure 1 Electron micrograph of PODP coated silica

phase. This confirmed that polypyrrole based polymers do not strongly interact with proton donors as was observed for PP/Cl previously (18). The polar compounds DMP, DEP, nitrobenzene and acetophenone had fairly high retention values. Presumably, this is due to the contribution of the hydrophilic charged polypyrrole backbone. The large difference in capacity factors obtained for aniline and DMA further confirmed that hydrophobic interaction due to $-CH_3$ groups on the DMA molecule was significant. Retention of the basic compounds caffeine and theophylline was not high but benzoic and toluic acids were infinitely retained indicating that although there was an increase in hydrophobic character due to introduction of the alkyl chain to the pyrrole backbone, the anion exchange capability

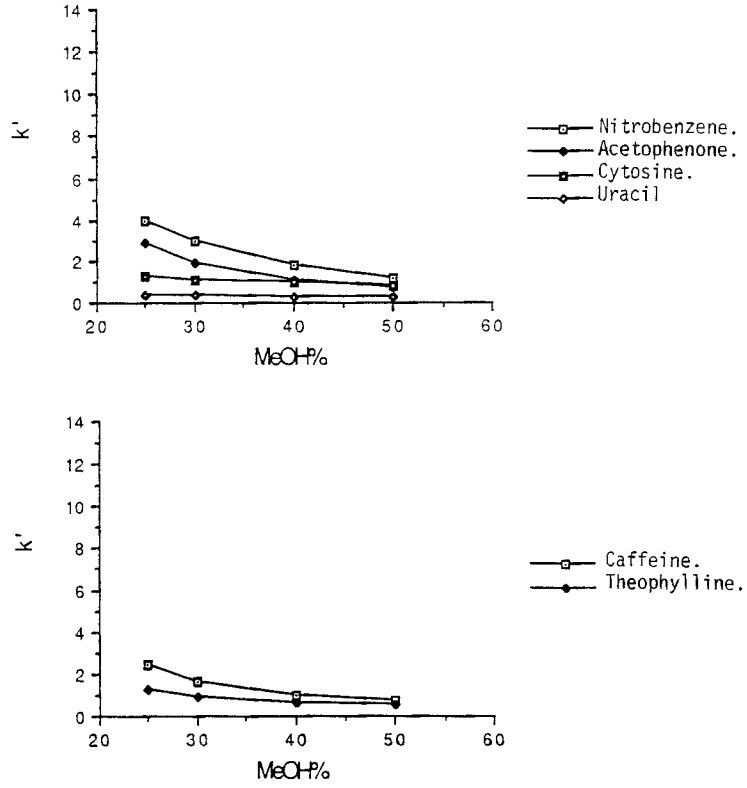


Figure 2 Plots of capacity factors vs methanol concentration in the eluents

Column: 4.6mm I.D.x50mm, 10μm PODP coated silica;
Eluent: Methanol and water (50/50) at 0.5ml/min.
Detector: UV-254 nm.

of the material is retained. Cytosine and uracil which are usually used to test cation exchange capability were weakly retained.

These results confirm that conducting polymer phases such as PODP possess multimodal chromatographic interaction capabilities with both hydrophobic and anion exchange character predominating but some cation exchange character also present.

TABLE 1 - Chromatographic retention characteristics
Comparison of PODP with Polypyrrole (PP)

Test Compound	Retention Index(1) On PP/Cl**	On PODP/Cl
Benzene	1.0	1.0
Toluene	1.6	1.8
DMP	2.0	0.6
DEP	2.1	1.4
Phenol	0.9	0.5
Aniline	8.0	0.5
DMA	22	2.1
Benzoic acid	∞	∞

* Eluent: 50% MeOH and 50% H_2O

** Data from reference (18)

Injection volume = 20µl

(1) Retention Index = $\frac{\text{capacity factor test compound}}{\text{capacity factor benzene}}$

Buffers have often been used to effect the separation of basic compounds. A comparison of the PODP phase with the Beckman ODS phase was made using pH=7 and pH=3 buffers. The retention characteristics on PODP were markedly different from those observed on ODS (Table 2). ODS strongly interacted with non-polar toluene due to the expected reversed phase interaction while PODP did not. The much higher capacity factors observed for phenol, caffeine and N,N-dimethylaniline on ODS compared to PODP may also be due to the same interaction. Under neutral conditions retention of procainamide and N-acetylprocainamide were much higher on the PODP than on the ODS column. This may be due to cation exchange interaction with uncovered SiOH groups on the

