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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF CARBOXYLIC ACIDS

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CONTENTS

1. INTRODUCTION

It is the purpose of this review to survey the separation systems applicable in high-performance liquid chromatography (HPLC) for the analysis of carboxylic acids. Many of these HPLC separation systems have been tested in our laboratories and the examples given demonstrate their performance and selective separation power.

The paper is subdivided into sections according to different modes of separation, although in reality such a classification does not exist. In a reversed-phase ion-pair separation system, for example, the neutral solutes will separate according to their hydrophobicity whereas, depending on the experimental conditions, the separation mechanism for ionic solutes may be ion-pair partitioning or ion exchange or both.

The applications cited are meant to be illustrative, not exhaustive. The selection was arbitrary, but preference was given to the separation of benzoic, cinnamic and organic acids present in fruits, spices, plant materials and food products. A review of liquid chromatographic determinations of phenolic acids of vegetable origin was recently presented by Roston and Kissinger[1].

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2. EXPERIMENTAL

The conditions specified below apply to the separations shown in Figs. 1–16.

2.1. *Apparatus*

The liquid chromatographic system was constructed from commercially available components. A high-pressure slurry-packing technique was used to pack the column at a pressure of 450 bar.

Injections were made on-stream through a loop injector. All separations were carried out at room temperature and no attempt was made to control the temperature of the mobile phase or the column.

2.2. *Materials*

All reagents were of analytical-reagent grade (E. Merck, Darmstadt, G.F.R., and Fluka, Buchs, Switzerland) and were used without further purification. The solvents used for the mobile phase were of HPLC grade (Rathburn Chemicals Ltd., Walkerburn, Great Britain, and Fluka) and were employed as supplied. The following column packing materials were used as received: Aminex-HPX 87 (Bio-Rad Labs., Richmond, CA, U.S.A.), Partisil 5 (Whatman, Clifton, NJ, U.S.A.), Radial Pak columns (Waters Assoc., Milford, MA, U.S.A.) and LiChrosorb RP-18 and LiChrospher Si 100 (E. Merck).

3. ION-EXCHANGE AND ION-EXCLUSION CHROMATOGRAPHIC SEPARATIONS

For a long period, the separation system published by Palmer and List[2] was standard for the ion-exchange chromatographic separation of organic acids. At about the same time Lehotay and Trailer[3] studied the separation of C_1–C_6 monocarboxylic and C_2–C_7 dicarboxylic acids on strongly acidic cation exchangers by the method of ion exclusion. The pressure stability and the overall performance of these resins (unreproducible swelling properties and exchange capacity) were unsatisfactory, however. The time required for the separation and the separation power did not deserve the expression "high-performance" liquid chromatography. A review of the ion-exchange chromatography of carboxylic acid was published by Jandera and Churáček[4].

Several workers have described the use of anion-exchange resins of different brands for the separation of carboxylic acids[5,6]. Nakajima *et al.*[7] constructed a highly sophisticated acid analyser and others used low-pressure liquid chromatography[8]. The applications of these separation systems were mainly in the wine industry[9–14], where a knowledge of the malolactic fermentation process is important in controlling the acidity of wines.

A great contribution to high-resolution and high-speed ion-exchange chromatography was the development of pellicular column packings[15–17]. The packing particle consists of an impervious central core coated with a thin shell of porous material where the exchange occurs. Pressure stability and swelling properties were no longer a problem and the analysis time was drastically reduced[18]. Pellicular packings have the

disadvantage of being relatively large (40–20 μm), therefore giving low plate performance. Several approaches have been reported of stable binding of organic layers to microparticulate silica gel, including the "brush-type", a ≡Si–C≡ binding, reported by Halász and co-workers[19,20] and Unger[21], the controlled surface porosity exchangers described by Kirkland and DeStefano[22,23] and the bonded phases reported by Locke *et al.*[24] and others[25–30]. However, disadvantages of these chemically bonded ion exchangers are the low exchange capacity, the sensitivity of the support material (silica gel) to high pH, the insufficient stability of the chemical bonding and the residual hydroxyl groups of the support. Styrene–divinylbenzene copolymer resins have therefore continued to be used. The separation power was improved by using materials of narrow particle size distribution and spherical beads.

3.1. Examples of applications

Tukelson and Richards[31] separated citric acid cycle acids by ion-exclusion chromatography using a dilute mineral acid as the mobile phase. The degree of cross-linking was increased to make the particles more rigid and the diameter of the beads was reduced to below 10 μm. With this material the resolution was significantly increased and the analysis time further reduced. Such materials are now commercially available from several suppliers.

An example is shown in Fig. 1. The acids elute in the order of increasing pK_a values. The use of dilute sulphuric acid as the mobile phase may cause minor problems with some LC equipment, but this drawback is more than compensated for by the separation power achieved. This separation system, often referred to as ion-moderated partition chromatography, has been described by several workers[32–35] and applied to the determination of acids in wines[36] and dairy products[37,38].

A disadvantage of this separation system is that some sugars and polyhydric alcohols are also retained and separated (Fig. 2). Unfortunately, those acids and sugars which are found together in nature elute at the same time. To ensure that all of the separated compounds are acids, a pre-separation or clean-up step is neces-

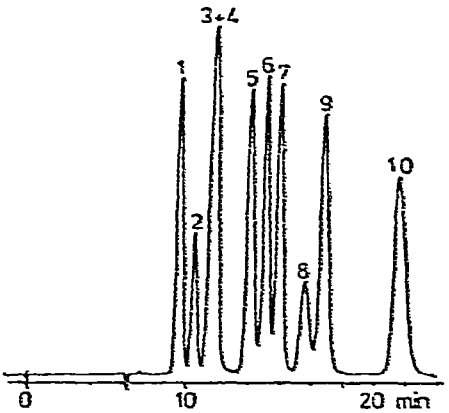

Fig. 1. Ion-moderated partition chromatography. Column: Aminex-HPX 87, 300 × 7.8 mm I.D., 22°C. Mobile phase: 0.01 *N* sulphuric acid, 0.6 ml/min. Solutes: 1 = citric acid; 2 = tartaric acid; 3 = malic acid; 4 = malonic acid; 5 = succinic acid; 6 = formic acid; 7 = acetic acid; 8 = glutaric acid; 9 = propionic acid; 10 = butyric acid.

