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Practical Aspects of Reverse Phase Ion Pair Chromatography*

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Abstract

Practical aspects of reverse phase ion pair chromatography are discussed and illustrated with various examples. The effects of counterion type, size, and concentration are illustrated on diverse solutes such as linear alkylbenzene sulfonates, catecholamines, and dyestuffs. A recommended approach to performing an RP-IPC separation is given.

Introduction

The development of ion pair chromatography (IPC) is generally attributed to G. Schill and his co-workers (1-6). These workers were the first to apply ion pair extractive techniques to modern liquid chromatography. They found that silica gel and cellulose columns could be coated with a reagent capable of forming ion pairs and using relatively nonpolar mobile phases, excellent separation of ionic species could be obtained. Before long, other workers expanded on this technique (7-12) illustrating its ability to separate a wide range of compounds (biogenic amines, drugs and metabolites, dyestuffs, carboxylic acids, etc.).

The IPC technique can, in many cases, be used for ionic or ionizable compounds in place of ion exchange. With proper use of the technique, complex separations can be readily achieved. Reverse phase IPC (RP-IPC), where one uses a hydrophobic stationary phase and aqueous buffers containing a low concentration of counterion, allows one to separate both ionized and un-ionized components under the same chromatographic conditions. Aqueous samples such as physiological fluids may be chromatographed by direct injection without removal of water. The selection and optimization of conditions to achieve RP-IPC separations are the subjects of this paper. Operational details on the use of bonded phase packings in RP-IPC will be discussed and illustrated. The major goal is to provide the chromatographer with some practical guidelines to assist the development of a RP-IPC separation. RP-IPC techniques are still in their infancy and many further developments will be forthcoming which may change these general guidelines.

Theoretical Considerations

The basic theory of ion pair techniques has been discussed by Schill (3). Although the theory for batch extraction of ion pairs has been thoroughly confirmed, the exact mechanism for IPC has not clearly been established to date. There are two fundamental proposed models. The first postulates that the solute molecule forms an ion pair with the counterion in

the mobile phase. This uncharged ion pair then partitions into the lipophilic stationary phase. The other mechanism postulates that the counterion partitions into the stationary phase with its ionic group oriented at the surface. The column then behaves like a liquid ion exchange column. In actuality, the true mechanism surely involves both postulates but is probably made more complex by *adsorption, micelle formation, and complexation of both the solute and the ion pair reagent*. In any case, for the practicing chromatographer, the formation of the ion pair in the mobile phase is conceptually the easiest model to understand. We will use this approach to develop the basic equations of RP-IPC for a cationic species.

A cation (R^+) may be partitioned into a nonpolar stationary phase as an ion pair (R^+C^-) formed with a counterion (C^-):

The overall equilibrium constant of this reaction is expressed as:

$$K_{IP} = [RC]_{org} / [R_{aq}^+][C_{aq}^-] \quad (2)$$

This may be rearranged to express the ratio of the solute in the two phases:

$$[RC]_{org} / [R_{aq}^+] = K_{IP}[C_{aq}^-] \quad (3)$$

The retention time (t_R) of a solute in HPLC is given by:

$$t_R = (L/\mu)(1 + k') \quad (4)$$

where L = length of the column,

μ = linear velocity of the eluent,

k' = capacity factor, the number of moles of solute in the stationary phase divided by the moles in the mobile phase,

$$\text{or} \quad k' = [RC]_{org} V_s / [R_{aq}^+] V_m \quad (5)$$

where V_s = volume of stationary phase

V_m = column void volume

Substituting into equation 4 we get

$$t_R = (L/\mu)[1 + (V_s/V_m)(K_{IP}[C_{aq}^-])] \quad (6)$$

For bonded phase columns V_s/V_m is constant, thus we may further simplify to:

$$t_R = (L/\mu)(1 + K[C_{aq}^-]) \quad (7)$$

where $K = K_{IP}(V_s/V_m)$.

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From equation 7 it is easily seen that retention may be controlled by the binding strength of the ion pair K_{IP} and the concentration of the counterion. The more soluble the ion pair in the hydrophobic stationary phase the longer it will be retained, i.e., k' is larger. Solubility in the stationary phase may be increased by using increasingly hydrophobic counterions. Equation 7 also states that, as the concentration of the counterion is increased, the retention time should also increase. It should, however, reach a limit and remain constant when all of the solute exists in the ion pair form. However, experimental results reported here and elsewhere (9) show that for certain counterions above a maximum concentration, t_R may actually decrease.

