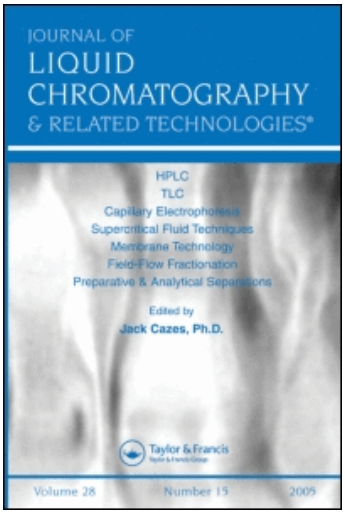

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Separation and Detection of Underivatized Amino Acids

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SEPARATION AND DETECTION OF UNDERIVATIZED AMINO ACIDS

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ABSTRACT

Amino acids (AA) are separated on a Hamilton PRP-1 polystyrene divinylbenzene column with a basic CH_3CN:H_2O mobile phase containing a tetraalkylammonium (R_4N^+) salt and a detector active counteranion. The underivatized AA are retained and separated as anions via a competing anion exchange process that occurs between the AA anion and the counteranion towards the R_4N^+ salt, an equilibrium amount of which is maintained on the stationary phase according to the mobile phase conditions. An indirect detection (ID) is possible because of displacement of the detector active counteranion. Indirect photometric (IPD), fluorometric (IFD), or electrochemical (IED) detection is possible by using a counteranion that absorbs, fluoresces, or is electrochemically active. Parameters that influence retention and resolution are AA side chain structure and mobile phase pH, R_4N^+ structure and concentration, organic modifier, and counteranion selectivity and concentration. These same parameters, in addition to the counteranion detector activity, also influence indirect detection. Detection limits for IPD are approximately 0.06 nmole (L-Ala) depending on the AA, mobile phase conditions, and counteranion chromophore for a 3:1 signal:noise ratio.

INTRODUCTION

Amino acids (AA) are most often separated and detected by either of two general approaches (1,2). In the first,

underivatized AA are separated by either anion or cation exchange and detection is achieved by a postcolumn reaction that converts the AA into detector active derivatives. The two most versatile and widely used derivatization reagents are ninhydrin and o-phthalaldehyde. In the second strategy derivatization is precolumn and the AA-derivative structure determines the chromatographic mobile phase conditions and column, usually reversed phase, in addition to enhancing the detection. Both strategies require a gradient to resolve multicomponent AA mixtures.

Whether derivatization is pre- or postcolumn reproducibility of the reaction conditions is required to obtain quantitative results. Other factors must also be carefully controlled. For example, in precolumn derivatization the presence of starting materials due to incomplete reaction can influence the chromatography and provide additional chromatographic peaks, while in postcolumn derivatization mixing of column effluent with derivatization reagents and providing suitable reaction time and temperature can lead to band broadening. AA detection limits in these strategies depend on sample cleanup and the separation and derivatization strategy; sub nmole detection limits are usually obtained in routine applications.

Underivatized AA can be separated but they are not easily detected. With the exception of the few AA that contain aromatic groups detection by UV absorption is possible only at low wavelength where sensitivity is not favorable and interference in background absorption is potentially high. A direct detection

method that has recently been developed for separated underivatized AA is pulsed amperometric detection (3).

A detection strategy that is being used with increasing frequency, particularly to detect analytes that do not absorb, fluoresce, or are electrochemically active is indirect detection (ID). In this approach a detector active component is purposely added to the mobile phase to provide a sufficient detector signal (4). The analyte in its chromatographic band displaces or enhances the amount of the detector active component in the band. Thus, the detector responds to the difference between the detector active component background signal, which is electronically offset by the detector, and the analyte band signal. Peaks can be positive or negative depending on whether the detector active component is enhanced or decreased in the band, respectively. The equilibria responsible for ID can be due to: an ion exchange selectivity as in the case where a detector active counterion competes with an analyte ion for the exchange site in ion exchange chromatography (4-6), an adjustment in the equilibrium amount of an ion interaction reagent that is maintained on a reversed stationary phase surface due to ionic strength and analyte selectivity factors (7,8), or a competition that can occur between the analyte and the detector active component for retention to the stationary phase (9). A postcolumn ID involving a chemical reaction is not only feasible but can be selective and sensitive (10). Another feature of ID recently recognized is that ID provides a way to study the complex equilibria occurring between the stationary phase and the ion interaction reagent (4,8,11).

