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Review

Chromatographic separations of aromatic carboxylic acids

Monika Waksmundzka-Hajnos

Department of Inorganic and Analytical Chemistry, Medical Academy, Staszica 6, 20-081 Lublin, Poland

Abstract

The purpose of this review is to present methods of chromatographic analysis of aromatic carboxylic acids. The separation, identification and quantitative analysis of aromatic carboxylic acids are necessary because of their importance as non-steroid antiphlogistic drugs, semi-products of biosynthesis of aromatic amino-acids in plants (phenolic acids), metabolites of numerous toxic substances, drugs and catecholamines. HPLC separation of ionic samples tends to be more complicated than separation of non-ionic compounds. The review describes the dependence of the retention of ionic solutes on pH and solvent composition as well as on the ionic strength of a mobile phase. The application of the ion-suppressing RP-HPLC method using organic modifiers (aqueous buffer solutions) as eluents in aromatic carboxylic acid analysis is also presented. In more difficult cases of analysis the addition of an ion-pairing reagent, such as the quaternary alkylammonium ion, is necessary to obtain satisfactory separations. Hypotheses of ion-pair formation in reversed-phase systems as well as the influence of various agents on the separation of ionic solutes in IP–RP systems are explained. Examples of the application of ion-pair liquid chromatography to the analysis of aromatic carboxylic acids have also been reviewed. The principles and application of ion-exchange chromatography to the purification, isolation and less frequently, to chromatographic analysis are discussed. Polar adsorbents and polar bonded stationary phases are also widely used in carboxylic acid separation in normal-phase systems, mainly by TLC, often coupled with densitometry. The review also shows examples of separation of chiral benzoic acids and their derivatives in LC systems. The possibilities of application of gas chromatography preceded by derivatisation or pyrolysis of acidic compounds and applications of GC–MS and Py-GC–MS coupled methods in identification and quantitation of aromatic carboxylic acids is also reviewed.

Keywords: Reviews; Carboxylic acids; Benzoic acids; Phenolic acids; Aromatic carboxylic acids

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1. Introduction

1.1. Role of benzoic acid derivatives as antiphlogistic, antiseptic, analgesic, antipyretic, antirheumatic and anaesthetic drugs

In the group of non-steroid antiphlogistic drugs there are, among others, derivatives of aromatic carboxylic acids [1]. Non-steroid antiphlogistic drugs (NSAD) are effective in principle for checking the metabolism of arachidonic acid (the precursor of prostaglandins), which causes increased sensibility of pain and temperature receptors.

A better known group of antiphlogistic drugs are derivatives of salicylic acid. Pure salicylic acid as well as benzoic acid are used only externally as irritative and keratolytic agents. The derivatives of salicylic acid such as acetylsalicylic acid, sodium salicylate, salicylamide are used as antiphlogistic, analgesic, antirheumatic, antipyretic drugs.

Similar therapeutic effects are caused by derivatives of phenylacetic and phenylpropionic acids widely applied in rheumatic diseases because of their low side-effects in comparison to salicylic acid derivatives. Derivatives of phenylpropionic acid such as: ibuprofen, fenoprofen, ketoprofen and naproxen and derivatives of phenylacetic acid such as: diclofenac and aclofenac can be given as examples.

Anthranilic (2-aminobenzoic) acid derivatives such as mefenamic acid (Mefacit), niflumic acid, flufenamic acid have also similar therapeutic effects and are widely used in gout diseases.

4-Aminobenzoic acid (PABA) is one of the components of the folic acid molecule (vitamin of B group). However, 4-aminobenzoic acid is also a bacteria increasing agent. The sulfonamides are used as its antimetabolites and therefore prove biological activity against numerous microorganisms.

4-Aminosalicylic acid (PAS) has bacteriostatic properties for tuberculosis bacillus and is used in combined therapy with other drugs.

Some derivatives of aromatic carboxylic acids are also in the group of anaesthetic drugs having influence on the nerve endings. The derivatives of benzoic acid such as cocaine, hexylcaine and others are applied only in topical anaesthesia in stomatology, laryngology and ophthalmology. The derivatives of 4-hydroxybenzoic acid such as cyclomethycaine and paretoxycaine as well as derivatives of acetylsalicylic acid, e.g. edan have similar applications. The derivatives of 4-aminobenzoic acid such as procaine and tetracaine, being less toxic, are also used as a nerve block and spinal anaesthesia.

Aromatic acids are synthesised in human organisms as metabolites of intoxication with toluene, xylene, ethylbenzene [2]. These compounds are easily absorbed by the skin and mainly by the respiratory system and are oxidized to aromatic acids. The acidic metabolism products as well as acidic drugs and food preservatives are removed from organisms mostly as glycine conjugates (hippuric acid), also as glucuronides and as free acids (for example, salicylic acid).

1.2. Phenolic acids: derivatives of benzoic and cinnamic acid, active components of plants. Their role in biosynthesis. Their biological activity in pharmacopoeia plants

Among natural compounds there are numerous aromatic substances containing carboxyl group, semi-products of the synthesis of aromatic amino acids. They are mainly phenolic acids and their variety arises from the ability to undergo various transformations catalysed by plant enzymes [3]. For example, shikimic acid, a biosynthetic precursor of aromatic amino-acids, by the loss of an –OH group can be transformed to protocatechuic acid and then to vanilline, and by the loss of all its –OH groups can be transformed to benzoic acid, which gives rise to such monophenolic acids as 4-hydroxybenzoic acid and salicylic acid. Further hydroxylation of salicylic acid gives such products of biosynthesis as gentisic acid and *o*-pirocatechuic acid. The transformations are catalysed by enzymes – hydroxylases, co-operating with oxygen and proving specificity to the attacked substrates and to the attack position.

Phenolic acids occur as universal plant components bonded to lignins by ester bonds and play the role of inhibitors of cellulase secreted by pathogens across the membrane of cells and prevent the penetration of pathogens across the cell membrane. It has been reported that phenolic compounds accumulate in parts of plants infected by fungi [4].

One of the most important phenolic acids is gallic acid which is the substrate of numerous condensations producing galotannins (tannins). Tannins play a protective role against pathogens and vermin. The synthesis of tannins takes place as the response of plants to attack from insects.

The next group of acids present in plants are derivatives of phenylpropenoic acids such as cinnamic acid and the products of its substitution with hydroxy and methoxy groups by such enzymes as hydroxylases and methylotranserases, e.g., caffeic, chlorogenic, coumaric, ferulic, synapic and other acids. It is asserted that the main physiological role of this group of acids is the inhibition of sprouting, by the inhibition of mitotic division of cells.

The cinnamic acid derivatives are the medial stage in the formation of the corresponding alcohols i.e. components of lignin structure in cell walls of old plants. The coumarins are products of lactonisation of *trans*-cinnamic acids.

Because of the varied pharmacological activity of phenolic acids such as biligenic and cholagogue activity [5,6], hypocholesteramic and hypolipidemic properties [5,7] and antibacterial and antivirus activity [5], plants containing these compounds are widely used in phytotherapy.

In the soil phenolic acids are formed mainly from decomposing plant materials [3] and may be responsible for phytotoxic activity especially towards seedlings and, when present in high concentration, have a negative effect on crop productivity [8]. In fact, phenolic acids may act as allelopathic agents, that is, chemicals excreted by the plant which may be autotoxic or affect the growth of other plants in the environment [9–11].

Phenolic acids are permanent constituents of body fluids, especially the phenylacetic and mandelic acids and their derivatives [12,13]. They are metabolites of catecholamines: adrenaline, noradrenaline and dopamine. Especially 3-methoxy-4-hydroxymandelic acid (vanillic–mandelic acid) and homovanillic acid, the main metabolites of catecholamines, are present in urine in detectable quantities. Their concentration level is an important indicator in some disease diagnoses [14].

1.3. Aromatic acids and their role in other spheres of industry

There are several acids widely used in the industry of gliptal resins: polyesters of two (or more) carboxylic acids and polihydroxy alcohols [15]. The glycol esters are semi-products in the production of varnish and dyes and glycerol esters in the production of polyester fibres.

Synthetic carboxylic acid esters are used in the perfume industry, for instance, the methyl ester of anthranilic acid, the component of essential oils of orange and jasmine flowers, and also the methyl ester of salicylic acid as well as the esters of phenyl acetic acid used as aromatic substances.

Benzoic acid is used as sodium salt in the food industry as a preservative agent because of its properties as an inhibitor of microorganisms.

2. Retention of ionizable weak acids in liquid chromatography in RP systems

HPLC separation of ionic samples tends to be more complicated and difficult to understand [16] than separation of non-ionic compounds. On the other hand, band spacing is much more easily manipulated for ionic than for neutral samples, which improves the likelihood of successful final separation. For regular ionic samples, we have a choice of three HPLC methods: reversed-phase, ion-pair or ion-exchange chromatography. Because of its simplicity, freedom from problems and better column performance RPC is usually the best starting point. If RPC separation proves inadequate, the addition of an ion-pairing reagent to the mobile phase or application of ion-exchange chromatography can be considered.

2.1. Selectivity of separation as a function of pH and mobile phase composition

Dependence of retention of different ionic substances on pH were often determined using liquid–liquid partition systems [17,18] and the results were applied for the determination of suitable buffer systems for countercurrent distribution [19].

From the theory [20] for RP retention of monoprotic acidic compounds as a function of pH it can be assumed that a given solute (for example carboxylic acid) exists in ionized (–) and non-ionized forms and its capacity factor k is given by:

$$k = k_0(1 - F^-) + k_{-1}F^- \quad (1)$$

where k_0 and k_{-1} refer to k values for non-ionized and ionic forms and F^- is the fraction of ionized solute molecules:

$$F^- = 1/\{1 + ([H^+]/K_a)\} \quad (2)$$

Several papers [21–23] have compared experimental data for acidic solutes with Eqs. (1) and (2) for a wide range of pH with good agreement. Significant deviations from the theory occur for pH values either $\gg pK_a$ or $\ll pK_a$.

The use of Eqs. (1) and (2) allows predictions of separation as a function of pH based on three initial experiments – three for each solute to determine k_0, k_{-1} and K_a.