Factors Controlling Retention

There are many other factors which enter into an RP-IPC separation. For clarity, Table I gives a brief description of each factor's role in an RP-IPC separation.

Table I. Variables of an RP-IPC Separation

Variable	Effect
Type of counterion	The better the ability to ion pair, the longer the retention.
Size of counterion	An increase in the size of the counterion will increase retention.
Concentration of counterion	Increasing concentration increases retention up to a limit (see text).
pH	Effect is dependent upon nature of solute. Retention increases as pH maximizes concentration of ionic form of solute.
Type of organic modifier	Retention decreases with increasing lipophilic nature.
Concentration of organic modifier	Retention decreases with increasing concentration.
Temperature	Retention decreases as temperature increases.
Stationary phase	More lipophilic or higher degree of coverage, more retention.

With this myriad of parameters available to effect a separation, it is somewhat difficult to select a reasonable starting point for investigation.

For samples containing strongly and weakly retained compounds, gradient elution is often necessary. In RP-IPC the following parameters may be changed to effect a gradient separation:

1. Change the lipophilic character of the counterion (i.e., by use of mixed counterions); decreasing the lipophilicity of the counterion with time will decrease retention.
2. Concentration of the counterion; decreasing with time will decrease retention.
3. pH; decreasing the degree of ionization will decrease retention.
4. Concentration of organic modifier; increase in organic concentration decreases retention.

In practice, only 1, 2 and 4 have been used with the change in concentration of organic modifier being the most popular because of its similarity to regular RP techniques. Reverse phase chromatography (RPC) on bonded phases is now a very familiar technique and the ability of the technique to separate a wide variety of both polar and nonpolar solutes has been shown in numerous examples. By controlling all of the variables excepting the concentration of the organic modifier, the IP separation can be considered a reverse phase separation, i.e., elution in order of decreasing ion pair "polarity."

The control of the pH is a most important parameter since it will dictate the concentration of the ionic form of the solute. Obviously, for greatest retention we desire maximum ionization. Table II presents general guidelines on the effect of pH. The major restriction to pH selection is the stability of the column packing. The reader is cautioned that these are only general guidelines for pH selection and that each case should be studied separately to determine the optimum pH necessary to achieve the desired result.

Table II. Selection of pH

Type of Solute	Example	pH (for RP-IPC)	Comment
Strong acid ($pK_a < 2$)	Sulfonated dyes	2 - 7.4	These solutes are ionized throughout the pH range; actual pH selected is dependent upon other types of solutes present.
Weak acids ($pK_a > 2$)	Amino acids, Carboxylic acids	6 - 7.4	Solutes ionized; retention dependent upon nature of ion pair.
		2 - 5	Ionization of solutes is suppressed; retention dependent upon nature of solute (not ion pair)
Strong bases ($pK_a > 8$)	Quaternary amines	2 - 8	Solutes are ionized throughout pH range; similar to strong acids.
Weak bases ($pK_a < 8$)	Catecholamines	6 - 7.4	Ionization is suppressed; retention dependent upon nature of solute.
		2 - 5	Solutes are ionized; retention dependent upon nature of ion pair.

Typical Counterions, Solvents and Columns of RP-IPC

To aid in successful operation of the technique, it is desirable at this point to briefly describe the reagents and columns available for RP-IPC.

Solvents

Just as in RP on bonded phases, the most commonly employed solvent combinations are water/methanol and water/acetonitrile. The major limitation is solubility of the ion pair reagent.

Typical Counterions

Type	Main Applications
Quaternary amines, e.g. tetramethyl, tetrabutyl, palmityl trimethyl ammo- nium ions	For strong + weak acids Sulfonated dyes, carboxylic acids
Tertiary amines, e.g., triethylamine	Sulfonates
Alkyl and aryl sulfonates, e.g. methane or heptane sulfon- ate, camphorsulfonic acid	For strong + weak bases Benzalkonium salts, catechol- amines
Perchloric acid	Forms very strong ion pairs with a wide range of basic solutes
Alkyl sulfates; lauryl sulfate	Similar to sulfonic acids; yields different selectivities.

Columns

Reverse phase packings with C_2 -, C_8 - and C_{18} - alkyl groups have all been employed (5,6,11,12); the length of the alkyl group may or may not be an important parameter since the mechanism of the separation may change with the length of the alkyl group. Adsorption can play a significant role. The major limitation is the workable pH range and lifetime of the columns. Optimum performance and lifetime is achieved in the pH range of 2 - 7.4.

