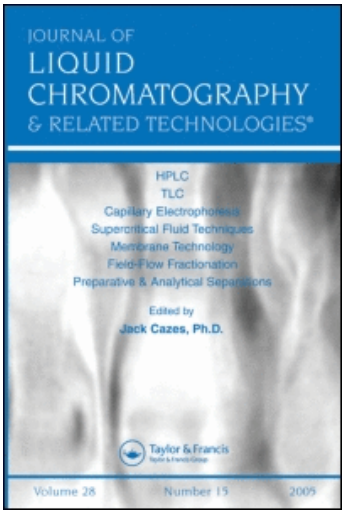

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SEPARATION OF FREE AMINO ACIDS ON REVERSE STATIONARY PHASES USING AN ALKYL SULFONATE SALT AS A MOBILE PHASE ADDITIVE

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ABSTRACT

Alkylsulfonate (RSO_3^-) salts were evaluated as mobile phase additives for the separation of free amino acids on reverse stationary phases using an acidic mobile phase where the amino acids are cations. The enhanced amino acid retention is the result of two major interactions, one being retention of the RSO_3^- salt on the stationary phase and the other an ion exchange selectivity between the amino acid analyte cation and the RSO_3^- countercation, or other countercations in the mobile phase. Major mobile phase variables are: type and concentration of RSO_3^- salt (the studies focused on $C_8SO_3^-$ salts), presence of organic modifier, type of countercation present, and mobile phase pH and ionic strength. Alkyl modified silica and polystyrenedivinylbenzene copolymeric reverse stationary phases were compared. A mobile phase gradient, increasing per cent organic modifier was shown to be best, is necessary for separating complex mixtures of polar and nonpolar or basic amino acids. The procedure is applicable to the identification and/or determination of amino acids in mixtures or in peptides after hydrolysis.

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INTRODUCTION

The accurate determination of amino acids (AA) has been long recognized as one of the most important types of analysis in biological and related sciences. Because of AA similarity, a separation strategy is almost always part of the procedure particularly when dealing with complex AA mixtures. In general, the separation is usually designed to take advantage of the differences in the AA side chain hydrophobicity and/or its influence on the charge that can be produced at the terminal $-NH_2$ and $-CO_2H$ group via pH control. Virtually all types of chromatographic techniques have been applied to AA separations. It is not surprising, therefore, that modern high performance liquid chromatography (HPLC), which offers many practical advantages, has also been studied extensively for this kind of application.

The more successful HPLC methodologies for AA separations employ either reverse or ion exchange type stationary phases; the former are generally alkyl modified silicas while the latter are sulfonated polystyrenedivinylbenzene copolymeric cation exchangers and to a lesser extent sulfonated alkyl modified silicas. Since most AA are modestly chromophoric only at a low UV wavelength, a favorable, sensitive detection is achieved by conversion of the AA to a derivative that either fluoresces or absorbs in the UV or visible. Derivatization can be performed by either a post-column or pre-column strategy. In the latter case, free amino acids are not being separated and mobile-stationary phase parameters are optimized according to the

structure of the AA derivative. Several recent reviews (1-3) have surveyed HPLC methodologies for AA separations in terms of chromatographic parameters, resolution, efficiency, detection sensitivity, accuracy, reproducibility, and areas of application.

AA analyses cover a wide spectrum of samples and applications and it is not likely that a single HPLC methodology will be ideal for all cases. Clearly, advantages and disadvantages are apparent for existing HPLC strategies. For example, underivatized AA separation on a reverse stationary phase is only modestly successful because retention of the more polar AA is low, thus, a favorable resolution for these AA cannot be obtained. For the less polar AA, where AA retention increases with side chain hydrophobicity (4-7) separation of many AA combinations are favorable. With pre-column derivatization strategies, where dansylsulfonamide, phenylthiohydantoin, and orthophthaldialdehyde (OPA) AA derivatives are probably the most widely used (1-3), reverse phase HPLC provides favorable resolution, analysis times, and detection limits but also requires a pre-separation derivatization step. This approach prevents recovery of the free AA and also requires that the derivatization step be quantitative and reproducible.

Polymeric type cation exchangers are favorable stationary phases for the separation of free AA (1-3). In this methodology the mobile phase pH is carefully altered on the acidic side and AA retention changes according to the cationic character of the AA terminal $-NH_2$ group. Acceptance of polymeric cation exchangers

for the HPLC separation of AA mixtures has been slow because of column cost, susceptibility to column pressure drop due to copolymer expansion-contraction, and the unavailability, until recently, of small particles of an uniform size distribution.

A general methodology that has been widely used to separate ionic analytes on reverse stationary phases is to use a hydrophobic ion of opposite charge as a mobile phase additive. The interaction between the hydrophobic ion, the analyte ion, and the reverse stationary phase enhances retention and increases the number of mobile phase variables which can then be altered to bring about the desired resolution. Although this technique is often referred to as ion-pair chromatography, many studies clearly indicate that, depending on the chromatographic conditions and degree of hydrophobicity of the additive and analyte, formation of ion pairs appears to play little or no role in the interactions. Both historical and recent advances in this area have been reviewed extensively (9-14 and references within).

AA and peptides, because of their zwitterion character, are analytes whose retention can be markedly altered by adjustment of mobile phase pH and the addition of suitable hydrophobic ion additives. This separation strategy has been particularly useful for small chain peptide separations (see for example 13-15 and references within). Several workers have reported enhanced AA retention in the presence of alkyl sulfonates or tetraalkylammonium salts as hydrophobic ion additives using predominately aqueous mobile phases (10, 11, 13, 14, 16-19) and combined

aqueous-immiscible stationary liquid phases (20-22), but only a few have attempted to identify (11, 13, 17-19) all the chromatographic variables and apply this strategy to the separation (13, 17, 19) of complex mixtures of AA. This report focuses on the use of alkyl sulfonates as hydrophobic ion mobile phase additives combined with a mobile phase pH that ensures that the AA are predominately in their cation form, the identification and optimization of all other mobile phase variables, and a comparison of two types of reverse stationary phases for the separation of underivatized AA.

