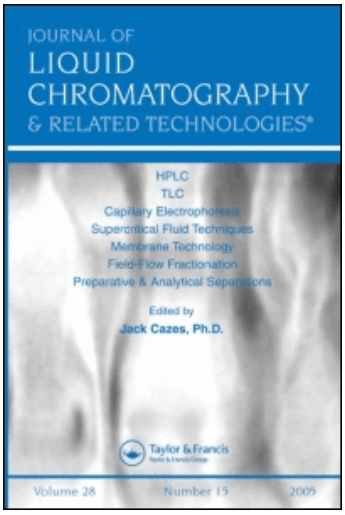

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Selection of Separation Conditions for HPLC and HPLC/MS of Aromatic Sulphonic Acids and Acid Azo Dyes

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Abstract: Liquid chromatography of organic anionic compounds usually cannot be performed without ionic additives to the mobile phase. Buffered mobile phases usually do not provide sufficient suppression of the ionization of strong acids, such as anionic dyes and aromatic sulfonic acids used in the dye production, or as whitening agents or detergents in various industrial and household applications. Hence, other modes are usually necessary for successful separation of these compounds, including ion-exchange chromatography, ion-interaction reversed-phase chromatography, or ion-pair chromatography. The retention mechanisms in these modes are still a controversial issue. In the present work, various theoretical models describing the retention of strong organic acids with bulky hydrophobic structural elements are reviewed.

Conventional ionic additives employed in these separation modes usually are not volatile enough for HPLC/MS applications, such as quaternary ammonium salts commonly used as ion-pairing reagents in ion-pair chromatography. This inconvenience can be overcome either by removing the nonvolatile additives from the mobile phase before introducing the column effluent to the ion source of the mass spectrometer, or by using more volatile compounds as their substitution. Suitable volatile reagents should be selected taking into account their effects on the retention, separation selectivity, efficiency, and band symmetry of various compounds differing in structures. Another important point that should be considered in method development is possible suppression of ionization by the additives, which should be avoided.

Keywords: Dyes, Sulfonic acids, Ion-pair chromatography, LC/MS, Ion-pairing additives

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INTRODUCTION

Anionic compounds are more difficult to separate by HPLC than nonionic solutes. Ion exchange chromatography was the first mode for separations of sulfonic acid dyes, intermediates, and surfactants, but it is not used frequently for this purpose in contemporary practice, because reversed-phase chromatography or ion-pair chromatography usually offer higher efficiency and better control of selectivity and resolution. Comprehensive reviews of the early applications of column liquid chromatography for separations of sulfonic[1] and carboxylic[2] acids can be found in the earlier literature.

Ionic additives to the mobile phase are necessary for successful separation of organic anions in all chromatographic modes, but may cause problems in HPLC/MS because of possible signal suppression and contamination of the ion source of a mass spectrometer. The selection of a suitable combination of the stationary and the mobile phase for HPLC/MS of carboxylic or sulfonic acids depends on the sample acidity, size, and polarity of the molecule backbone and often requires a compromise between chromatographic separation selectivity and performance of the mass spectrometer.

HPLC SEPARATION MODES FOR ORGANIC ACIDS

Ion-Exchange Chromatography

Ion exchange chromatography (IEC) was the oldest HPLC mode used for separations of organic acids.[1] Anion exchangers contain functional groups carrying a positive charge and retain acid anions by strong electrostatic interactions. Strong anion exchangers with -$N(CH_3)_3^+$ quaternary ammonium groups are completely ionized over the pH range 2–12 commonly used in HPLC, whereas weak anion exchangers carrying tertiary, secondary, or primary amine groups are ionized only in acidic mobile phases; outside this pH range the ionization of weak exchangers is suppressed and the retention of ions is significantly reduced.

Ion-exchange chromatography is based on the competition for the charged ion exchange functional groups between the sample ions and the counter-ions, which must be added to the mobile phase in the form of salts, buffers, ionized acids, or bases. The retention in IEC increases with increasing ion-exchange capacity of the column, while it decreases with increasing concentration of the counter-ions in the mobile phase. Depending on their dissociation constants, K_a, the ionization of weak acids is enhanced and the retention on anion exchangers increases at $pH > 6-7$. Varying pH of buffered mobile phases within the range of $pKa \pm 1.5$ can be used to adjust the separation selectivity, while the retention is adjusted by setting the ionic strength.[3]

Organic polymer ion exchangers with cross linked styrene-divinylbenzene or ethyleneglycol-methacrylate copolymer matrices provide hydrophobic

interactions with non-polar parts of organic molecules, much like in reversed-phase (RP) chromatography. Hence, most ion-exchange columns show mixed mode retention mechanisms, which often improves the separation selectivity for many organic acids and dyes. By adjusting the concentration of methanol or acetonitrile in the mobile phase, the retention can be controlled, much like in reversed-phase HPLC. Because the ionic strength of the mobile phase should fit the ion-exchange capacity of the column (1–5 meq/g for conventional ion exchangers with organic polymer matrices) adequate elution times require mobile phases with relatively high buffer concentrations ($10^{-2} - 10^{-1}$ mol/L), which are not compatible with HPLC/MS separations. Even though polymeric anion exchangers can be used for some simple separations of organic sulfonic acids,[1,4] usually the separation is slow, the efficiency poor and the selectivity insufficient for separation of isomeric compounds.