This report describes a procedure for the separation of underivatized AA and their detection by ID. Anionic AA are separated on a reversed stationary phase using a basic mobile phase containing a quaternary ammonium (R_4N^+) salt as an ion interaction reagent and a detector active counteranion which allows ID. If the counteranion absorbs, fluoresces, or is electrochemically active then indirect photometric (IPD), indirect fluorometric (IFD), or indirect electrochemical (IED) detection is possible.

EXPERIMENTAL

Materials. Tetraalkylammonium (R_4N^+) salts were obtained from Eastman Kodak Chemical Co. Amino acids (AA) were purchased from Sigma Chemical Co. Organic and inorganic compounds, acids, and bases (Fisher Scientific Co.) were analytical grade and organic solvents were LC quality. A Millipore Milli-Q Plus unit was used to prepare LC quality water from in-house distilled water. Prepacked Hamilton Co. (PRP-1) 4.1 x 150 mm ss columns containing 10 μm, spherical, polystyrene divinylbenzene copolymeric microparticles were used. LC instrumentation consisted of a Waters Associates M6000A or a Spectra Physics 8800 pump, a Rheodyne 7125 injector, a KRATOS 773 UV detector, an EG and G Princeton Applied Research M400 electrochemical detector, or a Kratos M900 fluorescence detector, a Bioanalytical Systems LC-22A column temperature controller, and a Spectra Physics 4270 recording integrator with Epson Equity I+ computer and WINner/LABNET software.

Procedures. Aqueous AA standards, about 0.2 mg/mL, and mixtures of standards were injected by syringe. Serial dilutions of

standards were used for the calibration curve. All mobile phase solvent mixtures were prepared as percent by volume. R_4N^+ salts containing specific counteranions were prepared by passing aqueous solutions of R_4NCl through an anion exchanger (Amberlite IRA-400, Sigma Chemical Co.) charged in the counteranion form of interest. Mobile phases were prepared by combining aliquots of standard solutions of R_4N^+, buffer, and ionic strength salts with LC water and organic solvent and diluting to volume. When necessary pH was adjusted with dilute NaOH prior to dilution to volume. Basic mobile phases were protected from CO_2 by passing displacement air through an air filter bottle containing a NaOH solution prior to entry into the mobile phase reservoir. PRP-1 column performance was established and compared to manufacture's certification using a phenol/benzene test sample and a 9:1 $CH_3CN:H_2O$ mobile phase at 0.5 mL/min. The equilibrium amount of $R_4N^+C^-$ on the stationary phase surface for a given mobile phase condition was determined from column breakthrough data which were obtained by detecting the appearance of the detector active counteranion C^- in the column effluent for a given flow rate and C^- concentration. The $R_4N^+C^-$ salt was removed from the stationary phase with about 200 mL (large excess) of 4:1 $CH_3CN:H_2O$ prior to equilibration with a different mobile phase composition. Flow rates were usually 1.00 mL/min, temperature was 25.0°C, inlet column pressures were 800 to 1200 psi, and column void volumes were 1.1 to 1.3 mL depending on mobile phase composition.