Experimental

Chemicals

The solvents employed were "distilled-in-glass" grade obtained from Burdick and Jackson, Muskegon, Michigan. The water was distilled in an all-glass still. The linear alkyl benzene sulfonates (LAS) sample is produced under the tradename Marlon A by Chem. Werke, Huels, Germany. The dyes and chemicals studied were obtained from commercial sources. The topical disinfectants Hyamine 10X and 1622 were gifts from FDA Western Regional Laboratory, San Francisco, California. The dansyl amino acids were obtained in purified form from Pierce Chemical. The catecholamines were obtained from Sigma Co.

Purification of Reagents

The ion pair reagents were obtained as reagent grade from either Eastman Kodak, Aldrich Chemical Co., or Pfaltz and Bauer. When low wavelength UV detection was used the reagents were purified by the following technique: A 0.1 or 0.5 M solution (depending upon solubility) of the selected reagent was prepared in distilled water. This solution was then passed through a reverse phase column at a flow rate of 1-2 ml/min. The eluent was collected and diluted to the desired concentration prior to subsequent chromatography. The effectiveness of this technique is illustrated in the Results section.

Most of the sulfonates and sulfates used were the sodium salt. In these cases, to obtain the desired pH, buffers or strong mineral acids were used. Tetraalkylammonium counterions were usually in the chloride form.

Apparatus

The HPLC utilized dual syringe pumps (Varian 8520) and was equipped with either a variable wavelength UV detector (Varichrom®; Varian) or a filter fluorometer (Fluorichrom®; Varian). Throughout the text, the "A" solvent is the weaker solvent placed in the A pump and the "B" solvent is the stronger solvent placed in the second or B pump.

Columns

The columns were 25 cm × 2.2 mm MicroPak® -MCH (Varian). The column packing is composed of a monomolecular layer of octadecylsilane bonded to the surface of the 10 μ m silica.

Conditions for LAS Study

For the initial work the aqueous phase was adjusted to pH of 6.5 with $NH_4H_2PO_4$ (0.001M) after making it 0.01M in the desired ion pair reagent. The proper amount of the ion pair reagent was dissolved in methanol to make it 0.01M. Gradients from 50 to 80% MeOH at + 1%/min were used.

Conditions for Hyamine Study

The A solvent was water adjusted to pH 2 with the proper acid and/or salt present. The B solvent was 90/10 v/v acetonitrile/water containing exactly the same concentration of acid and/or salt present. Thus, throughout the analysis, the concentration of the ion pair reagent was held constant. The analysis was started at 60%B and increased linearly at + 1%B/min to 80%B.

Equilibration Study

In these experiments the concentration of ion pair reagent was maintained constant. The type of counterion and the organic modifier (methanol) concentration were studied. Injections of a two-component mixture were made at the initial isocratic conditions until the k' 's for a two component mixture were constant. At this point a gradient of + 4%B/min was run for 10 minutes. This increased concentration was maintained for 10 minutes and then the eluent was returned to its initial composition by either a step change or a reverse gradient at -10%B/min. Injections were made within 5 minutes after achieving initial conditions. The injections were continued until k' 's equaled those obtained prior to the gradient run. In all cases a minimum of five column volumes were pumped before making injections.

Results and Discussion

Solubility of the Ion Pair Reagent

The solubility of the counterion is very important. If the counterion is not soluble in the organic modifier, precipitation may occur within the chromatographic system and plug columns or connecting lines. It is wise to check the solubility of the proposed counterion in the least polar chromatographic solvent to be used. The most useful solvent combination in RP-IPC is water/methanol since this combination readily dissolves a wide variety of ion pair reagents. Although use of acetonitrile offers better column efficiencies (due to its lower viscosity), its usefulness is limited by the poor solubility of many ion pair reagents.

The quaternary amines used as counterions are generally available as the chloride salts. They are for the most part readily soluble in methanol, but their solubility in acetonitrile is substantially lower. The bromides and iodides have higher solubilities, but have limited use with UV detection due to their high background absorbances at low wavelengths.

Perchlorate salts have excellent solubilities in acetonitrile but their general use is not recommended for two reasons: (a) perchlorate anion itself is a good ion pair reagent and may lead to undesirable side reactions such as complexation of the counterion, thus lowering the effective concentration available for the ion pairing of the solute; and (b) the salts in their solid form pose a potential explosion hazard.