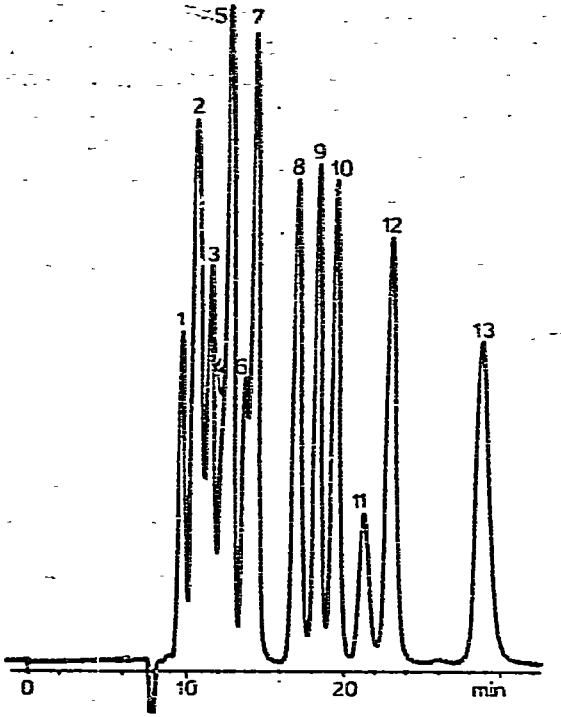

Fig. 2. Ion-moderated partition chromatography. Column: Aminex-HPX 87, 300 × 7.8 mm I.D., 22°C. Mobile phase: 0.01 *N* sulphuric acid, 0.6 ml/min. Solutes: 1 = sucrose; 2 = citric acid; 3 = glucose; 4 = tartaric acid; 5 = fructose; 6 = malic acid; 7 = malonic acid; 8 = succinic acid; 9 = formic acid; 10 = acetic acid; 11 = glutaric acid; 12 = propionic acid; 13 = butyric acid.

sary[39,40]. This co-elution of acids and sugars on ion-exchange resins shows that not only ion exchange or exclusion, but also partition plays a role in retention. The techniques for the separation of sugars[41] and other non-ionic organic compounds[42] on ion-exchange resins, both cation and anion exchangers, has been known for a long time. Partition chromatographic separations on ion-exchange resins of dicarboxylic acids from wine[10] and of phenols and aromatic carboxylic acids[43] have also been described. With aqueous alcohol as the mobile phase strongly polar solutes are retained by ion-exchange resins because the alcohol concentration in the resin phase is lower than that in the external mobile phase[44].

4. ION-PAIR CHROMATOGRAPHIC SEPARATIONS

Ion-pair extractions played a significant role in separation science for several decades. The term "ion pair" describes species formed between two ions of opposite electrical charge. Ion pairs have therefore a low net electrical charge and a low polarity. A review of the early years of ion-pair chromatography was published by Tomlinson *et al.*[45], with updates by Bidlingmeyer[46] and others[47].

The development of ion-pair chromatography is generally attributed to Schill and co-workers[48–52]. They used silica gel and cellulose, coated with a reagent capable

of forming ion pairs, and a relatively non-polar mobile phase and obtained excellent separations of ionic compounds. In normal-phase ion-pair chromatography the counter ion is a small molecule and is found in both the mobile and stationary phases. Persson and co-workers[53,54] separated carboxylic acids, such as homovanillic and vanillamandelic acids, using tetrabutylammonium hydrogen sulphate as the counter ion and stationary phase on silica gel and a mixture of butanol, methylenechloride and hexane as the mobile phase. Similar separations can also be achieved in reversed-phase ion-pair chromatography[51]. In reversed-phase ion-pair separation systems the ion-pair reagent often modifies the stationary phase. The non-polar counter ions are adsorbed on the stationary phase and can be seen as another means of preparing an ion-exchange column[55,56]. If, however, the counter ion is more polar it is present in the mobile phase and consequently the separation process is significantly different from that where a "liquid ion exchanger" is used as the stationary phase. The two techniques have to be clearly distinguished.

The theoretical aspects of reversed-phase ion-pair chromatography have been investigated by Horváth *et al.*[57]. They showed clearly that the degree of retention enhancement of a solute depends on the hydrophobic area of the counter ion and the charge of the solute, but is independent of the size of the solute if ion-pair formation occurs in the mobile phase. On the other hand, if ion-pair formation occurs on the stationary phase the enhancement of retention will depend on the charge of the counter ion and the charge and size of the solute. Practical aspects of reversed-phase ion-pair chromatography have been discussed by Gloor and Johnson[58] and illustrated with various examples. They presented very useful guidelines for the development of reversed-phase ion-pair chromatographic separations.

Knox and Laird[59] used a long-chain cationic detergent as a complexing agent for the separation of sulphonic acids of interest in the dyestuffs industry. They termed the technique "soap chromatography". The column packing may be either a reversed-phase material or an oxide gel. In reversed-phase separation the mechanism probably involves partition of the ion pairs between the water-rich eluent and the adsorbed layer rich in organic modifier and detergent. In the silica gel system ion pairs present in the mobile phase are adsorbed on the silica gel surface. The technique can equally well be applied to amines if an anionic detergent is used[60].

Soap chromatography proved to be very versatile and was used for many applications. Wall and co-workers[61] investigated porous ceria microspheres as a support for soap chromatography and developed a "duplex soap chromatographic" procedure by using silica gel columns modified by dynamic interaction with solutions of mixtures of ionic and non-ionic detergents[62]. Liao and Vogt[63] and Rotsch and Pietrzyk[64] carried out ion-pair partition chromatography on ion-exchange columns. A packing with a chemically bonded ion exchanger and non-polar alkyl groups gave combined properties of a reversed phase and an ion exchanger[63]. Solvophobic interactions, buffer type, buffer concentration, pH and the type and concentration of organic modifier in the mobile phase are parameters that can be used to select the best separation conditions[65]. Rotsch and Pietrzyk[64] studied the effect of tetraalkylammonium salts on the retention of mono- and diprotic weak organic acids and ampholytes on Amberlite XAD-2 as a function of pH in acetonitrile–water mixtures.

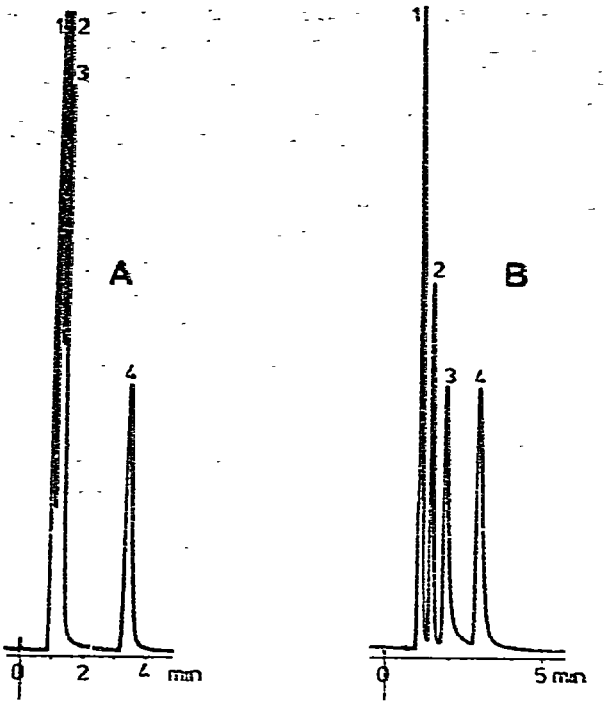

Fig. 3. Influence of pH on ion-pair chromatography. Column: LiChrosorb RP-18, 10 μm, 250 × 3 mm I.D., 21°C. Mobile phase: 0.05 *M* H_3PO_4–methanol (7:3) + 0.005 *M* hexylamine. (A) pH of mobile phase, 7.0; flow-rate, 1.0 ml/min. Solutes, dihydroxybenzoic acids: 1 = 3,5- + 3,4-; 2 = 2,5 + 2,4-; 3 = 2,3-; 4 = 2,6-. (B) pH of mobile phase, 2.5; flow-rate, 1.0 ml/min. Solutes, dihydroxybenzoic acids: 1 = 3,5- + 3,4-; 2 = 2,5-; 3 = 2,3- + 2,4-; 4 = 2,6-.