Ion exchangers with a porous layer of stationary phase on an impervious spherical glass core or ion exchange stationary phases chemically bonded on silica gel matrix have approximately one order of magnitude lower ion-exchange capacity in comparison to the classical ion-exchange resins, typically 0.3–1 meq/g and, therefore, require mobile phases with a lower ionic strength. On porous layer anion exchangers, some simple mixtures of aromatic sulfonic acids can be separated in 10–15 min.[5–9]

Special very low capacity ion-exchangers can be used with dilute mobile phases containing 10^{-3} mol/L or less buffers, allowing conductivity detection in ion chromatography.[10] Even though this method is mostly used for separation of simple inorganic ions and lower aliphatic carboxylic acids, mixtures containing methane- to octanesulfonic acids were also separated on a Dionex PAX column using a gradient of methanol in aqueous-organic mobile phase.[11] Ion exchangers with functional quaternary ammonium or alkylamino groups chemically bonded on a silica gel support were used in HPLC/MS of sulfonated dyes with mobile phases containing ammonium acetate and (or) acetic acid in aqueous acetonitrile.[11,12]

Reversed-Phase Chromatography

Many ionized organic acids often elute as strongly deformed peaks close to the column hold-up volume in reversed-phase chromatography with pure aqueous-organic mobile phases. Whereas most sulfonic acids are strong and, hence, completely ionized over the full pH range from $pH = 1$ to $pH = 14$, weak organic acids, such as carboxylic acids or aromatic aminosulfonic acids are completely ionized only at $pH > pK_a + 1.5$. Successful reversed-phase separations of weak organic acids with more or less bulk non-polar parts of molecules are often possible in aqueous or aqueous-organic mobile phases buffered to a low pH, which suppress the ionization, as non-ionized acid forms usually are more strongly retained than the

corresponding acid anions and yield regular peaks, like non-ionic compounds.[13,14]

As weak acids elute in the order of decreasing K_a constants, differences in the ionization can be utilized for their separation by adjusting pH of the mobile phase in the range of $\pm$ 1.5 units around the pK_a values. It should be noted that many chemically bonded silica based columns are less stable and not recommended for use outside the pH range from 2 to 8. The retention decreases as the ionic strength of the mobile phase increases and can be further adjusted by the addition of moderate concentration of organic solvent to the mobile phase (up to 30–40% acetonitrile or methanol). At very low buffer concentrations, the buffer capacity may be insufficient for reproducible separations; 10–50 mM phosphate (pH 2.1–3.1 and 6.2–8.2) or acetate buffers (pH 3.8–5.8) are usually suitable for most reversed-phase HPLC separations of weak acids on common alkylsilica columns.

However, the ionization of strong acids cannot be suppressed by adjusting pH of the mobile phase within practically useful limits and, hence, most sulfonic acids and dyes should be separated in the ionized forms. The difficulties experienced in reversed-phase separations of anionic compounds originate in the properties of the column packing materials. Even the sorbents, which do not possess ionizable surface groups can form an electrical charge on the surface in contact with an electrolyte solution, as a result of differences in the affinity of the sorbent and the bulk liquid phases for the ions of one charge or the other.[15] Chemically bonded silica gel stationary phases comonly used in reversed-phase HPLC contain a large proportion (usually 50% or more) of residual silanol groups that could not be modified during the silanization reaction because of limited sterical accessibility, even using end capping or sterical shielding surface modification procedures.[16] In pure water or in aqueous salt solutions, the residual silanols are partially ionized to $Si\text{-}O^-$ anions. In the liquid phase adjacent to the negatively charged stationary phase surface, there is an equivalent excess of counter ions having an ionic charge of opposite sign, which form a diffuse electrical double layer,[17,18] which interacts by attractive or repulsive forces with ionized sample solutes[19–22] and affects thus the retention of ionized samples. Fully ionized negatively charged compounds, such as aromatic sulfonic acids with two or more sulfonic acid groups, can be excluded from the stationary phase by silanophobic interactions and elute close to or even before the column hold-up volume in water or in aqueous organic solutions.[23–26] A combination of various attractive and repulsive interactions with different types of adsorption centers may result in significant band asymmetry.[27,28] This effect can probably explain strong peak tailing on some bonded alkylsilica columns in contrast to organic polymer columns.

The double layer thickness is usually less than 10 nm and it decreases when the ionic strength of the solution increases.[29] Hence, ionic mobile phase additives decrease the thickness of the electrical double layer on the adsorbent surface[15,29] and suppress thus the ionic exclusion. In addition to

suppressing ionic interactions in the stationary phase, increased ionic strength enhances the differences in the retention enthalpy arising from dipole-dipole and dispersion interactions of the non-ionic parts of the solute molecules, as well as the entropy change associated with a decrease in solvent structuring that occurs when the solute leaves the mobile phase (the salting-out or solvophobic effect).[30] Consequently, addition of a neutral salt such as sodium sulfate increases the ionic strength of the mobile phase and the retention of organic anions.