With acetonitrile the chloride form may be used if sufficient water is present to increase the solubility of the quaternary amine salt. Generally, 10-20% water is sufficient, the amount varying with each salt. The only drawback to this approach is that an upper limit is placed upon the range of organic modifier available for gradient analysis. If retention times are excessive at a concentration of 80-90% acetonitrile, for example, then a less hydrophobic counterion should be used.

Similar solubility problems exist for the sodium sulfonates and sulfates used in RP-IPC of cationic species. In general, the sulfonates are readily soluble in methanol. Again, water must be added to acetonitrile to obtain reasonable solubilities. Surprisingly, the detergent sodium lauryl sulfate displays markedly higher solubility in acetonitrile rather than methanol.

Since many of the counterions generally employed are obtained as neutral salts, it is necessary to add a buffer to obtain the desired pH. When selecting a buffer ensure that it, too, is soluble in the organic solvent under all chromatographic conditions to be used. To obtain pH values in the 2-3 range, perchloric or sulfuric acid may be used. If acetic acid or other weak acid is used, the larger concentrations necessary to achieve such an acidic pH may play a significant role in the elution behavior of the solutes. Remember that the buffer components may be ion paired also so low concentrations are desirable!

Purity of Ion Pair Reagent

For the most part, the commercially available reagents may be used as received without further treatment. However, we have found that when low wavelength UV detection (<230nm) is employed, many of these reagents contain impurities which have large absorptivities. The source and identity of these impurities is not known; however, in many cases, it appears to be residual solvent or reaction by-products from the manufacturing process.

We have found that the simple cleaning procedure described in the Experimental section is quite useful. The effectiveness of this cleaning procedure is depicted in Figure 1. The chromatogram in Figure 1a shows several spurious peaks obtained when 250 μ l of distilled water were injected into the system using 0.1M tetramethylammonium chloride (TMA) as the counterion and employing a detection wavelength of 224nm. When the same water was injected into a system using "cleaned" TMA, the chromatogram in Figure 1b is obtained. Note the much improved baseline. Apparently, the water displaces impurities in the TMA that are being deposited at the head of the column.

Types of Counterions

Previously we tabulated the various materials that have found use in RP-IPC. These materials range from strong acids such as perchloric and alkyl sulfonates to the significantly weaker alkyl sulfates used for pairing with basic solutes. For ion pairing with acidic solutes, quaternary amines ranging from TMA to palmityltrimethylammonium (PMA) have been used. If the pH is sufficiently low (<pH = 4) then tertiary amines may also be employed.

The choice of counterion type to use is rather straightforward.

Figure 1. Purification of the counterion solution for low wavelength UV detection: a) TMA "as is," b) TMA cleaned as described in text; 250 μ l distilled water injected in both cases; Conditions: 68/32 v/v MeOH/H₂O; 0.01M TMACl; Flow: 0.5 ml/min. Column: 25 cm x 2.2 mm MicroPak® -MCH. Detector: Varichrom® at 224 nm and 0.01 AUFS.

ward. For basic solutes, one could begin with an alkyl sulfonate (RSO_3^-). For acidic solutes use a quaternary amine. The actual selection of the alkyl radical, i.e., $\text{C}_1, \text{C}_2, \dots, \text{C}_{12}$, etc., is dependent upon the type of separation desired and this will be discussed in the next section.

The detergent sodium lauryl sulfate $\text{CH}_3(\text{CH}_2)_{11}\text{OSO}_3^- \text{Na}^+$ has been reported to be a useful ion pair reagent (10). Due to its low cost relative to the sulfonate, it is of interest to compare this sulfate to sodium lauryl sulfonate $\text{CH}_3(\text{CH}_2)_{11}\text{SO}_3^- \text{Na}^+$ for a given separation. The sulfate is a weaker acid and might be expected to be a much weaker ion pair reagent. The concentration at which micelles (aggregates of detergent molecules) form is different for the sulfate and the sulfonate and may play a role in the separation. A separation of four catecholamines was compared using lauryl sulfate or lauryl sulfonate as the ion pair reagent. Selectivities, as well as resolution, were completely changed as illustrated in Figure 2. These large changes in selectivity were not readily explainable but they did show that lauryl sulfate can be used to improve resolution for catecholamines.