RESULTS AND DISCUSSION

Previous studies (see 4, 7, 12-15 and references within) have shown that when a mobile phase containing an ion interaction reagent such as a tetraalkylammonium (R_4N^+) salt is passed over a reversed stationary phase, SP, an equilibrium amount of the R_4N^+ salt is maintained on the stationary phase surface according to the mobile phase composition as shown in eq. 1. When an analyte

$$SP + R_4N^+ + C^- \rightleftarrows SP\cdots R_4N^+C^- \quad (1)$$

$$SP\cdots R_4N^+C^- + X^- \rightleftarrows SP\cdots R_4N^+X^- + C^- \quad (2)$$

anion, X^-, is introduced into the column it competes with the counteranions, C^-, that are present at the R_4N^+ sites according to an anion exchange like selectivity as shown in eq. 2. If the counteranion is detector active, ID of the analyte is possible since an equivalent amount of C^- is displaced by X^- (4,6). A negative chromatographic peak (decrease in detector signal) is produced providing the column effluent is monitored at a detector setting where the counteranion is detector active. The detector electronic offset is used to compensate for the significant background signal. Thus, two major requirements for ID are: 1) the detector should have a suitable offset and 2) the detector active counteranion concentration in the mobile phase must not exceed the response characteristics of the detector. For example, for IPD the background absorbance should not exceed 0.7 absorbance units.

The enhanced retention, elution, and separation of analyte anions are a function of parameters which influence the equilib-

rium positions of eqs. 1 and 2. The major ones are the following. As the carbon number of the alkyl groups in the R_4N^+ salt increases the equilibrium amount of R_4N^+ salt maintained on the stationary phase surface increases. The amount retained also depends on the counteranion C^-. For a given ionic strength the greater the anion exchange like selectivity is for the selected counteranion the larger the equilibrium amount of R_4N^+ salt on the surface. Furthermore, the amount retained increases as ionic strength increases. If an aqueous-organic modifier mixture is used the equilibrium amount retained decreases as the organic modifier concentration increases and the effect follows the order CH_3CN > EtOH > MeOH. Elution time for the analyte anion decreases for a given R_4N^+ salt when the organic modifier, counteranion, or R_4N^+ salt concentration is increased or when a counteranion of higher anion exchange selectivity is used. The pH and buffer components are also variables, particularly when separating analyte anions derived from weak acids.

In optimizing the variables to achieve a particular separation opposing mobile phase effects must be adjusted. For example, increasing the R_4N^+ salt produces a maximum in analyte anion retention because the increased counteranion concentration, which accompanies the R_4N^+ salt and reduces analyte anion retention, eventually overcomes the increased retention due to the larger number of R_4N^+ sites maintained on the stationary phase. Similarly, increasing ionic strength yields a retention maximum because the added counteranion concentration eventually overcomes the effect of the increased number of R_4N^+ sites.

At a basic mobile phase pH, amino acids (AA) are anions, AA retention is enhanced, and separation is possible on a reversed stationary phase when the mobile phase also contains a R_4N^+ salt (14). In this study an organic polymeric stationary phase rather than a bonded silica was used because of the required basic conditions. If the counteranion used absorbs, fluoresces, or is electrochemically active then detection without derivatization is possible by IPD, IFD, or IED, respectively. Since ID is based on determining the difference between the background signal and the new signal in the presence of the analyte, mobile phase variables affecting retention and resolution are also the ones that affect the sensitivity of the detection as are the capabilities of the detector. Optimization, therefore, is a compromise between how these variables affect retention and resolution and ID.

Table I indicates that as the chain length (or carbon number) increases within the symmetrical R_4N^+ salts the equilibrium amount of R_4N^+ salt maintained on the PRP-1 stationary phase, as determined by breakthrough experiments, increases. For a given R_4N^+ salt increasing its concentration, the ionic strength (for example NaI concentration in Table I), switching to a counteranion of higher anion exchange selectivity (for example, salicylate anion rather than I^-), or decreasing the organic modifier increases the equilibrium amount of R_4N^+ salt on the PRP-1.

For a given mobile phase condition retention of AA increase as the R_4N^+ alkyl chain length increases and, in general, as AA side chain polarity decreases. For the more nonpolar side chain

TABLE I. Amount of R_4N^+ Salt Maintained on PRP-1 as a Function of Alkyl Chain Length

R_4N^+ Salt[a]	μeq R_4N^+/Column[b]
TBABr	22
TPABr	100
THxABr	205

a. Tetrabutylammonium bromide, tetrapentylammonium bromide, tetrahexylammonium bromide.

b. A 1.0 mM $R_4N^+Br^-$, 0.10 mM NaOH, 0.10 mM NaI, 5:95 $CH_3CN:H_2O$ mobile phase and a PRP-1 column.