The potential errors in the use of the Eqs. (1) and (2) result from the following facts [20]:

- retention of solutes by processes other than solvophobic interactions, e.g. with exposed silanols or metal contaminants [24]
- change in K_a values as a function of ionic strength: buffer concentration or buffer fraction
- solvophobic effect of ionic strength on solute retention (hydrophobic interaction)
- ion-pair interaction of sample ions with ionized buffer species
- change in the sorption properties of the stationary phase (C_8 or C_{18}) as a result of changing ionization of silanols
- a change in buffer type, when more than one buffer type is needed to cover a given pH range

It is maintained [16] that computer simulations based upon the theoretical model (Eqs. (1) and (2)) are able to predict accurately retention and resolution of acidic solutes as a function of pH. Fig. 1 [25] shows the dependence of the retention of benzoic acids on mobile phase pH. Three experimental runs with varying pH are required as input for the computer simulation [20]. Predicted retention time

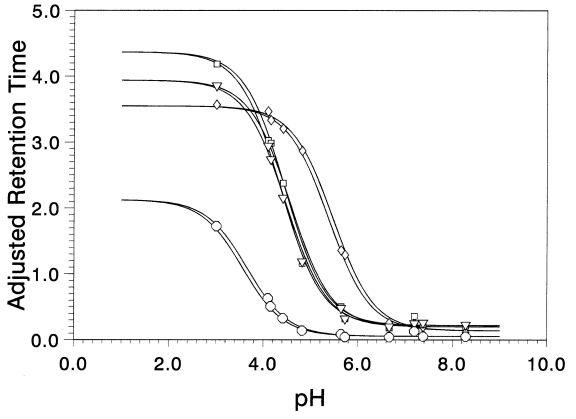


Fig. 1. Adjusted retention times of benzoic acids in 60% methanol against pH of mobile phase for acids: rhomb. = benzoic, circle = 2-nitrobenzoic, triangle = 3-nitrobenzoic, and square = 4-nitrobenzoic; for details see Ref. [25], reprinted with permission.

and α values were significantly more accurate for the case of benzoic acids than basic solutes due to silanol effects (more significant for basic solutes).

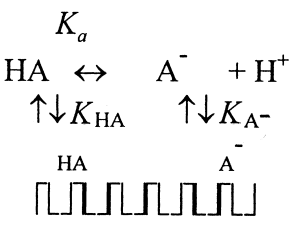
As retention factors (k) can decrease by a factor of ten or more for ionized versus a non-ionized compound, it is often necessary to combine pH optimization with variation of solvent strength (%B) in order to maintain a reasonable k range for the resulting separation ($1<k<20$) [20,26].

There are several papers [26–29] dealing with the combined variation of mobile phase pH and solvent strength. Marques and Schoenmakers [30] provide a detailed study of this problem.

The capacity factor k of an ionizable compound is a function of pH and concentration (volume fraction φ) of organic modifier in mobile phase (φ):

$$k = f([H^+],\varphi) \quad (3)$$

When we consider a weak monoprotic acid HA a simple reversible mechanism of retention can be assumed:



Several equilibria influence the concentration of various forms of acid in the stationary phase (S):

$$HA + S \overset{K_{HA}}{\leftrightarrow} HA(S) \quad (4)$$

$$A^- + S \overset{K_A}{\leftrightarrow} A(S)^- \quad (5)$$

$$HA \overset{K_a}{\leftrightarrow} H^+ + A^- \quad (6)$$

from which the following equilibrium constants can be derived:

$$K_{HA} = \frac{[HA(S)]}{\{[S_0] - [HA(S)] - [A(S)^-]\}[HA]} \quad (7)$$

$$K_{A^-} = \frac{[A(S)^-]}{\{[S_0] - [HA(S)] - [A(S)^-]\}[A^-]} \quad (8)$$

$$K_a = \frac{[H^+][A^-]}{[HA]} \quad (9)$$

where S_0 is total concentration of adsorbent active sites.

Because the concentration of occupied active sites in comparison to the total concentration of active sites is very low $[S]_0 \gg [HA(S)] + [A(S)^-]$ Eqs. (7) and (8) can be transformed as follows:

$$K_{HA} = \frac{[HA(S)]}{[S_0][HA]} \quad (10)$$

$$K_A = \frac{[A(S)^-]}{[S_0][A^-]} \quad (11)$$

The capacity factor of weak acid from the definition is $k_0 = \beta[HA(S)]/[HA]$ (β = ratio of stationary and mobile phases volumes) and thus:

$$k_0 = \beta \cdot [S_0][K_{HA}] \quad (12)$$

and for the anionic form analogously:

$$k_{-1} = \beta \cdot [S_0][K_A] \quad (13)$$

Because the capacity factor of weak acid is the average of the capacity factors of the individual species HA and A^-:

$$k = k_0\left(\frac{[HA]}{[HA] + [A^-]}\right) + k_{-1}\left(\frac{[A^-]}{[HA] + [A^-]}\right) \quad (14)$$

it can be transformed to the equation:

$$k = \frac{k_0 + k_{-1}K_a/[H^+]}{1 + K_a/[H^+]} \quad (15)$$

For each H_mA_n acids the global capacity factor is given by the equation:

$$k = \sum_m \sum_n k_{m,n} \frac{[H_mA_n]}{c_A} \quad (16)$$

where m may be zero for deprotonated solute, $n = 1,2,3\ldots$. c_A = concentration of A in all possible forms.

The problem is, however, more complex because the acidity constant K_a as well as the retention factor of the protonated form k_0 and the ionized form k_{-1} vary with the concentration of modifier in the aqueous mobile phase (φ), although the general form of Eq. (15) is maintained. This problem of the retention factor as the combined function of pH and modifier concentration in the aqueous mobile phase was analyzed by several researchers [26–32]. Some-

times the obtained mathematical models of such functions are complicated, operating with numerous parameters.

Marques and Schoenmakers made two approaches [30]:

1. At constant pH they describe k_0 and k_{-1} as a function of concentration of organic modifier in mobile phase $=\varphi$, taking from previous papers the dependence of the acidity constant as a function of [33]:

$$k = \delta + \frac{k_0(\varphi) + k_{-1}(\varphi)K_a(\varphi)/[\mathrm{H}^+]}{1 = K_\mathrm{a}(\varphi)/[\mathrm{H}^+]} \tag{17}$$

2. The second approach starts with:

$$\ln k = \mathrm{A} + \mathrm{B}\varphi + \mathrm{C}\varphi^2$$

where A is ln k for 0% modifier (methanol), which should be the sigmoidal function of $[\mathrm{H}^+]$, so that

$$\mathrm{A} = \ln\left[\frac{k_0^\mathrm{w} + k_{-1}^\mathrm{w}K_\mathrm{a}^\mathrm{w}/[\mathrm{H}^+]}{1 + K_\mathrm{a}^\mathrm{w}/[\mathrm{H}^+]}\right] \tag{19}$$

where k_0^w = capacity factor of HA, k_{-1}^w = capacity factor of A^- and K_a^w = acidity constant, all in pure water.

The authors assumed a linear relationship of ln k versus φ: $\ln k = \ln k_0 - S\varphi$. By measuring k at a fixed pH and two percent concentrations φ_α and φ_β of methanol the S value can be defined as:

$$S \approx \frac{1}{\varphi_\alpha - \varphi_\beta}(\ln k_\beta - \ln k_\alpha) \tag{20}$$

When pH is varying, the capacity factor at φ_α and φ_β can be expressed as sigmoidal relationships:

$$S([\mathrm{H}^+]) = \frac{1}{\varphi_\alpha - \varphi_\beta}\left[\ln\left(\frac{k_{0\beta} - k_{-1\beta}K_{\alpha,\beta}/[\mathrm{H}^+]}{1 + K_{\alpha,\beta}/[\mathrm{H}^+]}\right) - \ln\left(\frac{k_{0\alpha} - k_{-1\alpha}K_{a,\alpha}/[\mathrm{H}^+]}{1 + K_{a,\alpha}/[\mathrm{H}^+]}\right)\right] \tag{21}$$

After suitable transformations (when K_a is constant over the studied pH range):

$$S([\mathrm{H}^+]) = \frac{1}{\varphi_\alpha - \varphi_\beta}\ln\left(\frac{k_{0\beta} - k_{-1\beta}K_a/[\mathrm{H}^+]}{k_{0\alpha} - k_{-1\alpha}K_a/[\mathrm{H}^+]}\right) \tag{22}$$

The function derived from this approach has the form:

$$k = \delta + k^w([\mathrm{H}^+])\exp[B([H^+])\varphi + C([H^+])\varphi^2 \tag{23}$$

The first approach (Eq. (15)) is realised assuming k_0, k_{-1} and K_a as different functions of mobile phase composition: linear, quadratic, cubic (for K_a) and $\delta = 0$ or $\delta \neq 0$ (δ = constant shift parameter). All the models (class 1 models) were verified experimentally. The model approaching ln k_0, ln k_{-1} and ln K_a as quadratic functions of φ and $\delta = 0$ is in this authors' opinion the best compromise between precision and practicality.

The second approach (Eq. (23)) is realised assuming k_0 as a sigmoidal function of $[\mathrm{H}^+]$, the B parameter as a quadratic, cubic or sigmoidal function of $[\mathrm{H}^+]$ and $C = 0$ or C as a linear function of [H] and $\delta = 0$ or $\delta \neq 0$. All models were verified experimentally (class two models).