Size of the Counterion

The hydrophobic nature of the counterion can play a profound role in the resultant chromatography. In general, increasing the length (i.e., size) of the alkyl chain will produce a corresponding increase in retention of a given solute. How-

Figure 2. Comparison of lauryl sulfate and sulfonate counterions in the separation of catecholamines: a) sodium lauryl sulfate 0.005M; b) sodium lauryl sulfonate 0.005M. Eluent: 20/80 v/v acetonitrile/H₂O (pH 2.2 with HClO₄). Flow: 0.5 ml/min. Column: 25 cm x 2.2 mm MicroPak®-MCH; Detection: 254nm at 0.16 AUFS; Injection size: 2 µl. DOPA = 3,4-dihydroxyphenylalanine; Nor = Norepinephrine ADR = Epinephrine; SYN = Synephrine.

ever, selectivity may not be affected. If the chromatographer is attempting to separate solutes with only subtle molecular differences, then the ion pair reagent should be selected so as to not dominate the chromatographic behavior of the ion pair. Thus, an ion pair reagent of small size is desirable. On the other hand, if increased retention is desired, then a large ion pair reagent is the logical choice.

Linear Alkyl Benzene Sulfonates (LAS). The LAS are the major surfactant in household detergents at the present time. The detection of such substances in environmental samples is usually made by the methylene blue method (13) or by gas chromatography (14). However, the methylene blue method is unspecific while the GC method requires chemical treatment (desulfonation) prior to the separation step. High performance LC offers a potentially easier approach.

The LAS have the general structure:

where $x + y$ is usually equal to 7, 8, 9 and 10.

The goal was to separate the various LAS constituents based upon the alkyl chain length, thus it could be reasoned that a small counterion would be the most effective if ion pairing techniques are necessary.

One might expect these materials to exhibit retention by reverse phase techniques. First attempts using a methanol/water reverse phase system resulted in only two major peaks. Attempts to improve resolution by lowering the methanol concentration only broadened the peaks to such an extent that the data were not useful. The anionic sulfonate group would be expected to form an ion pair with a cationic counterion, therefore RP-IPC appeared to be an ideal separation technique. To investigate the effect of counterion size on retention, three different counterions were tried: ammonium, TMA, and tetrabutylammonium (TBA) chlorides.

Ammonium and TMA yielded very similar resolution but as expected TBA displayed a significant loss of resolution because its larger size dominated retention of the ion pair. TMA was selected as the optimum reagent since it yielded higher k' 's with no loss in resolution. Figure 3 shows the optimized separation of a 1ppm standard LAS solution under optimized conditions for their trace determination in water.

Figure 3. Optimized RP-IPC separation of LAS; concentration of LAS 1 ppm; same conditions as Figure 1.

ration of a 1ppm standard LAS solution under optimized conditions for their trace determination in water. The relative peak areas obtained for the four peaks show good agreement with those obtained by gas chromatography (14) as presented in Table III. As can be seen in Figure 4, the method can be employed for the analysis of an actual wastewater sample calculated to contain about 1ppm total LAS.

Topical Disinfectants. Topical disinfectants are often derived from benzalkonium salts. These large bulky quaternary amines generally have only minor structural differences so their separation is often difficult. For example, the structures of Hyamine 10X and Hyamine 1622, differing by only a

Table III. Comparison of LAS Analysis

Alkyl Chain Length	Relative Composition	
	RP-IPC	GC(14)
C ₁₀	1%	1%
C ₁₁	53%	54%
C ₁₂	36%	38%
C ₁₃	10%	7%

Figure 4. Chromatogram of 250 µl of wastewater; conditions same as Figure 1.

methyl group, are illustrated in Figure 5. As was observed previously for LAS, normal reverse phase techniques again yielded broad ill-defined peaks.

Figure 5. Structures of Hyamine 10X and 1622.

For the amines, a counterion capable of existing as an anion in acidic solution was required. The counterions studied were sulfuric, perchloric, methanesulfonic, and heptanesulfonic acids. Table IV shows that resolution was affected with a change in counterion. Sulfuric acid, known to be a poor ion pair reagent, had little effect upon the separation, the results being only slightly better than the poor reverse phase data. Perchloric and methanesulfonic acids both improved peak shapes, but methanesulfonic acid significantly improved resolution. A combination of perchloric and methanesulfonic acid yielded almost baseline resolution. The heptanesulfonic acid increased retention but resolution of the two components was completely lost, indicating that the minor chromatographic differences were masked by the more lipophilic C₇ counterion.

