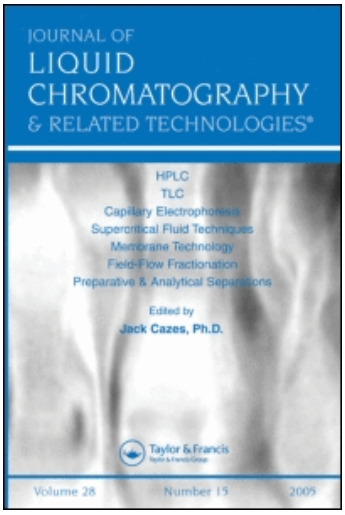

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B. Cañas Montalvo[a]; R. C. Izquierdo-Hornillos[a]
[a] Departamento de Química Analítica, Facultad de Química Universidad Complutense, Madrid, Spain

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COMPARATIVE BEHAVIOUR OF DIFFERENT REVERSED-PHASE PACKINGS WHEN EQUILIBRATED WITH CETYLTRIMETHYLAMMONIUM BROMIDE

B. CAÑAS-MONTALVO AND R. C. IZQUIERDO-HORNILLOS

Departamento de Química Analítica
Facultad de Química
Universidad Complutense
28040 Madrid, Spain

ABSTRACT

The saturation of different reversed-phase HPLC C_{18} packings with the cationic surfactant cetyltrimethylammonium bromide (CTA) and its subsequent elution in net aqueous eluants, has been studied. The distribution of the surfactant between the mobile and stationary phases indicates a mixed retention mechanism. Under conditions of saturation, the total amount of CTA retained in different commercial packings is inversely related to the density of coating (μmoles C_{18}/m^2), and for a given packing depends on the temperature and degree of ageing. When deionised water or a phosphate buffer was passed through the column saturated with CTA, part of it was eluted with the mobile phase, but there was always an amount of CTA that did not elute with aqueous eluants; this uneluted CTA was a constant quantity depending on the eluant, packing characteristics and temperature. When columns were equilibrated with CTA, there was a shift in the eluant pH which was related with the life of these packings working in the presence of CTA.

INTRODUCTION

The importance of silica type[1] and especially the packing surface characteristics, (residual hydroxyl groups, pore-size and surface alkyl concentration), on the chromatographic performance has been reported[2]. Differences in the stability of bonded-silicas, under hard eluent conditions, have been demonstrated for silicas of different procedences[3], and for the same silica using different alkyl substituents[3,4] or different chemistry for graft attachment[3-5].

Columns preequilibrated with CTA have been used in RP-HPLC for the separation and determination of compounds such as pterins[6], benzoates, phenylacetates[7], and inorganic anions[8]. Moreover, CTA has been used to dinamically modify bare silica gel columns to get reverse phase separations[9]. With aqueous eluants, the retention of CTA onto unmodified silica surfaces is mainly due to an ion exchange mechanism[9]. Ammonium quaternary salts have been used to block the effect of residual silanol that causes mixed-mode retention in reverse phase chromatography[10]. CTA itself is retained by both ion-exchange and hydrophobic interactions in RP-HPLC stationary phases[11,12]. The relative importance of both retention mechanisms has been discussed previously[13].

When reverse phase columns are equilibrated with ionic surfactants, the bound C_{18} chains are rearranged on

the surface of packings[14,15], with the consequent variation on the retention of analites, including neutral compounds[14]. Also, during CTA adsorption, the occurrence of pH-shifts in the eluant have been observed[9,11,16].

We present data on the adsorption of CTA by different commercial RP-HPLC packings, and a qualitative comparison of the stability of these packings when working in the presence of CTA.

MATERIALS AND METHODS

Reagents

Xanthopterin (X), isoxanthopterin (I), pterin (P), biopterin (B) and neopterin (N), were purchased from Sigma (St. Louis, MO, USA), and were of chromatographic grade. CTA was obtained from Serva (Heidelberg, Germany).

Sodium dihydrogenphosphate and disodium hydrogenphosphate, analytical-reagent grade, from Merck (Darmstadt, Germany). HPLC-grade methanol was purchased from Promochem (Wesel, Germany). Water was purified with a Milli-Q system (Millipore, Molsheim, France). Before use, all eluants were degassed under vacuum and filtered with an all glass apparatus through 0.45-μm filters (Millipore, Bedford, MA, USA). Other chemicals were of analytical reagent grade.