4.1. Examples of applications

In ion-pair chromatography many parameters can be varied in order to effect a separation. In addition to the stationary and mobile phases and the type, size and concentration of the counter ion, the pH is a very important parameter as it determines the concentration of the ionic form of the solutes. In Fig. 3 the separations of dihydroxybenzoic acids at different pHs are compared. 2,6-Dihydroxybenzoic acid has a pK_a of 1.0 and is ionized at both pH 2.5 and pH 7.0, and therefore k' does not change. All other dihydroxybenzoic acids, having pK_a values between 2.9 and 4.3, are ionized at pH 7.0 and the retentions will therefore depend on the nature of the ion pairs. At pH 2.5 the ionization of these acids is suppressed and the retentions depend on the nature of the solute. Van de Venne *et al.*[66] assume that hexylamine is predominantly adsorbed on the surface of the C_{18}-silica gel and the retention behaviour can be explained by an ion-exchange mechanism.

Kraak and Huber[67] used a tri-*n*-octylamine–aqueous perchloric acid system for separations of sulphonic and carboxylic acids. Quaternary ammonium salts (Fig. 4) dissolved in buffered water–methanol or water–acetonitrile as the mobile phase have become popular[68–71]. The more lipophilic the quaternary ammonium ion is, the more the acid is retained on non-polar stationary phases. Such separation systems

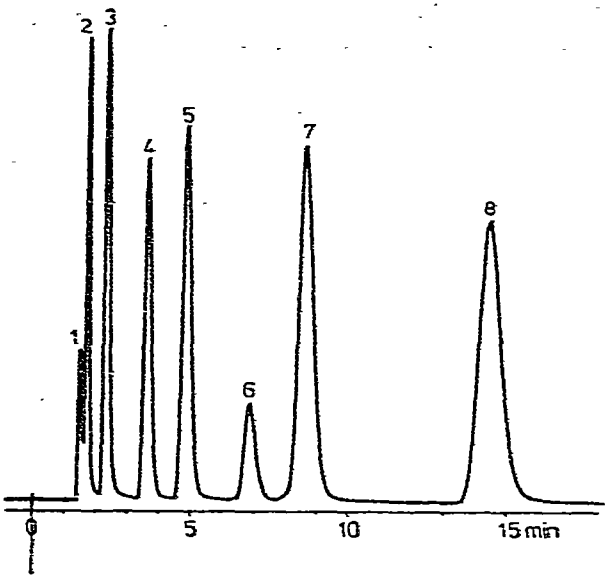

Fig. 4. Ion-pair chromatography. Column: LiChrosorb RP-18, 10 μm, 250 × 3 mm I.D., 21°C. Mobile phase: water–methanol (65:35) + 0.005 *M* tetrabutylammonium phosphate, pH 7.3. Flow-rate: 1.0 ml/min. Solutes: 1 = gallic acid; 2 = caffeic acid; 3 = ferulic acid; 4 = 3-hydroxycinnamic acid; 5 = benzoic acid; 6 = 2-hydroxybenzoic acid; 7 = cinnamic acid; 8 = 2,6-dihydroxybenzoic acid.

have been used, for example, for the determination of ascorbic acids in fruits and vegetables[72], in potatoes and potato products[73] and in foods and multivitamin products[74]. Branfman and McComish[75] described the separation of the three oxidation states of folic acid on C_{18}-silica gel using tetrabutylammonium phosphate as the counter ion.

Gradient elution ion-pair chromatography is also possible, but is mainly used in the reversed-phase mode[76]. In addition to the organic modifier concentration, the pH of the mobile phase can also be varied. Variation of pH is used to optimize the separation of ionic solutes in a given sample, while organic modifier gradients are mainly used to achieve optimum separation of the non-ionic components in a sample.

Reversed-phase ion-pair HPLC will become the preferred method, as this mode has the advantage that both the type and concentration of the pairing ion can easily be changed, even during an analysis (gradient elution), and large injection volumes are possible. This approach does not suffer from column instability due to bleeding, nor are peak asymmetries so apparent as with straight-phase methods.

5. SOLVOPHOBIC CHROMATOGRAPHY

The addition of acids or acidic buffers to the mobile phase lowers the pH and suppresses the ionization of the acidic functional groups of the solutes. Ionization suppression-aided separations are therefore based on the hydrophobicities of the solutes. The retention is the result of hydrophobic interactions of the hydrocarbonaceous moiety of the solute with the octadecyl chains of the stationary phase. The hydrophobic and solvophobic effects have been treated extensively theoretically[77–81].

There is a linear relationship between the logarithm of the capacity factors and the hydrocarbonaceous surface area of a class of solutes. Horváth *et al.*[82,83] found the slopes of each family of compounds to be nearly identical. Such a dependence of the capacity factor on the molecular surface area has been observed with a wide variety of homologous series and has also been reported as linear relationship between log k' and carbon number[84,85].

Relatively polar substances can be separated on C_{18}-silica gel columns with neat aqueous eluents of the appropriate pH[86]. This technique is termed hydrophobic chromatography. The capacity factors and the relative retention values are strongly influenced by the pH of the eluent, which controls the ionization of the solutes. Owing to changes in surface tension, the retention of a solute increases with increasing salt concentration of the eluent. When mixed solvents are used as eluents the retention decreases with decreasing water concentration. As the surface tension of aqueous salt solutions increases linearly with increasing salt concentration, log k' values will also increase linearly. With mixed solvents, however, this is no longer so. The increase in the capacity factor with increasing surface tension is very non-linear.

Deming and Turoff[87] studied the effect of aqueous solvent pH on separations of several benzoic acids and demonstrated a semi-empirical optimization strategy. A similar study was reported by Hanai *et al.*[88] on macroporous polystyrene–divinylbenzene copolymers and acetonitrile–water mixtures as eluents. At low pH, log k' of aromatic acids was linearly related to the logarithm of their partition coefficient in an octanol–water system. In combination with the dissociation constant of the acid it was possible to predict the retention of the acid at a given pH of the eluent.