EXPERIMENTAL

Reagents and Instrumentation. Amino acids, 2-mercaptoethanol and orthophthalaldehyde (OPA) were obtained from Sigma Chemical Co. Alkyl sulfonic acids ($RSO_3^-H^+$) or their Na salts were purchased from Eastman Kodak and Aldrich Chemical Co. Conversion of Na-salts to the acid was achieved by passing aqueous solutions of the RSO_3^- salt through a strong acid, H-form, cation exchanger (Dowex 50 x 8, 100 to 200 mesh). All organic solvents were LC quality. LC quality water was freshly prepared by passage of distilled water through a Sybron/Barnstead water purification unit. A DuPont 8800 gradient controller and 870 pump, and Altex 210 injector, and a Beckman 160 fixed wavelength detector (214 nm filter) for direct AA detection were used. For post column derivatization the detector (340 nm filter) was used as part of a post column apparatus which also included an Altex

100 pump, a SSI mixing T (0.62 µl internal volume) and 0.01 inch id connecting SS tubing. The OPA solution was delivered to the T at 1.0 mL/min. The reverse phase prepacked columns used were obtained from Hamilton Co. (PRP-1, a 4.1 x 150 mm, 10 µm, spherical, polystyrenedivinylbenzene copolymer) and DuPont Co. (Zorbax Octyl, C-8, and ODS, C-18, 4.6 x 150 mm and 250 mm, spherical, 5 to 6 µm, alkyl modified silica).

Procedures. Aqueous AA samples of about 1 mg/mL were used. Typical operating conditions involved a 0.5 to 1 hr column equilibration period after the RSO_3^- salt column breakthrough with the RSO_3^- salt mobile phase, 2 to 10 µL sample aliquots, 1.0 mL/min flow rates, inlet pressures of 500 to 1200 psi depending on column and mobile phase, and a column temperature of 25°C. Mixed solvents used in isocratic or gradient elution are percent by volume. Buffer salts and inert salts were used, when needed, to maintain mobile phase pH and ionic strength, respectively. Column breakthrough volumes were determined as described previously (12) and were used to calculate apparent cation exchange capacities.

The OPA solution required for post column detection (1-3, 23) was made by mixing 0.5 g OPA, KOH solution to yield pH = 10.0, and 10 mL of 1.43 M 2-mercaptoethanol with an aqueous solution containing 0.10 mole of K_3BO_3 and diluting the mixture to 1 L. The OPA reagent was refrigerated when not in use.

RESULTS AND DISCUSSION

Enhanced retention of inorganic analyte ions on reverse stationary phases from a mobile phase containing a hydrophobic

ion of opposite charge has been shown to be determined by two major equilibria (11-13). One accounts for the retention of the hydrophoic ion on the stationary phase while the second describes an ion exchange selectivity between the analyte ion and counterion associated with the retained hydrophobic ion. For an alkylsulfonate (RSO_3^-) salt as the additive these equilibria are represented by eqs. 1 and 2, respectively,

$$A + RSO_3^-C^+ \rightleftharpoons A \cdots RSO_3^-C^+ \quad (1)$$

$$A \cdots RSO_3^-C^+ + X^+ \rightleftharpoons A \cdots RSO_3^-X^+ + C^+ \quad (2)$$

where A is the stationary phase, C^+ is a counterion provided by the RSO_3^- salt, the buffer, and/or ionic strength salt, and X^+ is the analyte ion. Additional equilibria may become significant as the hydrophobic center within the mobile phase additive and/or within the organic analyte ion increases and as mobile phase additive concentration increases (11-13).

Since AA are converted into cations at acidic conditions, enhanced AA retention should take place from an acidic mobile phase containing a RSO_3^- salt additive according to eqs. 1 and 2. If it is assumed that retention of the RSO_3^- salt and ion exchange selectively are the major factors influencing the enhanced retention, the major mobile-stationary phase parameters that need to be adjusted are the type of stationary phase used, the hydrophobic character of the RSO_3^- salt additive, and mobile phase ionic strength, the mobile phase concentrations of organic modifier and RSO_3^- salt, and the kind of countercations provided by the buffer

and/or ionic strength salts added to the mobile phase. If the AA act as simple cations and retention due to interaction between the stationary phase and the AA side chains are minor, the effects of these variables should be consistent with those observed when using hydrophobic ion mobile phases for the separation of inorganic and other simple organic analyte ions (11-13). Thus, the following should be expected. 1) As RSO_3^- salt mobile phase concentration increases, its retention and the apparent cation exchange capacity (see eq. 1) should increase on the stationary phase and lead to higher AA retention up to a point where the countercation accompanying the RSO_3^- salt competes favorably with the AA cation for the exchange site (see eq. 2). 2) The RSO_3^- salt retention should increase as the hydrophobicity of R increases producing more apparent cation exchange sites and thus higher AA retention. 3) Increasing the organic modifier concentration in the mobile phase should reduce RSO_3^- salt retention and subsequently AA retention. 4) Countercations in the mobile phase should influence AA retention according to their cation exchange selectivity. 5) An increase in mobile phase ionic strength should increase RSO_3^- salt retention which produces more cation exchange sites and higher AA retention, however, this should be counterbalanced by the competitive action of the simultaneous increase in countercation concentration; over a broad and high ionic strength range the effect of the countercation concentration should be more significant thus reducing AA retention. 6) Mobile phase pH will influence AA retention by determining its cationic charge.

TABLE I. Retention of $C_8SO_3^-$ Salt on the Stationary Phase as a Function of $C_8SO_3^-$ Mobile Phase Concentration

	micromoles $C_8SO_3^-$ Salt Retained per Column					
	$C_8SO_3^-H^+$, M					
Column	0.0005[a]	0.001[a]	0.0025[a]	0.005[a]	0.010[b]	0.020[c]
C-8	26	35	56	76	110	170
PRP-1		100	140	190	240	300

a. The aqueous mobile phase contains $C_8SO_3^-H^+$, 0.010M HCl, and LiCl to give u=0.020M. All flow rates are 1.0 mL/min.

b. The mobile phase contains $C_8SO_3^-H^+$ and 0.010M HCl (u=0.020M).

c. The aqueous mobile phase contains $C_8SO_3^-H^+$ (u=0.020M).

RSO_3^- Salt. Zorbax alkyl modified silica and PRP-1, a polystyrene divinylbenzene copolymer, were evaluated as the stationary phases. Previous studies (11-13) had shown that both act as reverse phases towards hydrophobic ion retention. Breakthrough volumes were determined for the two columns as a function of mobile phase RSO_3^- salt concentration by passage of each mobile phase through a clean column. These data, which are summarized in Table I, indicate that RSO_3^- salt retention and subsequent apparent cation exchange capacity increases as mobile phase RSO_3^- salt concentration increases. The exchange capacities reported here are higher than previously reported (12, 13) because of the lower mobile phase pH and increased ionic strength used in Table I; as ionic strength increases RSO_3^- salt retention also increases.

