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Chromatography of substituted benzoic acids with methanol–water–carbon dioxide mixtures

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Abstract

The impact of the proportion of CO_2 concentration in methanol–water–CO_2 mobile phases on the separation of several substituted benzoic acids was explored by studying the variation of retention with mobile phase pH in these mixtures. As the amount of CO_2 in methanol–aqueous buffer–CO_2 mixtures increased, a more basic buffer was needed to control the dissociation of these acids. Differences in terms of retention, separation efficiency and peak asymmetry were shown for substituted benzoic acids with methanol–water–CO_2 and methanol–aqueous buffer–CO_2 mixtures. Variations of these chromatographic parameters with mobile phase pH were related to the dissociation of these acids and their interaction with methanol–aqueous buffer–CO_2 mobile phases and the stationary phase. The addition of a buffer into methanol–aqueous solution–CO_2 was an effective means to optimize separations of acidic analytes with high fluidity liquid mobile phases. The substituted benzoic acids had baseline separation in the least amount of time using the high fluidity liquid mobile phases.

Keywords: Mobile phase composition; Retention behavior; Benzoic acids; Carbon dioxide

1. Introduction

In reversed-phase high-performance liquid chromatography (HPLC) using conventional liquid mobile phases, the mobile phase pH is an important parameter to optimize the separation of ionizable analytes. For a weak acid, HA, its dissociation in the mobile phase is governed by the dissociation equilibrium. If the dissociation equilibrium is much faster than the adsorption–desorption equilibrium of analytes between the mobile phase and stationary phase, the retention factor (k) of the weak acid is the weighted average of that of the neutral (k_{HA}) and ionized species (k_{A^-}) [1]:

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

By combining Eq. (1) with the dissociation constant expression, Eq. (2) is obtained:

$$k = \frac{K_a 10^{pH} k_{A^-} + k_{HA}}{1 + K_a 10^{pH}} \quad (2)$$

Alternatively, the retention time of a solute can

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also be related to the dissociation constant of weak acid using Eq. (3):

$$t_R = \frac{K_a 10^{pH} t_R^{A^-} + t_R^{HA}}{1 + K_a 10^{pH}} \quad (3)$$

As the retention of analytes changes with mobile phase pH, other chromatographic parameters (retention factor, selectivity factor, resolution, etc.) will vary too. Extensive research has been conducted on separation optimization through manipulation of mobile phase pH [1–8].

The addition of liquid CO_2 carbon dioxide to conventionally used HPLC mobile phases increases the chromatographic efficiency significantly while lowering the analysis time [9]. The presence of CO_2 in the mobile phase causes the mobile phase viscosity to decrease significantly which also allows multiple columns to be coupled in-series to increase the total efficiency of the chromatographic system with a minimal increase in the pressure drop across the column.

Methanol–water–CO_2 mixtures were previously used to separate a range of polar analytes [10,11]. Methanol–water–CO_2 mixtures are acidic due to the reaction between water and CO_2 to form carbonic acid [12]. The acidity of methanol–water–CO_2 mixtures profoundly affects the chromatography when these mixtures are used as mobile phases. Chromatographic selectivity was significantly increased for the separation of several triazine compounds when a phosphate buffer was added to the mobile phase.

Methanol–water–CO_2 mixtures of different CO_2 content have different dielectric constants since CO_2 is a nonpolar molecule [12]. The Born equation (Eq. (4)) provides an approximate description of the variation of the dissociation constant as a function of dielectric constant variation:

$$-RT \ln K_a = \frac{C}{\varepsilon} \cdot \left[\frac{1}{r_{H_3O^+}} + \frac{1}{r_{A^-}} \right] \quad (4)$$

where R is the gas constant, T is absolute temperature, C is a constant, ε is the dielectric constant of the solvent, and r_i is the radius of the ion i. A 20% change in dissociation constant due to the dielectric constant variation can be obtained when the amount of CO_2 increases from 5.6 to 19.2 mol% in methanol–water–CO_2 [12]. As predicted by Eq. (2), the variation of the dissociation constant can influence the retention factor as well. Therefore, the chromatography of ionizable analytes with methanol–water–CO_2 will be influenced by the amount of CO_2 not only by the acidity of the mixture but also the ability to change the dielectric constant. Previous studies also illustrated that buffers of known pH can be generated in methanol–water–CO_2 mixtures [12].

Substituted benzoic acids are widely used as model compounds for fundamental studies on mobile phase pH [1–3,6,8]. Their chromatographic behavior is also important in biological studies [13–15], the pharmaceutical industry [16], and the food industry [17].

This study demonstrates the separation of several substituted benzoic acids in methanol–aqueous buffer–CO_2 mixtures. In previous studies it was unclear why the addition of a phosphate buffer was improving the selectivity. Questions such as, ‘‘Does the addition of a buffer affect the dissociation of the ionogenic analytes or is the buffer changing the surface charge on the chromatographic support?’’ remained unanswered. This study will clearly illustrate the change in solute dissociation methanol–water–CO_2 mixtures and the impact of CO_2 concentration on separation of substituted benzoic acids is investigated. In addition the isocratic separation of the substituted benzoic acids using conventional, buffered, mobile phases is compared to that obtained when using buffered, high fluidity liquid mobile phases, such as methanol–water–CO_2 mixtures.