The use of strong electrolyte solutions as the mobile phase for the separation of carboxylic acids have been described by Jandera *et al.*[89]. The role of the electrolyte in the separation has not been well defined. The increased ionic strength may enhance

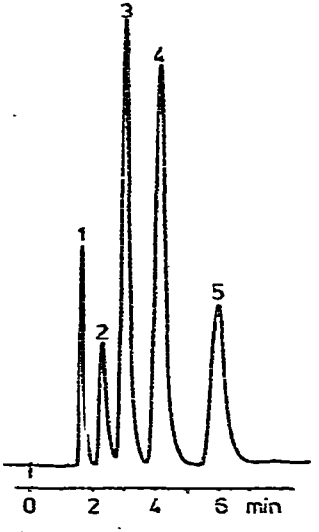

Fig. 5. Solvophobic chromatography with a strong electrolyte in the mobile phase. Column: Radial-Pak C_{18}, 10 μm, 100 × 5 mm I.D., 22°C. Mobile phase: 0.4 *M* aqueous sodium sulphate (pH 5.7)–methanol (9:1), 1.0 ml/min. Solutes: 1 = 3-hydroxybenzoic acid; 2 = syringic acid; 3 = 4-hydroxycinnamic acid; 4 = 3-hydroxycinnamic acid; 5 = 2-hydroxycinnamic acid.

the separation by decreasing intramolecular hydrogen bonding[90]. Capacity ratios increase with increasing number or size of aliphatic chains and with increasing number of aromatic rings. The selectivity of the system is sufficient to achieve separations of isomeric hydroxy acids (Fig. 5).

The acids commonly used as mobile phase modifiers are acetic and phosphoric acids. Citric acid[91] and different carboxylic acids[92] have also been used to lower the pH of the mobile phase. Fig. 6 shows the separation of hydroxybenzoic and -cinnamic acids using aqueous acetic acid and methanol as the mobile phase. The retention of the solutes decreases with increasing number of hydroxy and/or methoxy groups, with the exception of compounds where a hydroxyl group is *ortho* to a carboxyl group, so that an intramolecular hydrogen bond may be formed, which significantly increases the retention.

Phosphoric acid is often preferred to acetic acid because of its non-aggressive behaviour against the column and liquid chromatographic equipment. A disadvan-

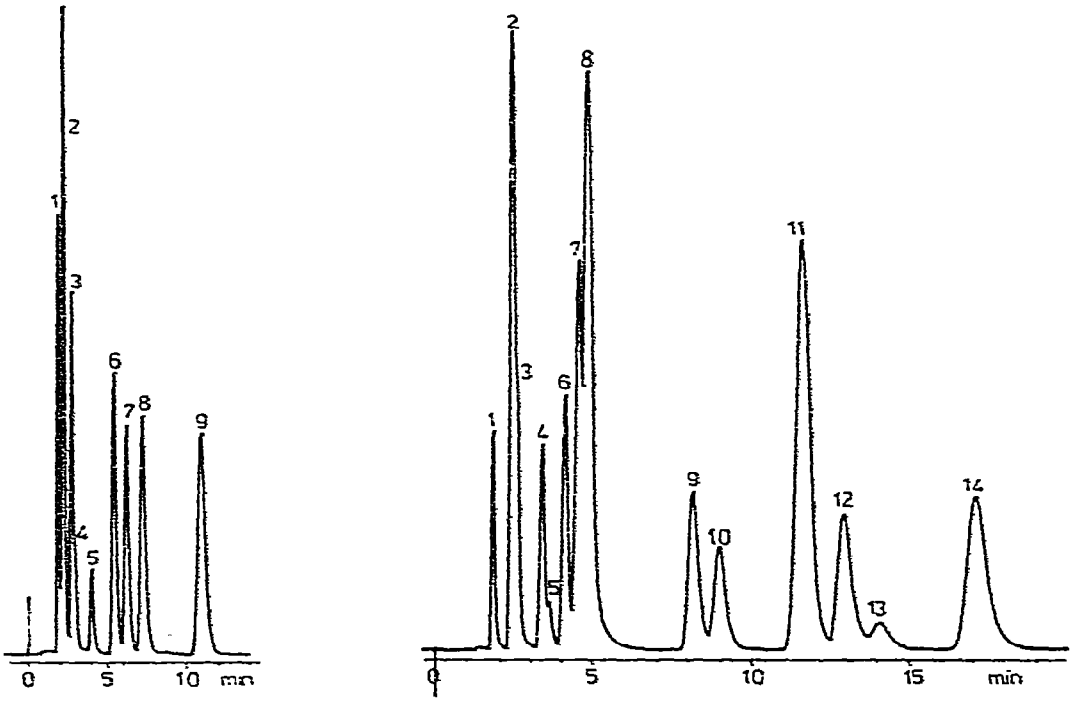

Fig. 6. Solvophobic chromatography with ionization suppressed by acetic acid. Column: Radial-Pak C_{18}, 10 μm, 100 × 5 mm I.D., 22°C. Mobile phase: 60% acetic acid–water (1:9) + 40% methanol, 1.5 ml/min. Solutes: 1 = gallic acid; 2 = protocatechuic acid; 3 = caffeic acid; 4 = syringic acid; 5 = ferulic acid; 6 = 3-hydroxycinnamic acid; 7 = benzoic acid; 8 = 2-hydroxybenzoic acid; 9 = cinnamic acid.

Fig. 7. Solvophobic chromatography with ionization supressed by phosphoric acid. Column: LiChrosorb RP-18, 10 μm, 250 × 3 mm I.D., 21°C. Mobile phase: 0.05 *M* H_3PO_4–methanol (7:3), pH 2.5, 0.9 ml/min. Solutes: 1 = gallic acid; 2 = 3,5-dihydroxybenzoic acid; 3 = 3,4-dihydroxybenzoic acid; 4 = 2,5-dihydroxybenzoic acid; 5 = mandelic acid; 6 = 4-hydroxybenzoic acid; 7 = caffeic acid; 8 = 2,3-dihydroxybenzoic acid; 9 = 4-hydroxycinnamic acid; 10 = ferulic acid; 11 = 3-hydroxycinnamic acid; 12 = benzoic acid; 13 = 2-hydroxybenzoic acid; 14 = 2-hydroxycinnamic acid.