TABLE 2 - Capacity factors on the PODP and ODS columns

	PODP		ODS	
Test compounds	pH = 7.0	pH = 3.0	pH = 7.0	pH = 3.0
toluene	7.88	7.12	>100	>100
phenol	1.44	1.17	8.59	7.91
caffeine	2.90	2.16	7.29	7.22
N,N-dimethylaniline	6.64	-*	93.4	-*
procainamide	24.94	1.38	1.78	0.80
N-acetylprocainamide	39.66	1.48	6.56	2.65

* No UV response obtained due to protonation

Column: ODS (5 µm, 80 Å pore size, 15 cm x 4.6 mm ID)

PODP (10 µm, 80 Å pore size, 15 cm x 4.6 mm ID)

Eluent = 20% MeOH, 80% H_2O, 20mM phosphate

Flow rate = 1.0 ml/min

Injection volume = 20µl ODS, 50µl PODP

Concentration of standards:

Phenol	8ppm
Caffeine	2ppm
DMP	2ppm
Toluene	80ppm
Procainamide	10ppm
N-acetylprocainamide	10ppm

PODP column. The greater retention of caffeine than that of phenol on the PODP indicated greater selectivity for basic compounds on the PODP.

Changes in the pH of the eluent had a slight influence on k' for toluene, phenol and caffeine on both phases. However, a significant decrease of k' for procainamide and N-acetylprocainamide was observed when the eluent was changed from 7.0 to 3.0. The capacity factors decreased by a factor of 2 on the ODS phase and a factor of 20 on the PODP phase. In the case of ODS this is probably due to protonation of residual silanol groups, preventing cation exchange interactions occurring. With the PODP phase this may also be the case. However as indicated above the conducting polymer phase is multimodal. This obviously results in more dramatic changes in retention as the result of pH is changed.

A further indication of the ion exchange capacity of the PODP phase were the observations recorded during changes in eluent pH. With the ODS column the effluent pH become constant after passing one column void volume while with the PODP column 10 times the void volume was required. The greater volumes required are a reflection of the ion exchange capabilities of the PODP column.

The column efficiency (N), in plates per metre, for selected test compounds (small molecules) are summarized in Table 3. The higher efficiency observed on the ODS column was at least in part due to the smaller particle size employed.

Following the above experiments with small molecules the chromatographic behaviour of selected proteins was investigated using this new polymer phase. The properties of the proteins used in this work and the corresponding chromatographic data obtained are summarised in Table 4. The retention sequence obtained on PODP was different from that observed on ODS based columns. For example, the retention sequence using PODP was MYO>BSA>OVA while it was found to be OVA>BSA>MYO using other ODS columns (9). The difference is probably due to the

TABLE 3 - Column efficiency(1)

	PODP	ODS
Test compounds		
phenol	9200	36200
caffeine	10000	37500

(1) Experimental conditions as in Table 2. (Eluent pH = 7.0).

N was calculated using = $5.54\ (\frac{t_r}{W_{1/2}})^2$

where - t_r = retention time.

$W_{\frac{1}{2}}$ = peak width at half peak height.

See Experimental section for test conditions.

TABLE 4

Protein	Molecular Weight (X 1000)	pI (Isoelectric Point)	t_R (min)	Recovery (%)
α- Lactalbumin	14	5.1	1.07	92
Ovalbumin	44	4.6	0.84, 1.14, 3.35	23
BSA	66	5.3	4.8	47
HSA	66	4.7	4.5	69
Myoglobin	18	7.0	1.43, 17.3	88
Transferrin	77	5.0-6.0	5.2, 7.5	76

Gradient from 0.1% TFA in H_2O to 0.1% TFA in CH_3CN in 20 minutes at 1.0ml/min.

Sample loading = 50μl of 0.05% protein aqueous solutions

contribution of the hydrophilic pyrrole ring which enhances the protein retention. With most proteins the elution of two or more peaks was observed. The first peak may be the native protein and the second the denatured protein as has been reported by others (4). Recovery, calculated from peak areas with and without the column, was low for most proteins especially for ovalbumin.