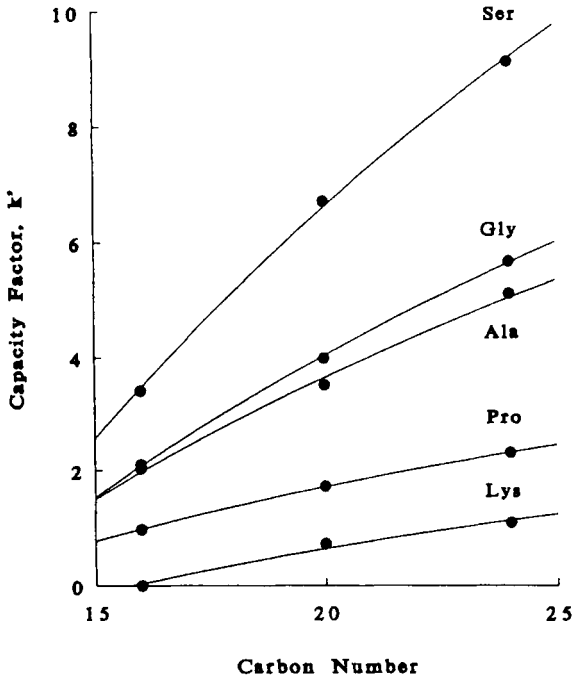

FIGURE 1. Effect of R_4N^+ Carbon Number on Amino Acid Retention. A 1.0 mM $R_4N^+Br^-$, 0.10 mM NaOH, 0.10 mM NaI, pH = 9.5, 5:95 $CH_3CN:H_2O$ mobile phase and IED (800 mV versus Ag/AgCl).

AA chromatographic selectivity also increases as side chain polarity decreases. These effects are illustrated in Figure 1 where retention data for more polar side chain AA are shown using the three symmetrical R_4N^+ salts listed in Table I. In Figure 1 the iodide counteranion is also electrochemically active and therefore detection of each AA is possible by IED (+800 mV versus Ag/AgCl or where I^- is oxidized).

A tetrapentylammonium (TPA^+) salt was selected for additional studies because this salt: 1) provides optimum modification of the PRP-1 surface in terms of retention and detection, 2) does not introduce undesirable surface active properties in the mobile phase, and 3) is readily converted into different counteranion forms which conveniently allowed a quick survey of the merits of ID. When the TPA^+ salt concentration is constant and counteranion concentration (NaBr) is increased AA retention decreases. This is shown in Figure 2 for TPA salicylate as the additive. The salicylate counteranion absorbs (298 nm) and fluoresces (λ_{ex} = 300 nm and λ_{em} = 410 nm) and either IPD or IFD, respectively, is possible. If TPA^+I^- is used, IED is possible. If NaCl or NaF is used instead of NaBr the effect on reducing AA retention follows the order $Br^- > Cl^- > F^-$ which is consistent with the anion exchange selectivity for these anions (16). Elution (counteranion selectivity) order for other counteranions was determined and AA retention was reduced according to the order:

naphthalenedisulfonate > naphthalenesulfonate > benzenesulfonate > salicylate > benzoate > $CO_3^{2-} > I^- > Br^- > Cl^- > OH^- > F^-$ (3)