Models approaching k_0 as a sigmoidal function of $[\mathrm{H}^+]$, B as a cubic function of $[\mathrm{H}^+]$, $C = 0$ and $\delta \neq 0$ or k_0 as sigmoidal function of $[\mathrm{H}^+]$, B as a quadratic and C as a linear function of $[\mathrm{H}^+]$ and $\delta \neq 0$ are adequate for practical purposes. The model equations proposed by Marques and Shoenmakers [30] are thus the following:

$$k = \frac{k_0^0 \exp(S_0\varphi + T_0\varphi^2)[\mathrm{H}^+] + k_{-1}^0 K_\mathrm{a}^0 \exp[(Q_1 + S_{-1})\varphi + (Q_2 + T_{-1})\varphi^2]}{[\mathrm{H}^+] + K_\mathrm{a}^0 \exp(Q_1\varphi + Q_2\varphi^2)} \tag{24}$$

$$k = \delta + \frac{k_0^\mathrm{w}[\mathrm{H}^+] + k_{-1}^\mathrm{w}K_\mathrm{a}^\mathrm{w}}{[\mathrm{H}^+] + K_\mathrm{a}^\mathrm{w}} \exp\{\varphi(S_0 + S_1[\mathrm{H}^+] + S_2[\mathrm{H}^+]^2 + S_3[\mathrm{H}^+]^3)\} \tag{25}$$

$$k = \delta + \frac{k_0^\mathrm{w}[\mathrm{H}^+] + k_{-1}^\mathrm{w}K_\mathrm{a}^\mathrm{w}}{[\mathrm{H}^+] + K_\mathrm{a}^\mathrm{w}} \exp\{\varphi(S_0 + S_1[\mathrm{H}^+] + S_2[\mathrm{H}^+]^2) + \varphi^2(T_0 + T_1[\mathrm{H}^+])\} \tag{26}$$

2.2. Retention as a function of ionic strength of eluent

The pH of the mobile phase is a major factor in the separation of ionizable compounds. As was mentioned in Section 2.1, the most widely used model [20] considers the retention factor as an average of k_0 and k_{-1} according to the mole fraction of neutral and ionic forms. The mole fraction depends on the pK_a and pH of mobile phase (Eq. (2)). The pH of the mobile phase is taken to be the same as that of the aqueous fraction and this implies a false assumption. Even when pH is measured after mixing the buffer with the organic modifier the potentiometric system calibrated with aqueous standards does not measure the true pH of the mobile phase.

The second problem is that [25,30,34]:

$$\mathrm{pH} = -\log a_{\mathrm{H}^+} = -\log[\mathrm{H}^+]\gamma_{\mathrm{H}^+} \quad (27)$$

and the effect of activity coefficients γ can be neglected in water which has a high dielectric constant, but when the percentage of the organic modifier in the mobile phase increases, the dielectric constant of the medium and the activity coefficients decrease and cannot be neglected. Similarly for dissociation constant:

$$K_a = \frac{[A^-][\mathrm{H}^+]\gamma_{A^-}\gamma_{H^+}}{[\mathrm{HA}]} \quad (28)$$

The Fig. 2 shows the variation of the pK_a values of acids with the methanol–water composition.

From the Debye–Huckel [35] definition an activity coefficient depends on the ionic strength I of the solution:

$$\log \gamma = -\frac{Az^2\sqrt{I}}{1 + Ba^0\sqrt{I}} \quad (29)$$

where z is the charge of the ion, a^0 a constant value and A and B are Debye–Hückel parameters.

The pH scale of any amphiprotic solvent is limited by zero and pK_{ap} values (K_{ap} = autoprotolysis constant of medium); it differs in a mixed solvent for example methanol–water, where different proton transfer equilibria occur. Ionizable solutes dissolved in these mixtures are differently solvated, show different dissociation constants and the pH scale of the medium changes with mobile phase composition.

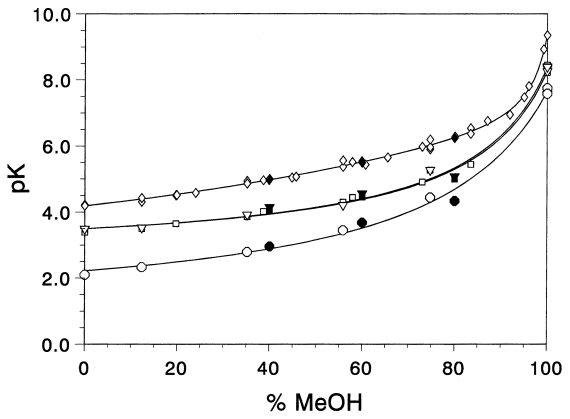


Fig. 2. Variation of the pK_A values of acids with the methanol–water composition for acids: rhomb = benzoic, circle = 2-nitrobenzoic, triangle = 3-nitrobenzoic, square = 4-nitrobenzoic; for details see Ref. [25], reprinted with permission.

Because the retention of ionic solutes depends on K_a, pH and solvent strength, it depends on the activity coefficients of ions in the medium and therefore on its ionic strength [25]:

$$t_R = \frac{t_{R(HA)}\gamma_{A^-} \cdot 10^{pK_a - pH} + t_{R(A^-)}}{\gamma_{A^-} \cdot 10^{pK_a - pH} + 1} \quad (30)$$

This Eq. (30) can be effectively used to calculate the pK_a value and retention time $t_{R(HA)}$ of neutral acid HA and $t_{R(A-)}$ of anionic base A^- from the measured retention times (t_R), pH and γ_A variables.

The assumption that the retention of ionic compounds depends only on the pH and mobile phase composition may be acceptable for the neutral form of the solute, but not for the ionic species. The retention of these species depends mostly on such processes as ion-pairing with other ions, solvophobic effects of the ionic strength and co-ion exclusion resulting from ionization of the residual silanol groups on the adsorbent surface [20].

2.3. Application of RP-HPLC systems for the carboxylic acids analysis

The optimization of RP separation and controlling the selectivity of acidic samples can be performed

similarly to non-ionic compounds by variation of the solvent strength (%B) to obtain a satisfactory k range ($1<k<10$) or by change of the column type (C_8, C_{18}, phenyl, cyano).

In applications of RP systems for the analysis of aromatic acids the choice of a suitable buffer is very important [16]. Several properties should be taken into consideration, such as: buffer capacity, UV absorbance and also solubility, stability, interactions with the sample and chromatographic systems.

Buffer capacity is determined by pH, pK_a and buffer concentration. The buffer is effective in controlling pH only in the range p$K_a \pm 1.5$, when buffer ionization occurs; a buffer concentration of 10–50 mM is usually adequate. Higher buffer concentrations provide increased buffer capacity but difficulties in solubility appear (salting-out effect), especially when high concentration of organic modifier are used. For RP separations when silica-based columns are used, the pH range of the mobile phase should be in the range 2–8. Therefore, for chromatographic analysis of aromatic acids the following buffers can be used: phosphate buffer, (2.1–3.1 and 6.2–8.2), acetate buffer (3.8–5.8), citrate buffer (2.1–6.4), carbonate buffer (3.8–4.8) and others. UV cutoff of a buffer is also very important. Ideally, the buffer should transmit light in the UV range.

The solubility of the buffer in the mobile phase is the next difficulty. Inorganic buffers such as phosphate are weakly soluble in solutions with high concentration of organic modifier. Methanol–water mobile phases provide better solubility than other aqueous eluents such as those containing tetrahydrofuran or acetonitrile; therefore, methanol should be the first choice organic solvent. Carbonate buffers are volatile because of the gradual loss of CO_2.

The preferred buffers in RP-HPLC are phosphate and acetate or phosphate–acetate in combination and can control pH over the range $2<\text{pH}<8$.

However, although temperature has a minor effect on band spacing for the RP-HPLC of neutral compounds, it has considerable influence on the separation of ionic samples, because it changes ionization of sample compounds, hydrophobic retention of ionized versus non-ionized molecules of a given compound and a change of pH and pK_a [16,36].

Table 1 presents examples of RP separations of aromatic carboxylic acids and Fig. 3 shows applications of RP-HPLC to the separation of phenolic acid mixtures [37].

3. Retention of carboxylic acids in RP ion-pair LC

Ion-pair and RP-HPLC share several features. The column and mobile phase used for these separations are generally similar, differing mainly in the addition of an ion-pairing reagent to the mobile phase for ion-pair chromatography (IPC). If the RPC method development is unable to provide an adequate separation due to poor band spacing, IPC provides an important additional selectivity option [16].

3.1. Mechanisms of ion-pair formation

The mechanism of ion-pair formation is still discussed [79]. There are several models explaining the mechanisms of processes occurring in ion-association systems [80–86]. Most of the proposed theories are derived from a few fundamental models:

- The ion-pair model assumes an association between the sample ion and an oppositely charged ion-pairing reagent in a liquid polar mobile phase before its adsorption on the hydrophobic stationary phase [87–91]. The ion-pair associate has a greater affinity for the non-polar stationary phase, which causes a stronger retention of the analyzed compound, adsorbing as an uncharged ion-pair. It can be assumed that the analyzed acidic anion A^- is adsorbed by the non-polar stationary phase as the ion-pair C^+A^-.

$$A_w^- + C_w^+ \leftrightarrow [AC_w] \leftrightarrow [AC_{org}] \tag{31}$$

The equilibrium constant of this process is as follows:

$$K_{IP} = \frac{[AC_{org}]}{[A_w^-][C_w^+]} \tag{32}$$

and from this:

$$\frac{[AC_{org}]}{[A_w^-]} = K_{IP}[C_w^-] \tag{33}$$

Table 1
Examples of application of RP-HPLC systems in the analysis of aromatic carboxylic acids

