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SEPARATION OF HYDROPHILIC THIOLS USING REVERSED-PHASE CHROMATOGRAPHY WITH TRIHALOACETATE BUFFERS

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SUMMARY

The reversed-phase retention behavior of several neutral and cationic hydrophilic thiols using trihaloacetic acid pairing agents is studied. Retention of cationic compounds increases with the size of the halogen substituent in the order: trifluoro- < trichloro- < tribromoacetic acid. The effect of pH, ionic strength, pairing ion and counter ion concentration on retention of cysteine and other thiols is measured. The formation of mobile phase ionic interactions is proposed as the mechanism of retention enhancement.

INTRODUCTION

Hydrophilic thiols of low molecular weight are involved in a variety of physiological and environmental processes. Clinical applications include heavy metal detoxification[1], the treatment of rheumatoid arthritis[2], and Wilson's disease[3]. Thiols are also important in the transport and bioavailability of metals in aquatic ecosystems[4]. Because of the significance of hydrophilic thiols, methods for their selective determination are needed. Compounds of interest in our work on marine sediment porewater are listed in Table I. Liquid chromatography (LC) is a technique well suited to the task of determining these water-soluble compounds in complex matrices.

Retention and separation of multicomponent mixtures of these thiols by LC are strongly influenced by their hydrophilic and ionic properties. For example, cysteine may be anionic, zwitterionic, or cationic depending on mobile phase pH; whereas methanethiol is neutral over most of the pH range (below pH 10). Various approaches have been used for the separation of mixtures of some of these compounds. The conjugate anions have been separated on a strong anion-exchange column[8], but with relatively low efficiency. Cation exchange has been used at low pH for the determination of cysteine, glutathione and penicillamine[9], but the neutral thiols were not well retained. Reversed-phase columns may be used directly, however, the more hydro-

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TABLE I

pK_a VALUES OF THIOLS IN THIS STUDY

Analyte	*Structure*	*Speciation category*	*pK_a value(s)*[5–7]			
			pK_1	*pK_2*	*pK_3*	*pK_4*
Methanethiol	CH_3-SH	Type A	10.33			
Ethanethiol	CH_3CH_2-SH	Type A	10.61			
1-Propanethiol	$CH_3CH_2CH_2-SH$	Type A	–			
2-Propanethiol	$CH_3CH(SH)CH_3$	Type A	10.06			
2-Mercaptoethanol	$CH_2(OH)CH_2-SH$	Type A	9.4			
1-Mercaptoglycerol	$CH_2(OH)CH(OH)CH_2-SH$	Type A	9.28			
2-Mercaptopropanoic acid	$CH_3CH(SH)-COOH$	Type B	3.5	10.1		
3-Mercaptopropanoic acid	$CH_2(SH)CH_2-COOH$	Type B	4.27	10.54		
Mercaptopyruvic acid	$HS-CH_2C(=O)-COOH$	Type B	(2.49)*	–		
N-Acetylcysteine	$HS-CH_2CH(HN-C(=O)-CH_3)-COOH$	Type B	3.2	9.7		
Cysteine	$HS-CH_2CH(NH_2)-COOH$	Type C	1.9	8.15	10.3	
Penicillamine	$(CH_3)_2C(SH)-CH(NH_2)-COOH$	Type C	1.9	7.9	10.6	
Glutathione	$HOOC-CH(NH)CH_2-C(=O)-NHCH(CH_2SH)-C(=O)-NHCH_2COOH$	Type D	2.1	3.5	8.7	9.5

* Pyruvic acid.

philic compounds (*e.g.* cysteine) show relatively little retention. Alkyl sulfate/sulfonate pairing agents have been used to increase the reversed-phase retention of three cationic thiols at pH 3[9]. However, the use of these long alkyl chain pairing agents requires long mobile/stationary phase equilibration time[10,11] and often results in nearly irreversible adsorption of the agent to the chromatographic column[11–13]. In addition, the retention of neutral compounds is decreased by the adsorption of the pairing agent to the stationary phase[10,11,14], which may complicate optimization of the separation of mixtures of ionic and neutral compounds.

Another approach to LC thiol determination is to derivatise the thiol moiety with orthophthalaldehyde (OPA) in the presence of excess amine to yield a fluorescent product that is easily separated by reversed-phase LC[15]. However, these OPA adducts exhibit poor stability and interferences may occur in samples that contain both primary amines and thiols, because OPA reacts with both moieties. Furthermore, cysteine and glutathione may undergo cyclization[16] in the OPA reaction and may not be determined by this approach. Because of these drawbacks, other LC approaches to the determination of thiols are needed.