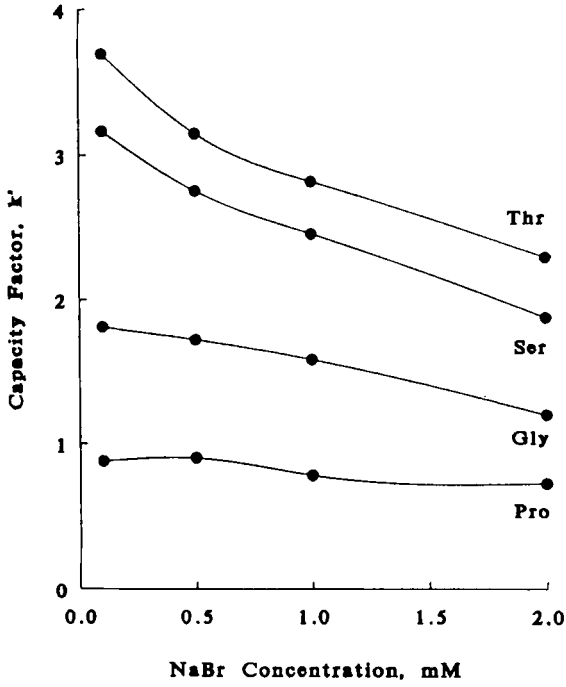

FIGURE 2. Effect of Counteranion Concentration on Amino Acid Retention. An aqueous 0.10 mM TPA salicylate, 0.10 mM Na_2CO_3, NaBr, pH = 9.5 mobile phase and IPD (λ = 298 nm).

While favorable eluting conditions can be obtained with multiple counteranions, one for ID and one for eluent strength, ID interference can occur because each counteranion will produce a system peak (4,7,8,16). The system peak elution time will depend on the counteranion, its concentration, anion exchange selectivity, and the effects of each on the equilibria in eqs. 1 and 2. From a practical viewpoint a counteranion of high anion selectivity is desirable since system peak elution time increases as selectivity increases. However, this kind of counteranion can

also produce an eluent that is too strong and a compromise between these two factors is often required.

Increasing the CH_3CN in a basic, TPA^+ salt mobile phase reduces the equilibrium amount of TPA^+ salt maintained on the stationary phase surface and consequently AA retention decreases (12-15). For example, for an aqueous pH = 9.5, 0.1 mM Na_2CO_3, 0.1 mM TPA salicylate mobile phase 124 μeq are maintained on the PRP-1 column compared to 6.2 μeq/column when the solvent is 4:1 CH_3CN:H_2O. Lowering the pH also decreases AA retention as the AA charge shifts from anion to a zwitterion. A pH of about 9.4 was used for data reported here. Most often Na_2CO_3 was the buffer salt since maintaining a CO_2-free basic mobile phase is difficult and therefore a carbonate system peak appears in the chromatogram. The carbonate system peak, which occurs at a high retention time, and its eluent strength was minimized by using a low carbonate mobile phase concentration. A higher pH, while desirable because it increases AA retention as the anion, also shifts the OH^- system peak to a lower retention time into the chromatographic region of interest and increases its peak area.

Table II lists retention data for several AA using benzoate, salicylate, and iodide as mobile phase counteranions that allow IPD, IFD (or IPD), and IED, respectively. AA elution order and level of retention is a function of the counteranion selectivity, its effect on TPA^+ salt retention, and the effect of AA side chain polarity. As side chain polarity decreases or becomes negatively charged retention increases. In Table II the low AA retention in

TABLE II. Retention of Amino Acids as a Function of Counteranion Using IPD, IFD, and IED

Amino Acid[a]	Benzoate[b]	Salicylate[c]	I^- [d]
Arg			0
Lys			0.72
Pro	1.87	1.22	1.72
Ala	3.11	2.20	3.51
Gly	3.35	2.44	3.98
Hyp	4.41	2.97	
Ser	6.88	4.08	6.69
Asn	7.83	4.16	8.00
Thr	8.74	4.69	7.69
Gln	9.39	5.21	7.00
His•HCl[e]			8.54
Val	10.5		
Ile	22.1	11.1	
Leu	27	12.7	
Met	36	18.6	
Asp	98	27	
Glu	130	33	
OH^- [f]	15.9	6.75	
CO_3^{2-}/HCO_3^- [f]	>180	51	

a. All L-amino acids.

b. An aqueous 0.10 mM tetrapentylammonium benzoate, 0.10 mM Na_2CO_3, pH = 9.4 mobile phase with IPD at 228 nm.

c. Same as (b) except 0.10 mM tetrapentylammonium salicylate and IPD at 298 nm or IFD at λ_{ex} = 300 nm and λ_{em} = 400 nm.

d. A 1.0 mM tetrapentylammonium bromide, 0.10 mM NaOH, pH = 9.5, 0.10 mM NaI, 5:95 $CH_3CN:H_2O$ mobile phase and IED at 0.500 V versus Ag/AgCl.

e. Overlaps with the OH^- system peak.

f. System peaks.