Sulfonated Dyes. For those solutes having different numbers and types of ionic groups present, selectivities can also be changed by varying the size of the counterion. Several dyes, whose structures are shown in Figure 6, were separated by gradient elution RP-IPC. The chromatograms obtained with TMA, TBA, and PMA chloride counterions are shown in Figures 7-9. Red #2, with three sulfonate groups available to form strong ion pairs, displayed quite different retention characteristics in the three different eluents. Yellow #5 also has three anionic sites available for ion pairing but one of them is a weak carboxylic acid group which under acidic conditions would not ion pair as readily as the sulfonate group of Red #2. This functional difference is reflected in its moderate variation of *k'* value with change in counterion.

With TMA and TBA, gradient elution could be carried out easily. However, with the extremely hydrophobic PMA, gradient elution could not be used effectively because of the extremely long equilibration times required. Therefore, an isocratic elution at high methanol concentration was used. Note that the Red #2 peak reversed its elution order relative to Yellow #5. It can be easily seen that *k'* not only increased with the size of the counterion but selectivity was also changed. The poor peak shapes observed with PMA probably indicates poor solute mass transfer.

Table IV. Comparison of Acids Used to Separate Hyamines

Counterion	Concentration	k'		
		Hyamine 1622	Hyamine 10X	Resolution
H ₂ SO ₄	0.005M (pH 2.0)	9.8	11.3	1.3
HClO ₄	0.01M	5.6	6.4	1.2
CH ₃ SO ₃ H	0.01M	6.0	6.8	1.6
H ₂ SO ₄	0.005M (H ₂ SO ₄);			
+ CH ₃ SO ₃ ⁻	0.01M CH ₃ SO ₃ ⁻ Na ⁺	6.5	7.6	1.8
HClO ₄	0.01M (HClO ₄);			
+ CH ₃ SO ₃ ⁻	0.01M CH ₃ SO ₃ ⁻ Na ⁺	10.0	11.3	2.5
CH ₃ (CH ₂) ₆ SO ₃ ⁻	0.005M (H ₂ SO ₄);			
+ H ₂ SO ₄	0.01M CH ₃ (CH ₂) ₆ SO ₃ ⁻	15.1	15.1	~0

Figure 6. Structures of dyes.

Amino Acids. The dansyl derivatives of amino acids are commonly used to obtain good fluorescent sensitivities for these biological significant compounds. Using reverse phase techniques, it is difficult to obtain adequate retention for the di-acids due to their high polarities. By ion pairing them with an organic amine, PMA, comparison of Figures 10 and 11 indicate that the k' 's of the di-acids aspartic and glutamic are significantly increased. In fact, their retentions are changed so significantly that they now elute after many of the mono-acids.

These four diverse examples show the importance of selecting the proper size counterion. If subtle differences in solute structures exist, then a relatively short counterion would be the first choice. To increase retention, improve

Figure 7. RP-IPC separation of dyes using TMA; Column: 25 cm x 2.2 mm MicroPak®-MCH. Solvents: A = 0.01M TMACI in MeOH; B = 0.01M TMACI in H₂O (pH 6.5 with 0.001M NH₄H₂PO₄). Gradient profile: 20% MeOH to 99% MeOH at + 3%/min. Flow: 0.5 ml/min. Detection at 254nm; 0.64 AUFS.

resolution, or eliminate interferences, then a large counterion is desirable. Under some conditions, mixtures of counterions may be used effectively (15).

Concentration of the Counterion

For a given counterion, equation 7 predicts that t_R or k' should increase with increasing concentration of the counterion up to a point where ion pair formation is

Figure 8. RP-IPC separation of dyes using TBA; conditions same as Figure 7 except 0.01M TBA.

Figure 9. RP-IPC separation of dyes using PMA; conditions same as Figure 7 except isocratic at 80% MeOH and 0.01M PMA.

quantitative and then should remain constant. This has been confirmed within a narrow concentration range (1-6). However, Knox and Laird (9) have shown that above a certain concentration, the k' 's of several sulfonated dyestuffs ion paired with the cation cetyl trimethylammonium, actually decreased as the concentration of the counterion was increased. They suggested that a combination of adsorption (partition) and micelle (clusters) formation may be the dominant factors in determining retention. At the lower concentrations, partition of the ion pair and the reagent into the stationary phase may be the major factor controlling the retention. When micelle formation occurs at higher concentrations the solubility of the ion pair is increased in the mobile phase thus resulting in a decrease of retention. We performed similar studies using the