In our study of the use of electrochemical detection for the LC determination of thiols[17], we observed increased retention of the cationic compounds on a reversed-phase column with the use of trihaloacetate (THA) buffers compared to inorganic

buffers such as phosphate. Retention also increased with increasing concentration and size of the THA halogen (Br > Cl > F). Other investigators have also noted buffer effects on the LC retention of ionic compounds[18–20]. In particular, the reversed-phase retention of cationic catecholamines was increased when a trichloroacetic acid buffer (TCA) was used[18]. Increases in retention similar to that obtained with octylsulfate were found with 0.1 mol/l TCA for these compounds. The authors suggested that increased retention of the cations resulted from the formation of ion pairs with the trichloroacetate anion.

In this paper, we describe a detailed examination of the reversed-phase retention of cationic and neutral thiols using trifluoro-, trichloro- and tribromoacetate buffers (TFA, TCA, and TBA respectively). The chromatographic variables that were investigated include pH, ionic strength, stationary phase material, counter cation size, and the concentration and size of the THA anion. A scheme for the isocratic separation of 12 low-molecular-weight, hydrophilic thiols is presented.

EXPERIMENTAL*

Chromatography

A liquid chromatograph consisting of two reciprocating dual-piston pumps with gradient controller was used to mix various mobile phase compositions during the isocratic separations (1 ml/min flow-rate). For all studies, 1% methanol was added to the mobile phase so that a catalytic oxygen-scrubber could be used to remove oxygen[21] which otherwise may oxidize the thiols[17]. The mobile phase was also sparged with nitrogen for 30 min and blanketed with helium to reduce the level of dissolved oxygen. Three commercial, octadecylsilyl-modified silica (C_{18}) columns with 5-μm particle size and 250 × 4.6 mm I.D. bed dimensions were used: Zorbax ODS (DuPont, Wilmington, DE, U.S.A.) and Vydac 201HS and Vydac 201TP (The Separations Group, Hesperia, CA, U.S.A.). Thiols were detected using a thin-layer electrochemical cell and is detailed elsewhere[17]. Briefly the detection conditions were: 1.0 mm gold–mercury thin-film working electrode; +200 mV applied potential (*vs.* Ag/AgCl, 3 mol/l KCl); 2-s time constant.

Reagents

Mobile phases were prepared with Burdick and Jackson Labs. distilled-in-glass grade methanol (Muskegon, MI, U.S.A.) and distilled water that was further purified with an ion-exchange/carbon adsorption system (Milli-Q, Millipore, Bedford, MA, U.S.A.). Buffers were prepared by addition of the conjugate acid followed by base titration to obtain the desired pH (measured *versus* standard buffers with a glass electrode). The acids/buffers included: phosphoric and perchloric acids (reagent grade, Mallinckrodt, Paris, KY, U.S.A.), monochloroacetic and monochloropropanoic acids (Alfa Products, Danvers, MA, U.S.A.), trifluoroacetic acid (Sequinal quality, Pierce, Rockford, IL, U.S.A.), trichloroacetic acid (ACS certified grade, Fisher

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* Certain commercial equipment, instruments , or materials are identified in this report to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

Scientific, Springfield, NJ, U.S.A.) and were used without further purification. Tribromoacetic acid (purum grade, Fluka, Ronkonkoma, NY, U.S.A.) was recrystallized from benzene before use and was limited to 0.05 mol/l concentration by its aqueous solubility. Buffers were prepared with bases of various counter cation size: lithium and ammonium hydroxides (Suprapur, Merck, Darmstadt, F.R.G.) and sodium and potassium hydroxides (analytical reagent, Mallinckrodt, Paris, KY, U.S.A.). The buffers served not only to control the pH of the mobile phase but also acted as supporting electrolyte for the electrochemical detector.

Thiols used in this study included cysteine, glutathione, *N*-acetylcysteine, mercaptoethanol, 2- and 3-mercaptopropanoic acid, 1-mercaptoglycerol and penicillamine (Sigma, St. Louis, MO, U.S.A.), methanethiol (Aldrich, Milwaukee, WI, U.S.A.), and mercaptopyruvate, 1- and 2-propanethiol, and ethanethiol (Fluka) and were stored under nitrogen at 4°C. Aqueous thiol stock solutions were prepared in a nitrogen-filled glove box using deoxygenated (1 h nitrogen sparge) mobile phase, standardized by iodometric titration[22], and stored with refrigeration ($\approx$ 4°C) under a nitrogen atmosphere. It was necessary to prepare dilute standard solutions ($\approx$ μmol/l) daily[17].