Sample	Compounds investigated	Mobile phase	Stationary phase	Dete ction	Remarks	Ref.
Italian wines	Phenolic acids	MeOH–phosphate buffer pH 2.7 (gradient)	ODS 5 μm	UV 280, 230 nm	SPE C_{18}–THF	[38]
Standards	11 phenolic acids	(1) MeCN–H_2O–1% AcOH (2) MeCN–THF–1% AcOEt (gradient)	C_{18}	UV	Computer simulation DrylabG	[37]
Canola-Meal	Phenolic glycoside	10% MeCN–H_2O+2% AcOH	C_{18}	UV	Semipreparative HPLC	[39]
Wine	Phenolic acids	MeOH–phosphate buffer pH 2.4 (gradient)	Microbore ODS 5 μm	UV	SPE CN cartridge	[40]
Standards	Benzoic and cinnamic acids	MeOH–AcOH–aq. ammonium acetate	ODS	ED	Comparison UV,ED, MS detection + chro matogr. systems	[41]
Eucalyptus spp., wood, bark leaf	Flavonoids phenolic acids, aldehydes	MeOH–H_2O, MeCN–H_2O +HCOOH, AcOH or H_3PO_4 (gradient)	ODS Hypersil	DAD	–	[42]
Echinacea spp.	Phenolic acids	MeOH–H_2O–AcOH-isocratic 25–75–1	ODS Hypersil	UV	SPE-anion exch. resin	[43]
Sherry wines	Phenolic acids polyphenolics	MeOH–AcOH–H_2O MeCN–H_2O (gradient)	C_{18}	PAD	SPE-C_{18}+SAX	[44] [45]
Plant extracts (barley straw, mixed grass)	*cis*, *trans*-Cinnamic acids	*n*-BuOH–H_2O–AcOH-isocratic 1.5–98–0.5	Nova-Pak C_{18} (T=35°C)	UV 270 nm	–	[46]
red wine	Phenolic acids + other compounds	2–100% MeCN aq. pH 2.6 gradient	C_{18}	UV	Fractionation C_{18} Seppak	[47]
Ruminant urine	Urinary aromatic acidic metabolites	15–100% MeOH–H_2O–AcOH pH 3.3 gradient	C_{18}	UV 210	Quantitation, animals' diets	[48]
Human urine containing *Gingko biloba* flavonol metab.	Substituted benzoic acids	H_2O–MeCN–MeOH–AcOH 88–5–5–2 88–10–2	Spherisorb ODS 5 μm	DAD	Flavone metabolism	[49]
Rat urine, blood containing *Gingko biloba* flavonol metab.	Substituted benzoic acids	H_2O–MeCN–AcOH 88–10–2	Spherisorb ODS 5 μm	DAD MS	Flavone metabolism	[50]
Vegetable products	Benzoic acids	MeOH–phosphate buffer pH 6.7 isocratic	Spherisorb ODS 5 μm	PAD	Food-preservation	[51]
Goat rumen fluid	Aromatic carboxylic acids	MeOH–sodium acetate buffer 8–92 (pH 6.5)	Spherisorb ODS 5 μm	UV 220 nm	–	[52]
Standards	Acidic drugs	MeOH–H_2O	C_{18}	UV	Hydrophobic interactions: acids-bovine serum albumin	[53]
Heart	Salicylic acid + products of hydroxylation	buffer 0.03 *M* sodium acetate + 0.03 *M* citric acid pH 3.6	ODS	ECD	Indirect measure of hydroxyl radical in heart	[54]
Standards	Substituted benzoic acids	MeCN–phosphate buffer pH 4–6 20–80	NH_2 polymer supports	UV	Good stability in aq. buffer solutions	[55]
Rat liver	Benzoic acids + glycine conjugates	MeOH–H_2O in different proportions + AcOH (0.2%)	C_8 5 μm	Ion-spray MS	Structure–metabolism relationship	[56]
Standards	Benzoic acids + glycine conjugates	MeOH–H_2O in different proportions + AcOH (0.2%)	C_8 5 μm	Ion-spray MS	Ion-mass spectral characterisation	[57]

Table 1. Continued.

Sample	Compounds investigated	Mobile phase	Stationary phase	Dete ction	Remarks	Ref.
Human plasma, urine	Salicylic acid + glycine, glucuronide conjugates	Gradient elution	C_{18}	UV	Separation + isolation by prep. HPLC	[58]
Wheat straw	Phenolic acids	MeOH–0.1% aq. perchloric acid 12–88	Viospher C_6 5 μm	ECD	Lignin, cellulose metabolism	[59]
Wheat flour Ground wheat	Ferulic acid	12% MeOH–citrate buffer pH 5.4 isocratic	Hypersil ODS 5 μm	DAD fluoresc.	–	[60]
Wine	Aromatic acids	MeOH–phosphate buffer pH 2.1 10–60% gradient	C_{18}	UV 254	–	[61]
Withe juices, wines	*cis*, *trans*-Cinnamic acids, benzoic acids	H_2O–AcOH pH 2.65–MeCN gradient	Nucleosil C_{18} 5 μm	DAD 280 nm	Changes during vinification	[62]
Cider brandies aged in oak barrels	Benzoic acids, cinnamic acids	2% AcOH + 0.02 *M* sodium acetate in H_2O–MeOH gradient	Spherisorb ODS	UV 280 nm	Classification of distillates	[63]
Red grape skins *Monarda* flower petals	Phenolic acids anthocyanins	1% formic acid–H_2O–MeOH gradient	Super Pak C_{18} 5 μm	PAD	–	[64]
Beer	Phenolic acids flavanols	MeOH–H_2O–phosphoric acid 35–64–1	Nucleosil C_{18} 10 μm	UV 228 nm	Invest. of stabil. agent (PVPP) on concentration of phenolics, flavanols	[65]
Barley Malt	Cellulose bound phenolic acids	Phosphoric acid H_2O pH 2.6–MeCN (gradient)	Adsorbosphere C_{18}	PAD	Antioxidant properties of phenolic acids	[66]
Maple products	Phenolic acids	H_2O + trifluoroacetic acid–MeOH 2–40% (gradient)	Econosil C_{18} 5 μm	DAD ECD	–	[67]
Sweet cherries	Neochlorogenic acid *p*-coumaroylquinic acid	5% formic acid–H_2O–MeOH gradient	Superpac C_{18}	PAD	–	[68]
Birds and rodents tissues	Ornithine conjugates of carboxylic acids	MeOH–10 m*M* ammonium acetate buffer	C_{18}	UV	Bird metabolism	[69]
Genus Althaea	Phenolic acids	MeOH–HCOOH–H_2O	RP-18	UV 254	Identification	[70]
Quercus petraea, *Castanea sativa*	Elagic acid gallic acid	MeOH–H_2O–H_3PO_4 gradient	RP18 5 μm	DAD	Plant metabolism in sapwood–heartwood	[71]
Additives in foodstuffs	Benzoic acid	MeCN–H_2O–phosphate buffer pH 3.9	Nova Pak C_{18} 5 μm	UV	Comparison CZE HPLC	[72]
Preservatives in beverages, jams	Benzoic acid	8% MeOH–phosphate buffer—H_2O, pH 6.7	Spherisorb C_{18}	UV 254 nm	–	[73]
Biological samples	Thiophenecarboxy benzoic acid + metabolites	30% MeOH–H_2O–phosphate buffer (0.01 *M*)	RP18 5 μm	UF 254	Drug metabolism	[74]
Ground water	Benzoic acid	MeCN–H_2O–0.2%H_3PO_4 isocratic and gradient	Econosph. C_{18} 3 μm	UV	Anaerobic degradation of toluene	[75]
Plasma	Benzoic acid hippuric acid	MeCN–H_2O–AcOH 12–38–0.25	Ultrasph. ODS 5 μm	UV 254 nm	Benzyl alcohol metabolites	[76]
Chrysanthemum maximum Ran, *Ch. segetum* L., *Rudbeckia laciniata* L.	Phenolic acids	MeOH–H_2O + 1% AcOH	RP-18	DAD	Identification	[77]
Standards	Chloro-, nitro-, bromobenzoic acid derivatives	MeOH (MeCN)–aqueous acetic buffer gradient	C-1, C-8, C-18	UV 280 nm	Multimodal elution	[78]

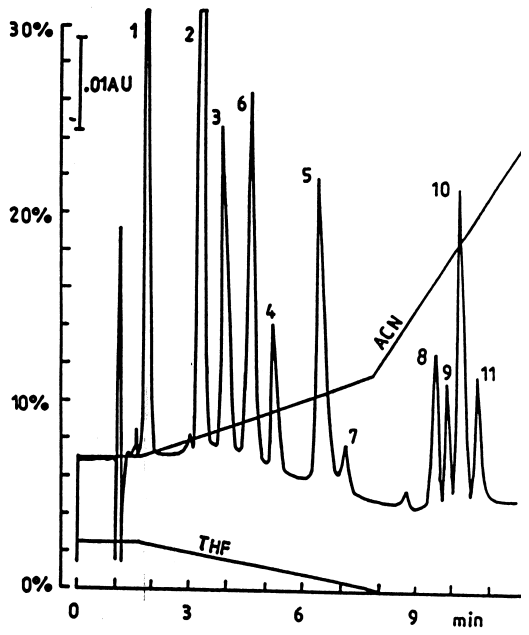


Fig. 3. Separation of phenolic acids by gradient elution with 7–11.6% acetonitrile and 2.5–0% THF during 0–6 min and 11.6—30% acetonitrile during 6–12 min. All solvents contained 1% acetic acid. Column packed with 7 μm Lichrosorb RP-18 (Merck). Acids: 1=gallic, 2=protocatechuic, 3=chlorogenic, 4=vanillic, 5=*trans*-caffeic, 6=syringic, 7=*cis*-caffeic, 8=*trans*-*p*-coumaric, 9=*cis*-*p*-coumaric, 10=*trans*-ferulic, 11=*cis*-ferulic. Reprinted with permission from Ref. [37].

Because the retention factor is defined by:

$$k = \frac{[AC_{org}] \cdot V_S}{[A_w^-] \cdot V_m} \quad (34)$$

where V_S and V_m are the stationary and mobile phases volumes, it can be transformed to the equation:

$$k = K_{IP}[C_w^-] \frac{V_S}{V_m} \quad (35)$$

Because the V_S/V_m ratio is constant for a given column, it results from Eq. (35) that the capacity factor depends on the kind and concentration of the counter-ion and in this way the retention of ionic compound can be controlled.

- The second model of the IPC mechanism is the ion-exchange model [92–97]. This model assumes the adsorption of lipophilic counter-ions on the non-polar surface of the stationary phase, which then behaves like an ion-exchanger. The molecules of the ion-pair reagent having a high affinity for the hydrophobic ligands on the stationary phase are easily adsorbed on it, forming the layer of ion-exchange sorbent. The processes which occur in such chromatographic system can be presented as follows: adsorption of ion-pair reagent with the co-ion Z^- on the sorbent surface:

$$C_w^+ + Z_w^- \leftrightarrow \{C^+, Z^-\}_{org} \quad (36)$$

adsorption of non-ionized analyzed compound on the ion-exchange sorbent-exchange of co-ion Z^- with an anion of chromatographed compound (aromatic acid)

$$A_w^- + \{C^+, Z^-\}_{org} \leftrightarrow \{C^+, A^-\}_{org} + Z_w^- \quad (37)$$

The increase of retention of the chromatographed ionic compounds may be caused by the increase of the concentration of the ion-pair reagent and by the increase of its hydrophobicity. Hansen et al. [98] have found that the coverage of the sorbent surface by a ion-pair reagent increases when the polarity of the eluent increases (lower concentrations of organic modifier). The retention of an acidic solute in IP systems also depends on the pH of mobile phase. When $pK_a - 2 < pH < pK_a + 2$, the solute molecules exist in ionic and non-ionic forms and the adsorption of both forms (ionic and non-ionic) and ion-pairing occurs [99].