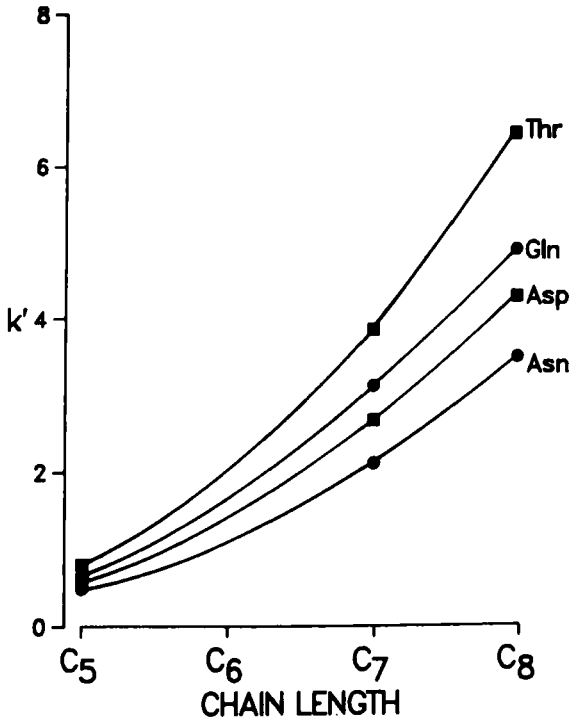

FIGURE 1

Effect of RSO_3^- Salt Alkyl Chain Length on Amino Acid Retention

A 4.1 mm x 150 mm, 10 μm, PRP-1 column and an aqueous 1.0 x 10^{-3}M $RSO_3^-H^+$, 0.010M HCl mobile phase at 1.0 mL/min.

The hydrophobic character of the RSO_3^- salt influences AA retention. Although not shown, as the R chain increases in length (C_5 to C_8 was studied) RSO_3^- salt retention (and apparent cation exchange capacity) increases causing AA retention to also increase. This effect is illustrated in Fig. 1 where retention of several AA on PRP-1 is plotted versus carbon number for the RSO_3^- salt. Of the salts studied the $C_8SO_3^-$ salt provides the

largest number of apparent cation exchange sites, the highest AA retention, and the most favorable selectivity for AA retention at a given condition. Thus, this salt was chosen for subsequent studies.

$C_8SO_3^-$ Salt Concentration. The mobile phase $C_8SO_3^-$ salt concentration plays a major role in the retention of AA. When the $C_8SO_3^-$ salt is absent from the mobile phase most of the AA are not retained while others are poorly retained on the two types of reverse phases. Only for the more nonpolar AA is retention significant enough to allow their separation in the absence of the $C_8SO_3^-$ salt. Table II lists retention data for several of the more polar AA on PRP-1 and C-8 as a function of $C_8SO_3^-$ salt concentration. These data show that both AA selectivity and retention increase as $C_8SO_3^-$ salt concentration increases. The enhanced AA retention correlates to the increase in apparent cation exchange sites on the stationary phase (see eq. 1) that was determined over this $C_8SO_3^-$ salt concentration range (see Table I). Subsequent studies with PRP-1 and C-18 indicate that even though the apparent cation exchange capacity continues to increase as $C_8SO_3^-$ salt concentration increases (studies were done up to 0.025 M $C_8SO_3^-M^+$) AA retention begins to level off and appears to pass through a maximum. Furthermore, the selectivity between AA appears to be constant above 0.001 M $C_8SO_3^-$ salt concentration. The leveling off of AA retention is consistent with Eq. 2. As $C_8SO_3^-$ concentration increases, its co-cation, in this case H^+, also increases and, thus, because of mass action and the cation exchange selec-

TABLE II. Retention of Amino Acids as a Function of $C_8SO_3^-$ Concentration

Amino Acid	Capacity Factor, k' $C_8SO_3^-H^+$, M[a]					
	0.0005	0.0010	0.0025	0.0050	0.01	0.02
			Zorbax C-8			
L-Asn	0.75	0.98	1.33	1.65	2.10	3.79
L-Asp	0.73	0.94	1.32	1.64	2.08	3.79
L-Ser	0.73	0.94	1.32	1.65	2.07	3.79
Gly	0.77	0.99	1.35	1.70	2.16	3.96
L-Gln	0.93	1.23	1.71	2.10	2.68	4.83
L-Thr	0.97	1.32	1.89	2.35	2.96	5.26
L-Glu	0.98	1.33	1.87	2.27	2.78	5.26
L-Ala	1.06	1.45	2.11	2.67	3.33	6.15
L-Met	5.54	8.58	12.7	15		
			Hamilton PRP-1			
L-Asn	1.76	2.24	2.97	3.66	4.18	4.87
L-Asp	1.85	2.82	3.65	4.48	4.89	5.18
L-Ser	1.80	2.37	3.18	3.99	4.46	5.18
Gly	1.80	2.40	3.20	4.00	4.59	5.50
L-Gln	2.30	3.27	4.27	5.20	5.83	6.67
L-Thr	3.29	4.36	5.66	6.88	7.51	7.98
L-Glu	3.69	4.76	6.00	7.10	7.38	7.98
L-Ala	3.72	5.01	6.66	8.29	9.10	10.6

A. An aqueous $C_8SO_3^-H^+$ mobile phase containing 0.01M HCl and LiCl to give ionic strength of 0.020M (0.02M $C_8SO_3^-H^+$ did not contain HCl or LiCl) at a 1.0 mL/min flow rate.

tivity of H^+, the equilibrium in eq. 2 begins to shift to the left. Also, rapid column deterioration was found for the C-18 column, but not the PRP-1, in repeated column uses with the more concentrated $C_8SO_3^-$ salt mobile phases. For these reasons a 1.0×10^{-3}M $C_8SO_3^-$ salt mobile phase was used in subsequent AA separation studies.

Mobile Phase pH. As mobile phase pH increases toward the AA zwitterion pH, AA retention on the stationary phase sharply decreases in the presence of a RSO_3^- salt. These trends are shown in Fig. 2 where retention of several lesser retained AA are shown only for PRP-1 and a $C_8SO_3^-$ salt mobile phase. For more nonpolar AA the change in retention is much greater. If the AA contains a basic side chain, retention is also greater because at the more acidic mobile phase pH both the terminal $-NH_2$ group and the basic side chain group are converted into cation charge sites. While AA retention is possible at zwitterion pH conditions, particularly for nonpolar AA, the more favorable levels of retention, selectivity, and resolution of complex AA mixtures are obtained at an acidic pH where the AA are cations. Thus, acidic pH mobile phase conditions were used in subsequent studies.