Reversed-phase techniques for the separation of ionic solutes employing ionic additives in the mobile phase are often called "ion-interaction reversed-phase chromatography". The separation is usually sensitive to the character of the electrolyte used as the mobile phase additive and to pH, sometimes even in the range of complete dissociation of the sample solutes. The retention behavior of organic anions also strongly depends on the column type, i.e., on the chemistry of the stationary phase and on the number, spacing, and shielding of the residual silanol groups.[31] Sometimes, the separation selectivity of isomeric aromatic sulfonates is significantly improved in mobile phases containing high concentrations of electrolytes, e.g., sodium sulphate,[23–26,32] ammonium sulphate,[33] or various other salts,[25] so that surprisingly good separations of strong organic acids are often possible, with sharp symmetrical peaks, in mobile phases containing inorganic or organic salts. However, this approach is useful only for acids containing a bulky hydrophobic part in their molecules. For example, the separation factors of 1,5- and 1,3- naphthalene disulfonic acids significantly differ between the individual columns, so that this parameter can be used as an indicator of the column silanophilic activity.[31]

The columns showing the greatest retention of naphthalene disulfonic acids provide sufficient selectivity for the separation of some industrial sulfonated dyes and intermediates. On such columns, separation of most isomeric naphthalene disulfonic acids and of some naphthalene trisulfonic acids can be achieved using gradient elution with simultaneously increasing concentration of the organic solvent and decreasing concentration of sodium sulphate (Fig. 1).[31] Mobile phases containing 0.02–0.15 M sodium perchlorate in aqueous methanol or acetonitrile were employed for separation of linear alkylbenzene sulfonates in commercial and environmental samples according to the length of alkyl chains, even though the selectivity was not sufficient to allow the separation of all positional isomers.[34–36]

Unfortunately, sodium sulfate and sodium perchlorate are not volatile salts and, hence, are not suitable for HPLC/MS applications. There are only a few volatile simple salts available as suitable mobile phase additives to adjust the ionic strength and the double layer thickness at the adsorbent surface, e.g., ammonium acetate or ammonium formate. Reversed-phase HPLC with aqueous-organic mobile phases containing ammonium acetate and (or) acetic acid has been frequently used for the analysis of aromatic sulfonic acids and sulfonated azo dyes and their degradation products, both

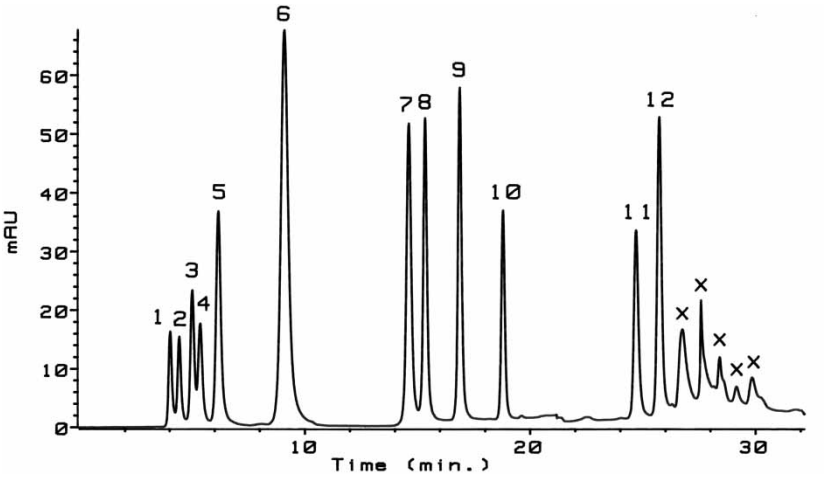

Figure 1. Separation of twelve naphtalene sulfonic acids by gradient-elution RPC on a Separon SGX RPS column, 7 μm (250 × 4 mm I.D.). Solvent program: 5 min isocratic, 0.4 mol/L Na_2SO_4 at 0.5 mL/min, followed by linear gradient from 0.4 mol/L Na_2SO_4 to 40% (v/v) methanol in water in 15 min at 1 mL/min. Detection: UV, 230 nm; column temperature 40°C. Compounds: naphthalene-1,3,5,7-tetrasulfonic acid (1), naphthalene-1,3,6-trisulfonic acid (2), naphthalene-1,3,5-trisulfonic acid (3), naphthalene-1,3,7-trisulfonic acid (4), naphthalene-1,5-disulfonic acid (5), naphthalene-2,6-disulfonic acid (6), naphthalene-1,6-disulfonic acid (7), naphthalene-2,7-disulfonic acid (8), naphthalene-1,3-disulfonic acid (9), naphthalene-1,7-disulfonic acid (10), naphthalene-1-sulfonic acid (11), naphthalene-2-sulfonic acid (12), unidentified less polar impurities (X).

in the production control and in the water analysis.[37–47] The retention times in the mobile phases containing ammonium acetate generally increase with decreasing number of sulfonic acid groups and decrease with increasing number of amino- and hydroxy- groups in the acid molecules. Some isomeric acids also differ in retention, e.g., the 1-aminonaphthalene sulfonic acids elute in the order: 1-amino-5-sulpfo- < 1-amino-6-sulfo- < 1-amino-7-sulfo- < 1-amino-8-sulfo; 6-amino-1-hydroxynaphthalene-3-sulfonic acid elutes earlier than 7-amino-1-hydroxynaphthalene-3-sulfonic acid and naphthalene-1-sulfonic acid is less retained than naphthalene-2-sulfonic acid.[38] However, isomeric naphthalene di- and tri- sulfonic acids cannot be resolved under these conditions. Ammonium acetate or formate are weak electrolytes, which usually do not increase enough the ionic strength for adequate retention and separation of benzene-or naphthalene sulfonic acids with more than two sulfonic acid groups. Larger hydrophobic parts in the molecules of sulfonated azo dyes usually provide sufficient retention for dyes with two sulfonic acid groups, so that HPLC/MS techniques can be successfully applied for the analysis of some mono- and disulfonated dyes.[38–47] Also,

linear alkylbenzene sulfonates, which are widely used anionic surfactants, have hydrophobic chains long enough to accomplish reversed-phase separations according to the alkyl chain length in mobile phases containing salts.[48,49] However, their carboxylated degradation products require ion-pair additives for sufficient retention.[47]