2. Experimental

2.1. Material

HPLC-grade methanol was obtained from J.T. Baker (Phillipsburg, NJ, USA). Water was distilled and deionized by a NANOpure II system (SYBRON/Barnstead, Boston, MA, USA). The resistivity of the deionized water was 17.3–17.8 MΩ. SFE/SFC-grade CO_2 was obtained from Air Product and Chemicals (Allentown, PA, USA). The benzoic acids (>95%, Table 1) were purchased from Aldrich (Milwaukee, WI, USA) and used as received and were dissolved in methanol to form sample solutions.

Table 1
Substituted benzoic acids and pK_a values in water at 25°C

Substituted benzoic acid	pK_a*
Benzoic acid	4.19
3-Hydroxybenzoic acid	4.06
4-Hydroxybenzoic acid	4.48
2-Nitrobenzoic acid	2.16
2-Chlorobenzoic acid	2.92

*From Ref. [18].

The concentration for benzoic acid, 2-chlorobenzoic acid and 3-hydroxybenzoic acid was 2.5 mg/ml, for 2-nitrobenzoic acid and for 4-hydroxybenzoic acid, was 0.75 mg/ml.

2.2. Chromatographic system

The chromatographic system consisted of an ISCO 260-D syringe pump (ISCO, Lincoln, NE, USA), a Valco W-series high-pressure injection valve with an injection volume of 200 nl (Valco Instruments, Houston, TX, USA), a Hypercarb, porous graphitic carbon (PGC) column (100×2.0 mm, 5 μm) from Shandon HPLC (Runcorn, UK), and a Spectra-Physics UV2000 UV–Vis absorption detector equipped with a capillary flow cell (Model 9550-0155). A 10 cm×1/16 in. O.D.×0.004 in. I.D. polished stainless steel tube (Alltech, Deerfield, IL, USA) connected the injector to the column (1 in.= 2.54 cm). The flow cell for detection was created by removing the polyimide coating from 5 mm section of 100 μm I.D. fused-silica tubing (Polymicro Technologies, Phoenix, AZ, USA) and centering it in the capillary flow cell. An Omega Model PX931-5KSV pressure transducer (Omega Engineering, Stamford, CT, USA) was placed in-line to monitor the outlet pressure of the column after the detector and before a post-detection restrictor. This restrictor, which is a piece of 30 μm I.D. fused-silica tubing of an appropriate length, was used to control the flow in the chromatographic system. The ISCO 260 D pump was operated in constant pressure mode to control the pressure at the head of the column. The average flow-rate was controlled by the pressure drop across the fixed length restrictor at the end of the column.

Methanol–aqueous buffer–CO_2 mixture preparation and their pH measurements were outlined in a previous study [12]. The chromatography was isocratic and conducted at room temperature. The inlet pressure was maintained at 204 atm (1 atm = 101 325 Pa). The outlet pressure was maintained above 136 atm to prevent the methanol–water–CO_2 mixture from separating into two phases [19]. The flow-rate was maintained at 0.100 ml/min by adjusting the length of the fix diameter restrictor. The column hold up time (t_M) was determined by the disturbance at the baseline due to the solvent front. The detection wavelength was at 254 nm. Each individual acid was analyzed in duplicate to determine the elution order and then the acid mixture was separated.

2.3. Data analysis

Chromatographic data were collected by a Pentium-90 computer with Ezchrom chromatography data system (Scientific Software, San Ramon, CA, USA). Data analysis was performed with Peakfit v4.0 for Windows (Jandel Scientific, San Rafael, CA, USA). The chromatographic parameters were determined by using an exponentially-tailed Gaussian Model for the chromatographic peaks. This was done because most of the peaks were slightly tailed so a Gaussian model was not appropriate. The asymmetry factor was calculated as the ratio of the width to the right over that to the left of the peak apex taken at 10% of the peak maximum.

3. Results and discussion

3.1. Retention

The retention factors of the substituted benzoic acids with different methanol–aqueous buffer–CO_2 mixtures over various pH values were determined from the chromatography of each individual acid and graphed in Figs. 1–3. In any of the three methanol–aqueous solution–CO_2 compositions, the largest changes in retention time and retention factors with mobile phase pH were observed for 2-nitrobenzoic acid and 2-chlorobenzoic acid (Figs. 1–3). 2-Nitrobenzoic acid was the most retained analyte when no buffer was added to the mobile phases and became the least retained with addition of a phosphate buffer (pH 6.62, ionic strength 28.8 m*M*) in the mobile phases. The absolute values for the retention factor

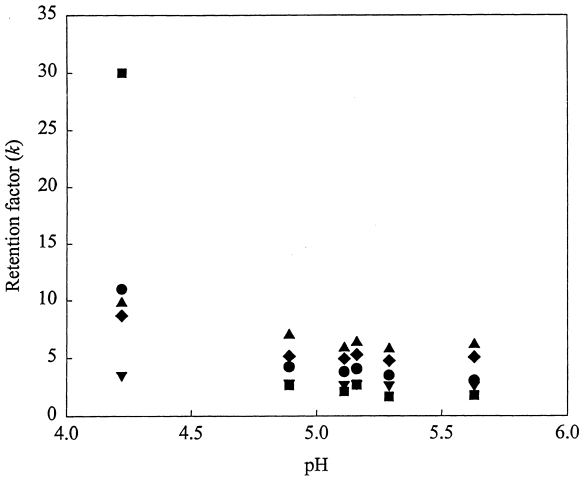

Fig. 1. Variation of retention factor with mobile phase pH for benzoic acids in methanol–aqueous buffer–CO_2 (65.1:29.3:5.6 mole ratio) mixtures. (▼) Benzoic acid; (◆) 3-hydroxybenzoic acid; (▲) 4-hydroxybenzoic acid; (■) 2-nitrobenzoic acid; (●) 2-chlorobenzoic acid. (The relative standard deviation, RSD, for the retention factors was ≤2%).