RESULTS AND DISCUSSION

The retention of ionic compounds in reversed-phase systems may be enhanced by the addition of oppositely charged, hydrophobic pairing ions to the mobile phase, often termed "ion-pair" chromatography. Three basic mechanisms have been proposed to model the observed increase in retention: (1) an ion pair forms in the mobile phase, increasing the lipophilicity of the ion followed by partitioning into the stationary phase[23], (2) the stationary phase is modified by the adsorbed pairing agent to form a "dynamic ion exchanger"[10,24] and (3) modification of the stationary–mobile phase interface with non-stoichiometric interaction of the pairing ion and analyte[25,26]. It is often very difficult to prove the dominance of one of the three mechanisms for any given separation system. As noted by Knox and Hartwick[10], the chromatographic retention factor k' is a thermodynamically derived quantity, and its measurement does not *directly* shed any light on the kinetics or mechanism of the retention process. In this work, the increased retention of cationic thiols by the use of trihaloacetate buffers as pairing agents was investigated.

Effect of chromatographic variables on the retention of thiols

Strategies for the separation of biogenic thiols must take the proton equilibria of the compounds into account. Fig. 1 illustrates the four classes of equilibria exhibited by the thiols used for this study, containing combinations of carboxylate ($pK_a \approx 2$–4), amine ($pK_a \approx 8$–9), as well as the thiol ($pK_a \approx 9$–11) functionalities. Note that at the lowest pH normally used for silica-based column materials, pH = 2, type A and B are neutral, and type C and D are about half zwitterionic and half cationic. We will refer to these latter types as "net-cationic", since they show retention behavior expected for cationic species in the presence of oppositely charged pairing agent, *vide infra*.

We initially tested the addition of four commonly-used pairing agents for the separation of cations: pentane- and heptanesulfonate, as well as octane- and dodeca-

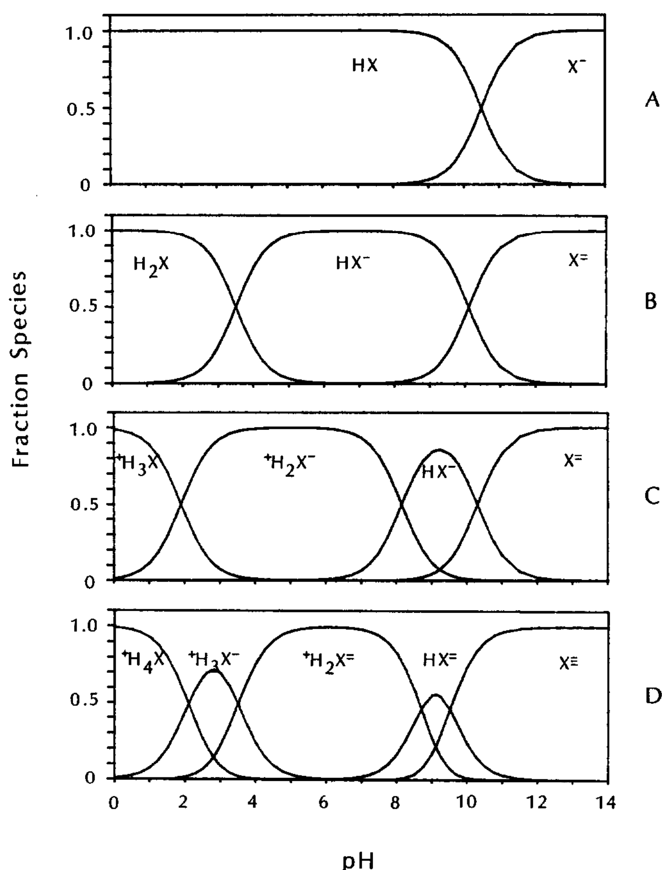

Fig. 1. Protic equilibria of the four classes of thiols from Table I.

nesulfate all in 0.1 mol/l monochloroacetate buffer with a pH of 3. Although the retention of the net-cationic thiols was increased by the addition of these reagents, the retention of the neutral components was decreased. This effect has been observed by others[10]. For example in our system, k' for cysteine was increased from 0.1 to 0.9 by the addition of 1 mmol/l dodecanesulfate at pH 3; whereas, the retention of neutral 3-mercaptopropanoic acid was decreased from a k' of 5 to 2.1. This made the simultaneous multicomponent separation of the net-cationic thiols from the hydrophilic neutral thiols more difficult. Employing alkyl sulfate/sulfonate pairing agents resulted in co-elution of thiols at low k' values.

We also examined the reversed-phase retention of the thiols as a function of mobile phase pH and buffer-type without the addition of alkyl sulfate/sulfonate pairing agents. The acid form of the buffers are listed in Table II and were neutralized with lithium hydroxide to achieve the desired pH. We found that the reversed-phase retention of cysteine was higher at pH 2 than at pH 3 for a given buffer, as shown in Table III. According to Fig. 1, cysteine (type C) is net-cationic at pH 2 and primarily zwitterionic at pH 3. The anionic salt of the buffer apparently acts as a pairing agent

TABLE II

pK_a VALUES OF BUFFERS EMPLOYED[6,27]

Acid	*pK_a value*
Acetic	4.76
Monochloroacetic (MCA)	2.86
2-Chloropropanoic (MPA)	2.88
Trifluoroacetic (TFA)	0.23
Trichloroacetic (TCA)	0.63
Tribromoacetic (TBA)	0.66

with the net-cationic cysteine (pH 2), thus increasing hydrophobicity and retention over the zwitter-ionic form (pH 3). The buffer anions used here only enhance the retention of the net-cationic form, not the zwitterion form.