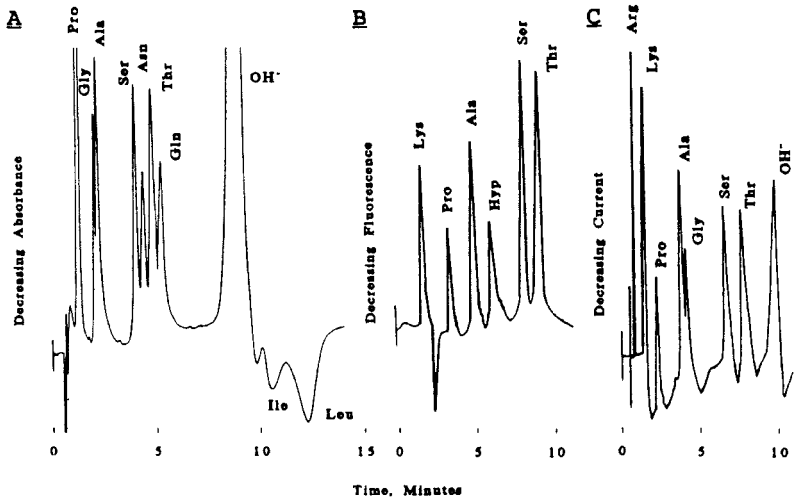

FIGURE 3. Separation of Several Polar and Intermediate Polar Amino Acids. (A) Mobile phase as in (b) in Table II except 0.05 mM Na_2CO_3, 2.5:97.5 $CH_3CN:H_2O$ and IPD. (B) Mobile phase as in (c) in Table II and IFD. (C) Mobile phase as in Figure 1 and IED.

the I^- mobile phase is due to the high Br^- concentration since I^- eluent strength (see eq. 3) under identical conditions is weaker than either benzoate or salicylate as the counteranion.

Figure 3A to C illustrate the separation of several polar AA using a TPA benzoate, TPA salicylate, and a TPABr/NaI mobile phase with IPD, IFD, and IED, respectively. The more polar AA peaks in the IPD chomatogram (Figure 3A) are negative because in the IPD strategy the detector active counteranion concentration is reduced in the analyte band by an amount stoichiometrically equivalent to the amount of AA anion present (see eq. 2). However, after the major OH^- system peak the ionic strength and AA^- counteranion exchange selectivity become major factors and the amount of TPA

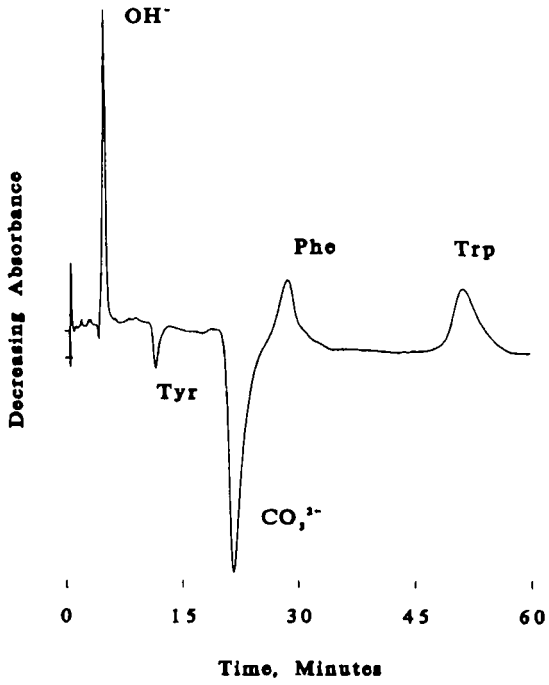

FIGURE 4. Separation of Nonpolar Amino Acids. An aqueous 0.10 mM TPA salicylate, 0.20 mM Nasalicylate, 0.10 mM Na_2CO_3, pH = 9.4 mobile phase and IPD (298 nm).