Mobile Phase Solvent Composition. As organic modifier concentration increases in the mobile phase, RSO_3^- salt retention on the stationary phase decreases reducing the apparent cation exchange capacity. Thus, the AA retention also decreases. This is illustrated in Table III where retention of several AA on C-8

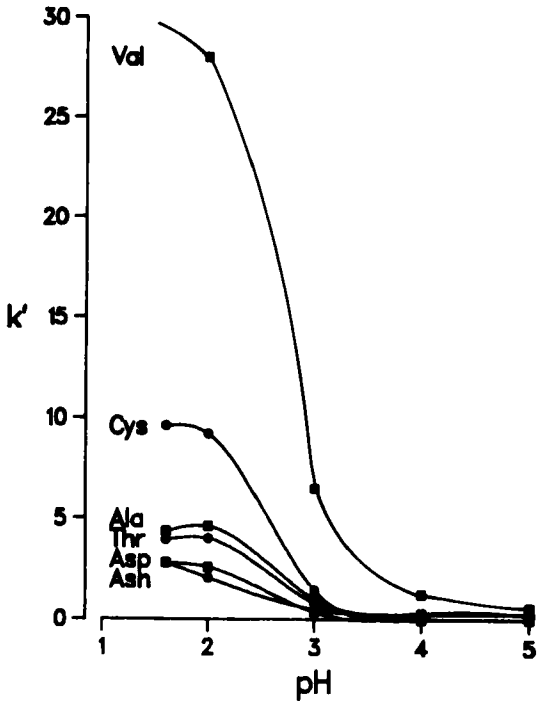

FIGURE 2

Effect of Mobile Phase pH on Amino Acid Retention

Same as Fig. 1 using $C_8SO_3^-H^+$ and HCl and/or LiH_2PO_4 to yield the mobile phase pH and 0.025M ionic strength.

from a mobile phase containing a $C_8SO_3^-$ salt are shown as a function CH_3CN-H_2O composition. If alcohols are used as the organic modifier the retention effect is less where the order is CH_3CN > EtOH > MeOH.

Mobile Phase Ionic Strength. The ionic strength affects AA retention in two competing ways. One, increasing ionic strength

TABLE III. Effect of Organic Modifier on Amino Acid Retention

Amino Acid	Capacity Factor, k' — Percent CH_3CN[a]					
	1.0	2.5	5.0	7.5	10.0	12.5
L-Asn	2.09	1.71	1.37	1.20	0.95	0.74
L-Ser	2.15	1.73	1.30	1.22	0.94	0.74
L-Asp	2.16	1.75	1.36	1.19	0.92	0.72
Gly	2.25	1.89	1.50	1.32	1.04	0.81
L-Gln	2.66	2.06	1.55	1.35	1.04	0.80
L-Thr	3.00	2.28	1.69	1.44	1.09	0.83
L-Glu	3.28	2.44	1.76	1.50	1.12	0.86
L-Ala	3.40	2.58	1.90	1.63	1.22	0.92
L-Cys	3.68	2.73	1.93	1.63	1.19	0.90
L-Pro	5.15	3.70	2.45	2.02	1.43	1.05
L-Val	15.3	9.86	5.54	4.70	2.54	1.72
L-Lys					2.81	1.72
L-His			7.31	5.92	3.14	2.02
L-Met	22.5	13.3	7.49	6.31	3.35	2.20
L-Arg					4.43	2.62
L-Leu			12.4	9.82	5.65	3.36
L-Tyr		30	13.5	9.90	4.39	2.61
L-Ile			13.7	9.18	4.46	2.95
L-Phe			31.8	24	11.2	5.83
L-Trp					28	14.6

a. A H_2O-CH_3CN mobile phase containing 1.00×10^{-3}M $C_8SO_3^-H^+$ and 0.010M HCl at a 1.0 mL/min flow rate on a C-8 column.

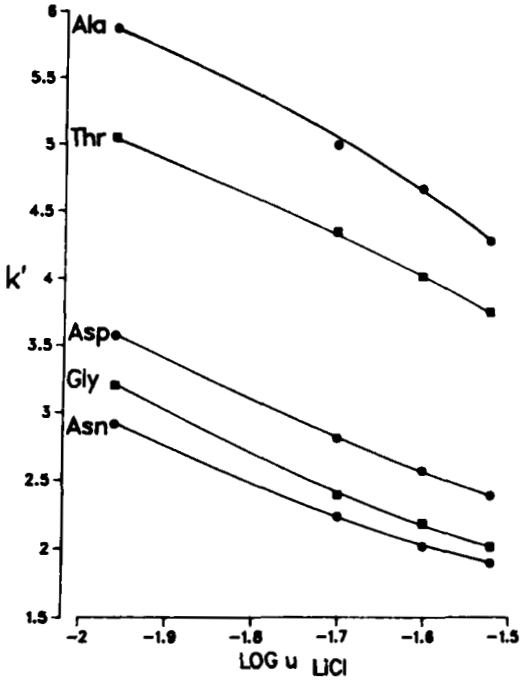

FIGURE 3

Effect of Mobile Phase Ionic Strength on Amino Acid Retention

Conditions are the same as Fig. 1 using $C_8SO_3^-H^+$ with added LiCl to obtain the desired ionic strength.

causes RSO_3^- salt retention to increase producing a higher apparent cation exchange capacity which should lead to higher AA retention. Opposing this is the countercation effect, whereby increasing the ionic strength increases the countercation concentration causing a decrease in AA retention due to a shift in the equilibrium in eq. 2 to the left. Since different cations will exhibit their own cation selectivity the magnitude of the

effect will depend not only on concentration but also on the type of countercation. Fig. 3 shows that AA retention decreases as ionic strength increases indicating that the countercation effect is more dominant than the increase in apparent cation exchange sites. If a K^+ salt is used as a source for ionic strength the decrease in retention is greater. For the countercations studied their effect on AA retention is in the order $M^{+2} > K^+ > Na^+ > Li^+$ which is consistent with previous studies involving organic and inorganic cation analytes (12,13) and with cation exchange selectivity exhibited by classical sulfonated strong acid cation exchangers (24).