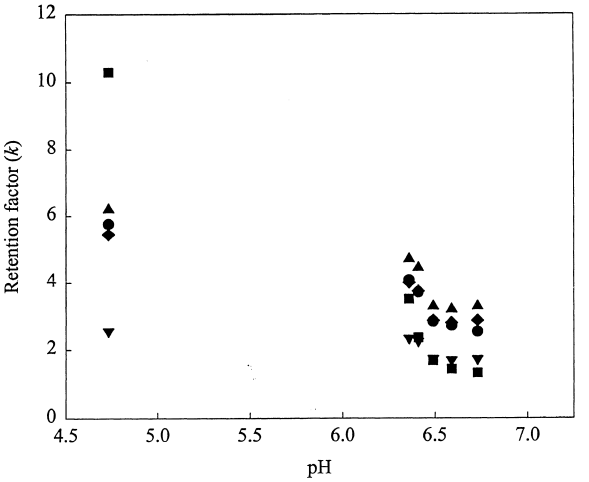

Fig. 3. Variation of retention factor with mobile phase pH for benzoic acids in methanol–aqueous buffer–CO_2 (55.7:25.1:19.2 mole ratio) mixtures. (▼) Benzoic acid; (◆) 3-hydroxybenzoic acid; (▲) 4-hydroxybenzoic acid; (■) 2-nitrobenzoic acid; (●) 2-chlorobenzoic acid. (The RSD for the retention factors was ≤2%).

and retention time variation of 2-chlorobenzoic acid were second only to those of 2-nitrobenzoic acid. The retention change with mobile phase pH for benzoic acid, 3-hydroxybenzoic acid and 4-hydroxybenzoic acid was also noticeable (Figs. 1–3), yet not comparable to that of 2-nitrobenzoic acid and 2-chlorobenzoic acid. The longest retention was always exhibited when there was no buffer added to methanol–water–CO_2 mobile phases for all the acids. Upon the addition of the five aqueous buffers listed in Table 2, the pH of the mobile phase increased and the retention of benzoic acids decreased substantially. For example, the retention factor of benzoic acid experienced a 20% decrease when water in the mobile phase was replaced with an acetate buffer of pH 3.00 and ionic strength of 10.0 m*M* (mobile phase A-I changed to A-II as in Table 2). However, it only decreased 6% when the acetate buffer was replaced by a more basic phosphate buffer in the mobile phase (mobile phase A-II changed to A-VI as in Table 2).

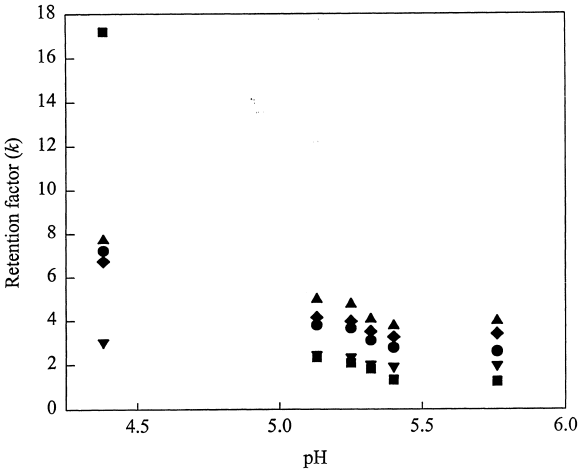

Fig. 2. Variation of retention factor with mobile phase pH for benzoic acids in methanol–aqueous buffer–CO_2 (66.7:27.7:10.6 mole ratio) mixtures. (▼) Benzoic acid; (◆) 3-hydroxybenzoic acid; (▲) 4-hydroxybenzoic acid; (■) 2-nitrobenzoic acid; (●) 2-chlorobenzoic acid. (The RSD for the retention factors was ≤2%).

The pK_a values in H_2O (see Table 1) for benzoic acid, 3-hydroxybenzoic acid and 4-hydroxybenzoic acid were near 4 (4.19, 4.06, 4.48), while 2-nitrobenzoic acid and 2-chlorobenzoic acid have pK_a values near 2.5 (2.16 and 2.92). Acids with lower pK_a are more affected as their [HA]/[A^-] ratio changes significantly. As a result, their retention varies substantially. Increasing ionic strength of the mobile phase would also cause the retention to

Table 2
The make up of methanol–aqueous solution–CO_2 mixture mobile phases with a fixed composition for methanol–aqueous solution (69.0:31.0 mole ratio)