benzoate in the analyte band becomes larger relative to the background resulting in a positive peak. This effect of the system peak and the equilibria involved on analyte peak direction is consistent with previous studies (8). Peak reversals also would occur in Figures 2B and 3C if AA of higher retention were in the mixture. Separation of nonpolar AA, such as Phe and Trp, at favorable elution times requires a stronger eluent. This is illustrated in Fig. 4 where Tyr, Phe, and Trp are separated and detected by IPD using a higher counteranion concentration.

Separation of AA covering a range of side chain polarity, requires a significant gradient change to obtain a resonable analysis time. This type of gradient produces a large change in the background signal since the amount of detector active counteranion changes appreciably during the gradient. Even if the gradient involves only a solvent change, while all other factors such as the detector active counteranion are held constant, the background signal changes since the solvent change affects the equilibrium amount of $R_4N^+C^-$ salt on the stationary phase. While gradients can be used with ion interaction reagents and ID by a dual column strategy (17) and/or computer gradient baseline substraction (18) the procedures are complex and detection limits are less favorable. Suitable isocratic eluents for AA group separation and IPD for the more polar AA (Lys to Val), intermediate polarity (Ile to Asp) and more nonpolar (Glu to Trp) on PRP-1 are aqueous - 0.10 mM TPA salicylate - 0.10 mM Na_2CO_3 - pH = 9.2, 2.5:97.5 $CH_3CN:H_2O$ - 0.10 mM TPA benzoate - 0.050 mM Na_2CO_3 - pH = 9.2, and aqueous 0.10 mM TPA salicylate - 0.20 mM Na salicylate - 0.10 mM Na_2CO_3 - pH = 9.4, respectively.

The application of R_4N^+ salts and ID using a detector active counteranion is particularly suited to the separation and detection of AA mixtures within each of these subsets since the derivatization steps are eliminated and detection limit is still favorable. For example, the determination of L-Gln, γ-aminobutyric acid (GABA) and L-Glu mixtures are vital in studies of neurotransmitters and related diseases including Huntington's disease,

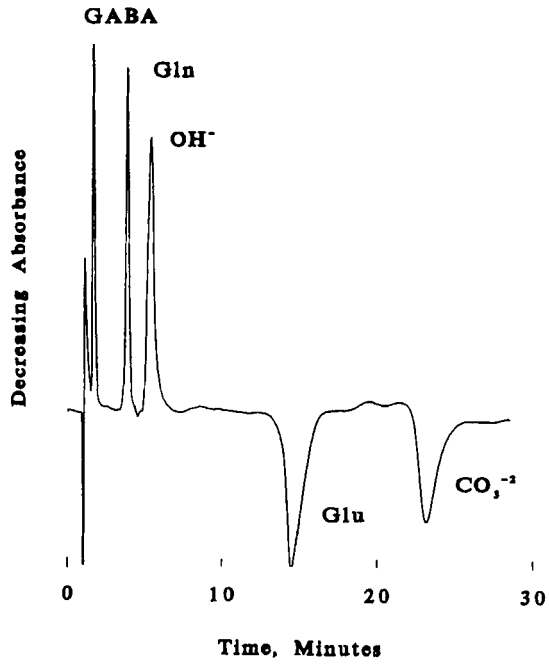

FIGURE 5. Separation of Amino Acids of Neurological Importance. Mobile phase same as Figure 4 and IPD.

Alzheimer dementias, and Parkinson's disease. Figure 5 illustrates the separation of the three AA using a salicylate mobile phase and IPD. The separation is selective for L-Gln, GABA, and L-Glu and only L-Thr (k' = 4.69) overlaps the L-Gln (k' = 5.21) peak. Also, system peaks do not interfere. Detection limit for L-Glu in Fig. 5 is about 10 ng for a 3:1 signal:noise.

