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Chromatographic separation of 3,4-difluorophenylacetic acid and its positional isomers using five different techniques

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Abstract

The separation of five positional isomers from 3,4-difluorophenylacetic acid was investigated using normal- and reversed-phase high-performance liquid chromatography, capillary zone electrophoresis, gas chromatography and supercritical fluid chromatography. Operating parameters of each technique, such as temperature, type of stationary phase, mobile phase pH, ionic strength, organic modifiers and additives were varied in order to elucidate the separation mechanisms. Based on the advantages and disadvantages of each methodology, a simple and practical RPLC method was selected. The method was validated in terms of linearity, limit of detection, accuracy, recovery, ruggedness and precision.

Keywords: Positional isomer separation; Validation; Difluorophenylacetic acids; Organic acids

1. Introduction

Since isomers usually possess similar physical and chemical properties, isomeric resolution is also one of the most challenging areas of separation science. Positional isomer separation can be achieved through a number of different analytical techniques such as gas chromatography (GC) [1], high-performance liquid chromatography (HPLC) in reversed-phase mode (RPLC) [2–8] and with normal-phase mode (NPLC) [9,10], supercritical fluid chromatography (SFC) [11], and capillary zone electrophoresis (CZE) [12]. Separation mechanisms of positional isomers have been extensively studied for both normal- and reversed-phase HPLC. Interpretation of elution orders on alumina columns were first reported in the literature by Snyder [13]. The separation was interpreted in terms of a number of physicochemical effects, including intramolecular hydrogen bonding, electronic activation, steric repulsion and solute molecular orientation. Although NPLC was considered better for separating positional isomers [14], interest in the separation of positional isomers in RPLC has increased in the last decade [7–11]. The chromatographic materials investigated in these studies have included cyclodextrin-bonded silica [15], crown ether-bonded silica [3], octadecyl-bonded silica (ODS) [4–7], porous graphitic carbon (PGC) [4–7], and carbon-clad microporous zirconia [8]. GC is another popular technique for positional isomeric separation. The reported choices of stationary phases have varied from the more common types such as dimethyl polysiloxane-coated columns and polyethylene glycol columns to the more specialized

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ones like crown ether-bonded polysiloxane columns [1]. In recent years, SFC has been applied successfully to a number of isomeric separations due to the unique physical property of its mobile phase [11]. However, most of the applications have been focused on the separation of enantiomers and geometric isomers [11,16]. Examples of using SFC for positional isomeric separations are less frequent. Separation of positional isomers in CZE has been the newest reported technique for this type of separation [12]. In many cases, the separations have involved micellar or cyclodextrin additives.

In this paper, we compare the separation behaviors of the difluorophenylacetic acid (DFPAA) isomers with five different techniques: RPLC, NPLC, GC, CZE and SFC. 3,4-DFPAA is an important key raw material in the synthesis of a pharmaceutical compound. In order to ensure the quality of a synthetic product, an analytical method is needed to separate these five isomeric impurities from the 3,4-DFPAA. Various operating parameters such as temperature, type of stationary phase, mobile phase pH, ionic strength, organic modifiers and additives were studied in order to understand the separation mechanisms. Based on the advantages and disadvantages of each methodology, a simple and practical RPLC method was selected and validated.

2. Experimental

2.1. Reagents

All organic solvents used in the study were HPLC grade and purchased from Fisher Scientific (Fairlawn, NJ, USA). Phosphoric acid (85%), sodium hydroxide (50%) and acetic acid solutions were obtained from Aldrich (Milwaukee, WI, USA); tris-(hydroxymethyl)aminomethane (Tris) was obtained from Bio-Rad (Richmond, CA, USA). The helium gas used was of high purity grade from JWS Technologies (Piscataway, NJ, USA) and SFC-grade carbon dioxide was purchased from Scott Specialty Gases (Plumsteadville, PA, USA). Water used in the study was deionized water purified through a Millipore deionization device (Milford, MA, USA). Chemicals including sodium nitrate, the positional isomers of difluorophenylacetic acid, the positional isomers of difluorophenylbenzoic acid and phenylacetic acid were purchased from Aldrich, except 2,3-difluorophenylacetic acid which was purchased from Lancaster (Whitelund, Morecambe, UK).