Apparatus

A Waters (Milford, USA) liquid chromatograph, consisting of a 590 model solvent-delivery pump, a 481 model UV variable-wavelength detector, a 420 model fluorescence detector and a 730 model data module, was used. The injector, with a 10 μl loop was from Rheodyne (Cotati, CA, USA). A Selecta (Barcelona, Spain) thermostated bath was used to control the column temperature. For pH measurements, a Crison (Barcelona, Spain) digital pH-meter with an Ingold 10-402-253 glass combined electrode was used.

Sep-Pak C_{18} cartridges were obtained from Waters. Nucleosil-CN 10μm (250x4 mm) was from Knauer (Berlin, Germany), μBondapack 10μm (300x4 mm) from Waters, Ultracarb 5μm (250x4.6 mm) from Phenomenex (Torrence,CA,USA), Nucleosil-120 5μm (120x4 mm) from Knauer, Hypersil 5μm (100x4 mm), Nucleosil-100 5μm (150x4 mm) and a Spherisorb-ODS2 10μm (300x4 mm) were packed by Technokroma (Barcelona, Spain). Other technical characteristics from these columns are summarized in Table I.

Procedures

To saturate the columns with CTA [the term saturation is used to specify the CTA adsorpted on the

TABLE I.

Technical Characteristics of the C_{18} Packings Used.

	A	B	C	D	E	F
Total volume, ml	4.15	3.58	3.58	1.88	1.51	1.26
Dead volume, ml	3.00	2.72	2.10	1.38	1.05	1.26
Porosity	0.72	0.76	0.58	0.73	0.70	0.64
Mass (g)	2.53	1.89	3.26	1.10	1.01	0.98
Particle size, μm	5*	10**	10*	5*	5*	5*
% Carbon	22	10	12	14	11	8.8
Surface area (m^2/g)	370	350	220	350	220	200
Coating, μ-mol C_{18}/m^2	4.05	1.55	3.26	2.33	3.03	2.70
Coating, μ-mol C_{18}/g	1567	542	673	815	606	467

A: Ultracarb ODS; **B:** μ-Bondapak C_{18}; **C:** Spherisorb ODS-2; **D:** Nucleosil 100-C_{18}; **E:** Nucleosil 120-C_{18}; **F:** Hypersil C_{18}.
* Spheric. ** Irregular.

stationary phase when it is in equilibrium with the CTA in the mobile phase at the critical micellar concentration (CMC)], a 5 mM aqueous solution of this compound was passed through them at a flow rate of 1.5 ml/min, until CTA was present in the efluent. The amount of CTA retained was called CTA-T (i.e. the maximum quantity of CTA that can be retained by a column at a given temperature). Then 200-300 ml of water or 1.5 mM phosphate buffer (pH 6.5) without CTA were passed through the columns at the same flow-rate. After this aqueous washing there was some uneluted CTA that was called CTA-P

(the amount that remains in the column when there is no CTA in the mobile phase).

CTA was determined chromatographically with UV indirect photometric detection. For this procedure[17] a Nucleosil-CN column thermostated at 20°C was used; the mobile phase was (55:45) methanol:water containing 5 mM p-toluensulphonic acid at a flow-rate of 1 ml/min. The UV detector was set at 250 nm. To determine CTA at concentrations below 0.1 mM, the solution was concentrated using a C_{18} Sep-pak cartridge. The diluted aqueous solution, up to 450 ml, was passed through the cartridge; CTA was eluted with 5 ml of (90:10) methanol:1.2 M HCl.

The distribution isotherm of CTA between the mobile and stationary phases was studied at 22°C with a Hypersyl column using different aqueous solutions of CTA ranging from 0.1 to 1.5 mM. When the CTA concentration was the same in the mobile phase and in the efluent, the retained CTA was calculated. The amount of CTA retained was obtained by measuring the difference between the total CTA passed through the column and the CTA in the efluent.

Pterins were separated using a Hypersil C_{18} column, pre-equilibrated with 50 mg of CTA; 1.5 mM phosphate buffer (pH 6.5) was used as mobile phase at a flow rate of 1.5 ml/min. Fluorescence detection was used (excitation filter: 365nm; emision filter: band pass 420 nm).