Figure 10. RP-IPC separation of dansyl derivatives of amino acids using TMA. Column: 25 cm x 2.2 mm MicroPak®-MCH. Eluent: 15/85 v/v acetonitrile/H₂O; 0.01M TMAClO₄; water was pH 6.5 with 0.001M NH₄H₂PO₄; Flow: 0.5 ml/min. Detector: Fluorichrom®, $\lambda_{exc} = 365$ nm; $\lambda_{em} = >430$ nm.

anionic counterion lauryl sulfate to ion pair several catecholamines. Figure 12 illustrates the effect of increasing concentration of counterion of the k' values for three catecholamines. All amines displayed similar behavior. In agreement with the data of Knox and Laird, the curves showed a definite concentration maximum of about 0.005M. This appears to be the optimum concentration for lauryl sulfate (C₁₂) and cetyl (C₁₆) trimethylammonium chloride for maximum retention.

A word of caution is advisable at this point. The chromatographer should not conclude that 0.005M is the optimum concentration for all ion pair reagents. The optimum concentration may be dependent upon the strength of the ion pair formed, the extent of adsorption on the stationary phase, and the extent of micelle formation by the counterion. In general, it may be concluded that the optimum concentration for larger counterions is 0.005M or below. For smaller counterions the optimum concentration is probably greater than 0.005M. In fact, for smaller counterions the upper concentration limit may be their solubility or an actual change in the mechanism by a salting out effect. Table V gives an example of increasing k' with increasing concentration for selected catecholamines and two different concentrations of the counterion heptane-sulfonic acid. Thus, even at 0.1M concentration of a smaller counterion retention has been further increased. The concentration of perchloric acid added to establish a pH of 2 was held constant at 0.01M.

Equilibration and the Use of Gradients

In order to compare to the reverse phase technique, only gradients of organic modifier concentration are considered here. An important consideration for the routine use of RP-IPC is the time necessary to achieve re-equilibration after mobile phase composition changes. If equilibration times were

Figure 11. RP-IPC separation of dansyl derivatives of amino acids using PMA. Eluent: 40/60 acetonitrile/H₂O; 0.005M PMACI; All other conditions same as Figure 10.

Figure 12. Capacity factor (k') vs. concentration of lauryl sulfate.

Table V. k' vs. Concentration of Sodium Heptane Sulfonate

Compound	k'		
	0.0	0.01M	0.1M
Noradrenalin	0.2	1.7	2.1
Adrenalin	0.5	2.9	3.6
DOPA	0.7	3.8	5.0

Conditions:

Column: 2 MicroPak M-CH columns, 25 cm $\times$ 2.1 mm, coupled in series.

Mobile Phase: Water; pH = 2.0 with HClO₄ 0.01M sodium heptane sulfonate concentration varies as shown above.

Flow Rate: 0.5 ml/min.

excessive, i.e., greater than 50-60 column volumes, then the routine use of gradients would be greatly restricted.

The data of Table VII show that equilibration time was quite dependent upon the size of the counterion employed. The larger the counterion the longer the time required to achieve initial equilibrium conditions. However, in contrast to other modes of LC (16,17) no significant difference in the time to achieve initial equilibrium conditions was observed when a step change back to initial eluent composition was compared to a programmed reverse gradient back to initial conditions.

Column Limitations

Reverse phase packings of the SiO₂-based type are predominantly used in RP-IPC. The major limitation of these

Table VI. Counterion Size vs. Equilibration Volume

Counterion	Counterion Concentration	Solute	Column Volumes to Achieve Equilibration
None	—	Naphthalene, Anthracene	10
TMA	0.01M	Benzene sulfonic acid, <i>p</i> -toluenesulfonic acid	20
TBA	0.01M	Benzene sulfonic acid, <i>p</i> -toluenesulfonic acid	26
Palmityltrimethyl ammonium	0.005M	Benzene sulfonic acid, <i>p</i> -toluenesulfonic acid	52
Lauryl sulfate	0.005M	Noradrenalin, adrenalin	36
Heptane sulfonate	0.01M	Noradrenalin, adrenalin	20
Methane sulfonate	0.01M	Noradrenalin, adrenalin	15

Test column: 25 cm × 2.2 mm MicroPak-MCH.

Mobile Phase: Water-methanol gradient as described in Experimental.