Mobile phase	CO_2 content (mol%)	Aqueous solution	Apparent mobile phase pH*
A-I	5.6	Water	4.22
A-II	5.6	Acetate buffer, pH 3.00, ionic strength 10.0 m*M*	4.89
A-III	5.6	Acetate buffer, pH 3.45, ionic strength 10.0 m*M*	5.11
A-IV	5.6	Sodium acetate buffer, ionic strength 10.0 m*M*	5.16
A-V	5.6	Phosphate buffer, pH 6.62, ionic strength 28.8 m*M*	5.29
A-VI	5.6	Sodium carbonate buffer, ionic strength 10.0 m*M*	5.63
B-I	10.6	Water	4.38
B-II	10.6	Acetate buffer, pH 3.00, ionic strength 10.0 m*M*	5.13
B-III	10.6	Acetate buffer, pH 3.45, ionic strength 10.0 m*M*	5.25
B-IV	10.6	Acetate buffer, ionic strength 10.0 m*M*	5.32
B-V	10.6	Phosphate buffer, pH 6.62, ionic strength 28.8 m*M*	5.40
B-VI	10.6	Acetate buffer, ionic strength 10.0 m*M*	5.76
C-I	19.2	Water	
C-II	19.2	Acetate buffer, pH 3.00, ionic strength 10.0 m*M*	6.36
C-III	19.2	Acetate buffer, pH 3.45, ionic strength 10.0 m*M*	6.41
C-IV	19.2	Sodium acetate buffer, ionic strength 10.0 m*M*	6.49
C-V	19.2	Phosphate buffer, pH 6.62, ionic strength 28.8 m*M*	6.59
C-VI	19.2	Sodium carbonate buffer, ionic strength 10.0 m*M*	6.73

*pH measured spectrophotometrically following the procedure outlined in Ref. [12]

decrease [20]. Replacing water with a buffer will increase the ionic strength of methanol–aqueous solution–CO_2 mobile phase mixtures, which also should cause retention factor difference between non-buffered and buffered mobile phases for these benzoic acids in this study.

When 5.6 mol% CO_2 was added into the mobile phases, the variation of the retention factor with mobile phase pH did not fully exhibit the typical sigmoidal pattern predicted by Eq. (2) with the exception of the most acidic compounds, 2-nitrobenzoic and 2-chlorobenzoic acids. With the 61.7:27.7:10.6 mole ratio methanol–aqueous solution–CO_2 mixtures a sigmoidal pattern was still not completely established for all solutes except the most acidic compounds (2-nitrobenzoic acid and 2-chlorobenzoic acid) had obvious sigmoidal curves (Fig. 2). Fig. 3 shows the variation of the retention factors when using 55.7:25.1:19.2 mole ratio methanol–aqueous solution–CO_2 mixtures as the mobile phase. Under these conditions, all of the substituted benzoic acids showed the characteristic sigmoidal curves.

According to Eq. (2), the retention factor de-

creases as the mobile phase pH increases for acidic compounds. Retention changes most when the mobile phase pH is within the range of $pK_a \pm 1$ for the analyte. Outside this region, reducing or increasing mobile phase pH has limited impact on the retention factor. The correlation between retention factor and mobile phase pH can be used to determine the dissociation constant of acidic analytes. When the mobile phase pH is equal to the pK_a of the analyte, the absolute value of the slope of the k versus pH curve reaches the maximum (the inflection point). The approximate pK_a values for 2-nitrobenzoic and 2-chlorobenzoic acid were 4.5 to 4.75, in the 65.1:29.3:5.6 mole ratio methanol–aqueous buffer–CO_2 mixture as the mobile phase. For 66.7:27.7:10.6 mole ratio methanol–aqueous buffer–CO_2 mobile phases, the approximate pK_a values for 2-nitrobenzoic acid and 2-chlorobenzoic acid were 4.8 and 5.1, respectively, and for 55.7:25.1:19.2 mole ratio mobile phases, the pK_a values for all of the substituted benzoic acids were approximately 6.4.

In order to determine the retention factor, column hold up time (t_M) must be measured accurately. However, the experimental determination of t_M is complicated by many factors, e.g., ionic exclusion, adsorption and size exclusion effect [21]. The mobile phase composition can cause variation in measured t_M value as well [8]. The determination of retention factor thus will be affected by the error of t_M measurements. As shown in Eq. (3), the retention time (t_R) of the analyte should also show sigmoidal variation with mobile phase pH. In order to obtain a better understanding of the retention behavior of analytes with buffered mobile phases, the retention factor and retention time should both be monitored. Figs. 4 and 5 display the variation of t_R with pH for 4-hydroxybenzoic acid and 2-nitrobenzoic acid, respectively. Similar pK_a values were obtained from the t_R versus pH curves compared to the retention factor versus pH curves.

The convergence of the dissociation constants to similar values for the whole group of benzoic acids standards in methanol–aqueous solution–CO_2 (61.7:27.7:10.6 and 55.7:25.1:19.2 mole ratio) mixtures was initially surprising, as their pK_a values in water (Table 1) are very different. Systematic error in the mobile phase pH determination should not be the reason as it would only shift pK_a values up or down simultaneously for all acids. The dielectric constant of the mobile phase decreases with the addition of CO_2 in methanol–aqueous solution–CO_2 mixtures [12] which as described by Eq. (4) will cause the pK_a values to increase and potentially converge.