RESULTS AND DISCUSSION

Saturation of C_{18} packings

The distribution isotherm (Fig.1) between mobile and stationary phases reached saturation at 0.8-0.9 mM CTA, which is the CMC of CTA. This has been described before[13,18]. Table II shows the amount of CTA retained at different temperatures for a new and old Hypersil 5-ODS column, depends on temperature and degree of ageing of the column. It is observed that for both, new and old packing, the retention of CTA changed very little with temperature variations around 20°C (room temperature). The new packing retained at 18°C an amount close to 30 % higher than the old packing. Since there was no void volume in this Hypersil column, the decrease in CTA retention by the old column must be due to a decrease in C_{18} coverage. There were important variations in the amount of CTA retained when the temperature was lowered to $14^{o}C$.

The amounts of CTA-T retained by the packings tested were very different (Table III) and were mainly related to the specific C_{18} coating of packings. From the comparison between CTA-T, in $\mu mol/m^2$, and the specific C_{18} coating of the packing, also expressed as $\mu molC_{18}/m_2$, the total CTA retention can be explained by taking into

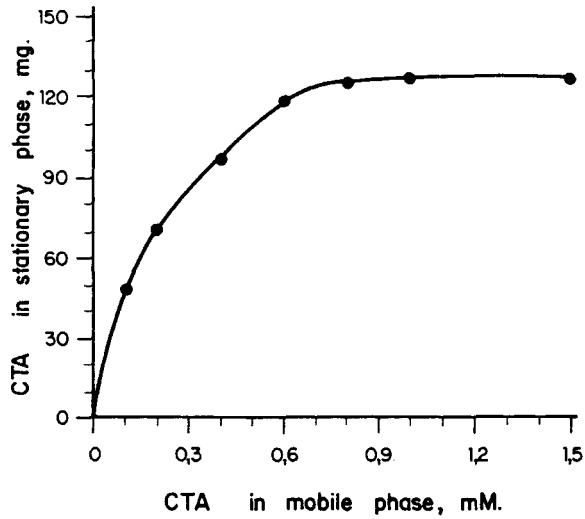

FIGURE 1. Distribution isotherm of CTA at 22°C using a Hypersil column and different aqueous solutions of CTA.

TABLE II

Influence of the Temperature and Degree of Ageing on the Retention of CTA on Reversed-Phase Hypersil 5-ODS (100 mm x 4 mm i.d.) Packing.

	T,°C	CTA, mg
New column	14	170
	20	127
	22	125
Old column	10	168
	17	109
	28	102

TABLE III

CTA Retained by Different Reversed-phase Packings Under Saturation (CTA-T) and After Equilibration with 1.5 mM Phosphate (pH 6.5) (CTA-P).

CTA-T	mg	μmol	$\mu mol \cdot m^{-2}$	$\mu mol \cdot g^{-1}$
Ultracarb	545	1487	1.59	587
μ-Bondapak	432	1185	2.09	626
Spherisorb	377	1034	1.44	317
Nucleosil-100				
- New	291	798	2.07	725
- Aged	256	702	1.82	638
Nucleosil-120	151	414	1.86	410
Hypersil ODS	127	348	1.76	355

CTA-P	mg	μmol	$\mu mol \cdot m^{-2}$	$\mu mol \cdot g^{-1}$
Ultracarb	360	960	1.02	379
μ-Bondapak	168	460	0.69	243
Spherisorb	250	658	0.95	210
Nucleosil-100				
- New	180	494	1.28	449
- Aged	135	369	0.96	335
Nucleosil-120	55	150	0.68	148
Hypersil ODS	50	137	0.69	140

account steric effects, i.e. Ultracarb, with higher C_{18} coverage, retained lower CTA-T/m^2 of packing. However, the μ-Bondapack packing, which has lower density of coverage, retained higher CTA-T/m^2 . These results were in agreement with other studies about the steric effects that limit the maximun C_{18} coating for a given packing[19].