Amino Acid Concentration. The analyte concentration will be an important variable if the analyte consumes a significant portion of the apparent cation exchange capacity generated by the retention of the RSO_3^- salt (see eq. 1-2). Previous studies (11-13) have shown that the analyte retention will decrease as analyte concentration increases in the region where significant cation exchange capacity is utilized. For the data reported here 0.6% or less of the total apparent cation exchange sites are utilized for retention of the AA cationic analyte, which is within an analyte concentration range where analyte retention is independent of its concentration. Thus, under these conditions AA retention is influenced by the equilibrium in eq. 1, or retention of the RSO_3^- salt, and by the equilibrium in eq. 2, or the exchange selectivity for the analyte cation and any other cation in the mobile phase. It should be noted that the analyte concen-

tration range that provides retention independent of analyte concentration also depends on those mobile phase variables that influence RSO_3^- salt retention, since these will determine the apparent cation exchange capacity, and the mobile phase pH, since this determines the cationic character of the AA. For mobile phase conditions that provide 100 μmole of retained RSO_3^- salt per 15 cm column length, injection of 5 μg of a single AA analyte will consume approximately 0.5% of the cation exchange capacity assuming molecular weight of 115.

Stationary Phase. While investigating the effects of the mobile phase variables several differences between the C-8, C-18, and PRP-1 stationary phases were noted. In general, the apparent cation exchange capacities, which were calculated from the breakthrough volumes and are due to RSO_3^- salt retention, were nearly the same for the C-18 and PRP-1 columns and larger than for the C-8 column for a given mobile phase condition. Since prepacked columns were used, no attempt was made to determine the loading per gram of stationary phase and the apparent exchange capacities reported here are for 15 cm columns. Although AA retention is greater and selectivity appears to be more favorable on the PRP-1 column, resolution, particularly for complex AA mixtures, is not as favorable as with the C-18 column because the band shapes are much broader on the PRP-1 column. PRP-1 is stable below pH = 2 unlike the C-8 or C-18, however, this more acidic mobile phase which should ensure a more complete conversion of the AA into a cation does not lead to an improved

chromatography on the PRP-1. In the more acidic mobile phase the countercation effect of H^+ (see eq. 2) is also increased.

The conditioning period with the RSO_3^- salt mobile phase was found to be similar for the three columns, however, removal of the retained RSO_3^- salt with mixed solvent free of the RSO_3^- salt was much slower on the C-8 and C-18 columns suggesting that the sorption-desorption of the RSO_3^- salt on the PRP-1 is more reversible than on the C-8 and C-18. It was also noted that the chromatographically useful time period for the PRP-1 column was significantly longer than for the C-8 and C-18 column. Because of the more favorable efficiency on C-18 the focus of the separation studies, particularly for more complex AA mixtures, was with the C-18 column. For simpler AA mixtures resolution on the PRP-1 column can be as favorable, and in some cases more favorable, as that obtained with the C-18.

Separations. Tables II, III and other data not reported here (13, 17-19) indicate that nonpolar AA and those with basic side chains are highly retained in the presence of the $C_8SO_3^-$ salt while the more polar AA are less retained and resolution of mixtures of the latter group is more difficult. Fig. 4 illustrates the effect of mobile phase $C_8SO_3^-$ salt concentration and type of stationary phase on the separation of the more polar AA. As $C_8SO_3^-$ salt concentration increases resolution does not improve continuously because of the competing effect of the countercation, H^+, which also increases in concentration. Thus, even though the apparent cation exchange capacities increase due

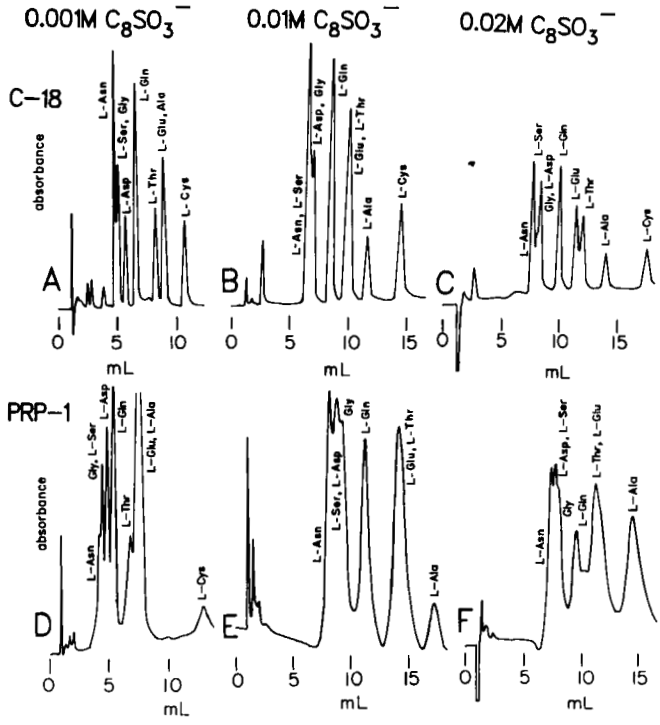

FIGURE 4

Effect of $C_8SO_3^-$ Salt Concentration on the Retention of Polar Amino Acids on C-18 and PRP-1 Stationary Phases

A to C: C-18 column and (A) 0.0010M $C_8SO_3^-H^+$, 0.010M HCl, μ = 0.011M, (B) 0.010M $C_8SO_3^-H^+$, 0.0047M HCl, 0.010M LiCl, μ = 0.021M, and (C) an 0.020M $C_8SO_3^-H^+$, μ = 0.020M mobile phase at 1.0 mL/min. D to F: PRP-1 column and (D) 0.0010M $C_8SO_3^-Li^+$, 0.001M HCl, 1:99 $CH_3CN:H_2O$, μ = 0.011M, (E) 0.010M $C_8SO_3^-H^+$, μ = 0.010M, and (F) 0.020M $C_8SO_3^-H^+$, μ = 0.020M mobile phase at 1.0 mL/min.

to increased amount of retained $C_8SO_3^-$ salt, optimum resolution is obtained at about 0.001M $C_8SO_3^-$ salt concentration. Comparison of the chromatograms also indicates a more efficient column performance for the smaller particle, C-18 column. A 5 μm PRP-1 column, which subsequently became available, was not evaluated in these studies.

The mobile phases used in Fig. 4 were free of buffer salts and inert, inorganic electrolyte. If either of these is added to the mobile phase, AA retention and resolution is decreased due to the mass action effect and the cation exchange selectivity due to the type of countercations that are also added. Similarly, adding organic modifier to the mobile phase reduces retention and resolution.