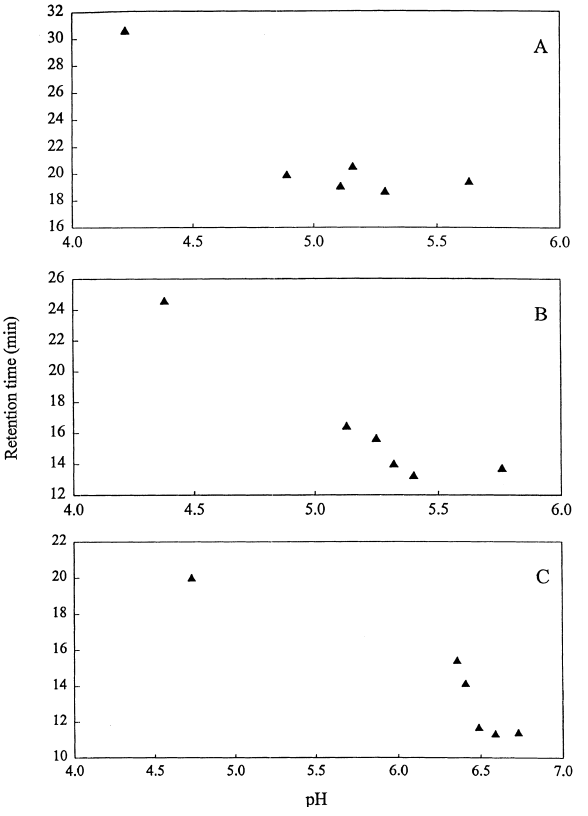

Fig. 4. Variation of retention time with mobile phase pH for 4-hydroxybenzoic acid in methanol–aqueous buffer–CO_2 mixtures. (A) 65.1:29.3:5.6 mole ratio; (B) 66.7:27.7:10.6 mole ratio; (C) 55.7:25.1:19.2 mole ratio. (The RSD for the retention times was $\leq$2%).

3.2. Plate number and peak symmetry comparison

The plate number, n, and peak asymmetry, Asy_{10}, for the five substituted benzoic acids with various mobile phases were measured and are listed in Tables 3–8. Lower plate number and higher Asy_{10} values were obtained for the separation of benzoic acids using unbuffered methanol–water–CO_2 mixtures as mobile phases. Higher plate numbers and

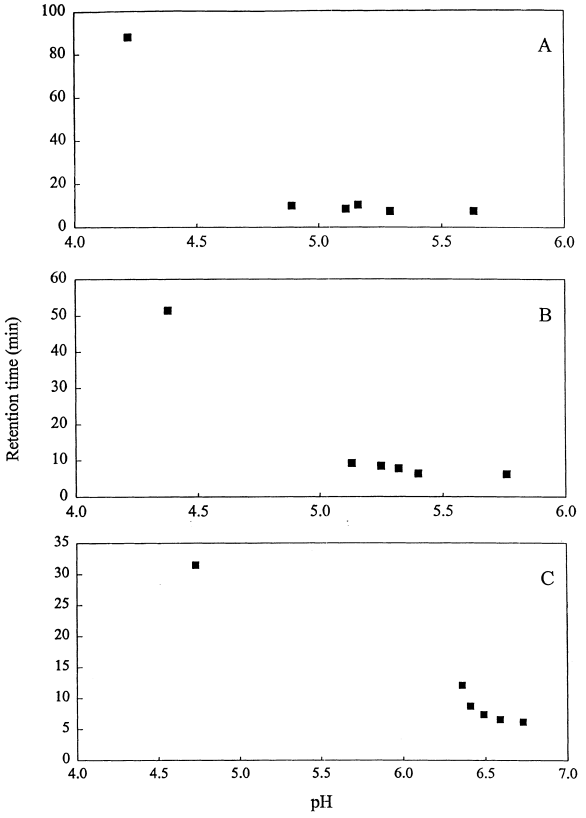

Fig. 5. Variation of retention time with mobile phase pH for 2-nitrobenzoic acid in methanol–aqueous buffer–CO_2 mixtures. (A) 65.1:29.3:5.6 mole ratio; (B) 66.7:27.7:10.6 mole ratio; (C) 55.7:25.1:19.2 mole ratio. (The RSD for the retention times was ≤2%).

increased peak symmetry were obtained with mobile phases of higher pH value. With 5.6 mol% CO_2 in the mobile phases, a noticeable improvement of separation performance was observed for 2-nitrobenzoic acid and 2-chlorobenzoic acid when a buffer was added. With 10.6 and 19.2 mol% CO_2 in the buffered mobile phases, the plate number and peak symmetry were improved for all the acids, especially when a phosphate or carbonate buffer was used. With mobile phases of a fixed methanol–aqueous solution–CO_2 ratio (varying pH), 4-hydroxybenzoic acid had the best peak symmetry and highest plate number, while 2-chlorobenzoic acid had the lowest plate number and least peak symmetry.

The addition of more CO_2 to the mobile phase was expected to improve peak symmetry and increase the plate number [9]. Noticeable improvements were observed for the substituted benzoic acids with methanol–water–CO_2 mixtures when the CO_2 content was increased from 5.6 to 19.2 mol%, especially for 2-nitrobenzoic acid and 2-chlorobenzoic acid (Tables 3–8). Plate number and peak symmetry can also be affected by the presence of a buffer in the mobile phase [22]. Significant changes of plate number and peak symmetry were reported with mobile phase pH variation [23]. Previous work showed that the variation of separation efficiency and peak symmetry may not be consistent with mobile phase pH for HPLC separation of acids [24]. A similar conclusion can be reached in this study (Tables 3–8). Each benzoic acid did not have the best plate number and the best peak shape with the same mobile phase. For example, 2-chlorobenzoic acid had higher plate numbers with mobile phase B-V than with mobile phase B-VI; yet the Asy_{10} value was lower with mobile phase B-VI than with mobile phase B-V (Tables 5 and 6). Nonetheless, peak symmetry and plate numbers generally improved with the addition of buffers with increasing pH.