The elution of CTA from C_{18} packings

Once the different packings were saturated with CTA, a mobile phase without CTA was passed through the column,

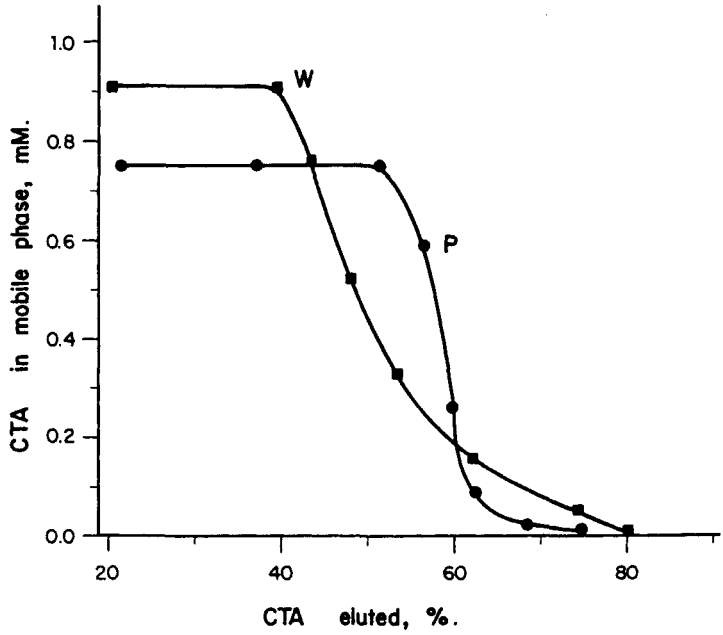

FIGURE 2. Dynamics of CTA elution from a Hypersil column, when CTA was suppresed from the mobile phase. W: Deionised water and P: 1.5 mM phosphate buffer (pH 6.5) mobile phases.

and the elution of CTA was studied. Two mobile phases were used: deionised water and a 1.5 mM phosphate (pH 6.5) buffer. The results are shown in Fig. 2 for a Hypersil column and a similar pattern was observed for the other columns studied. In these curves it is possible to distinguish three intervals. In the first one the CTA concentration that eluted from the column remained constant and equal to the CMC of CTA in these eluents. This indicates that the aqueous mobile phase is able to remove CTA from the stationary phase, but without

exceeding the CMC. In other words, the association of CTA with the stationary phase is more stable than the intermolecular association of CTA to form micelles.

In the second interval the slope is higher with the phosphate buffer than with deionised water as mobile phase. This result could be expected, since CTA is more soluble in water than in this buffer. This different solubility can be used to manipulate the selectivity of the column: small amounts of CTA can be eluted from the column with water until the desired separation is obtained with the buffer.

In the third interval the elution of CTA from the column with buffer was negligible and the CTA amount remaining in the stationary phase was constant. So, a reverse phase columnn, that also works as an anion exchanger, without adding CTA to the mobile phase, is obtained. When this equilibrium was reached, large volumes of mobile phase (2-3 liters) could be passed through the column and the retention time of the analites remained constant.

The amount of CTA retained by the stationary phase without eluting was called CTA-P (phosphate buffer as mobile phase) and was characteristic for each packing (Table III). CTA-P was related to the packing's superficial C_{18} coating, i.e., the more coated packing (Ultracarb), retained higher amount of CTA, and the lower

value of CTA-P corresponded with the less coated packing (μBondapak) (Table III). However, other factors must contribute because there is not a good correlation between these two magnitudes. In three of the columns used, CTA-P is one third of CTA-T, while in the others it is about two thirds. A different organization of the C_{18} chains must be responsible of these differences.

pH effects during the saturation and equilibration of the packings

When the C_{18} packings were saturated with 5 mM CTA in water, the pH of the eluent was measured. During the saturation process there was a decrease in the pH of the efluent. There was a displacement of hydronium ions from the acidic silanols of the silica matrix by CTA, with a consequent acidification of the mobile phase. So, it is possible to understand why, using these columns with CTA, it was necessary to wait for a long time to obtain reproducible retention times for ionic compounds. A mixture of pterins was used to illustrate this phenomena. Fig.3 shows the variation of the retention times of these compounds during the equilibration of the column with phosphate buffer. For all pterins, after passing 200 ml of phosphate buffer, the retention times and peak heights were stabilized. Xanthopterin(X) and Isoxanthopterin(I),