- The next retention model in IP systems is the model proposed by Bidlingmeyer and co-workers [100] –an ion interaction model– model of a double electric layer. This model was elaborated on the basis of several chromatographic investigations of a retention of neutral and ionic compounds in eluent systems containing positively and negatively charged lipophilic counter-ions [101–103]. According to this theory the dynamic equilibrium of the lipophilic ion in the double electric layer formed on the sorbent surface occurs. The retention of the analyte is caused by the charge of the double layer formed by the ions of the ion-pairing reagent. The ions of the analyte compete for the access to the external charged layer, from which they are attracted by electrostatic and Van der Waals forces to the oppositely

charged internal layer and they can also interact with the nonpolar ligands of the stationary phase. The process is a dynamic one. The double layer produced has a definite surface potential which determines solute retention [104]. The surface potential depends on the electric permittivity, ionic strength of the mobile phase and hydrophobicity of the organic modifier. The model also explains the role of the addition of a competing ion of the same charge as the analyte ion. Since the system is in a dynamic equilibrium, the ions of both solutes compete in electrostatic and Van der Waals interactions and therefore the part of the adsorbed ions e.g. the analyte is eliminated from the surface more rapidly, which reduces the retention times and improves the peak shapes.

- The electrostatic model proposed by Stahlberg and co-workers [105–108] assumes that the ion-pairing reagent is fully ionized in the applied pH range and influences first of all the retention of the ionized form of the solute. The basic assumption of this model is the adsorption of the ion-pair and counterions on the charged layer formed on the surface of the stationary phase, which leads to the formation of a difference in electrostatic potential (Ψ_0) between the stationary and mobile phase. The capacity factor of the fully ionized molecules (k_i) of charge z_i is determined by the following equation:

$$k_i = k_{0i} \exp\left[\frac{-z_i F \Psi_0}{RT}\right] \quad (38)$$

where k_{0i} is the capacity factor of the fully ionized form in the absence of an ion-pairing reagent, F is the Faraday constant, R is the gas constant and T the absolute temperature. For monoprotic weak acids ($z_i = 1$):

$$k = \frac{k_{HA} + K_A/[H^+]k_{0A^-}\exp(F\Psi_0/RT)}{1 + (K_a/[H^+])} \quad (39)$$

where k_{HA} is the capacity factor of the non-ionized form of the weak acid, and k_{0A^-} is the retention factor of the ionized form when the ion-pairing reagent is absent.

The electrostatic potential has positive values for positively charged counterions and negative values for negatively charged counterions.

3.2. Parameters influencing the retention and selectivity in IP systems

In the analysis of acidic compounds cationic ion-pairing reagents have been employed such as alkylammonium compounds [109–111], organic amines and other basic compounds [112–114]. Crommen [115] reported that elongation of the alkyl chain of quaternary amines applied as ion-pairing reagents by a single methylene group increases the log k value by about 0.2 units. Similarly, an increase of the degree of substitution of the amines' nitrogen atom increases the retention; introduction of a methyl group to primary, secondary or tertiary amine increases the log k value by about 0.4 units [115,116].

When the concentration of ion-pairing reagent is gradually increased, then a distinct increase in retention of the analytes is observed and in a limited range of concentrations a linear relationship of log k and log of concentration of counterion is obtained [79,117,118]:

$$\log k = \text{const} - m \log[X] \quad (40)$$

where [X] is mole (or percentage) concentration of ion-pairing reagent.

However, the linear relationship of k versus concentration of ion-pairing reagent (in logarithmic scale) is observed only up to the moment of approaching the saturation of surface concentration of hydrophobic counterions, after which further increase of concentration does not lead to significant changes in retention (see Fig. 4) [119,120]. Moreover, a decrease in retention is sometimes observed [121,122].

The change of type and concentration of the counterion often causes variations in the selectivity of separation [120,123].

Additionally, the retention and selectivity in IP–RP systems can be controlled by a change of type and concentration of the organic modifier in the aqueous mobile phase [120,123]. The retention of solutes decreases with the increase in concentration of the organic modifier and the log k values are

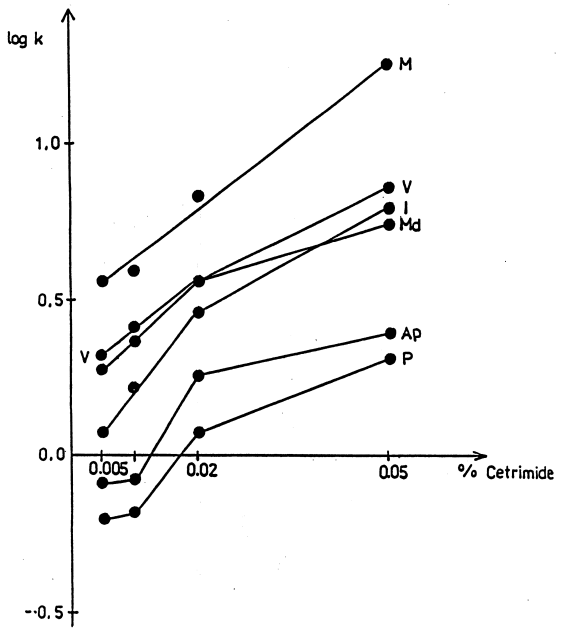


Fig. 4. log k versus % cetrimide in the mobile phase containing 70% methanol and 0.05 M phosphate buffer, pH 7.38. Column 10×3.8 cm, 10 μm ODS. Solutes: M=mefacit, V=voltaren, I=ibuprofen, Md=metindol, Ap=apranax, P=profenid. Reprinted with permission from Ref. [119].

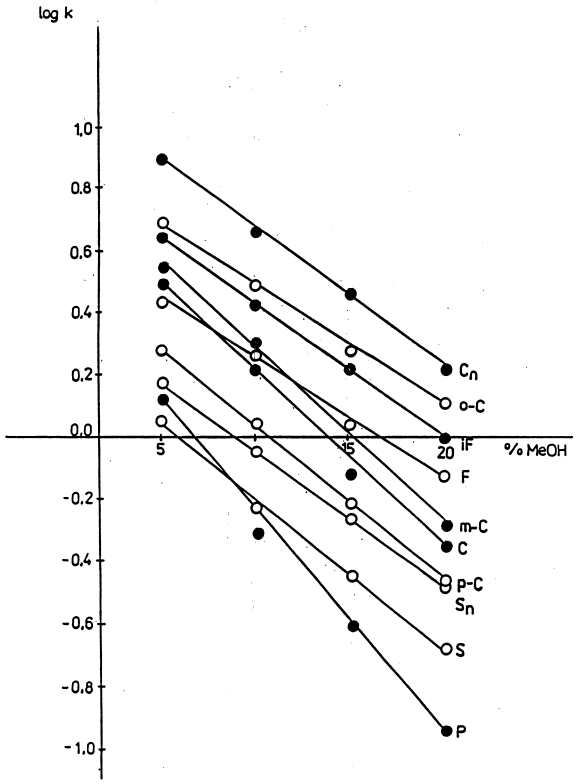


Fig. 5. Plots of log k versus % methanol in the mobile phase containing 0.01 M TEA-Cl and 0.005 M phosphate buffer of pH 7.17. Column: 10×3.8 cm, 10 μm ODS. Acids: Cn=cinnamic, o-C=o-coumaric, i-F=isoferulic, F=ferulic, m-C=m-coumaric, C=caffeic, p-C=o-coumaric, Sn=sinapinic, S=syringic, P=protocatechuic. Reprinted with permission from Ref. [119].

linear functions of the volume concentration of the modifier in accordance with equation [79]:

$$\log k = \log k_w - bC_{mod} \quad (41)$$

where k_w is capacity factor for pure water or aqueous buffer solution, b is constant (see Fig. 5 [119]).

The possibility of using a gradient of polar modifier concentrations at a constant concentration of ion-pairing reagents as well as gradient of concentration of ion-pairing reagent at constant concentrations of modifier for the separation of ionic compounds with various polarity has also been reported [124].

The next determinant playing an important role in IP–RP chromatography of ionic compounds is the pH of the mobile phase [83,125,126], which should be selected to obtain maximal ionization of solute molecules and ion-pairing reagent molecules to possibly form an ion-pair. For acidic solute analyses the pH range 7.0–7.5 is often applied, because extreme pH values can cause the destruction of an alkylosilane adsorbent.

The stationary phase, i.e. the length of the alkyl chains bonded to the silica support also influences the retention of hydrophobic ion-pairs.

Similar to RP-HPLC separations, selectivity can be additionally varied by solvent type (methanol, tetrahydrofuran, acetonitrile), buffer concentration and temperature [16].

3.3. RP-IP systems in analysis of carboxylic acids

Some problems that can occur in RP separation of ionic samples are also applicable to ion-pair HPLC: pH sensitivity, silanol effects (less serious), temperature sensitivity (more serious) and peak tracking are a few [16]. However, some special problems also occur. For example, sometimes positive and negative

artifact peaks appear in a blank run. It is usually the result of differences in composition of the mobile phase and sample solvent as well as the use of impure buffer or ion-pair reagents. These peaks can interfere with the development of the method and its routine use and it is necessary to carry out blank runs before development and after separation in IP systems. The next problem in IP separations is a slow column equilibration when ion-pair reagent is a component of the mobile phase. For this reason it is imperative to confirm reproducibility of sample separation when the eluent used contains an ion-pair reagent. Column equilibration is slower when the ion-pair reagent is more hydrophobic and in the case of quaternary ammonium reagents [95]. When the ion-pair reagent has to be removed from the column it should be previously removed by washing with a solvent followed by equilibration of the column with the next eluent. The slow equilibration of the column with many ion-pair reagents can create problems if a gradient elution is used under these conditions (more erratic base line, less reproducible retention). The gradient elution in IP systems is therefore not recommended except for small ion-pair reagent molecules like triethylamine. Another problem is the poor peak shape in some IP systems, sometimes corrected at higher temperatures [127].