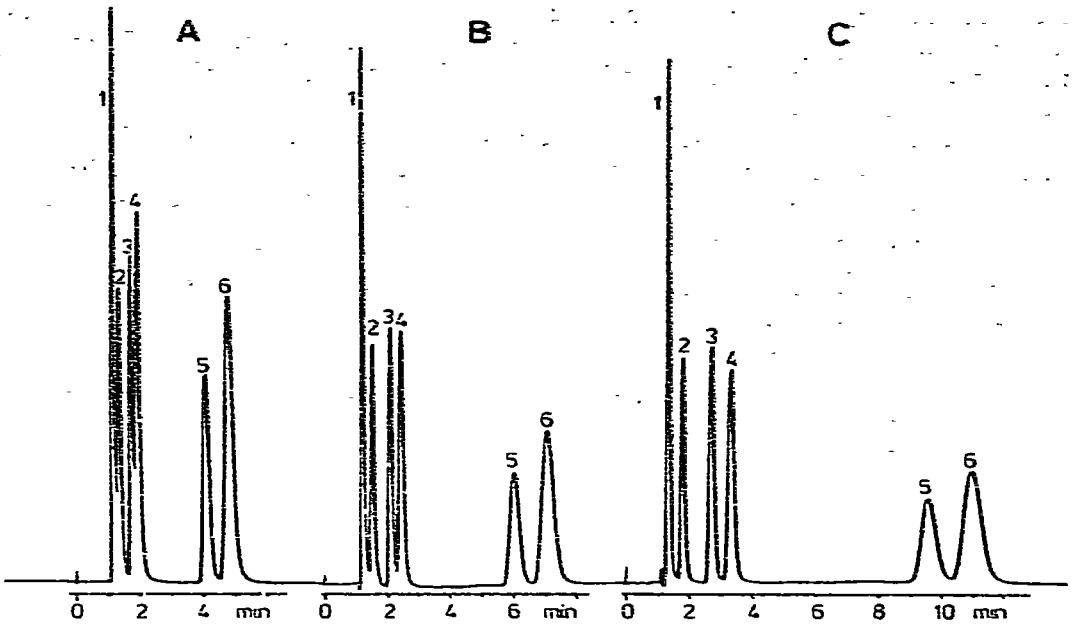

Fig. 8. Influence of the concentration of the organic modifier in the mobile phase on solvophobic chromatography. Column: Radial-Pak C_{18}, 10 μm, 100 × 5 mm I.D., 22°C. Mobile phase: 10% aqueous acetic acid–methanol, (A) 6:4, (B) 7:3, (C) 8:2; flow-rate, 1.5 ml/min. Solutes: 1 = 3,4,5-trihydroxybenzoic acid; 2 = 3,4-dihydroxybenzoic acid; 3 = 4-hydroxybenzoic acid; 4 = 3-hydroxybenzoic acid; 5 = benzoic acid; 6 = 2-hydroxybenzoic acid.

tage is its high viscosity, which leads to a lower plate performance of the column. The retention behaviour for acids, however, is the same as with acetic acid (Fig. 7).

The capacity factor of a solute is determined by the concentration of the organic modifier in the mobile phase, as demonstrated in Fig. 8. The separation selectivity can be influenced by either the pH of the mobile phase (Fig. 9) or the nature of the organic modifier (Fig. 10).

By changing the pH of the mobile phase[92] the equilibrium of dissociation of the solute is changed and with it its chromatographic behaviour. Changing the type of organic modifier affects the solubility of the solutes in the mobile phase and hence their retention characteristics. The effects of ternary mobile phases on the separation of cinnamic acid derivatives has been described by Roggendorf and co-workers[93–95].

To suppress the dissociation of di- and tricarboxylic acids, acetic acid and phosphoric acid are replaced by buffers (Fig. 11)[96–100]. In addition to sodium or potassium phosphate, sodium hydrogen sulphate, sodium chlorate and other acidic buffers have also been used successfully as reagents. The use of a buffer solution does, however, restrict the use of gradient elution.

In this context, it is useful to remember that the different reversed phases commercially available are alike in name only. Differences in their surface chemistries, due to different manufacturing processes, can cause great differences in their separation capabilities.

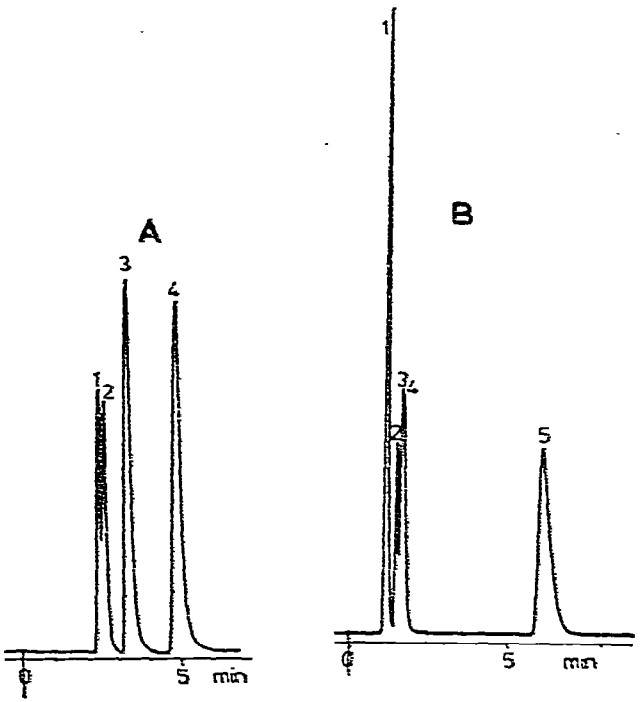

Fig. 9. Influence of pH on separation selectivity in solvophobic chromatography. Column: LiChrosorb RP-18, 10 μm, 250 × 3 mm I.D., 21°C. Mobile phases: (A) 0.05 *M* H_3PO_4–methanol (70:30), pH 2.5, 0.9 ml/min; (B) 0.05 *M* H_3PO_4–methanol (85:15), pH 7.0, 0.9 ml/min. Solutes: (A) 1 = 3,5-; 2 = 3,4-; 3 = 2,5- + 2,6-; 4 = 2,3- + 2,4-dihydroxybenzoic acid; (B) 1 = 3,5- + 3,4-; 2 = 2,3-; 3 = 2,4-; 4 = 2,5-; 5 = 2,6-dihydroxybenzoic acid.

5.1. Examples of applications

Separation systems for acids using ionization suppression are used frequently and in numerous fields of analysis, and undoubtedly there will be an increase in the use of these systems in the future.

A reversed-phase high-performance liquid chromatographic method described by Coppola *et al.*[101] uses 2% aqueous potassium dihydrogen phosphate solution adjusted to pH 2.4 with phosphoric acid for the determination of organic acids in cranberry juice. Distler[102] used sulphuric acid to adjust the pH of the pure aqueous mobile phase for the separation of short-chain carboxylic acids on C_{18}-silica gel. A similar system was used by Bigliardi *et al.*[103] to separate organic acids in fruit juices. The separation of sorbic acid from benzoic and cinnamic acids and its determination in beverages and food products has been described by several workers[104–109].