Nonpolar and basic side chain AA retention is more enhanced than for polar AA from a $C_8SO_3^-$ salt mobile phase and increased eluting power is required to reduce analysis time when separating mixtures of the former AA. This is achieved by increasing mobile phase organic modifier, pH, and/or ionic strength, choosing a countercation of greater selectivity, or decreasing the $C_8SO_3^-$ salt concentration. In general, a modest increase in organic modifier, (see Table III) which has the net effect of reducing the apparent cation exchange capacity by shifting the equilibrium in eq. 1 to the left, provides favorable chromatography and analysis times. This is illustrated in Fig. 5A-B where CH_3CN is added to the aqueous, $C_8SO_3^-$ salt mobile phase to bring about resolution of the more highly retained AA in a

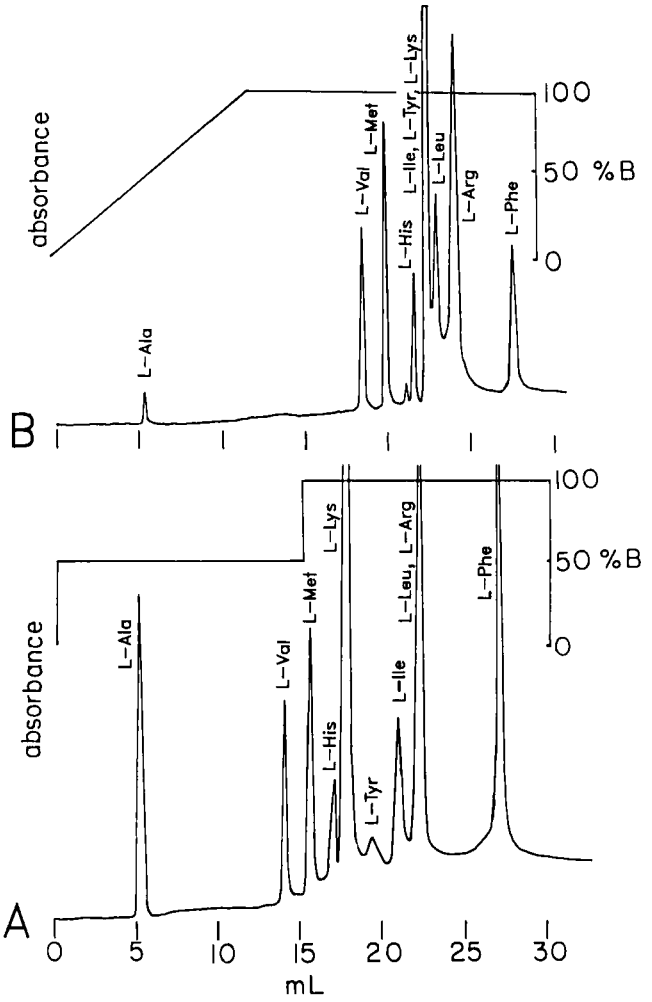

FIGURE 5

Effect of $CH_3CN:H_2O$ Gradient on Retention of Nonpolar-Basic Side Chain Amino Acids on C-18 Column

A. A step gradient with an A solvent of 0.0010 M $C_8SO_3^-H^+$, 0.010 M HCl, and 0.5:99.5 $CH_3CN:H_2O$ and a B solvent of the same except 15:85 $CH_3CN:H_2O$ at 1.0 mL/min.

B. An isocratic-linear gradient using the same A and B solvent.

reasonable analysis time. In Fig. 5A the column was first conditioned with an aqueous $C_8SO_3^-H^+$ mobile phase which should provide the largest number of exchange sites due to the retained $C_8SO_3^-H^+$ on the stationary phase (see eq. 1). As the AA mixture is introduced, the CH_3CN is simultaneously increased to 7.5% CH_3CN and subsequently stepped to 15% CH_3CN. Resolution of L-His, L-Lys and L-Leu, L-Arg appears to be the most critical in adjustment of the time and percent CH_3CN for the elution. For a simpler mixture of nonpolar AA the steps can be adjusted accordingly. L-Ala was in the mixture to indicate how rapidly the more polar side chain AA would be eluted under these conditions. L-Trp was omitted in Fig. 5A because its retention time would be about 35 min; L-Phe and L-Trp separation times can be reduced by increasing the percent CH_3CN in an additional step. L-Cys and L-Pro retention time in Fig. 5A would be about 7 and 10 min, respectively. When a linear gradient of the slope shown in Fig. 5B is used, resolution of L-Ile, L-Tyr, L-Lys is not obtained; adjusting the gradient slope will alter this resolution as well as resolution for other AA. The elution order for the basic AA differs in the two chromatograms due to the effect of the gradient on the number of exchange sites retained on the column as the percent CH_3CN is increased. Thus, the elution order can be altered and optimized according to the basic-nonpolar AA being separated by adjusting the rate of addition of CH_3CN to the mobile phase.

The overall AA elution order in Fig. 5 is not identical to the order obtained when using typical strong acid cation exchangers

as the stationary phase (1-3). In the early stages the elution order is the same while in the latter stages the order is typical of reverse phase AA chromatography (4-7). For example, in Fig. 5 the AA with basic side chains, which are partially di-cations at the conditions used, are eluted before the very nonpolar AA; on a typical cation exchanger (1-3) the basic side chain AA are the most retained from an acidic mobile phase. As the CH_3CN is stepped or the gradient is started the retained RSO_3^- salt on the stationary phase decreases as the mobile phase CH_3CN concentration increases. At 15% CH_3CN the retention of the $C_8SO_3^-$ salt is minor (12). During this stage of the chromatography the cation exchange sites are being removed and the stationary phase is converted into a reverse phase; the faster the CH_3CN is introduced in the gradient the more rapid the conversion takes place and the greater the effect on AA elution order since reverse phase AA retention is not only solvent dependent but is also influenced differently by AA side chain structure (4-7).

A single, isocratic elution scheme will not provide favorable resolution and/or analysis time if the AA mixture is composed of both polar-acidic and nonpolar-basic AA. The more favorable elution scheme is one which provides a constant, weak eluting power for the less retained AA followed by a gradient for the more highly retained AA or a combination of conditions used in Figs. 4 and 5. Using a test mixture of polar-nonpolar AA, gradients involving either an increase in organic modifier, pH, eluent countercation of greater ion exchange selectivity at fixed ionic strength, and ionic strength, while all other variables were held constant, were

evaluated. A RSO_3^- salt concentration gradient was not studied because of the equilibration period required for RSO_3^- salt retention onto the stationary phase (see eq. 1). These studies indicated that a solvent gradient was the best. For simple AA mixtures other gradients can be optimum depending on the type of AA present.