Initial experiments should be carried out without ion-pair reagents in the mobile phase, which should only be added to achieve better control over the retention range and band spacing. Table 2 presents some examples of IP–RP separations of aromatic carboxylic acids.

4. Chromatography of weak acids using ion-exchange chromatographic systems (IEC)

Nowadays ion-exchange chromatography is used infrequently in comparison to other chromatographic methods. It is, however, used for the separation of inorganic salts, organometallics and sometimes for mixtures of biological origin. In most cases the separations possible in IP systems can also be achieved by IEC because of the similarity of both retention mechanisms [16]. In most cases IPC is more convenient because of higher column efficiency, more stable and reproducible columns and easier control over selectivity and resolution. There are, however, cases for using IEC instead RP or IP HPLC:

- When organic ions have poor UV absorptivity and need conductivity detection. Also when a mass spectrometer detector has to be used, completely volatile components of the mobile phase are required. In such a case, IEC with volatile buffers fulfils this requirement, whereas ion-pair reagents are not sufficiently volatile in most cases.
- When compounds are isolated or purified by HPLC separation, the removal of the mobile phase is necessary. In such a case, it is much easier with volatile components of mobile phases used in IEC separations. The removal of the ion-pair reagent is more difficult and therefore, for isolation of ionic components, IEC is used more frequently.
- When multi-step separation is necessary, the aqueous buffer-salt mobile phase used for ion-exchange allows direct injection of a sample fraction onto a RP column for the next step of separation. It may be difficult with IP systems.

For the separation of organic acids, anion-exchange columns are used, having positively charged groups (e.g.quaternary ammonium groups) attached to the stationary phase. Two kinds of anion-exchange column can be used: Weak Anion Exchanger (WAX) or Strong Anion Exchanger $-N(CH_3)_3^+$ (SAX). The retention of carboxylic acids X^- on such stationary phases (R^+) can be represented by the equilibrium of an ion-exchange:

$$X^- + R^+Cl^- \leftrightarrow X^-R^+ + Cl^- \quad (42)$$

where Cl^- plays the role of a counterion in the mobile phase and the effect of its concentration on retention of sample ion of charge z is:

$$k = \frac{\text{constant}}{(\text{counterion concentration})^z} \quad (43)$$

The increase of salt or buffer concentration in the mobile phase results in a decrease in retention of sample compounds. Moreover, the effect is greater for highly charged samples.

Table 2
Examples of application of IP–RP systems in the analysis of carboxylic acids

Ion-pairing reagent	Acids analysed	Method	Stationary phase	Mobile phase	Reference
Tetrabutylammonium bromide (TBA)	Salicylic acid, acetylsalicylic acid	HPLC	RP 18	MeCN–buffer pH 6	[89]
Terabutylammonium iodide (TBA)	Aspirin, antipyrine, codeine, phenobarbital	HPLC	Bondapak C_8	MeCN–phosphate buffer pH 3 and 6	[110]
Tetrabutylammonium hydroxide	*p*-Aminosalicylic acid	HPLC	RP 18	MeOH–phosphate buffer pH 7.7	[128]
Cetyltrimetyl-ammonium bromide	Nalidixic acid	HPTLC	RP 18	MeOH–phosphate buffer	[129]
Cetyltrimetyl-ammonium bromide	Naproxen	HPLC	RP 18	Methanol–formic buffer	[130]
Dodecylethyldimethyl-ammonium bromide	Glucuronides	HPLC	ODS	MeOH–phosphate buffer	[131]
Bromides: Tetramethylammonium, tetrabutylammonium, cetyltrimethylammonium, and trioctylmethylammonium chloride	Benzoic acids	TLC	Silanized silica and silica impregnated with ion-pair reagents	MeOH–H_2O	[132]
Bromides: Tetramethylammonium, tetrabutylammonium, cetyltrimethylammonium, and trioctylmethylammonium chloride	Substituted benzoic acids	OPLC	Silica impregnated with ion-pair reagents	MeOH–H_2O	[133]
Tetraethylammonuim iodide	Phenolic acids	HPLC + MS	Phenyl column	MeOH–H_2O 0.01% AcOH	[134]

The retention of ionic solutes can be controlled by changing pH. It is comprehensible that only the ionized form of the acid will be retained on the oppositely charged sorbent. The increase in pH leads to stronger ionization and retention of acidic sample. Varying pH is usually a way to change the selectivity in IEC separations. The next way to change retention in IEC systems is the use of different counterions (displacers). The relative strength of various displacers in an anion-exchange process is [16]:

$$F^-(\text{weak}) < OH^- < \text{acetate} < Cl^- < SCN^- < Br^- < CrO_4^{2-} < NO_3^- < I^- < \text{oxalate} < SO_4^{2-} < \text{citrate}$$

Sometimes the addition of organic modifiers such as methanol or acetonitrile is applied in IEC. It causes decreased retention of ionizable compounds.

A less common but interesting form of IEC is carried out with zirconia. Zirconia is an amphoteric material with anion-exchange properties in neutral and acid solutions [135]. Since the zirconia surface contains many adsorption sites and has the ability to ion and ligand exchange, it should be in most cases modified, e.g. by silylation of the surface or coating the zirconia surface with a polymer or carbon layer. The eluotropic series of Lewis bases created for benzoic acid derivatives at pH≈6 is presented by Blackwell et al. [136,137].

Synthetic (soft) ion-exchangers are not suitable for

Table 3
Examples of application of IEC systems in the analysis, isolation and purification of carboxylic acids

Sample	Compounds investigated	Mobile phase	Stationary phase	Remarks	Reference
Juices, wines	Phenolic acids	MeOH–H_2O–AcOH	SAX-quaternary amine cartridge	Isolation	[62]
Standards	*ortho*-Benzoic acid derivatives	60% MeOH–acetate buffer	Zirconium oxide, polymer based Zr-gel-5	Optimization	[63]
Wine tannin	Phenolic acids		Sephadex LH-20	Purification	[138]
Echinacea sp.	Phenolic acids	MeOH–H_2O–phosphoric acid	AE quaternary amine Bakerbond SPE	Purification, isolation	[43]
Bovine liver mitochondria	Glycine conjugates of aromatic and arylacetic acids		AnEx	Purification	[139]
Standards	Homologous series of *p*-alkoxybenzoic acids	K_2HPO_4 + NaCl acidified by HCl pH 7.4	Polyethylenimine coated zirconia	HP anion-exchange chromatography	[140]
Standards	*p*-Benzoic acid derivatives	Sodium acetate pH 7 + 100 μm NaF	Polyethylenimine coated zirconia	HP anion-exchange chromatography	[141]
Maize bran	Ferrulic acid, diferrulic acid		Amberlite XAD-2 Sephadex LH-20	Isolation, purification	[142]
Wine	Phenolic acids, shikimic acid	Na(HCOO) at 70°C	Aminex A25	Isolation	[61]
Standards	Salicylic acid, 5-sulphosalicyclic acid	H_2O solution of invest. acids	SiO_2 impregnated with Aliquat 336	Solid–liquid extraction	[143]
Standards	Phenolic acids	ammonia–ammonium sulphate buffer pH 8	SiO_2 impregnated with Aliquat 336	Extraction mechanism	[144]
Standard	*o*-Phthalic acid	acetate buffer-MeOH pH 6	Zirconia, polymer based zirconia	–	[145]
Standards	Substituted benzoic and naphthoic acids	1 *M* HCl + 1 *M* Tris	TSKgel IC-Anion-SW	Determination of pK_a values	[146]

HPLC; special ion-exchange sorbents on the basis of silica are used.

There are several examples of the use of IEC systems for purification, isolation, separation of benzoic acid derivatives presented in Table 3.

5. Normal phase systems in carboxylic acid analysis

The possibility of achieving different selectivity of separation on various polar adsorbents causes wide application of the normal-phase systems to the separation of acidic compounds. Ternary (or more component) mixtures containing polar modifiers from different classes of selectivity [147], nonpolar or moderately polar diluents and acetic or formic acid (to suppress dissociation of chromatographed acids) are used as eluents. There are several inorganic adsorbents like silica and Florisil, organic adsorbents such as cellulose and polyamide as well as polar bonded stationary phases like diol–silica, cyanopropyl–silica or aminopropyl-silica widely used mainly in thin layer chromatography, often followed by densitometry.

5.1. Retention as a function of mobile phase composition

Polar inorganic adsorbents are often used with non-aqueous non-polar solvent mixtures as eluents. According to the Snyder–Soczewiński's model of

adsorption [148,149], displacement of solvent molecules (S) occurs by solute molecules (Z) from adsorbent active sites (A):

$$AS + Z \Leftrightarrow AZ + S$$

Sometimes it is coupled with competitive solvation [150]. For a simple 1:1 displacement it leads to the equilibrium constant equation:

$$K = \frac{X_{AZ} X_S}{X_Z X_{AZ}} \quad (44)$$

and

$$k = \frac{X_{AZ}}{X_Z} = K \frac{X_{AS}}{X_S} \quad (45)$$

and to a simple general relationship(for1:*n* displacement or competitive solvation of solute):

$$\log k = \text{const} - n \log X_S \quad (46)$$

The relationships of log $k(R_M)$ versus log X_S (X = mole fraction of polar modifier) obtained for systems of the type: polar adsorbent/polar modifier + nonpolar diluent are linear with high regression coefficients for different groups of substances, among other aromatic acids and aldehydes [151–153]. Fig. 6 shows R_M (log k) versus log c relationships for phenolic acids obtained in the system: cellulose/benzene + acetic acid + methanol [154].