Ion-Pair Chromatography

Ion-pair chromatography (IPC) should be used for separation of organic acids too strong to be sufficiently retained in reversed-phase HPLC with simple buffer or salt additives. In IPC of strong acids, ion-pair additives can be used, either in the mobile or in the stationary phase. The ion-pairing reagents are essentially surfactants containing a completely ionized strongly basic group (quaternary, tertiary, secondary or even primary amino- groups) and a bulky hydrophobic part, usually containing alkyls with 4–18 carbon atoms, such as tetrabutyl ammonium or hexadecyl trimethyl ammonium salts, which can form strong associates (ion pairs) with the anionic samples.[2] Generally, IPC can be used either in normal-phase (NP) or in reversed-phase (RP) modes, but RPIPC techniques are more common nowadays.

In normal-phase ion-pair chromatography, an ion-pairing reagent is coated on a polar adsorbent support as a stationary phase, which consequently has properties similar to a liquid ion exchanger. The separation of ionic compounds is based on the differences in partition between a buffered aqueous-organic bulk mobile phase and a polar liquid stationary phase, where ion associates are formed. In some early applications, n-octylamine or another aliphatic amine[50,51] or a quaternary alkyl ammonium, such as hexadecyl trimethyl ammonium salts[52–54] in the liquid stationary phases on a silica gel support were used for separations of aromatic sulfonic acids with acetate buffers[55,56] or crown ethers[57] in aqueous-organic or in pure non-aqueous mobile phases. However, long time is necessary to attain equilibrium between the adsorbent, the adsorbed liquid stationary phase and the mobile phases.[2] More reproducible separations of strong organic acids can be obtained with bonded amino stationary phases and ion-pairing reagents, such as hexadecyl trimethyl ammonium salts added to binary non-aqueous organic mobile phases, but the separation efficiency is usually low and the analysis may take a long time.[58] With aqueous-organic mobile phases containing an ion-pair reagent, the efficiency of separation on bonded amino phases often considerably improves, for example rapid separation of sulfated ethyleneglycol nonylphenyl ether surfactants according to the number of repeat oxyethylene units is possible (Fig. 2).[59]

In reversed-phase ion-pair chromatography, an ion-pairing reagent is added to aqueous-organic mobile phases usually containing methanol or acetonitrile. The effects of ion-pairing reagents on the retention in reversed-phase

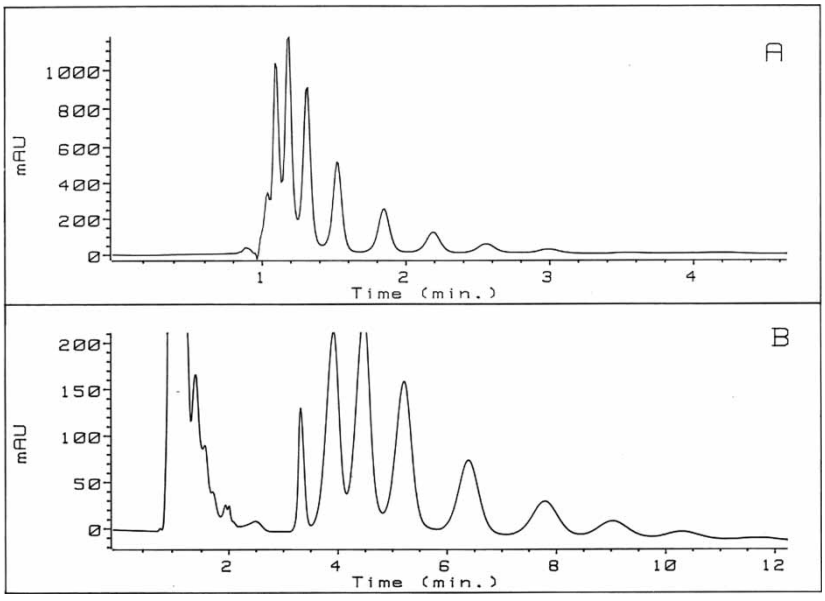

Figure 2. Separation of the individual non-sulfated (first group of peaks) and sulfated anionic (second group) oligomers in a partially sulphated Serdox NNP 4 sample of ethoxylated nonylphenol on a Separon SGX Amine, 7 μm, column (150 × 3.3 mm I.D.) with the mobile phase containing 0.04M cetyl trimethylammonium bromide (CTAB) in acetonitrile:water:dichloromethane 68.6:1.4:30 at 0.5 mL/min. Detection UV, 230 nm.