At a mobile phase pH of 3, there was no difference in the retention of cysteine when different buffers were used, but retention was influenced by the nature of the buffer at pH 2. These results are shown in Table III. Given the pK_a values for MCA and MCP (Table II), these buffers will be largely protonated at pH 2. Thus it is surprising to see the retention of cysteine increase significantly from pH 3 to pH 2 for these buffers. The protonation of the thiol to the net-cationic form appears to be much more important than deprotonation of the pairing agent in enhancing reversed-phase retention. Although the hydrophobicity of the pairing agent should increase by using MCP instead of MCA, substituting a single methyl group on the monochloroacetic acid resulted in only a modest increase in the retention of the net-cationic cysteine. This contrasts results obtained with 4–10 carbon alkyl sulfates, where increasing the carbon length of the alkyl chain substantially increases cation retention[23]. An additional disparity between these two types of pairing agents is that alkyl sulfonates/sulfates were found to enhance the retention of the zwitterion form of cysteine as well. This has also been observed by others for cysteine[9] and longer chain peptides[10].

The higher retention of the net-cationic cysteine with the TCA buffer prompted further investigation of the pairing agent properties of this buffer anion. The effect of the mobile phase pH on the retention of 4 thiols (cysteine, glutathione, 2-mercaptopropanoic acid and methanethiol) over the range of 1.25 to 5.0 using the trihaloacetate TCA, is shown in Fig. 2. The maximum k' value was achieved at pH values near 1.8, although in solution, half of cysteine and glutathione are still in the zwitterion

TABLE III

CYSTEINE RETENTION WITH DIFFERENT BUFFERS

Mobile phase pH	*k' for cysteine with acid (0.05 mol/l)*			
	Unbuffered $HClO_4$	*MCA*	*MCP*	*TCA*
3	0.14	0.13	0.12	0.12
2	0.36	0.39	0.44	0.71

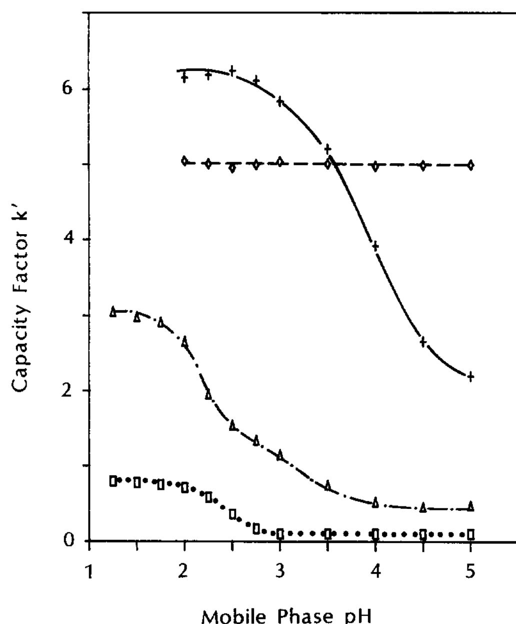

Fig. 2. Effect of pH on the reversed-phase retention of four thiols. Compounds: (□) cysteine, (+) 2-mercaptopropanoic acid, (◇) methanethiol, (△) glutathione. Mobile phase: 0.05 mol/l TCA buffer.

form. However, since the additional LC partition equilibria favors the more neutral form, the effective "chromatographic pK_a" (pK_a') may be shifted substantially from the solution value[24,28]. These pK_a' values were higher than the solution pK_a (at μ = 0.1) values for cysteine (2.4 *vs.* 1.9) and 2-mercaptopropanoic acid (3.9 *vs.* 3.5) but were similar for glutathione (2.1 for both). In the case of glutathione the retention behavior and hence the pK_a' measurement is complicated by a second proton equilibrium with pK_a = 3.5. As expected, methanethiol, which is neutral over this pH range shows no dependance of retention on mobile phase pH.

We also compared three commercially available C_{18} columns for their retention of the net-cation cysteine with the pH 2 TCA mobile phase, the results are listed in Table IV. Although the Zorbax ODS column did not have the largest silica surface area, the high % C loading produced the highest k' values for cysteine. Since reten-

TABLE IV

REVERSED-PHASE C_{18} COLUMNS TESTED FOR CYSTEINE RETENTION

All data are from manufacturer's specifications.