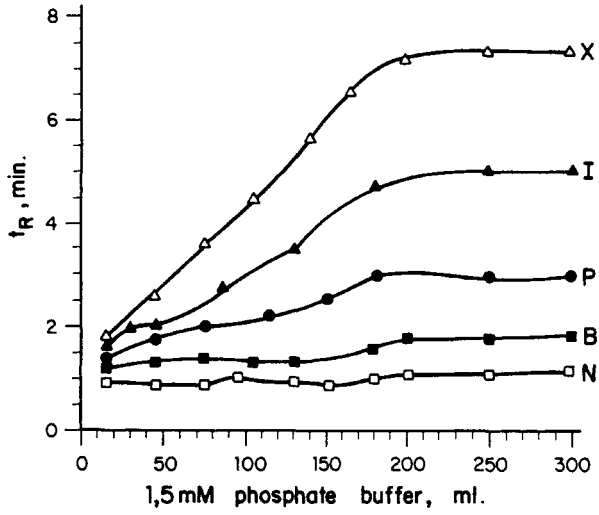

FIGURE 3. Variation of the t_r of pterins during the Hypersil column equilibration with 1.5 mM phosphate buffer (pH 6.5), when the column was previously equilibrated with 50 mg of CTA.

with lower pK values than the other pterins, were the most affected compounds by this equilibration process. That means that the real pH of the separation during the equilibration process was lower than the pH of the mobile phase. Using different flow rates it was observed that this process was not affected by kinetic parameters, but only by the total volume of eluant passing through the column.

Table IV shows the hydronium ions liberated, by the different columns used, during their saturation with CTA. These values were different and depend on the packing used. It was possible to observe:

TABLE IV.
Hydronium Interchange During the Saturation of Different C_{18} Packings with CTA, Using an Aqueous Solution.

Packing	Surface area $m^2.g^{-1}$	H^+ μmol	H^+ $\mu mol \cdot m^{-2}$	H^+ $\mu mol \cdot cm^{-3}$	H^+ $\mu mol \cdot g^{-1}$
Ultracarb	370	100	0.107	33.3	39.5
μ-Bondapack	350	80	0.121	29.4	42.3
Spherisorb	220	43	0.060	20.5	13.2
Nucleosil-100	350	33	0.086	23.9	30.0
Nucleosil-120	220	11	0.050	10.5	10.9
Hypersil	200	4	0.020	4.9	4.1

i) the packings with higher specific surface area (m^2/g) exchanged more hydronium ions per volume that the packings with lower specific surface-area; so, the packings with lower specific area needed less volume of eluant for their equilibration.

ii) When the hydronium ions liberation was related to the surface area of the packing, the differences between packings still remained, and those with higher specific surface area liberated more hydronium ions per m^2 than the packings with less specific surface area.

The stability and chomatographic performance of reverse-phase packings vary depending on the surface population of acidic and hydrogen bond associated silanols groups[20,21]. Acidic silanols have been described to cause peak tailing for basic compounds[20]. Moreover, silicas with an homogeneous distribution of associated

silanols have better stability and performances[21]. According to the results herein presented, packings with lower surface area seemed to have a better distribution of silanol groups than those with higher surface area, since they exchanged less quantity of hydronium ions when they were equilibrated with CTA. This can be due to accesibility problems: packings with a high specific surface area have a small pore-size and, due to steric effects, the C_{18} coating reached in the silanization process may be less homogeneous than the coating in higher pore-size materials. This kind of coating may provide a higher amount of isolated silanol groups and, as a consequence, the modified silica has higher acidity.

Duration of the packings used with CTA

The special aggressivity of cationic surfactants for silica packings has been described[10,22]. This behaviour was corroborated herein. Table V shows the qualitative results about the relative deterioration of packings which were conditioned with CTA. There was a group of packings which suffered a ready deterioration because silica was dissolved with formation of a void volume; its uses with CTA are restricted. The second group presents a decrease in the capacity factor (k') during the early uses with CTA. After that, the capacity factor values

TABLE V.
Estimation of the Duration of Different C_{18} Packings when Used with CTA.

Packing	Surface area $m^2.g^{-1}$	H^+ $\mu mol \cdot cm^{-3}$	Durability
Ultracarb	370	33.3	short (void volume)
μBondapack	350	29.4	short (void volume)
Spherisorb	220	20.5	long
Nucleosil-100	350	23.9	medium (k'decrease)
Nucleosil-120	220	10.5	long
Hypersil	200	4.9	long

were stabilized and there was no variation with time. The third group presents better stability: there were no void volumes and the capacity factor values were constant after iterative processes using CTA and only after a long time did the capacity factor values decreased.

With the packings tested, there is a correlation between stability and specific surface-area. When they are equilibrated with CTA, those with less specific surface area used to be the more stable. This relation between pore-size and stability has been described before[23]. That implies that the higher production of hydronium ions during the equilibration with CTA, may be responsible of the lower stability of the high specific surface area packings.

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