systems have been explained by several, rather controversial, theories. In the original ion-pairing retention model, association of the sample acid anions with alkylammonium counter-ions in the mobile phase was assumed, even though there is little experimental evidence for such behaviour. The ion associates (ion pairs) are much less polar than the original acid anions and, hence, are much more strongly retained on non-polar stationary phases.[60,61] In this model, no special role was attributed to the ion-pair reagent ions adsorbed on the surface of the column packing material. However, the distribution isotherm measurements give evidence for a strong adsorption of the ion pairing reagent from the bulk mobile phase on non-polar column packing materials, following the Langmuir adsorption model.[62] The adsorbed amount increases with the size of the non-polar part in an ion-pair reagent molecule and with increasing concentration in the mobile phase up to the critical micellar concentration (CMC).[63] At further increasing surfactant concentrations, only the concentration of the micelles in the mobile phase increases, but the contents of the free surfactant ions in the mobile phase and the adsorbed amount in the stationary phase remain constant.[64] The adsorbed ion-pairing reagent molecules essentially

change the properties of the stationary phase, which contains positively charged groups and plays an active role in the retention mechanism according to the "dynamic ion-exchange" model.[65] Further, the electric potential gradient at the interphase between the non-polar column packing and the bulk liquid is strongly changed in the presence of an ion-pairing reagent, and the charge and thickness of the diffuse part of the double layer strongly depend on the adsorbed ions. The free energy of retention of an ionic solute depends on its charge and on the local electric potential, so that the ion-pairing reagent would affect the retention even in the absence of ion-pair formation in the mobile phase or ion-exchange effects.[15] Hence, the exact role of the ion pairing reagent in reversed-phase chromatography is difficult to determine on the basis of chromatographic data alone.

Regardless of the actual retention mechanism, the ion-pair additives greatly improve the peak symmetry. The retention generally increases when more hydrophobic ion-pair reagents are used at a constant concentration of the ion-pair reagent. Various theoretical models provide very similar results concerning the effects of increasing concentration of the ion-pair reagent in the mobile phase on increasing retention until the capacity of the non-polar column packing for the ion-pair reagent is saturated.[26,64]

The retention and selectivity in IPC can be varied by changing the type or the concentration of the ion-pair reagent, the type and concentration of one or more organic solvent(s) and by controlling the pH of the mobile phase.[2] The effects of the organic solvent in the mobile phase on the retention is much the same as in RPC of non-ionic solutes.[26] The ion-pair reagent increases significantly the retention of ions carrying opposite charges, but also decreases, to some extent, the retention of non-ionized molecules or of ions with the charges of the same sign.[15,64] Hence, the retention of weak acids in IPC can be enhanced at a higher pH, in contrast to the reversed-phase behavior.

When the concentration of the ion-pair reagent in the mobile phase is so high that the surface of the stationary phase is fully saturated with the reagent, an increase in the concentration of the ion-pair reagent in the mobile phase may decrease the retention because of the competition for the adsorption sites in the stationary phase with the adsorbed ion-pairs of the analytes.[62,64,65] The mobile phase concentration of the ion-pair reagent required for full column saturation is usually in the range $10^{-2} - 10^{-1}$ mol/L and decreases with increasing size of the non-polar part of the reagent molecule and concentration of the organic solvent in the mobile phase.[15,26,63] The most useful range of the ion-pair reagent concentrations in IPC is in between $10^{-4} - 10^{-2}$ mol/L, depending on the sample, column, and other components of the mobile phase.[2]

An advantage of IPC with respect to RPC without ion-pair additives consists in suppressed silanol effects by stronger interactions between the ionic reagent and the analytes on one hand, and between the reagent and the ionized silanol groups on the other. However, ion-pair techniques suffer

from numerous disadvantages. Artifactual positive and negative peaks, which may complicate the evaluation of chromatograms, can be observed in IPC when the sample dissolved in a solvent that does not contain ion-pair additives is injected.[65] The column equilibration after changing the mobile phase in IPC may be slow, increasing the time necessary for method development and often causes reproducibility problems.[2] Removing of adsorbed ion-pair reagent from the column is tedious and time demanding and complete wash out may be difficult to achieve. Hence, it is not advisable using a column in RPC without ion-pair reagents once it was run in the IPC mode. Because of the variety of the effects controlling the retention, the development of an IPC separation method is rather complex. For these reasons, it is recommended to use IPC for separation of ionic compounds only if RPC with buffered mobile phases does not yield adequate retention range or band spacing.[2]

Reversed-phase ion-pair chromatography with UV detection has been widely used for the analysis of aromatic sulfonic acids, sulfonated azo dyes, and degradation products. The retention is enhanced with increasing number of carbon atoms in the alkyl chains of the alkylammonium IP reagents. Tetrabutyl ammonium[26,32,65–74] or hexadecyl trimethyl ammonium[75–78] salts have been applied most frequently. Symmetrical tetrabutyl ammonium (TBA) salts are today preferred to asymmetrical IP reagents or to long-chain primary alkylamines, such as octylamine[79] or nonylamine,[80] as they enable rapid and efficient separations of various non-substituted naphthalene- and anthraquinone sulfonic acids and their hydroxy- and amino-derivatives (letter acids) and acid azo dyes;[24–26,32,66,72,81–88] however, tetramethyl- and tetraethyl ammonium salts often do not increase sufficiently the retention of aromatic sulfonates for successful separation.[26] The retention can be controlled by adjusting the concentrations of the ion-pairing agent and of the organic solvent in the mobile phase[26,89,90] pH.[77,79,80,89,90] The ionic strength of the mobile phase can be also fine tuned to optimize the separation.[91] In addition to conventional C_{18} and C_8 columns, stationary phases with an incorporated polar amide group intended for use in highly aqueous mobile phases were also employed for separations of mono- and disulfonated aromatic compounds and azo dyes.[92]

The retention behavior of (poly)sulfonated compounds in IPC is more complex than in reversed-phase chromatography with mobile phases containing simple salts, where the retention increases as the number of acid groups in the molecule decreases. One could expect the inverted elution order in IPC, because the size of ion associates increases with the number of paired sulfonic acid groups, but this is the case only at high ion-pair reagent concentrations. At low reagent concentrations, naphthalene monosulfonates are retained more strongly than di- and trisulfonates, but the retention of trisulfonates increases more steeply with increasing concentration of TBA ions than the retention of mono- and disulfonates (Fig. 3). This behavior suggests that the aromatic sulfonic acids are not completely paired at low TBA