The variation of peak symmetry with mobile phase

Table 3
Separation efficiency (n) for the substituted benzoic acids determined with methanol–aqueous buffer–CO_2 (65.1:29.3:5.6 mole ratio) mixtures

Substituted benzoic acid	Mobile phase A-I	Mobile phase A-II	Mobile phase A-III	Mobile phase A-IV	Mobile phase A-V	Mobile phase A-VI
Benzoic acid	2300	2350	2400	2500	2500	2400
3-Hydroxybenzoic acid	2800	3300	3100	3400	3500	3400
4-Hydroxybenzoic acid	3450	4200	3900	4010	4300	4000
2-Nitrobenzoic acid	*	2000	2400	2600	3000	3000
2-Chlorobenzoic acid	*	1200	1660	2200	2200	3400

*Peak not fully resolved.

Table 4
Asymmetry factor (Asy_{10}) for the substituted benzoic acids determined with methanol–aqueous buffer–CO_2 (65.1:29.3:5.6 mole ratio) mixtures

Substituted benzoic acid	Mobile phase A-I	Mobile phase A-II	Mobile phase A-III	Mobile phase A-IV	Mobile phase A-V	Mobile phase A-VI
Benzoic acid	2.1	2.2	2.2	2.2	2.2	2.3
3-Hydroxybenzoic acid	2.17	2.1	2.1	2.2	2.2	2.2
4-Hydroxybenzoic acid	1.6	1.4	1.6	1.6	1.4	1.5
2-Nitrobenzoic acid	*	2.6	2.5	2.3	2.1	2.1
2-Chlorobenzoic acid	*	5.0	3.6	2.7	2.5	2.3

*Peak not fully resolved.

Table 5
Separation efficiency (n) for the substituted benzoic acids determined with methanol–aqueous buffer–CO_2 (61.7:27.7:10.6 mole ratio) mixtures

Substituted benzoic acid	Mobile phase B-I	Mobile phase B-II	Mobile phase B-III	Mobile phase B-IV	Mobile phase B-V	Mobile phase B-VI
Benzoic acid	1700	2500	2100	3400	3800	3200
3-Hydroxybenzoic acid	3100	3400	3800	4100	4000	3800
4-Hydroxybenzoic acid	4300	3700	3900	5400	5300	5100
2-Nitrobenzoic acid	1500	2600	2400	3200	3900	3300
2-Chlorobenzoic acid	1600	2800	1700	3600	3300	2600

Table 6
Asymmetry factor (Asy_{10}) for the substituted benzoic acids determined with methanol–aqueous buffer–CO_2 (61.7:27.7:10.6 mole ratio) mixtures

Substituted benzoic acid	Mobile phase B-I	Mobile phase B-II	Mobile phase B-III	Mobile phase B-IV	Mobile phase B-V	Mobile phase B-VI
Benzoic acid	2.2	2.2	2.2	1.9	1.7	1.8
3-Hydroxybenzoic acid	2.2	2.1	2.0	1.8	1.8	1.8
4-Hydroxybenzoic acid	1.6	1.6	1.6	1.5	1.4	1.5
2-Nitrobenzoic acid	2.1	2.1	2.3	1.8	1.5	1.7
2-Chlorobenzoic acid	3.2	2.6	3.4	1.9	2.1	1.8

pH could be related to the dissociation of the benzoic acids and their interactions with the PGC stationary phase and methanol–aqueous solution–CO_2 mobile phases. At low mobile phase pH, the dissociation of benzoic acids is suppressed. Stronger interaction between the acid and PGC surface may cause peak tailing. With an increase of mobile phase pH, dissociation occurs. The interaction between the acid

Table 7
Separation efficiency (n) for the substituted benzoic acids determined with methanol–aqueous buffer–CO_2 (55.7:25.1:19.2 mole ratio) mixtures

Substituted benzoic acid	Mobile phase C-I	Mobile phase C-II	Mobile phase C-III	Mobile phase C-IV	Mobile phase C-V	Mobile phase C-VI
Benzoic acid	3000	2900	3000	4000	3700	3500
3-Hydroxybenzoic acid	3100	3600	3100	4000	4100	4100
4-Hydroxybenzoic acid	4600	5500	5100	5200	5600	5400
2-Nitrobenzoic acid	2000	2900	2500	3100	4200	3300
2-Chlorobenzoic acid	2300	1900	2600	3200	4200	4100

Table 8
Asymmetry factor (Asy_{10}) for the substituted benzoic acids determined with methanol–aqueous buffer–CO_2 (55.7:25.1:19.2 mole ratio) mixtures

Substituted benzoic acid	Mobile phase C-I	Mobile phase C-II	Mobile phase C-III	Mobile phase C-IV	Mobile phase C-V	Mobile phase C-VI
Benzoic acid	1.8	1.8	2.1	1.6	1.7	1.8
3-Hydroxybenzoic acid	2.1	2.0	2.0	1.9	1.8	1.7
4-Hydroxybenzoic acid	1.6	1.3	1.4	1.5	1.5	1.5
2-Nitrobenzoic acid	2.2	2.2	2.6	1.8	1.6	1.7
2-Chlorobenzoic acid	2.2	3.2	2.3	1.8	1.8	1.7

and the mobile phase is strengthened and peak shape improves. Reducing the retention factor contributes to the increase of plate number for acidic analytes [25].