Figure 6A-B shows the separation of a complex AA mixture on a 15 and 25 cm C-18 column, respectively, using an isocratic elution for the polar, less retained AA and a linear nonpolar gradient for the nonpolar, basic, more retained AA. When a 15 cm PRP-1 column was used with a combined isocratic-gradient condition as in Fig. 6A using HOAc instead of HCl, AA retention order was the same, peaks were broader, and resolution due to peak broadening was not as favorable particularly for the early eluting AA. Increasing the column length improves the resolution of the weakly retained AA. A more gradual gradient change in percent CH_3CN was used on the 25 cm column (Fig. 6B) versus the 15 cm column (Fig. 6A). Thus, the removal of the retained RSO_3^- exchange sites and the conversion to a reverse type stationary phase is less rapid on the former column. This causes a significant alteration in the AA elution order in the nonpolar-basic AA range of the separation, particularly for the basic AA where their elution relative to the nonpolar AA is much earlier. The best resolution for a complex mixture of AA, while also maintaining a favorable analysis time, is obtained by using a step gradient. This separation is shown in Fig. 7 where an aqueous mobile phase is used to elute the polar AA and the percent CH_3CN is stepped (see Fig. 5A) to remove the nonpolar-

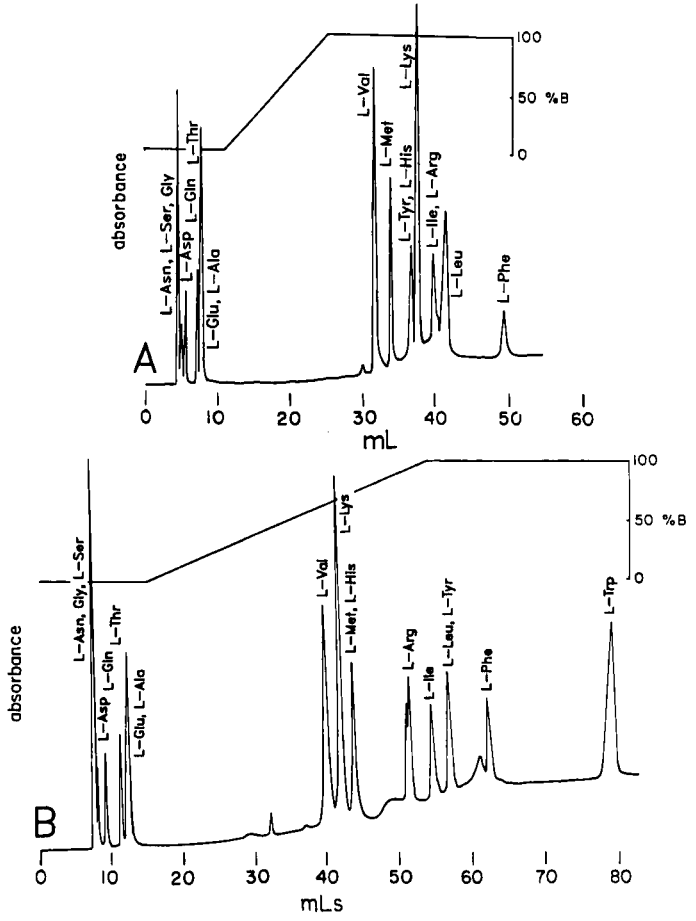

Figure 6

Separation of a Complex Mixture of Amino Acids on a C-18 Column

A. An isocratic-linear gradient with an A solvent of 0.0010 M $C_8SO_3^-H^+$, 0.010 M HCl, 0.5:99.5 $CH_3CN:H_2O$ and a B solvent of the same except 15:85 $CH_3CN:H_2O$ at 1.0 mL/min and a 4.6 x 150 mm C-18 column.

B. Same as chromatogram A except for 0.089 M NaCl in the B solvent and a 4.6 x 250 mm C-18 column.

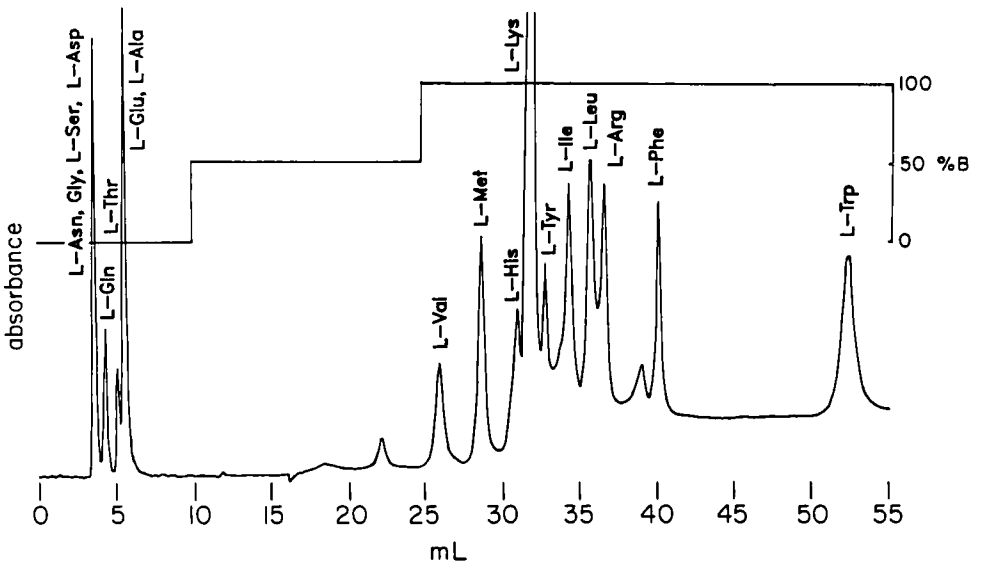

Figure 7

Separation of Amino Acids on C-18 Using a Step Gradient

A step gradient with an A solvent of 0.0010 M $C_8SO_3^-H^+$, 0.010 M HCl 100% H_2O and a B solvent of the same except 15:85 CH_3CN:H_2O at 1.0 mL/min.

basic AA. Since removal of the RSO_3^- exchange sites and conversion to a reverse phase is gradient dependent, the elution order for the basic-nonpolar AA in Fig. 7 is different than the order shown in Fig. 6. A third step can also be used in Fig. 7 to reduce the retention time for L-Trp.