The ID detection limits for the separation of free amino acids depend primarily on the AA (lower retained AA have lower detection limits), the experimental conditions, the magnitude of the background signal due to the detector active counteranion, and

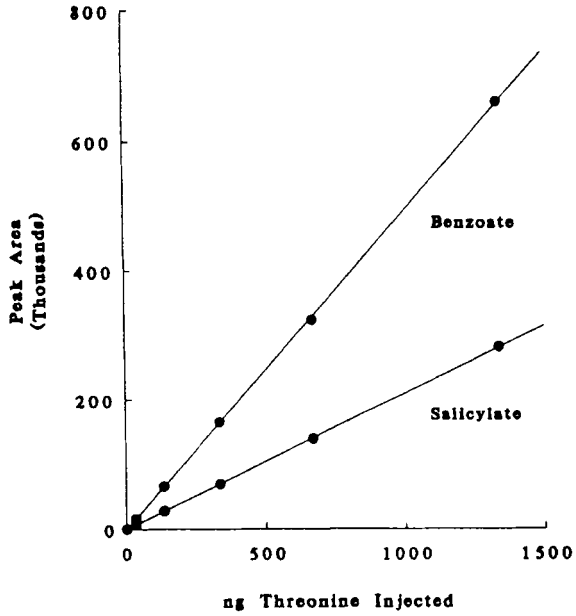

FIGURE 6. Effect of Counteranion Absorptivity on Calibration Curve for Thr. Mobile phases as in (b) and (c) in Table II and IPD.

the detector offset capabilities. Figure 6 compares the calibration curves obtained for Thr using IPD and benzoate and salicylate as the detector active counteranions. The curves are linear over a wide Thr range (upper limit not determined) and the sensitivity is greater for the benzoate mobile phase because at the conditions of the measurement benzoate yields the higher molar absorptivity. This also means that the background absorbance is higher and therefore the maximum amount of benzoate in the mobile phase that can be offset by the detector is lower which in turn limits the maximum eluent strength that can be obtained.

The Thr peak occurs in the earlier portion of the AA separation (see Fig. 3 and Table II) and its calibration curve and detection limits are characteristic of other AA in this region. A calibration curve for Leu, which is more highly retained, was also determined using IPD and the benzoate eluent as in Fig. 6. Leu's calibration curve with a slope similar to Thr corresponded to: Peak area = 262 (ng Leu) - 1360. The Leu peak is opposite in direction to Thr (see Fig. 3) and this does not influence the ability to quantitate the Leu.

A summary of detection limits using IPD, IFD, and IED and Ala as the analyte are listed in Table III. The detection limits were determined at chromatographic conditions which provided baseline resolution from neighbor eluted polar AA. A lower detection limit can often be achieved at the expense of chromatographic resolution which is an option when only a few AA are being separated.

In IPD sensitivity and detection limits are directly related to molar absorptivity. For example, for Ala in Table III detection limit is halved for an increase in molar absorptivity from 3800 to 8500 (benzoate) $cm^{-1}mol^{-1}L$. The detection limit using IED with I^- as the detector active counteranion is lower than either IPD or IFD. However, IED, which is discussed in detail elsewhere (12), requires very careful control of the detector and mobile phase conditions in order to ensure reproducible results. Detection limit using the available fluorescence detector appeared to be limited by the background fluorescence and noise associated with this signal which must be offset. While improvements are

TABLE III. Typical Detection Limits Using Indirect Detection Strategies

	Detection Limits	
Counteranion	Detection	ng Ala
salicylate	IPD	10
benzoate	IPD	5
salicylate	IFD	17
iodide	IED	0.8

possible with other fluorescence detectors IPD is recommended because the major advantages of IPD are: detection limit is favorable, counteranions differing in absorptivity and eluent strength are available, reproducibility is good, IPD is easy to use and requires low maintenance, and absorbance detectors offering favorable offset and sensitivity are available.

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