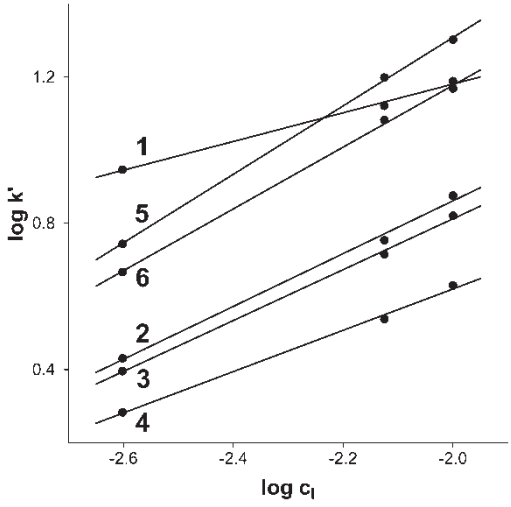

Figure 3. Effect of the concentration, c_I (in mol/L), of tetrabutlammonium sulfate in mobile phases methanol-water (35:65) on the retention factors, k, of aromatic sulphonic acids on a Silasorb C8 column. Compounds: 1: Naphthalene-2-sulfonic acid, 2: Naphthalene-1,6-disulfonic acid, 3: Naphthalene-2,7-disulfonic acid, 4: Naphthalene-2,6-disulfonic acid, 5: Naphthalene-1,3,7-trisulfonic acid, 6: Naphthalene-1,3,6-trisulfonic acid.

concentrations and is in better agreement with a dynamic ion exchange model than with mobile-phase ion pairing retention.[26]

Reversed-phase ion-pair chromatography was also used to separate anionic sulfated surfactants from their non-sulfated parent fraction,[72] oligoethylene alkylphenyl ether nonionic surfactants from the carboxylic acids formed by their biodegradation[93] and the individual sulfated oligomers according to the oxyethylene units distribution.[94] Linear alkylbenzene sulfonates, lignosulfonic acids, and polyaromatic sulfonic acids in wastewater samples could be separated in a single run.[71]

SELECTION OF MOBILE PHASES FOR HPLC/MS AND HPLC/MS/MS OF ACID DYES AND INTERMEDIATES

For successful application of HPLC/MS to the analysis of acid dyes and intermediates, non-volatile tetraalkylammonium salts are not suitable, because they form stable adducts with sample ions, suppress signal, and contaminate the ion source.[47,95] To remove non-volatile mobile phase additives from the column effluent, an ion exchange suppressor column, fiber, or ultra thin membrane can be inserted in between the analytical column and the mass spectrometer,[96,97] much like in ion chromatography. However, the

suppressor adds additional extra-column volume and contributes to band broadening, which often significantly impairs the resolution achieved on the analytical column. Further, it is inconvenient to change regularly the cartridge for a fresh one when its ion-exchange capacity is exhausted. Finally, the suppressor columns designed for aqueous ion chromatography may cause problems when used with mobile phases containing an organic solvent. These inconveniences can be avoided by using more volatile reagents at low concentrations instead of the non-volatile tetraalkylammonium salts, as a compromise between the chromatographic and mass spectrometric performance.

Acetate or formate salts of mono-, di- or trialkylamines are most frequently used for this purpose,[98] such as butylammonium[99] or longer monoalkylammonium,[83,84] salts. Monosulfonic aromatic acids and dyes often can be separated in mobile phases with ammonium acetate or ammonium formate,[44,98] but compounds with two or more sulfonic acid groups are usually very weakly retained under these conditions, if at all, and their separation is not possible in such mobile phases.

Di- or triethylammonium cations in the mobile phase often improve the retention and separation of mono- and disulfonic aromatic acids and dyes and in some cases of compounds containing more than two sulfonic acid groups with respect to ammonium acetate additive to the mobile phase.[44,98] Alkylammonium acetates with longer alkyls usually further increase the retention and improve the separation selectivity of (poly)sulfonated dyes. Triethylammonium acetate[99–105] has been often used for the separation of aromatic monosulfonic acids and mono- or disulfonated azo dyes. Mobile phases containing triethylammonium acetate also provided sufficient retention for the separation of linear[97] and branched[106] alkylbenzene sulfonates[97] and sulfophenyl carboxylates occurring as their degradation products in aqueous environmental samples.[107,108] HPLC/MS/MS with triethylammonium acetate as ion-pairing reagent was used to discriminate and quantify linear alkyl benzenesulfonates with different positions of the phenyl group on the alkyl chain on the basis of the MS-MS responses, which are higher for the external isomers and the longer alkyl chain homologues.[109]

The separation of simple naphthalene, benzene, and anthraquinone sulfonic acids, including two naphthalene trisulfonic acids, improves when dimethylbutylammonium acetate or tributylammonium acetate are used as ion-pairing additives instead of triethylammonium acetate.[47,98,110,111] Tributylammonium acetate (TBAA),[95,111,112] dihexylammonium acetate (DHAA),[101] or triethylammonium acetate (TEAA)[111,113] ion-pairing additives have been successfully applied in the HPLC/MS analysis of various sulfonated dyes. Improved separation of isomeric naphthalene disulfonic and trisulfonic acids was obtained in reversed-phase ion-pair chromatography on packed capillary C_{18} columns using mobile phases comprised of aqueous solutions of sodium sulfate or triethylammonium acetate with

β-cyclodextrin additive forming inclusion complexes with the analytes,[100] however, cyclodextrin additives are incompatible with MS.