Stability Test

Using a thermostatted column (50°C) and an acidic eluent containing 0.1% TFA the stability of the column under acidic conditions was tested using procedures described previously (10-12). The column was found to be stable. Even after passing 15000 column volumes of eluent, the retention of the small molecular probes listed in Table 2 had not changed.

The column resolution also did not change for these small molecules. Using the same conditions, the column was shown to be stable with respect to protein separations (Figure 3). The column performance was again unchanged after passing 15000 column volumes. The lack of stability of conventional carbon bonded silica phases when using strong acids such as TFA is well documented (22, 23). Obviously the pyrrole ring - C_{18} bond is not so susceptible to acid attack as the silane bond used to couple carbon chains to silica.

The stability of the PODP phase and ODS phase in basic media was then investigated using the procedure outlined in the experimental section. At certain intervals the flow of the high pH eluent was stopped and the above mentioned (Experimental) chromatographic eluents with pH = 3 or pH = 7 buffer were employed.

Retention of two test compounds, caffeine and phenol was used to test the column stability in basic media. On the PODP phase it was found that using a pH = 7 buffer

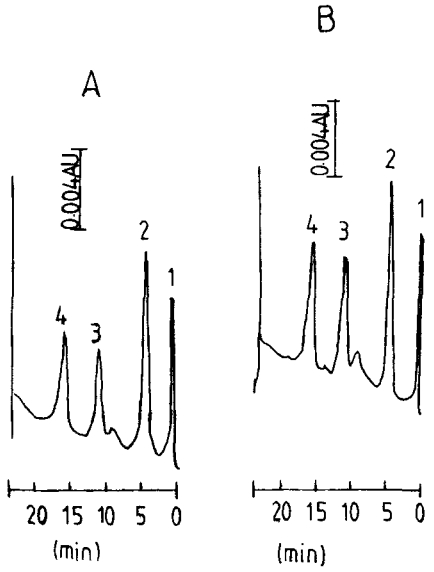


Figure 3 Separation of proteins after passing (A) 1170 and (B) 15300 column volumes stability test eluent.

(1) 25 μg oval bumin, (2) 50 μg BSA, (3 & 4) 25 μg myoglobin.

Column: 4.6 mm I.D x 50 mm, 10 μm PODP coated silica.

Stability test solvent: 50% H_2O and 50% CH_3CN with 0.1% TFA at 50°C at 1.0 ml/min;

Separation Eluent: Gradient from 0.1% TFA in H_2O to 0.1% TFA in CH_3CN in 20 minutes at 1.0 ml/min;

Detection: UV - 280 nm.

in the chromatographic eluent that the capacity factor for caffeine remained relatively constant while that for phenol decreased from 2.8 to approximately 2.2 after 500 hours exposure to the basic pH = 9 eluent. This resulted in a concomittant loss in selectivity over the period of the test. These changes were not apparent with the ODS phase over the same test period. It is possible that exposure to higher pH eluents causes some chemical transfomation in the conducting polymer stationary phase as has been discussed previously (24, 25).

The basic stability test was then repeated using a pH 9 buffer of higher buffering capacity (25 mM phosphate 50 mM borate) containing 40% MeOH. Retention of caffeine on PODP declined during the course of this test (as with the previous test) whereas it remained relatively stable on the ODS column. Column efficiency declined overall for both columns, but the effect was more significant on the ODS column, with approximately 55% loss in efficiency for phenol and caffeine compared with only a 20% loss of efficiency on the PODP. Silica based columns are know to be prone to dissolution at high pH. It appears that the conducting polymer protects against this. However, the exposure to high pH does cause some other chemical transformation to occur that influences chromatographic performance.

CONCLUSIONS

PODP coated silica has been synthesised and characterised using a group of test compounds. The novel properties of this new stationary phase have been revealed. The elution sequence of both small molecules and proteins revealed that the PODP phase has different selectivity to that of conventional ODS stationary phases. The stationary phase was stable under acidic reversed phase conditions but not basic. However the mechanism causing the changes observed at high pH appears to be different from that observed with silica. Separation of proteins was carried out using this stationary phase.

ACKNOWLEDGEMENTS

The financial assistance of the Beckman Corporation via the GIRD Scheme is gratefully acknowledged.

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Received: June 20, 1993
Accepted: October 19, 1993