The slopes of R_M (log k) versus log c correlation lines (factor n in Eq. (46)) correspond in the simplest case to the number of polar groups responsible for the adsorption or the number of displaced solvent molecules. For the relationships obtained on polar adsorbents they are about two for two functional compounds (especially with functional groups in the 1,4 position) and about one for monofunctional compounds [153].

Surface-modified sorbents, especially the polar bonded stationary phases, increase in importance as stationary phases in both TLC and HPLC. This can often be attributed to the extended range of selectivity possible when such phases are used in combination with simple eluents. Surface-modified sorbents have a moderate polarity and can be used both in normal-phase and reversed-phase chromatography.

Linear relationships showing the effect of concentration of organic modifier on the retention of active compounds (R_M versus log C) have been

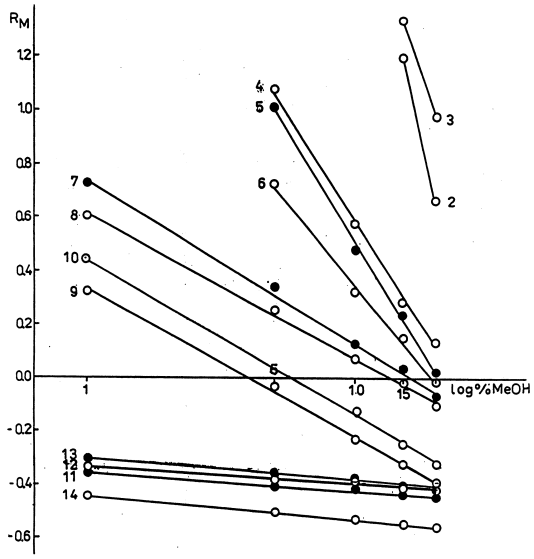


Fig. 6. Dependence of the R_M (log k) values of phenolic acid standards on the logarithm of the concentration of methanol in the eluent (benzene–acetic acid–methanol); sorbent = cellulose. Acids: 2 = gallic, 3 = chlorogenic, 4 = protocatechuic, 5 = caffeic, 6 = gentisic, 7 = *p*-hydroxyphenylacetic, 8 = *p*-hydroxybenzoic, 9 = *p*-coumaric, 10 = *o*-hydroxyphenylacetic, 11 = syringic, 12 = vanillic, 13 = synapic,14 = ferulic. Reprinted with permission from Ref. [154].

obtained for polar bonded stationary phases [155,156] according to Snyder–Soczewiński's displacement theory for normal-phase systems [148,149].

5.2. Comparison of the retention behaviour of aromatic acids on different adsorbents by TLC and HPLC

The differences of selectivity of separation on polar adsorbents are mainly caused by their different active sites. Adsorbents with specific surface areas and high densities of coverage with active sites fulfil the requirement for highly active solids. Such adsorption centres on silica are silanol (Si–OH) groups of various types [157]. Apart from silica, there are multi-active-site-type inorganic adsorbents such as alumina (rarely applied to the analysis of acidic substances) and Florisil having strong acidic properties because of their –OH groups which are proton-donor centres and Mg^{2+} ions which serve as strongly electron accepting centres [158]. Cellulose also

belongs to multi-active-site-type adsorbents; for aqueous eluents it acts primarily as a cellulose gel (liquid–liquid partition). Polar bonded stationary phases have different properties as polar sorbents because of their different surface active sites, although, the partition–adsorption mixed mechanism on NP–CN, NP–Diol and NP–NH_2 stationary phases in most eluent systems predominates [159].

Fig. 7 illustrates differences in the selectivity of separation of phenolic acids on Florisil and silica presented as R_{MI} versus R_{MII} correlation diagrams [152]. The points are widely dispersed, which indicates high differences in the retention behaviour of investigated ionic compounds on both adsorbent surfaces. Similar correlations have been obtained for derivatives of benzoylbenzoic acids in some eluent systems for silica and Florisil by HPLC [160].

5.3. Planar chromatography of phenolic acids

Examples of the use of thin-layer chromatographic technique and its modifications for the separation of aromatic carboxylic acid are presented in the Table 4 and Fig. 8 shows densitogram of phenolic acids separated on an NH_2–silica HPTLC plate using a gradient mode of development [161].

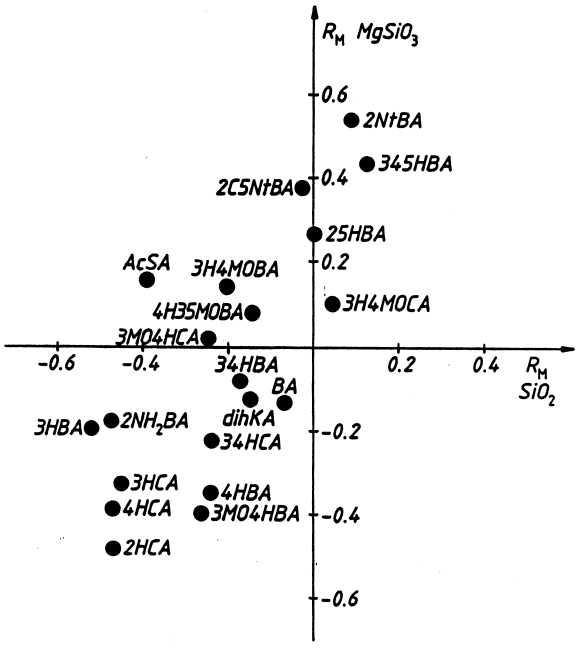


Fig. 7. Correlation between R_M values of aromatic acids on Florisil and silica; mobile phase: 2-propanol–*n*-heptane + 2% acetic acid. For details see Ref. [152], reprinted with permission.

6. Separation of chiral benzoic acids and their derivatives

The separation of chiral compounds is important because of the differences in biological activity of enantiomers (amino acids, drugs and their metabolites). Carboxylic acids are one of the most important classes of chiral compounds such as non-steroid antiphlogistic drugs (NSAD) and their metabolites as well as catecholamine metabolites.

It is well known that HPLC and TLC techniques are useful for the separation of carboxylic acid enantiomers. Derivatives of cyclodextrin, cellulose, amylose and proteins have been used as chiral stationary phases (CSPs) for this purpose [175–177].

Brush-type CSPs are unsuitable for the direct separation of racemic carboxylic acids and these compounds have usually been resolved in the form of amide derivatives [178]. It was found [179] that the separation of some aromatic carboxylic acids is possible with typical brush type CSP as *N*-3,5-dinitrobenzoyl-D-1-(α-naphthyl)glycine, 3,5-dinitrophenylaminocarbonyl-D-phenylglycine, 3,5-phenylaminocarbonyl-L-valine and 3,5-dinitrophenylaminocarbonyl-L-*tert*.-leucine covalently bonded to silica gel. Direct separation of various aromatic carboxylic acid enantiomers (NSAD, aromatic amino acids and derivatives) was accomplished with these chiral stationary phases [179]. The influence of the composition of the mobile phase (ammonium acetate–water–methanol; ammonium acetate–water–acetonitrile; hexane–dichloromethane–ethanol–acetic acid) was investigated.

Amylose tris(3,5-dimethylphenylcarbamate) chiral stationary phase (AD–CSP) was used for the separation of a series of 28 chiral α-alkyl arylcarboxylic acids. The retention data were correlated to a series of molecular descriptors to produce a quantitative structure-enantioselective retention relationships (QSERR) incorporating the hydrogen bonding ability and aromaticity of the solutes [180].

Direct resolution of optically active isomers of

Table 4
Examples of application of NP systems in the analysis of aromatic carboxylic acids by TLC

Sample	Compounds investigated	Stationary phase	Mobile phase	Remarks	Reference
Standards	Phenolic acids	Silica	16 binary eluents	R_M versus log C relationships	[162]
Standards	Phenolic acids	Silica	Homologous series of ethers and ketones	eluent strength of homologous series	[163]
Chelidonium majus L.	Hydroxycinnamic acid derivatives	Silica	CH_2Cl_2–toluene–formic acid; AcOEt–MeOH–H_2O–formic acid; AcOEt–acetic + formic acid–H_2O;	–	[164]
Piper nigrum L.	Aromatic carboxylic acids	Silica imp. ammonium sulphate	Toluene–ethanol–formic acid	Quantitative identification, preparative TLC	[165]
Standards	Natural phenol carboxylic acids	Silica	MeOH–CH_2Cl_2–H_2O–formic acid (gradient)	AMD, densitometry	[166]
Eupatorium cannabium	Caffeic, chlorogenic acid	Silica	Ethyl acetate–acetone–formic acid–H_2O	Qualitative identification	[167]
Standards and *Polygonum amphibium* extract	Phenolic acids	Cellulose	(1) MeOH–MeCN–benzene–acetic acid (2) H_2O–formic buffer	2D-TLC identification	[154]
Ribes nigrum *Ribes grossularia*	Phenolic acids	Cellulose	(1) MeOH–MeCN–benzene–acetic acid (2) H_2O–formic buffer	2D-TLC identification	[168]
Ginko biloba extract and preparation	Phenolcarbonic acids	Silica	Ethyl acetate–acetic acid–formic acid–H_2O	Qualitative identification	[169]
Urine	Phenolic acids	Silica cellulose	*n*-BuOH–AcOH–H_2O	Phenyloketonuria metabolism	[170]
Zizyphus jujuba Mill	Phenolic acids	Silica	AcOEt–HCOOH(AcOH)–H_2O	Optimization	[171]
Standards	Aspirin, salicyclic acid, metabolites	Silica	MeOH–*n*-hexane	AMD	[172]
Standards	*o*-Aroylbenzoic	Silica	DX–AcOH–toluene	Retention behaviour, comparison with RP-HPLC	[173]
Standards	Benzoic acid derivatives	Cellulose	*i*-BuOH sat. ammonia	Structure–activity relationships	[174]

carboxylic acids (among others NSAD, α-hydroxy carboxylic aromatic acids) was examined on chiral packings containing ergoline skeletons [181]. Optimization of the enantioseparations was attained through the study of the influence of the organic modifier content (methanol, acetonitrile), pH and ionic strength of the buffer (acetate, phosphate) in the aqueous eluent. Electrostatic and hydrophobic interactions between the ergot alkaloid and the analyte contribute to a large extent to the retention and chiral discrimination.