The effects of the number of aromatic sulfonic acid groups on the retention of dyes and intermediates in aqueous-methanolic dihexylammonium acetate (DHAA)[95] are similar as in ion-pairing chromatography with tetra-alkylammonium salts,[26] and differ from the behavior in the mobile phases with ammonium acetate. The elution order of isomeric acids is similar to the mobile phases containing TBA ion-pair reagents or ammonium acetate: naphthalene 1,5-di < 1,6-di <1,3-di <1,7-di-sulfonic acids (Fig. 4). Strong retention of 8-aminonaphthalene-1-sulfonic acid can be attributed to its decreased polarity by the formation of an intramolecular hydrogen bond between the NH_2 and SO_3^- groups.[95]

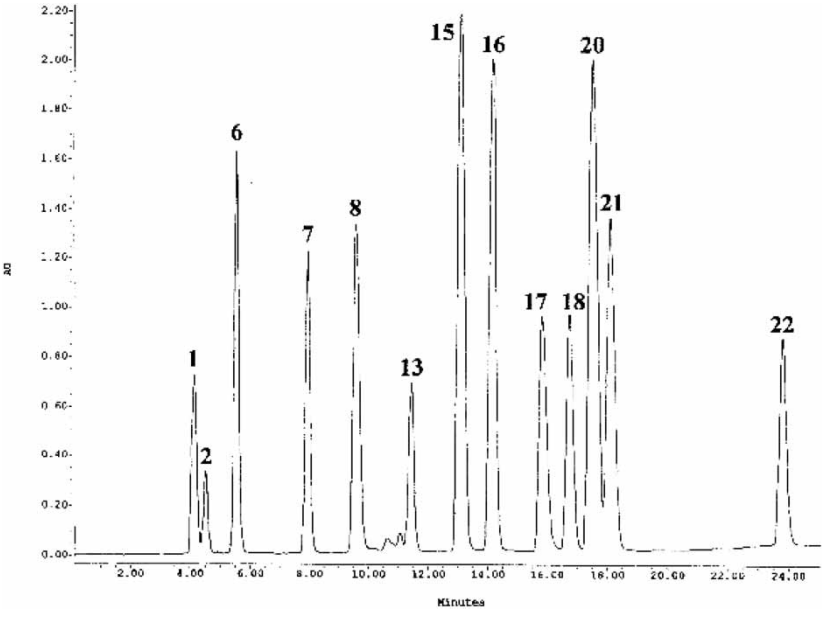

Figure 4. HPLC separation of naphthalene sulfonic acids on a C_{18} column with a mobile phase containing dihexylammonium acetate as a volatile ion-pairing reagent. Gradient elution, 2.5 mM dihexylamnonium acetate in water as solvent A and 2.5 mM dihexylammonium acetate in methanol as solvent B, 50%–80% B in 35 min. Detection, UV, 230 nm. Peak numbers: 1: 7-Amino-1-hydroxynaphthalene-3-sulfonic acid, 2: 5-Aminonaphthalene-1-sulfonic acid, 6: 1-Aminonaphthalene-6-sulfonic acid, 7: 1-Aminonaphthalene-7-sulfonic acid, 8: 1-Amino-8-hydroxynaphthalene-3,6-disulfonic acid, 13: 8-Aminonaphthalene-1-sulfonic acid, 15: Naphthalene-1,3-disulfonic acid, 16: Naphthalene-1,7-disulfonic acid, 17: 1,8-Dihydroxynaphthalene-3,6-disulfonic acid, 18: Naphthalene-1,3,7-trisulfonic acid, 20: Naphthalene-1,3,6-trisulfonic acid, 21: Naphthalene-1,3,5-trisulfonic acid, 22: Naphthalene-1,3,5,7-tetrasulfonic acid.

Mobile phases with 0.0025 mol/L dialkyl- or trialkylammonium acetate provide sufficient retention and separation selectivity even for complex dyes with molecular weights over 1000 and can effectively substitute tetraalkylammonium ion-pairing reagents in HPLC/MS. For this purpose, triethylammonium acetate is suitable as an ion-pairing additive.[114] Mobile phases containing a volatile dihexylammonium acetate ion-pairing reagent provide good resolution of metal complex disulfonated dyes of the Rylan type, whilst these dyes are not retained in the mobile phase with 5 mM ammonium acetate. This is also the case also with most Egacid and Saturn type dyes.[95]

The retention of the acid dyes generally increases with the size of the ion-pairing reagent and with the molecular weight of the dyes, but decreases with the number of the acid groups in the dye molecules. These structural effects should be taken into account when developing appropriate HPLC or HPLC/MS separation methods for sulfonated dyes.[114] For separation of complex mixtures of various acid dyes, gradient elution usually yields better resolution and covers a broader range of the molar masses of dyes that can be separated in a single run than isocratic elution. Gradient elution with mobile phases containing 2.5 mM DHAA is suitable for separation of a variety of sulfonic acid dyes containing 1–5 sulfonic acid groups; this reagent can be used for a limited time period without impairing significantly the mass spectrometric performance. Some (poly)sulfonated dyes or metal complex disulfonated dyes of the Rylan type are not fully resolved even in gradient elution with DHAA containing mobile phases, but chromatograms of coeluting dyes can be reconstructed by monitoring appropriately selected ions in HPLC/MS.[95]