Flow: 0.5 ml/min.

packings is their limited pH range. The columns of HPLC, whether self-made or obtained prepacked, are rather expensive and it is desirable to have a reasonable lifetime. Wahlund (13) makes note of the relatively short lifetime (1-2 weeks) of a C₂ bonded phase at pH's of 7.9. Our experience has been that the use of pH's greater than 7.4 should be avoided on the bonded phase columns. In addition, as the concentration of counterion increases the pH limit should be decreased since the lifetime appears to be a function of the concentration of the counterion. For example, a C₁₈ column exhibited 2 months of stable operation at a pH of 7.2 and 0.01M TBA. An identical column performed only 2 days at a pH of 7.2 and 0.1M TBA. In both cases, failure of the column was characterized by a decrease in *k'* and a catastrophic increase in the backpressure. It appears that the failure of the column is due not only to hydrolysis of the stationary phase but also due to dissolution of the silica gel substrate as well. Iler (18) has shown that the solubility of silica is enhanced by the presence of quaternary amines which apparently can complex silicates (the soluble form of silica), thus increasing its solubility. This pH limitation on the basic side decreases the usefulness of RP-IPC for the ion pair chromatography of weak organic acids.

On the acid side, pH's as low as 2 have been used successfully (10). In general, the columns display significantly longer lifetimes when used in the pH range of 2-4. This may reflect the decreased solubility of silica gel in these acidic solutions. In acidic media, columns have been used, so far, in excess of 3 months. The utility of RP-IPC could be furthered if high efficiency columns stable to high pH's were available (XAD resins and neutral polystyrene-divinylbenzene resins are candidates).

It appears that packings containing monolayers of C₈- or C₁₈-alkyl groups are good starting choices for the chromatographer beginning RP-IPC. Packings having polymeric coverages should be avoided since mass transfer is slow and peak shapes are generally poor.

Practical Guidelines in Performing RP-IPC Separations

1. Use RP-IPC when other techniques such as RP and ion suppression fail, or if the desired solutes are composed of both nonionic and ionic species.
2. Use a water-methanol mixture; problems with counterion solubility will be minimized. If better efficiencies are required use acetonitrile but recognize that solubility properties may differ.
3. Select a counterion consistent with the desired results. If the solutes are ionic and have only subtle molecular differences, then select a short-chain counterion. If increased retention is necessary, use a large hydrophobic counterion.
4. Ensure the complete solubility of the counterion throughout the range of solvent conditions likely to be encountered during the analysis.
5. Select a pH such that ionization of the solutes is maximized provided that the pH is compatible with the silica-based columns (pH 2-7.4).
6. A good first choice of bonded phase column would be a monolayer C₁₈ (octadecyl) or a C₈ (octyl) RP column. Short-chain bonded phases appear to be less stable (13).
7. Degas the solvents (if necessary) prior to addition of ion pair reagents to minimize foaming problems, especially with long-chain detergent-like compounds.
8. Start with a 0.01M concentration of shorter counterion or if the counterion contains docetyl groups or larger alkyl groups prepare a 0.005M solution. In a two-pump chromatographic system, if a buffer is required to achieve a certain pH, add exactly the same amounts of buffers to both the aqueous and methanol solutions. The concentrations of buffer and/or counterion may require modification based on the initial experiment. A reasonable concentration of buffer is 0.001M to 0.005M. Try to select buffers with poor ion pair properties but good solubilities. (The term buffer is

used rather loosely; our main goal is to ensure the pH does not change significantly when the sample is added).

9. Optimize the separation by usual RP techniques. For those samples containing both nonionic and ionic solutes, optimize the separation of the nonionic solutes first, then select a counterion that will cause the ionic solutes to elute in the desired time window.

Always keep in mind the following operational points:

1. To achieve rapid column equilibrium initially or after a mobile phase composition change, pump the eluent at a flow of 2-3 times that to be used for the analytical separation. Pump a minimum of 20 column volumes before attempting separations.
2. Always maintain a flow of eluent whenever IP reagents are in the chromatographic system. For overnight operation, maintain a slow flow of 3-5 ml/hr to avoid precipitation of counterions and/or buffer or evaporation at detector exit.
3. In many cases, it is not necessary to dissolve the sample in the mobile phase. For those applications where sample is limited direct injection into the chromatographic system yields acceptable results.
4. If low wavelength UV detection is to be used, check the background absorbance of the ion pair reagent. If necessary, purify by using technique described earlier.

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