Column	*% C*	*Pore size (Å)*	*End capped*	*Surface area (m^2/g)*	*Particle size (μm)*	*k'* *k'*
Vydac TP	9	300	No	100	5	0.36
Vydac HS	13	80	Yes	500	5	0.42
Zorbax ODS	16	60	No	330	5–6	1.09

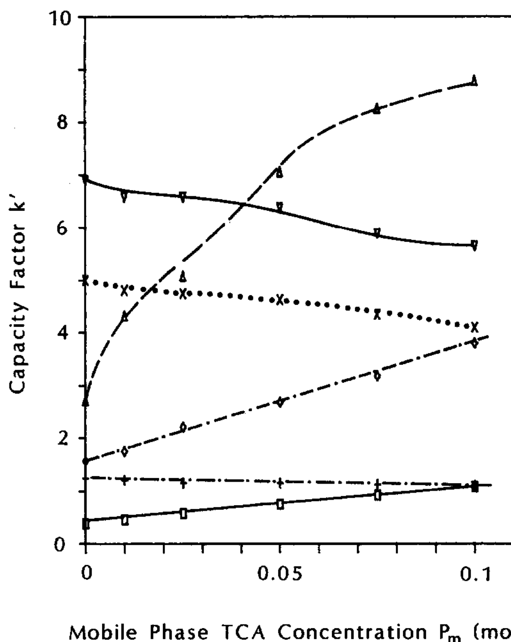

Fig. 3. Effect of P_m of TCA on retention of thiols at constant pH 2 buffer concentration. Compounds: (□) cysteine, (+) 1-mercaptoglycerol, (◇) glutathione, (△) penicillamine, (×) 3-mercaptopropanoic acid, (▽) 2-mercaptopropanoic acid.

tion of this compound was essential to our work, all further studies were performed on the Zorbax ODS column.

To investigate further the possibility of buffer ion–analyte interactions, retention of the thiols was measured as a function of TCA mobile phase concentration (P_m) at a constant pH of 2.0. To minimize the effect of ionic strength, test amounts of TCA were substituted for equal amounts of perchlorate. Fig. 3 shows the change in k' with the mobile phase concentration of pairing agent. A marked increased retention is observed for the net-cationic analytes (cysteine, glutathione, penicillamine) with increasing P_m, with a slight decrease in retention observed for the thiols that are neutral at this pH (1-mercaptoglycerol, 2- and 3-mercaptopropanoic acid). Since the pH and ionic strength remain constant in this experiment, the increased retention of the net-cations with P_m must be a result of solvophobic ion interactions between the TCA anions and the analytes. The slightly decreased retention of the neutral compounds with increasing P_m must be primarily a result of displacement by the increased adsorbed pairing agent on the stationary phase[10,11] or by lowering of the surface tension of the stationary phase by the adsorbed surfactant[14,29]. However, the slight decrease in retention for polar neutral compounds using TCA is much less than that observed for alkyl sulfates/sulfonates[10] and is consistent with the low surface coverages found for the THA in these experiments (*vide infra*).

The effect of increasing ionic strength at constant P_m using TCA on the retention of 3 net-cationic and 1 neutral thiol is presented in Fig. 4. For the net-cations, the

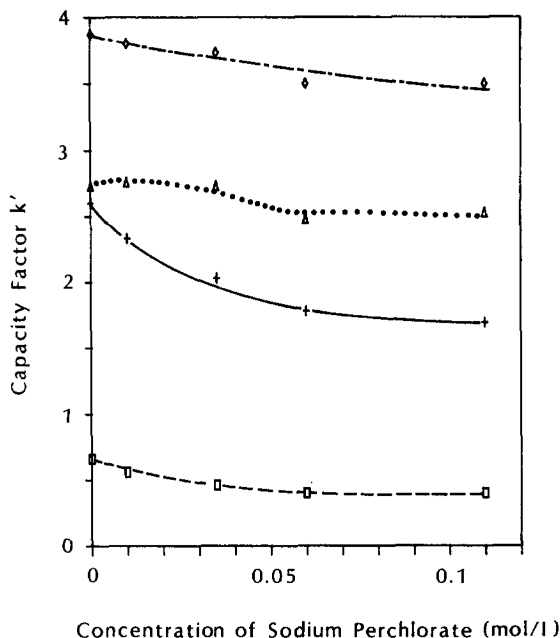

Fig. 4. Ionic strength effect on the retention of four thiols. Compounds: (□) cysteine, (+) glutathione, (◇) 3-mercaptopropanoic acid, (△) N-acetylcysteine, Mobile phase: 0.05 mol/l TCA with added $NaClO_4$.

slight decrease in k' must result from competition of the added cation for interaction with the TCA buffer anion. Little change is noted for the retention of 3-mercaptopropanoic acid, a neutral compound at this pH.