A disadvantage of DHAA and TBAA ion-pairing reagents is a relatively high mass of the alkylammonium cations (m/z 186), which represents a more important potential interference in mass spectra than the TEAA cation (m/z 102).[96] TEAA offers sufficient chromatographic separation and improves signal suppression in HPLC/MS. The negative-ion electrospray ionization mass spectrometry (ESI-MS) is the most suitable technique for the analysis of anionic dyes,[38,95,98,101,102,111,113] as it enables the determination of the molar masses and the number of sulfonic acid groups in the dye molecules. Studying the fragmentation behaviour in tandem mass spectrometric (MS/MS) experiments extends the possibilities of the structure identification.[111,115–117] Practical environmental analysis applications of HPLC/MS of sulfonated azodyes include monitoring of the degradation products from electrochemical purification of waste waters containing synthetic dyes.[118]

Another very important point that should be considered when developing an HPLC/MS or HPLC/MS/MS method for sulfated compounds is signal suppression, which may strongly affect the results of the analysis. The ionization suppression in LC/MS is attributed mainly to a decrease of the evaporation efficiency due to the presence of interfering compounds (such as ion-pairing reagents) in high concentrations, which can increase the viscosity and the

surface tension of the droplets produced in the electrospray (ESI) interface and reduce the capability of the analytes to reach the gas phase. The coprecipitation of the analytes with nonvolatile mobile phase additives can also limit their transfer into the gas phase. Further, analytes and interfering compounds may compete for the maximal ionization efficiency in the ion source of the mass spectrometer. Finally, interfering substances may neutralize the ions formed in the gas phase or impair their stability.[119] The interferences present in the sample matrix can be minimized by using a cation-exchanger suppressor,[120] by using selective extraction procedures, or by increasing the chromatographic retention of the analytes.[121] Ion-pair additives to the mobile phase cannot be removed in this way and may form characteristic adducts with anions of sulfonated dyes and other sulfonic acids. The signal suppression effects can be reduced and the mass sensitivity improved by using a nanosplitting device with a high splitting ratio.[122] However, competitive coions of other sulfonated compounds present in the sample may also show strong signal suppression effects. That is why sufficient chromatographic resolution of all sample compounds is very important, even if it may seem that the analytes could be distinguished by mass spectrometry alone.

The signal suppression effects are more significant with tetraalkylammonium ions than with di- and trialkylammonium acetates.[123] This is another reason for substituting tetraalkylammonium salts by di- or trialkylammonium ion-pairing additives in the analysis of sulfonated compounds. Further, ammonium acetate, which is frequently used for HPLC/MS of ionic compounds, causes similar signal suppression as di- and tri-alkylammonium

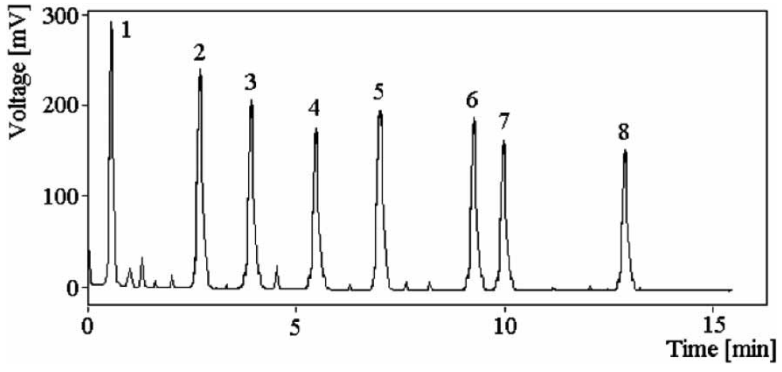

Figure 5. Ion-pairing HPLC separation of synthetic dyes on a LiChrospher C18 column (125 × 4 mm I.D., 5 μm). Gradient elution with 0.0025 mol/L TEAA in water as the solvent A and 0.0025 mol/L TEAA in methanol as the solvent B, 40% B-70% B in 30 min, 1 mL/min. 1 - Egacid Orange GG ($M_r = 408$, 2 acid groups), 2 – Rylan Red 3G ($M_r = 887$, 2 acid groups), 3 - Saturn Blue L4G, ($M_r = 967$, 4 acid groups) 4 – Saturn Green LB ($M_r = 1222$, 5 acid groups), 5 – Rylan Bordeaux B ($M_r = 897$, 2 acid groups), 6 – Egacid Yellow M ($M_r = 353$, 1 acid group), 7 – Egacid Blue A2G ($M_r = 451$, 1 acid group), 8 – Midlon Red E ($M_r = 540$, 1 acid group).

ion-pairing reagents. Moreover, di- and trialkylammonium ion-pairing reagents provide a better separation selectivity than ammonium acetate for the separation of (poly)sulfonic acids.[117,123] The concentration of mobile phase additives has a significant influence on the abundance ratios of multiply charged ions in the mass spectra of (poly)sulfonated compounds. In the mobile phases with 2.5 mM tributylammonium acetate (TBAA), a reasonable compromise was obtained between the chromatographic performance, which improves with increasing concentration of TBAA, and undesired ESI-MS signal suppression occurring at higher concentrations of TBAA. For separation of large dyes, triethylammonium acetate (TEAA) offers sufficient chromatographic separation (see Fig. 5 showing the separation of various types of dyes with 1–5 acid groups and molecular weights in the range 350–1220) and provides only moderate signal suppression in HPLC/MS.

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