L-Cys and L-Pro elute in this order after L-Ala in Figs. 6 and 7. These are not shown because OPA post-column detection was used; L-Cys has poor sensitivity and L-Pro requires a modified reagent for OPA detection (1,3,23). Since they are

separated from each other and neighbor AA, for example in Fig. 7 L-Cys and L-Pro have retention times of 12 and 15 min, respectively, resolution is favorable. UV detection of underrivatized AA is possible at 214 nm. However, we found that several interferring narrow and broad chromatographic peaks were produced during the CH_3CN gradient. These peaks were subsequently shown to be due to removal of the retained $C_8SO_3^-$ salt and sulfonated impurities in the $C_8SO_3^-$ salt (see eq. 1) from the column. Purification of $C_8SO_3^-$ salts by prep LC reduced these background peaks but did not eliminate them. Detection at 340 nm using OPA post-column derivatization showed no signs of this background and special purification of the $C_8SO_3^-$ salts was not necessary for this detection. Adding electrolyte to the mobile phase in Figs. 6-7 reduces analysis times but also decreases resolution for closely related peaks. If the mobile phase $C_8SO_3^-$ salt concentration is increased, resolution increases for some adjacent peaks and decreases for others. For example, at 0.01M $C_8SO_3^-H^+$ L-Ser, Gly and L-Glu, L-Ala are resolved while for Gly, L-Asp and L-Thr, L-Glu resolution is less favorable.

This separation scheme should be applicable to peptide AA identification and analysis since for a given gradient the appearance of the peaks of a hydrolyzed peptide can be correlated to retention times and peak areas for AA standards and AA concentration-area calibration curves, respectively. Figure 8 shows the separation of AA derived from three hydrolyzed peptides. All peptide AA are accounted for and their presence was verified by

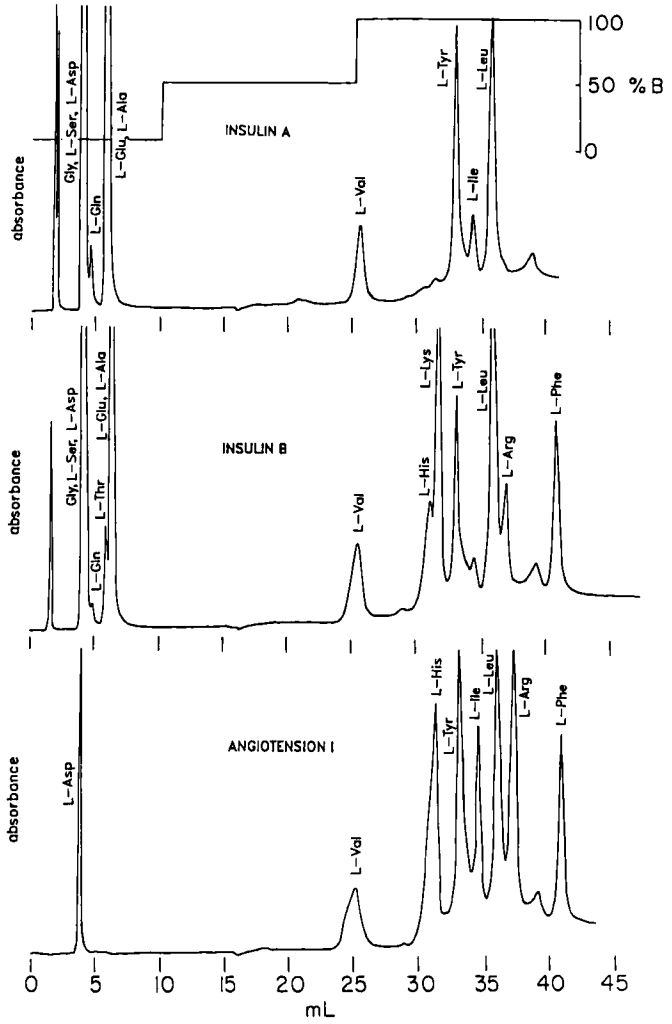

FIGURE 8

Separation of Amino Acids Derived From Hydrolyzed Pepides on a C-18 Column

Conditions are the same as Figure 7 except 0.5:99.5 CH_3CN:H_2O in the A solvent.

comparison to AA standards. In Fig. 8, mg quantities of peptide was hydrolyzed with 6M HCl in a sealed, N_2 purged glass tube that was heated at 110°C for 24 hours, and dissolved (3). An aliquot of this solution was injected into the LC so that the amount of AA introduced never exceeded 0.1% of the available exchange capacity provided by the retained $C_8SO_3^-$ salt (see eq. 1). A stepwise gradient, like Fig. 7, was used; for a linear gradient, like Fig. 6A, resolution of several AA pairs is not complete for the peptides studied. When insulin was hydrolyzed its chromatogram was the sum of insulin A and insulin B.

Calibration Curve. Analyte standards were prepared containing nearly identical amounts of L-Ser, L-Val, and L-Leu and covered the range of 20 to 1000 ppm in each AA. A 20 µl aliquot was injected and the mixture separated using the gradient elution conditions outlined in Fig. 6A. A linear calibration curve (OPA detection) of peak area versus AA amount injected was obtained over the range of an injection of 20 µL of 20 to 500 ppm AA. A linear least squares fit of these data provided a correlation factor of 0.997 or greater. Two major factors could strongly influence the linear calibration. First, during the gradient the amount of retained $C_8SO_3^-$ salt on the stationary phase, as discussed previously, is gradually reduced converting it to a reverse phase. This change in retention mode could influence peak area and consequently calibration. Second, since the apparent exchange capacity due to the retained RSO_3^- salt is low an overload, which will influence peak area and position, could occur because of

competition between the AA for the available exchange sites. The three AA used in the study are examples of early, middle and late eluters, respectively. Since the calibration curve for each AA is favorable, these data indicate that these two factors are not limiting in terms of the calibration curve and that the gradient is not harmful during any point of the elution. Fluorescence detection of the post-column OPA derivative (440 nm emission) was also evaluated since this has a lower detection limit and is often used in AA analysis (1-3, 23). A 5 ng to 500 ng range of the three AA were studied. Each provided a favorable calibration curve indicating that the effect of the $C_8SO_3^-$ salt and gradient on fluorescence is either negligible or reproducible. Lower fluorescence detection is still possible depending on the quality of the LC fluorescence detector.

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