3.3. Optimum chromatographic performance among different buffers and comparison with conventional mobile phase conditions

Baseline separation of the substituted benzoic acids was achieved using some buffered mobile phase mixtures for all three levels of added CO_2 (5.6–19.2 mol% CO_2). However, the buffer that was optimum varied. With 5.6 mol% CO_2 the phosphate buffer provided the highest selectivity and baseline separation of the analytes. When 10.6 mol% CO_2 is added in the mobile phase, complete separation of benzoic acids was achieved with the addition of phosphate buffer and carbonate buffer (Fig. 2). However as noted above, peak symmetry and plate numbers were better with the phosphate buffer (Tables 5 and 6). With 19.2 mol% CO_2 in the mobile phases, 2-chlorobenzoic acid and 3-hydroxybenzoic acid co-eluted when the acetate and phosphate buffers were added in methanol–water–CO_2 mixtures (Fig. 3), while mobile phases using a carbonate buffer provided the best separation of these benzoic acids with comparable separation performance (Tables 7 and 8).

Figs. 6–8 compares the best separation of the substituted benzoic acids to that achieved without the addition of a buffer. Clearly the addition of a buffer was necessary. When the best separations (Figs. 6B,

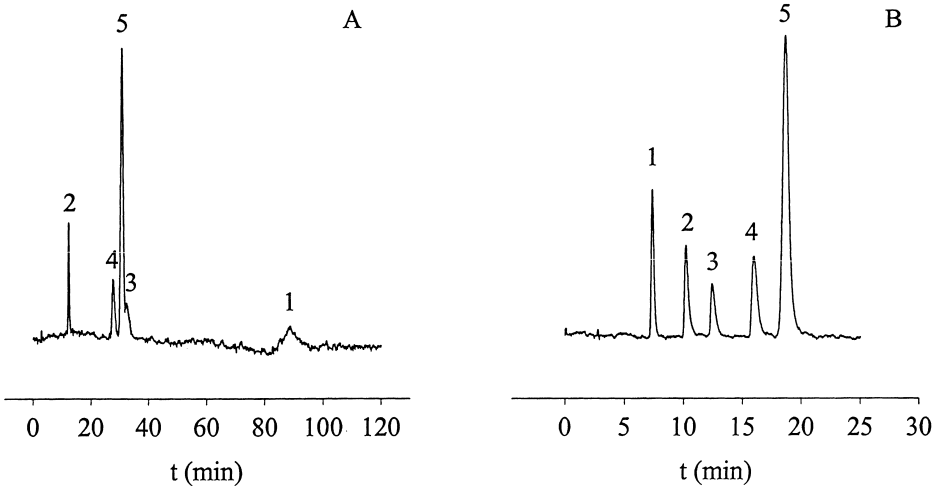

Fig. 6. The chromatograms of benzoic acids with (A) methanol–water–CO_2 (65.1:29.3:5.6 mole ratio) mixture and (B) methanol–phosphate buffer–CO_2 (65.1:29.3:5.6 mole ratio) mixture. 1 = 2-Nitrobenzoic acid; 2 = benzoic acid; 3 = 2-chlorobenzoic acid; 4 = 3-hydroxybenzoic acid; 5 = 4-hydroxybenzoic acid.

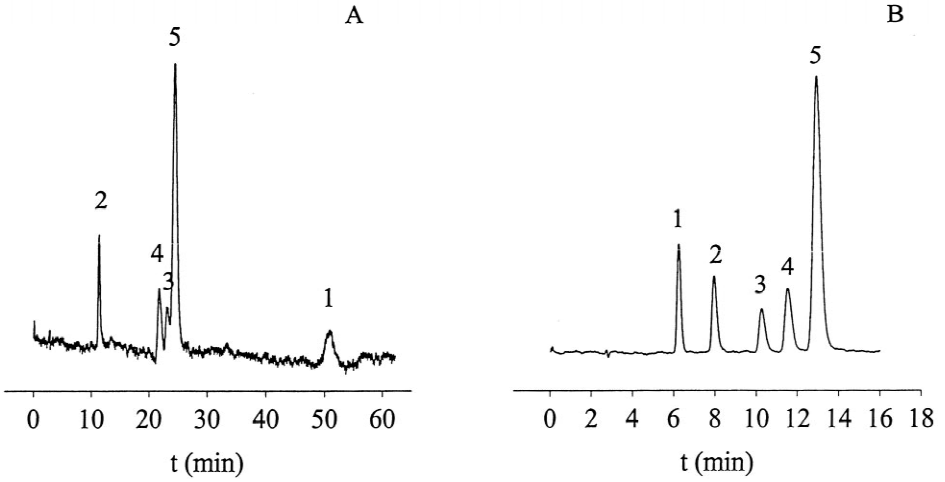

Fig. 7. The chromatograms of benzoic acids with (A) methanol–water–CO_2 (61.7:27.73:10.6 mole ratio) mixture and (B) methanol–phosphate buffer–CO_2 (61.7:27.7:10.6 mole ratio) mixture. 1=2-Nitrobenzoic acid; 2=benzoic acid; 3=2-chlorobenzoic acid; 4=3-hydroxybenzoic acid; 5=4-hydroxybenzoic acid.