Flavones, flavanones and flavanoids are another important group of related compounds, which often have to be separated from phenolic acids. Similar systems to those used for benzoic and cinnamic acid separations can be successfully employed for the chromatography of flavones and flavanoids, as shown in Fig. 12. Reversed-phase chromatographic systems were used by several workers for this type of separation[110–119]. In some instances addition of acid to the mobile phase was not necessary for tailing-free elution.

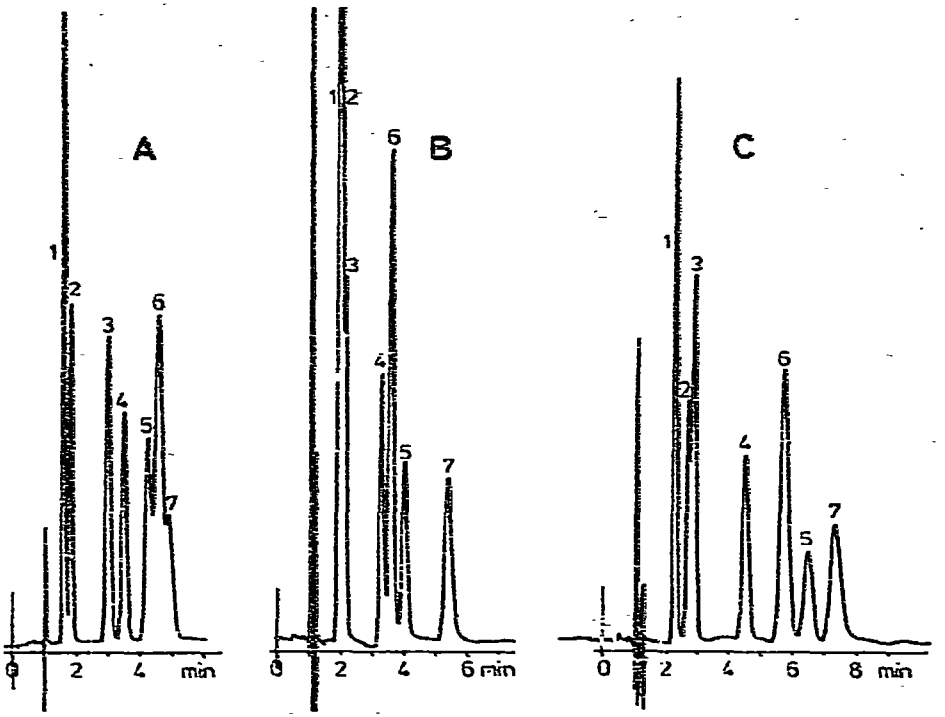

Fig. 10. Influence of organic modifier on separation selectivity in solvophobic chromatography. Column: Radial-Pak C_{18}, 10 μm, 100 × 5 mm I.D., 22°C. Mobile phases: (A) 0.05 *M* H_3PO_4–methanol (70:30); (B) 0.05 *M* H_3PO_4–acetonitrile–tetrahydrofuran (80:10:10); (C) 0.05 *M* H_3PO_4–methanol–acetonitrile–tetrahydrofuran (80:12:4:4). Flow-rate: 2.0 ml/min. Solutes: 1 = 4-hydroxybenzoic acid; 2 = 3-hydroxybenzoic acid; 3 = 2-methoxybenzoic acid; 4 = benzoic acid; 5 = 3-methoxybenzoic acid; 6 = 4-methoxybenzoic acid; 7 = 2-hydroxybenzoic acid.

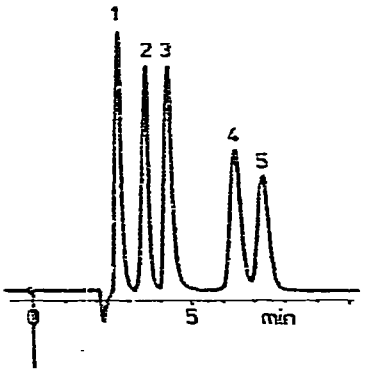

Fig. 11. Solvophobic chromatography with aqueous buffer solution as mobile phase. Column: Radial-Pak C_{18}, 10 μm, 100 × 8 mm I.D., 22°C. Mobile phase: 0.01 *M* aqueous K_2HPO_4, pH 2.5, 1.5 ml/min. Solutes: 1 = tartaric acid; 2 = malic acid; 3 = lactic acid; 4 = citric acid; 5 = fumaric acid.

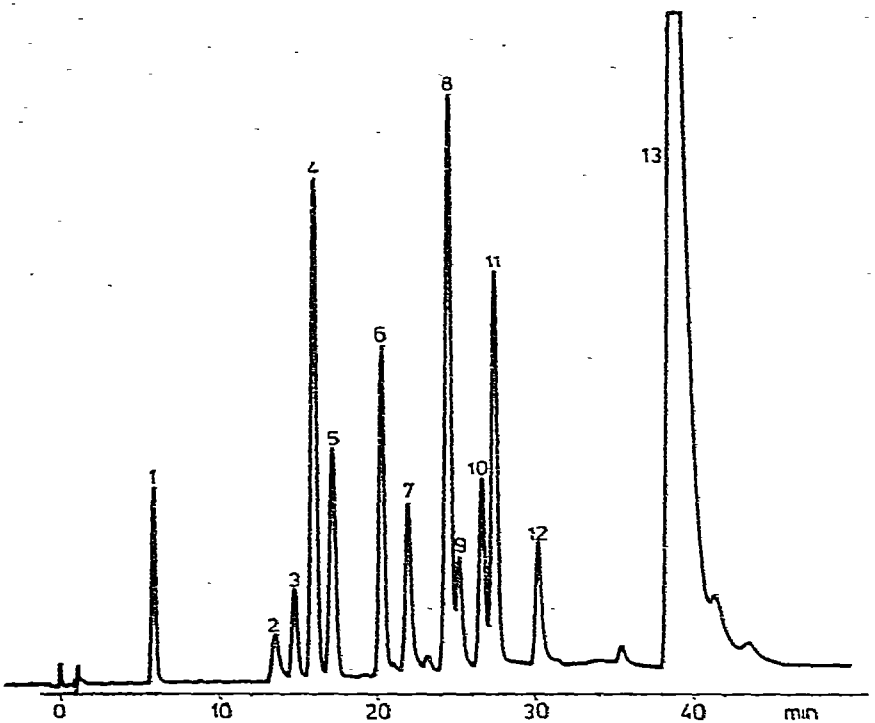

Fig. 12. Solvophobic chromatography with gradient elution. Column: Radial-Pak C_{18}, 10 μm, 100 × 5 mm I.D., 22°C. Mobile phase: 1% aqueous H_3PO_4–methanol, 1.5 ml/min. Gradient: linear from 30% to 70% methanol in 30 min. Solutes: 1 = epicatechin; 2 = hesperidin; 3 = naringin; 4 = quercetin; 5 = rutin; 6 = quercitrin; 7 = morin; 8 = naringenin; 9 = kaempferol; 10 = hesperetin; 11 = luteolin; 12 = galangin; 13 = flavone.