2.2. Instrumentation

In the RPLC and NPLC modes, a system consisting of Shimadzu (Princeton, NJ, USA) SCL-10AS pumps, an APD-10AV UV–Vis detector, and SCL-10A sample controller with a 10-μl sample loop was used. In the CEC mode, a Hewlett-Packard ^{3}D capillary electrophoresis (HP ^{3}D, HPCE, Model G1600A) system with a CE model was used. In the SFC mode, a Berger system consisting of a dual fluid control module, a thermal control module, an automatic liquid sampler, and a HP 1050 diode array detector was used. In the GC system, a Hewlett-Packard 5890 Series II plus with flame ionization detection (FID) system was used. All chromatograms were processed by a PE Nelson data system equipped with Access*Chrom software (version 1.9) (PE Nelson, Cupertino, CA, USA).

2.3. Chromatographic columns

The columns used in the reversed-phase mode were Zorbax C_8, Zorbax Rx C_8, Zorbax XDB C_8, Zorbax SB phenyl and Zorbax CN. The columns used in the normal-phase mode were Zorbax silica, Zorbax CN, Zorbax SB phenyl and Zorbax diol. The columns used for SFC were Zorbax CN, Zorbax SB phenyl and Zorbax silica. All columns were 25 cm×4.6 mm I.D. with a particle size of 5 μm. They were purchased from Rockland Technologies, distributed by MAC-MOD Analytical (Chadds Ford, PA, USA). The columns used for GC were Rtx Stabilwax–DA (60 m×0.53 mm, film thickness: 1.5 μm) from Restek (Bellefonte, PA, USA). The capillary used in CZE was poly(vinyl alcohol) (PVA)-coated and had an effective length of 56 cm (total length, 63 cm) (Hewlett-Packard, Wilmington, DE, USA).

2.4. Chromatographic conditions

All LC separations, except where specified, were performed at an ambient temperature of 25°C. The

mobile phases were isocratically pump-mixed at specified compositions. The flow-rate was 1.0 ml/min; the injection volume was 10 μl; the detection was UV at 220 nm in all cases. The retention factor k for the two bands (formerly referred to as the capacity factor k') was determined as $k=(t_R-t_0)/t_0$, where t_R and t_0 were retention times of retained and unretained compounds, respectively. In RPLC, the t_0 was determined by injecting a concentrated solution of sodium nitrate (detection at 210 nm) [14,23]. In CZE, except when systematically varying the composition, the background electrolyte (BGE) used was a 60 m*M* Tris–acetate buffer. In all cases, the capillary temperature was controlled to 20±0.1°C. A diode array detector was operated at 200 nm. Hydrodynamic sample injection was used with a 3-s injection time at 50 mbar pressure. The applied voltage was 20 kV. In GC, the mobile phase used was helium, and the flow-rate was 14.5 ml/min. A FID system was used and the temperature was 250°C. The injector temperature was 250°C and the injection volume was 0.1 μl as the temperature was programmed from 180°C to 200°C at the ramp rate 5°C/min for the optimized chromatogram. In SFC, the mobile phase was a mixture of carbon dioxide (CO_2)–isopropanol (IPA)–trifluoroacetic acid (TFA) (98:1.975:0.025, v/v), the flow-rate was 1.0 ml/min; the column temperature was maintained at 40°C and a diode array UV detector was used at 220 nm for the optimized chromatogram.

2.5. Preparation of solutions

The samples were dissolved in isopropanol for NPLC and SFC, in deionized (DI) water for GC and CZE and in DI water–acetonitrile (ACN) (95:5, v/v) for RPLC. The concentrations of all samples were 0.5 mg/ml for method development.

3. Results and discussion

3.1. Selection of suitable separation modes

The initial separation attempts were made in five common chromatographic separation techniques: RPLC, NPLC, GC, CZE and SFC. The method development was accomplished by varying both compositional and operational parameters. SFC using carbon dioxide–IPA mixture not surprisingly gave similar retention characteristics to NPLC. GC would intuitively be a good choice since separation is derived through the difference in the boiling point and the polarity of the positional isomers. However, using a deactivated column to reduce the strong hydrogen bonding interactions [18], no resolution of the 2,3- and 3,4-DFPAA isomers could be achieved. Based on preliminary separation results, RPLC, NPLC and CZE techniques were selected for both separation optimization and mechanism investigation.