We also examined the effect of changing the charge density of the counter cation used to prepare the TCA buffer on the retention of the net-cation cysteine. Any ionic interaction between the counter cation and the buffer anion should cause a decrease in retention of the net-cation thiols, by decreasing the "free" anion available for cysteine pairing. Using a 0.01 mol/l TCA buffer at pH 2, cysteine retention was highest for the counter cations with the lowest charge density, in the order $K^+ > NH_4^+ \approx Na^+ > Li^+$. This order is expected based on the predicted strength of the interaction of the counter cations with the TCA anion, with Li^+ possessing a high charge density and forming stronger ionic interactions than Na^+ and the other cations (in order of decreasing charge density).

Effect of the halogen substituents of the trihaloacetic acid

Since increased retention of the net-cations was found using trichloroacetic acid over monochloroacetic acid, we decided to investigate the pairing agent properties of the series of trifluoro-, trichloro- and tribromoacetic acid buffers, all at pH 2.

The adsorption of the three pairing agents to the reversed-phase stationary phase was studied by breakthrough experiments using a minimum dead-volume solvent switch and ultraviolet absorbance detection at 235 nm. The concentration of the pairing agent adsorbed to the stationary phase (P_s) was calculated using the method of Knox and Hartwick[10]. A plot of P_s as a function of P_m for the three THAs is

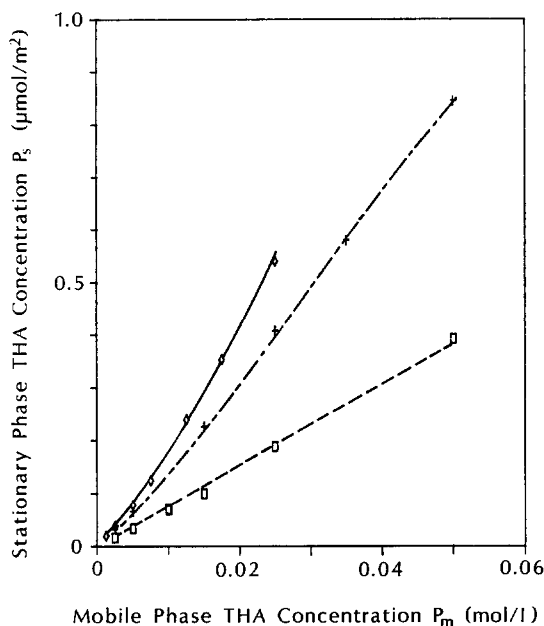

Fig. 5. P_s as a function of P_m for the THA pH 2 buffers. (□) TFA, (+) TCA, (◇) TBA.

presented in Fig. 5. Adsorption of all three compounds is in the linear region of the adsorption isotherm. The comparitively low reversed-phase adsorption of these pairing agents is expected since the haloacetic acids are nearly 100% ionized at this pH and have small hydrophobic R groups. In the THA systems, the P_s values found are all at least one order of magnitude lower than for octylsulfate, which shows a curvilinear isotherm with stationary phase saturation occurring at P_m above 0.02 mol/l[10]. The adsorption of the THA occurs in the order TBA > TCA > TFA, as expected from the relative sizes of the hydrophobic trihalomethyl moiety. Rapid reversibility of the adsorption of all of the THA buffers was observed upon switching the mobile phase to pure phosphate buffer, requiring elution of 2–5 column volumes. This is in sharp contrast to the unfavored reversed-phase desorption observed for alkyl sulfate/sulfonate pairing agents, especially the longer chain C_8–C_{12} compounds, which may require elution of up to 20 l of aqueous solution to return the column to the original condition[11].

A comparison of TFA, TCA, and TBA buffers was made on the retention of the net-cation cysteine. Since retention was maximized at low pH, pH = 2 was chosen for the study. A plot of the capacity factor of cysteine as a function of P_m, at constant ionic strength, is shown in Fig. 6. For all three buffers the retention increases with P_m, but the increase was most dramatic for the larger THAs. The reversed-phase retention of cysteine is plotted as a function of the stationary-phase pairing agent concentration (P_s) in Fig. 7. Equal net-cation retention is not obtained for the three THAs at any given P_s. This contrasts results found for alkyl sulfate/sulfonates, where cation retention is equivalent for equal stationary phase concentrations, independent of the size of the hydrophobic moiety of the pairing ion, and depends only on the surface con-