6. NORMAL-PHASE SEPARATIONS

6.1. *Partition chromatography*

Because of operational difficulties, partition chromatography is being used less frequently. Small changes in temperature can drastically change solubilities and hence the separation. A major disadvantage is that gradient elution cannot be employed. The technique of partition chromatography, however, remains of value and serves as a powerful alternative, as demonstrated in Fig. 13.

Stahl and Laub[120] described the separation of acids of interest in food analysis on microcrystalline cellulose and applied the method to samples of wine and fruit juice[121]. Similar separations were achieved by using silica gel as the support material[122,123] (Fig. 13). The alcoholic and aqueous part of the mobile phase are adsorbed at the silica gel surface and serve as the stationary phase. The partition of the solutes takes place between this water-rich stationary phase and the more organic mobile phase. As pointed out earlier, this equilibrium is very sensitive to changes in temperature and flow-rate.

Other partition systems use pure water[124] or a buffer solution[125] as the

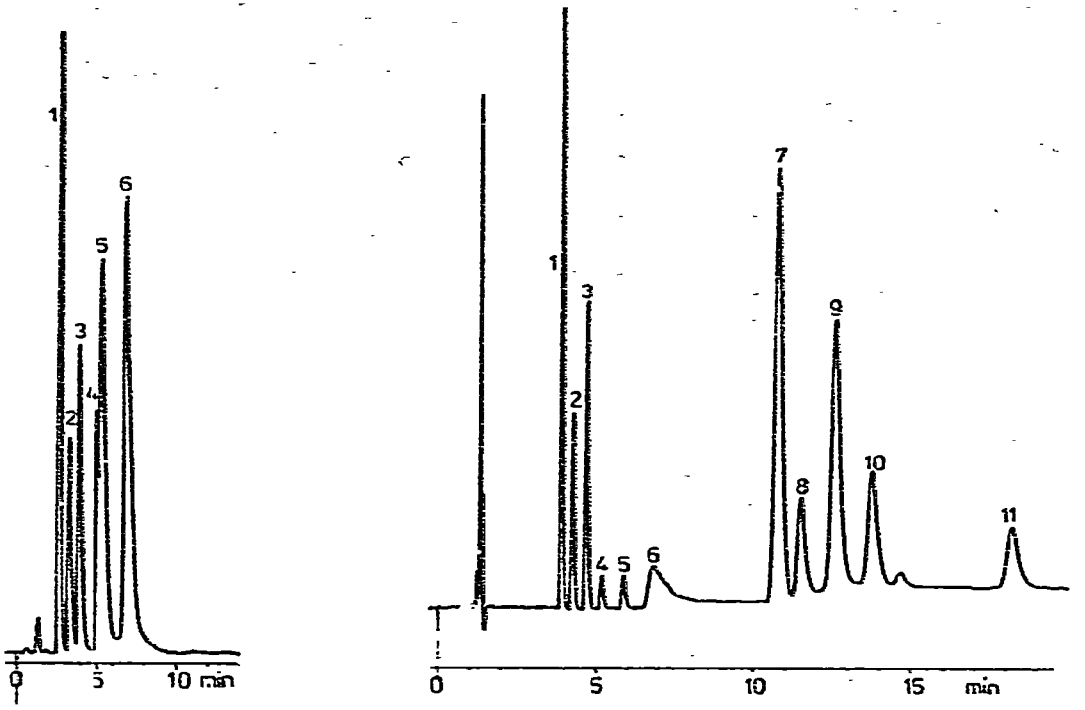

Fig. 13. Normal-phase partition chromatography. Column: Partisil 5, 5 μm, 250 × 3 mm I.D., 22°C. Mobile phase: dichloromethane–ethanol–water (82:14:4). Solutes: 1 = cinnamic acid; 2 = benzoic acid; 3 = ferulic acid; 4 = 2-hydroxycinnamic acid; 5 = syringic acid; 6 = 4-hydroxybenzoic acid.

Fig. 14. Normal-phase partition chromatography. Column: Partisil 5, 5 μm, 250 × 3 mm I.D., 22°C. Mobile phase: dichloromethane–methanol–water H_2O (78:20:2) + 0.025 *M* acetic acid + 0.025 *M* potassium acetate. Solutes: 1 = 4-methoxycinnamic acid; 2 = 3-methoxycinnamic acid; 3 = cinnamic acid; 4 = 3-methoxybenzoic acid; 5 = benzoic acid; 6 = 2-hydroxybenzoic acid; 7 = 4-hydroxycinnamic acid; 8 = 2-hydroxycinnamic acid; 9 = 3-hydroxycinnamic acid; 10 = 4-hydroxybenzoic acid; 11 = 3-hydroxybenzoic acid.

stationary phase. The support, in most instances silica gel, is coated with water or aqueous buffer solution before packing the column or *in situ* (Fig. 14). The mobile phase is a mixture of non- and medium-polar organic solvents. The addition of acids or buffers to the stationary phase improves the chromatographic process by blocking hydrogen-bonding sites, which cause slow mass transfer and result in tailing.

The retention mechanism on silica gel is classically thought to involve direct competition of solutes and solvents for active adsorption sites[126]. Some workers[127] therefore term this type of separation adsorption chromatography. However, studies by Scott and Kucera[128,129] indicated that when the mobile phase contains solvents of high polarity one or more layers of polar solvent molecules are adsorbed on the silica gel surface. A discussion about the possible retention mechanism was also given by Green[130], who used such separation systems for the characterization of fossil fuels[131].

Brugman *et al.*[132] investigated very systematically the influence of the type, concentration and pH of the buffer added to the mobile phase on the separation of organic acids on bare silica gel. For some mobile phase compositions the composition

of the adsorbed layer on the silica gel surface was also determined. The results showed that the distribution process in the examined phase systems is very complex.

6.2. *Adsorption chromatography*

We restrict the term normal-phase adsorption chromatography to systems where the support is a water-free adsorbent and the mobile phase is strictly non-polar organic in nature. On bare silica gel the organic acids would elute with strong tailing owing to interactions between different polar functional groups on the solutes and the active sites of the silica gel surface. By coating the surface silica gel with a crystalline buffer this interaction can be controlled and the tailing eliminated.

The preparation of such a silica gel was described by Schwarzenbach[133,134]. Fig. 15 shows the separation of various hydroxy- and methoxybenzoic and -cinnamic acids. The possible applications of these buffered silica gel systems are numerous. For the separation of the different bitter acids present in hop, hop products and beer, this system is about ten times faster than traditional ion-exchange chromatography[135].