3.2. Separation in NPLC mode

3.2.1. Effect of TFA

Silica, diol, cyano and phenyl columns with hexane–IPA or hexane–ethanol or hexane–ACN mobile phases were used. No separation was achieved from any of these columns and mobile phases initially, as severely tailing peaks were observed. However, a trace amount of TFA as mobile phase additive improved the peak shape and resolution significantly. This behavior was repeatedly observed on the silica, diol, cyano and phenyl columns regardless of the nature of column packing materials. A typical comparison chromatogram obtained from a silica column is shown in Fig. 1. These observations can be accounted for in terms of the competition of hydrogen bonding. Without the addition of TFA, the acidic functionality of DFPAA interacts strongly with the silanol groups of the silica gel packing material through hydrogen bonding [17], resulting in the tailing and co-eluting peaks. When 0.025% of TFA was added to the mobile phase, the analytes with acidic groups hydrogen bonding to the silanol sites of stationary phase were displaced by the presence of TFA in the mobile phase. Thus, the tailing was eliminated and the resolution was improved.

A group of isomeric compounds with structures closely related to DFPAA, difluorophenylbenzoic acid, were used to validate this effect. As one can see in Fig. 2, similar separation behaviors were obtained for these compounds with the peak efficiency significantly improved with the addition of TFA in the mobile phase.

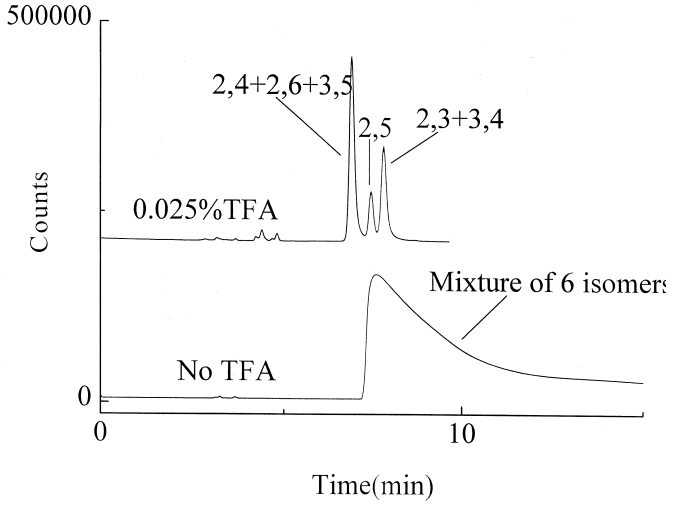

Fig. 1. Optimized chromatographic separations of DFPAA isomers in NPLC. Column: Zorbax silica 25×0.46 cm. Column temperature: ambient. Mobile phase: upper: hexane–IPA–TFA (98:1.975:0.025); bottom: hexane–IPA (98:2). Flow-rate: 1.0 ml/min; UV detection: 220 nm.

3.2.2. Effect of polar organic solvents

The effect of organic modifiers also indicated the existence of the hydrogen bonding competition. As shown in Fig. 3, the resolution completely disappeared when 2% acetonitrile was used as mobile phase modifier instead of IPA or ethanol (EtOH) even with the presence of TFA, because acetonitrile does not provide any hydrogen bonding competition due to its aprotic nature.

Although the addition of TFA showed a great

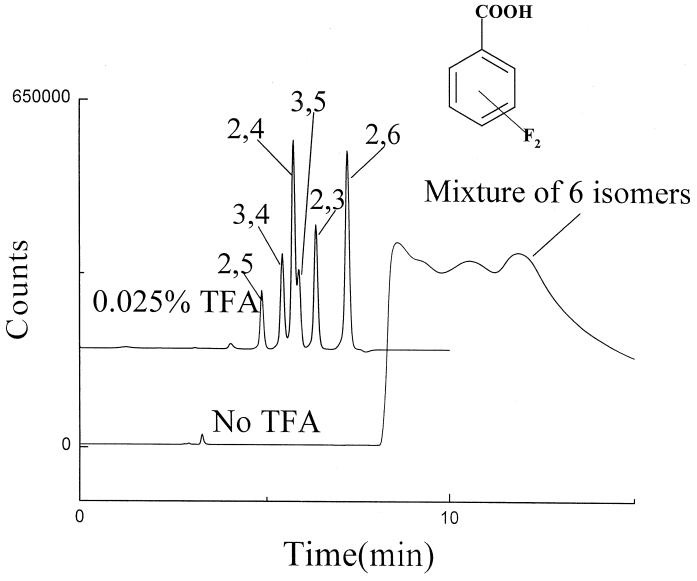

Fig. 2. Chromatographic separations of six difluorobenzoic acid isomers. Mobile phase: upper: hexane–IPA–TFA (98:1.975:0.025); bottom: hexane–IPA (98:2). Other conditions as in Fig. 1.