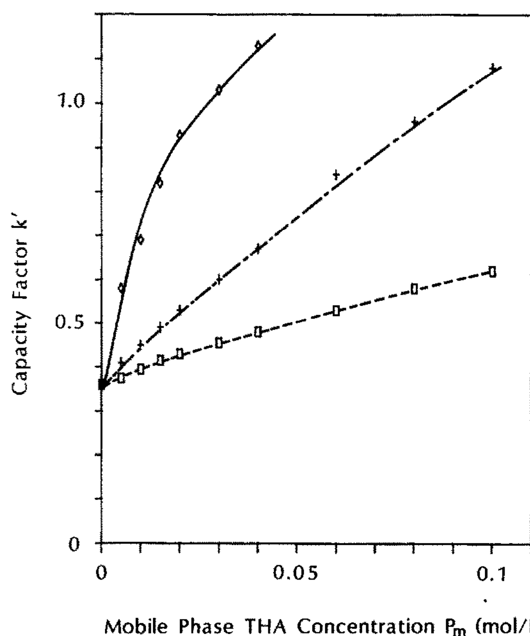

Fig. 6. Effect of P_m of the THA pH 2 buffers on cysteine reversed-phase retention. (□) TFA, (+) TCA, (◇) TBA.

centration of adsorbed pairing ions[10]. In the THA pairing system, the surface concentration of adsorbed pairing agent is not the dominant parameter in net-cation retention.

Since the retention enhancement appeared to be a strong function of the size of

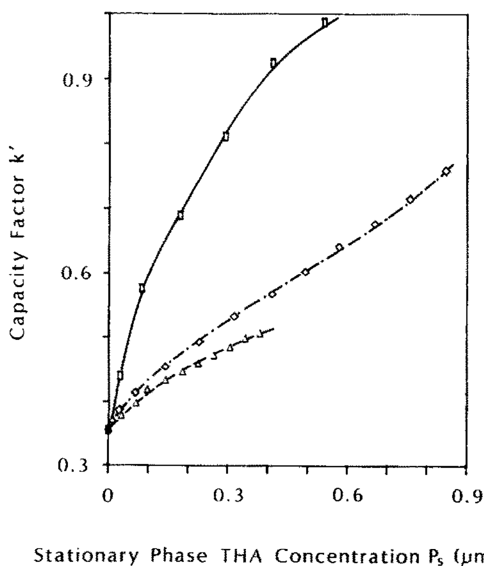

Fig. 7. Cysteine retention as a function of P_s. (△) TFA, (◇) TCA, (□) TBA.

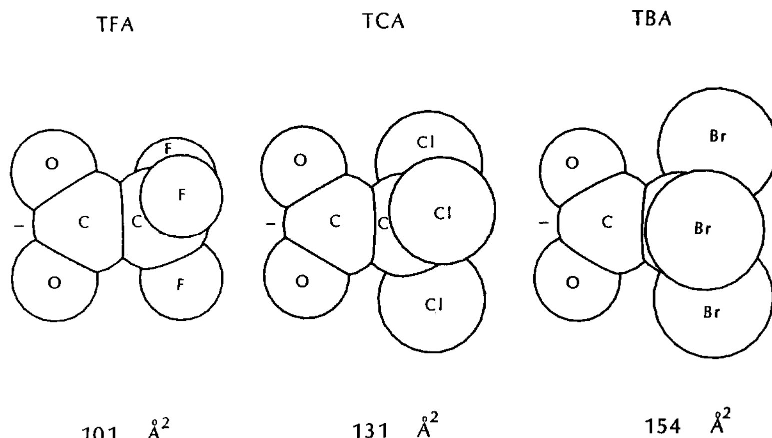

Fig. 8. Space filling models of THA anions.

the halogen moiety of the THA, we decided to examine this relationship. The size differences of the three anions are graphically represented by the space filling models shown in Fig. 8. A plot of the log of the retention enhancement factor[30] *versus* the molecular surface area of the three THA (calculated from known bond lengths and Van der Waals' radii) shows an excellent linear relationship (Fig. 9). This retention behavior is most consistent with solvophobic ionic interactions with the pairing agent that enhance reversed-phase partitioning[30]. It is also possible that hydrogen bonding

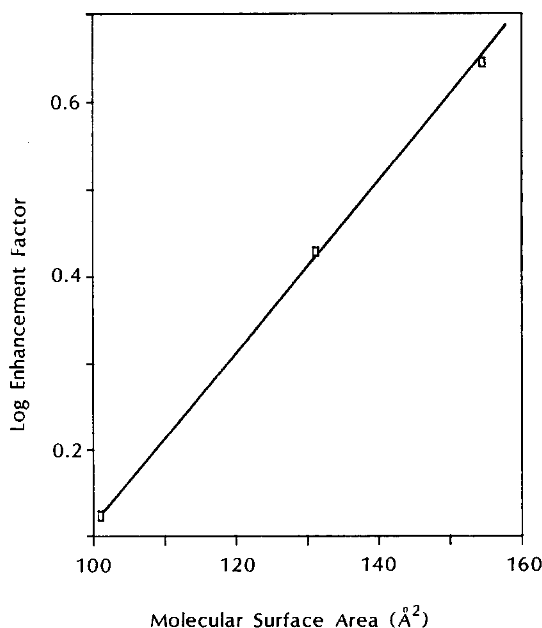

Fig. 9. Retention enhancement factor for cysteine as a function of pairing agent surface area.

between the pairing anion and the protonated primary amine of the cysteine may enhance the tendency to form structure-specific ionic interactions.