The power of adsorption chromatography in the separation of isomers is shown in Fig. 16; compared with ion-pair chromatographic system, all of the dihydroxybenzoic acid isomers are separated. The stability of a buffered silica gel system is comparable with that of a normal silica gel system. Flow and gradient programme can be used and for preparative separations of polar compounds the buffered silica

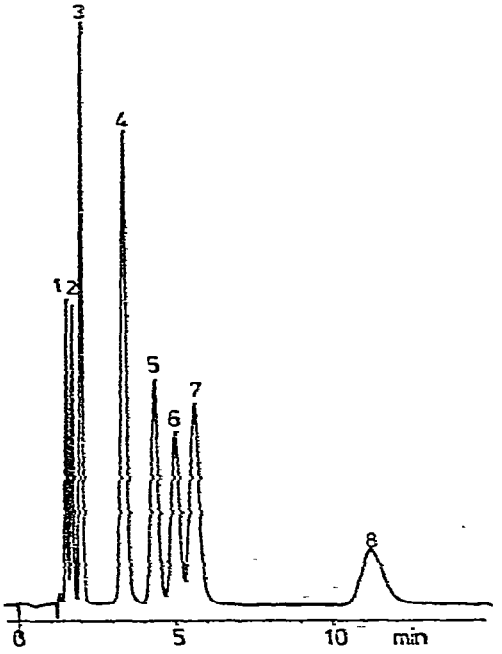

Fig. 15. Adsorption chromatography on buffered silica gel. Column: LiChrospher Si 100, 5 μm, 250 × 3 mm I.D., 22°C, buffered to pH 1.2 (0.1 *M* tartaric acid). Mobile phase: *n*-hexane–diethyl ether (1:1), 1.0 ml/min. Solutes: 1 = benzoic acid; 2 = 2-hydroxybenzoic acid; 3 = cinnamic acid; 4 = 4-hydroxybenzoic acid; 5 = 2-hydroxycinnamic acid; 6 = ferulic acid; 7 = syringic acid; 8 = gallic acid.

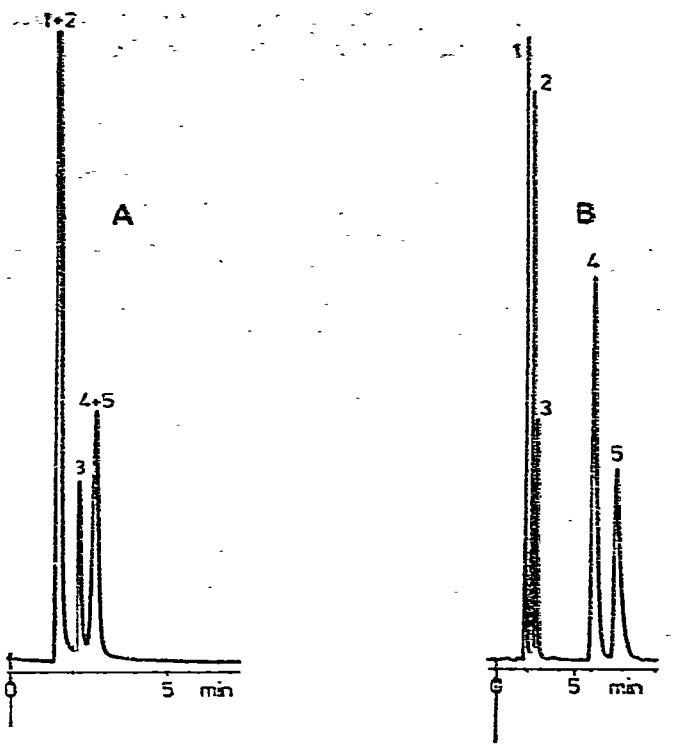

Fig. 16. Comparison of ion-pair and adsorption chromatography. (A) Ion pair chromatography. Column: LiChrosorb RP-18, 10 μm, 250 × 3 mm I.D., 21°C. Mobile phase: water–methanol (65:35) + 0.005 *M* tetrabutylammonium phosphate. Solutes: 1 = 3,4-; 2 = 3,5-; 3 = 2,3-; 4 = 2,4-; 5 = 2,5-dihydroxybenzoic acid. (B) Adsorption chromatography. Column: Partisil 5, 5 μm, 250 × 3 mm I.D., 22°C, buffered to pH 1.0 (0.1 *M* tartaric acid). Mobile phase: *n*-hexane–diethyl ether (1:1). Solutes: 1 = 2,3-; 2 = 2,4-; 3 = 2,5-; 4 = 3,4-; 5 = 3,5-dihydroxybenzoic acid.

gel system is preferred to reversed-phase systems because the mobile phase can easily be removed from the collected fractions. The only disadvantage is the limited choice of the mobile phase; the buffer salt, coated on the surface, must not be dissolved by the mobile phase.

7. FUTURE PROSPECTS AND CONCLUDING REMARKS

HPLC techniques will undoubtedly have an important place in acid analysis. The trend is away from classical ion-exchange chromatography[4] and special techniques such as salting-out chromatography[136], complex chromatography[137], ligand-exchange chromatography[138] and others, to reversed-phase ion-pair or ion-suppression chromatography and to reversed-phase partition chromatography. The special techniques[139], however, also have applications, and may in some instances provide the only solution to a particular separation problem.

Of great interest will be the development of separation systems for enantiomers of acids[140,141]. Davankov *et al.*[142] developed a system for the separation of amino acid enantiomers by ligand-exchange and reversed-phase chromatography. Engel-

hardt and Kromidas[143] and Lindner[144] used a different approach for the separation of enantiomers.

There is in general no satisfactory solution to detection problems in HPLC. With aromatic acids their UV absorption at *ca.* 280 nm can be used and allows sensitive detection. Aliphatic acids can be detected by UV absorption at 205–220 nm with higher sensitivity than by differential refractometry. For the more specific detection of acids electrochemical detectors are available[98], which also provide high sensitivity and selectivity can be enhanced by derivatization[145]. UV-absorbing derivatives[146–148] are mainly prepared off-line, prior to injection, whereas fluorescence labelling is used as an on-line technique[149].

The requirements for a successful HPLC method in acid analysis will always be speed, sensitivity, selectivity and efficient resolution from complex matrixes.

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9. SUMMARY

The development of different separation systems for carboxylic acids are discussed. Techniques are considered that have the greatest significance and references are given to liquid chromatographic systems for the separation of carboxylic acids, particularly the phenolic acids and fruit acids found in foods and food products.

Ion-exchange chromatography on microparticulate exchange materials gives excellent separations with short analysis time. The separation mechanism, however, is no longer pure ion exchange but is often referred to as ion-moderated partition chromatography.

The use of ion-pairing reagents in reversed-phase chromatography facilitates the HPLC separation of acidic solutes and gives the largest number of parameters for the selection of separation selectivity.

Reversed-phase partition chromatography, using an acid or a buffer in the mobile phase to suppress the ionization of the solutes, is the simplest separation system for carboxylic acids.

Normal-phase separation systems, partition, and adsorption are used for special selectivity. In many instances they are a good alternative to ion-exchange and ion-pair chromatographic systems.

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