7B and 8B) using high-fluidity liquids are compared, all show excellent separations. However, complete separation was achieved in 20, 14 and 12 min when 5.6, 10.6 and 19.2 mole% CO_2 was present in the mobile phase, respectively. Therefore, the addition of the largest proportion of CO_2 is the optimum condition in terms of speed of analysis.

Without CO_2, using buffered methanol–water mobile phases, the best separation of the substituted benzoic acids was achieved with a methanol–acetate buffer (pH 3.00 and ionic strength 10.0 m*M*) at 69.0:31.0 mole ratio. The best isocratic separation using conventional liquid conditions had much longer analysis time (50 min) compared to the best

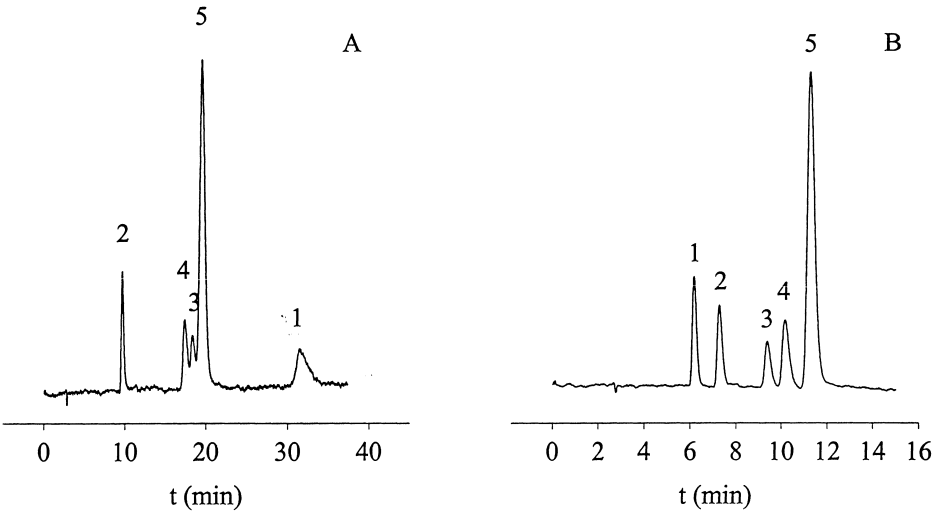

Fig. 8. The chromatograms of benzoic acids with (A) methanol–water–CO_2 (55.7:25.1:19.2 mole ratio) mixture and (B) methanol–carbonate buffer–CO_2 (65.1:29.3:5.6 mole ratio) mixture. 1=2-Nitrobenzoic acid; 2=benzoic acid; 3=2-chlorobenzoic acid; 4=3-hydroxybenzoic acid; 5=4-hydroxybenzoic acid.

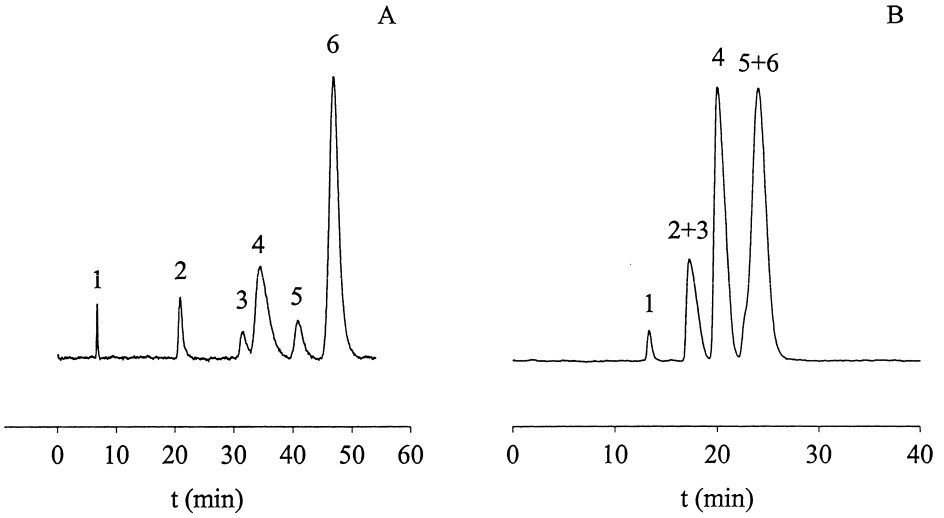

Fig. 9. The chromatograms of benzoic acids with methanol–aqueous buffer (69.0:31.0 mole ratio) mixtures. (A) Acetate buffer, pH 3.00, ionic strength 10.0 m*M*; (B) phosphate buffer, pH 6.62, ionic strength 28.8 m*M*. 1=Benzene, 2=benzoic acid; 3=2-chlorobenzoic acid; 4=2-nitrobenzoic acid; 5=3-hydroxybenzoic acid; 6=4-hydroxybenzoic acid.

separation using high fluidity liquid mobile phase conditions which had an analysis time of 12 min. Also, the band dispersion for using the conventional methanol–water mixtures was markedly greater than when using the high fluidity mobile phase. It was necessary to add more basic buffers to the mobile phase when using the mobile phases containing CO_2. The phosphate buffer (pH 6.62, ionic strength 28.8 m*M*) that was optimum for the high fluidity separation provided poor selectivity with methanol–water mixtures (Fig. 9).

Acknowledgements

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