Although an excellent correlation of the retention enhancement factor and the surface area of the THA was found, differences in ionic interactions between the three anions, resulting from inductive effects of the halogens on the electron density at the carboxylate anion, are not accounted for. The interaction formation constants of the THA anions with the net-cation cysteine may be calculated[30]. Although a number of tenuous assumptions need to be made about the retention mechanism with this approach, the following values were calculated for the THA–cysteine interaction formation constants: 30 $\pm$ 2 for TFA, 39 $\pm$ 2 for TCA and 41 $\pm$ 5 for TBA ($\pm$ 1 S.D., n = 9). The trend of these chromatographically-derived formation constants is consistent with the intuitively expected inductive effect that the halogens have on the electron density of the carboxylate anion; *i.e.*, the trifluoride is most electronegative, withdrawing electron density from the anion, and it has the lowest interaction constant with the net-cation. Note that the tendency of the THAs to form ionic interactions may also reflect the effect of the hydrophobicity of the THA anions as expected from solvophobic theory[30]. Since the inductive and solvophobic effects both favor the tendency to form ionic interactions in the order TBA > TCA > TFA >, it is impossible to sort out which is the more important parameter. Nevertheless, the linear relationship between the retention enhancement factor and the molecular size of the THAs is most consistent with the solvophobic effect being the more dominant factor in the retention of the net-cations in this system.

For the application of the reversed-phase THA pairing separation system to the determination of the biogenic thiols in marine sediment pore-water samples[17], we chose to use TCA buffer rather than the more retentive TBA buffer because of the noise-generating, electroactive impurities in the commercial TBA and because of its limited aqueous solubility. Development of an analytical reversed-phase separation of the neutral thiols (mercaptopyruvic acid, 2-mercaptoethanol, 1-mercaptoglycerol,

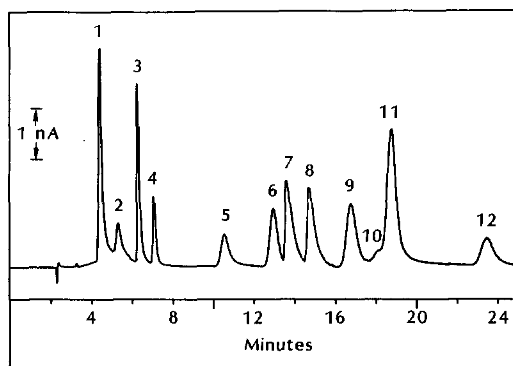

Fig. 10. Separation of several biogenic thiols. Mobile phase: 0.05 mol/l TCA buffer– methanol (99:1) (pH 2). Compounds: 1 = cysteine (0.55 nmol), 2 = mercaptopyruvic acid (0.07 nmol), 3 = 2-mercaptoethanol (0.05 nmol), 4 = 1-mercaptoglycerol (0.16 nmol), 5 = glutathione (0.65 nmol), 6 = 3-mercaptopropanoic acid (0.11 nmol), 7 = methanethiol (0.19 nmol), 8 = ethanethiol (0.16 nmol), 9 = 2-mercaptopropanoic acid (0.12 nmol), 10 = 2-propanethiol (0.05 nmol), 11 = 1-propanethiol (0.25 nmol), 12 = penicillamine (0.10 nmol).

2- and 3-mercaptopropanoic acid, methanethiol, ethanethiol, 1- and 2-propanethiol, was simply a case of choosing a mobile phase pH low enough to ensure that all ionizable functional groups were protonated. At pH 2, using the methanol–water (1:99) mobile phase, all of these components could be baseline separated. Adjustment of the concentration of TCA to 0.05 mol/l (see Fig. 3) provided the appropriate retention of the net-cations cysteine, penicillamine and glutathione for resolution from the other thiols of interest. This optimization process was greatly facilitated by the lack of appreciable change in retention of the neutral components with changing TCA concentration. The TCA concentration required for optimum selectivity of compounds not studied here could easily be obtained from a plot of k' *vs.* P_m like shown in Fig. 3. Fig. 10 shows the simultaneous separation of twelve biogenic thiols using the TCA buffer system. One advantage of the TCA mobile phase was in the qualitative identification of the thiols in sediment pore-water samples. The retention of the net-cationic thiols could be easily varied by a change in the mobile phase concentration of TCA; whereas neutral thiols showed no change in retention. These experiments were facilitated by the rapid system response to mobile phase changes, where elution of only 2–5 column volumes was required for column–mobile phase equilibration. Using this separation, twelve biogenic thiols were identified and determined at the nanomolar level in sediment pore-water samples[17].

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