TLC techniques for the separation of α-hydroxy carboxylic aromatic acids (mandelic acid and its derivatives, vanillic acid, hydroxyphenylalanine) and aromatic amino-acids by means of ligand exchange on a RP-silica gel covered with a chiral selector (copper complexes of 4-hydroxyproline) have been described [182]. Most racemate separations can be

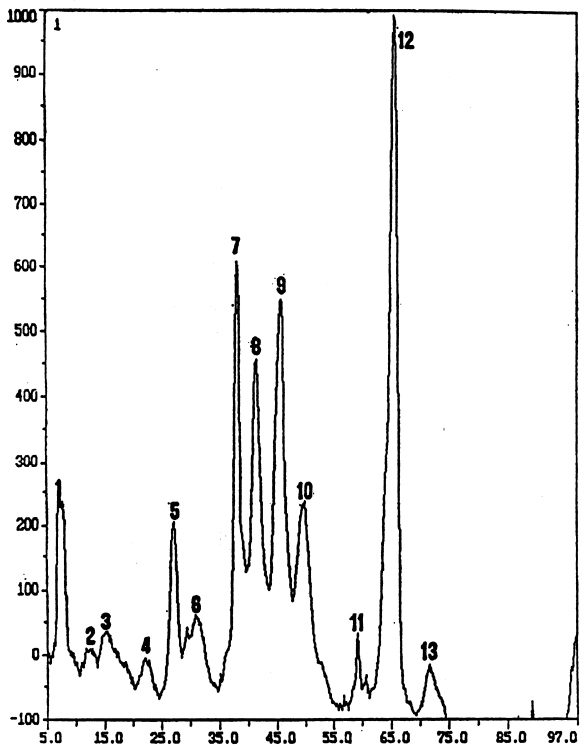


Fig. 8. Densitogram of the separated phenolic acids mixture. System: NH_2-silica/-ethyl acetate–chloroform–6 *M* acetic acid (multiple gradient development). Acids: 1=chlorogenic, 2=2,5-dihydroxybenzoic, 3=caffeic, 4=3,4-dihydroxybenzoic, 5=4-hydroxybenzoic, 6=*m*-coumaric, 7=*p*-coumaric, 8=isoferulic, 9=ferulic, 10=syringic, 11=protocatechuic, 12=*o*-hydroxybenzoic, 13=3,4-dimethoxycinnamic. Reprinted with permission from Ref. [161].

accomplished using eluent systems containing methanol–water–acetonitrile in various proportions and dichloromethane–methanol for the separation of α-hydroxyacids. Similar chiral stationary phases were used in ligand exchange column chromatography by the use of surface modified porous graphite [183].

The addition of β-cyclodextrin to the aqueous mobile phase (phosphate buffer (pH3) solution - organic modifier) improves the separation of *cis-trans* cinnamic acids in RP systems using TLC and HPLC techniques [184]. It is also possible to apply β-cyclodextrin as a mobile phase (ethanol–water) modifier in ion-exclusion chromatography on an Aminex HPX-87H column. This modifier permits the attainment of selectivity effects (separation of benzoic acid derivatives and other solutes) which differ from those achieved with the conventional method of varying the pH of the mobile phase [185].

7. Chromatography of aromatic acids using GC methods

Chromatography of free carboxylic acids in GC systems is difficult because of their low volatility, strong adsorption on solid supports and/or dimerisation of acid molecules. Nevertheless, the GC method is widely used for the chromatography of acidic compounds (aromatic carboxylic acids) because of the possibility of easy identification of separated unknown compounds when gas chromatography is coupled with mass spectrometry (GC–MS). Both analytical methods are easily coupled because of several similarities in the analysis conditions [186]:

- both methods analyze the sample in the gaseous phase
- quantitity of sample can be less than 10^{-6} g
- velocity of both methods is comparable; it seems that in the time of elution of chromatographed sample components several mass spectra can be obtained.

However, some differences exist in the conditions of analysis of both methods such as different gas pressures (much lower for MS analysis), which are technically solved by a suitable interface.

The samples containing aromatic carboxylic acids need transformation into a more volatile form by derivatisation or pyrolysis.

7.1. Derivatisation methods in carboxylic acids analysis. Pyrolysis

The general method of transformation of the sample consists of obtaining more volatile compounds e.g., their trimethylsilyl derivatives. The following derivative reagents are used for this purpose [187]:

- hexamethyldisilazane (HMDS) used initially requires the drastic conditions of reaction i.e. requires 16 h of heating. The reaction is accelerated by anhydrous HCl, which reacts with HMDS forming trimethylsilyl chloride (TMSiCl). The catalyst in the reaction is pyridine, dimethylsulfoxide or tetrahydrofuran. The use of a HMDS–TMSiCl mixture in a proportion 2:1 is more profitable.
- *N*,*O*-bis(trimethylsilyl)acetamide (BSA) is a stronger reagent than combined HMDS–RMSiCl. Moreover, there are no by-products of the reaction like ammonium chloride in the first case. BSA can be used with or without the solvent and the reaction is catalysed by pyridine. The use of a derivative of BSA-bis(trimethylsilyl)trifluoroacetamide (BSTFA) is more effective.
- *N*-trimethylsilyldiethylamine (TMS) is used in the case of non-reactive compounds. The by-product diethylamine can be removed by distillation. The variations of the reagent are derivatives containing one methyl group, chlorine or bromine atom.

Carboxylic acids can be chromatographed as their esters. These compounds are of middle polarity and are more volatile as substrates. The esterification can be performed in different ways. The main ways are the following:

- by the use of diazomethane (obtained in laboratory). The solutions of diazomethane are stable for only a few days, and are toxic and explosive but very reactive and react with carboxylic acids rapidly and effectively

Table 5
Examples of the application of GC in the analysis of carboxylic acids

Sample	Acidic compounds	Derivatising agent	Column	Temperature program	Detection	Remarks	Reference
Wheat, grasses	Monomeric and dimeric phenolic acids	TMS BSTFA	Capillary column – MePh polysiloxane film 40 m×0.25 mm	80–320°C	FID MS	Separation, identification	[188]
Standards	Benzoic acid	–	Capillary column QF1 – on	200°C	FID	Penetration and dermal retention	[189]
Urine of rats	Acidic metabolites of 1,4-diethylbenzene and conjugates	BSTFA, diazomethane	capillary column QV-1	35–55–250°C	MS	–	[190]
Egyptian mummy tissues	Phenolic acids	TMS methanolysis	Capillary column – phenyl methyl siloxane	40–290°C	MS	Invest. the botanical source of the tannin	[191]
Lysimachia nummularia	Phenolic acids	BSTFA	Capillary column SE 52 25 m×0.25 mm	80–260°C	MS	Identification, quantitation	[192]
L. vulgaris							
Patulin pathway metabolites	Gentisic acid, 6-methyl salicyclic acid, 3-hydroxybenzoic acid	TMCS HMDS	Stainless-steel column 10% QF1on Gas Chrom Q	120–200°C	FID	Quantitation	[193]
Plant tissues, soils	Phenolic acids	TMS	Capillary column SPB-1	138–150°C	FID	Qualitative, quantitative analysis	[11]
Standards	Phenolic acids	Methyl, ethyl chloroformate	Capillary column CP-SIL 5 CB 25 m×0.32 mm	Different temperature gradient	FID	Conditions of chloroformate derivatisation	[194]
Propolis	Phenolic acids	BSTFA	Capillary column SE-54	80–280–300°C	FID ECD	–	[195]

- by the reaction of silver salts of carboxylic acids with methyl iodide. The reaction takes a few hours but with 100% yield.

Pyrolysis of carboxylic acids can also be used to obtain more volatile fragments of the analytes. In the pyrolysers, the analyzed substance is thermally decomposed in the stream of carrier gas and is introduced directly to the sample injector. As a result, a characteristic pyrogram for a particular carboxylic acid is obtained by the chromatography of the pyrolytic products of compound decomposition.

7.2. Application of gas chromatography systems in analysis of carboxylic acids

Examples of the use of the GC technique in the separation of aromatic carboxylic acids are presented in Table 5.

Moreover, the GC–MS technique was used in the identification of phenolic acids in plants: lupine seeds [196], in *Populus-Trichocarpa* [197], in stem bark of *Amphypterygium Adstringens* [198], in wheat straw [199], in the investigations of metabolism of drugs [200], and in environmental analysis [201–204].

Pyrolysis–gas chromatography–mass spectrometry coupled techniques are used in the following analysis: ester and ether linked phenolic acids in cell walls of Coastal bermudagrass [205], wheat straw lignin [206], phenolic acids in wines [138,207], *p*-coumaric and ferrulic acids in paddy rice (*Oryza sativa* L.) [208], and aromatic carboxylic acids in organic matter of agricultural soils [209].

8. Symbols

a	activity
β	ratio between stationary and mobile phase volumes
C_A	sum of the concentrations of a solute A in all its possible forms
δ	constant shift parameter included in some retention models
F	Faraday constant
F^-	fraction of solute molecules that carry a negative charge
γ	ionic activity coefficient
I	ionic strength of the solution
k	observed retention factor
k_0	retention factor of neutral species
k_0^w	retention factor of neutral species in pure water
k_{0i}	retention factor of fully ionized form in the absence of ion-pairing reagent
k_{-1}	retention factor of negatively charged species
k_{-1}^w	retention factor of negatively charged species in pure water
k_α, k_β	observed retention factor of a solute at α%, β% of organic modifier respectively
$k_{m,n}$	retention factor of H_mA_n
k^w	observed retention factor of a solute in water
K_a	acidity constant of HA
K_a^w	acidity constant of HA in pure water
K_A	equilibrium constant for the partition of A^- between mobile and stationary phases
K_{HA}	equilibrium constant for the partition of HA between mobile and stationary phases
K_{IP}	equilibrium constant of ion-pairing reaction
φ	fraction of organic modifier in the mobile phase
Ψ_0	electrostatic potential
R	gas constant
S_0	total concentration of surface active sites
S	slope of a linear model for ln k as a function of φ
t_R	retention time of the solute
V_S	volume of stationary phase
V_m	void volume of column
z	charge of a ion

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