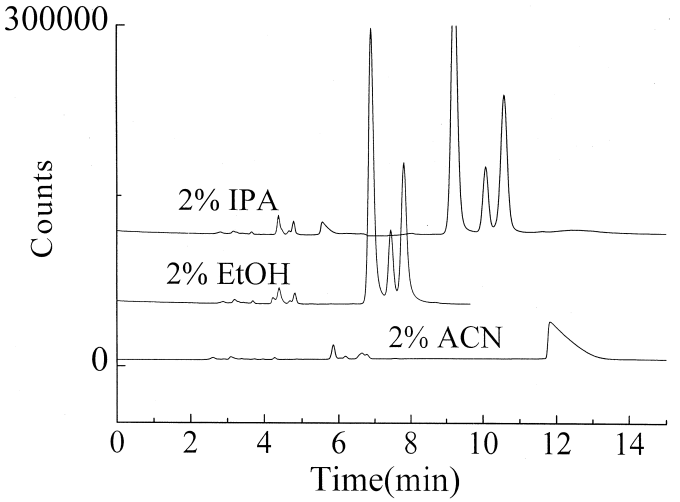

Fig. 3. Effect of organic modifier in NPLC. Mobile phase: upper: hexane–IPA–TFA (98:1.975:0.025); middle: hexane–ethanol–TFA (98:1.975:0.025); bottom: hexane–ACN–TFA (98:1.975:0.025). Other conditions as in Fig. 2. Peaks are (from left to right): first peak: 2,4-, 2,6- and 3,5-DFPAA; second peak: 2,5-DFPAA; third peak: 2,3- and 3,4-DFPAA.

improvement in separation, the six isomers still could not be completely resolved by any of the normal-phase systems that we tried. Therefore, alternate techniques were explored to improve the separation.

3.3. Separation in CZE mode

CZE separation of the six DFPAA isomers was accomplished through background electrolyte pH_{app} (apparent pH) control on a PVA-coated capillary. In such a capillary, the electroosmotic flow (EOF) is significantly eliminated because the PVA shields the silanol groups on the surface of the fused-silica [19]. Using reversed polarity of the applied voltage, anionic analytes can migrate electrophoretically to the anode in a wide pH range.

3.3.1. Effect of pH_{app}

For a weak acid such as DFPAA, only the deprotonated form can migrate electrophoretically. The effective electrophoretic mobility μ_e of a solute in the buffer solution can be expressed as $\mu_e=\alpha\mu$ [20], where μ is the electrophoretic mobility of the fully ionized species, and α is the fraction of the solute ionized which is directly related with buffer pH_{app}. Thus, as observed in Fig. 4, the mobilities (the difference between apparent mobilities and effective mobilities of the solutes can be negligible since EOF is suppressed by the PVA-coated capillary), of DFPAA isomers were a function of pH_{app}. At an elevated pH_{app}, $\alpha\rightarrow1$, all isomers were fully charged and the six isomers migrated at the same rate because they have equal charge and are similar in size. As the buffer pH_{app} decreased, the α decreased, resulting in a reduction of the overall charges on each isomer. Since the pK_a of each isomer is slightly different, which is a common phenomenon for ionic positional isomers [21], the optimal separation occurred as the buffer pH_{app} decreased. Well-separated peaks were obtained when the pH_{app} approached 3.2 with extensively long run time (Fig. 5). In consideration of both runtime and resolution, a good compromise was reached at a pH_{app} of 3.8.

3.3.2. Effect of organic modifier

The addition of organic modifiers not only increased the migration time of solutes due to increased viscosity and decreased dielectric constants [22], but also affected the separation, as shown in Fig. 6. The resolution between 2,3- and 2,5-DFPAA isomers decreased while the resolution of others increased.

3.3.3. Effect of buffer concentration

The mobility of each isomer increased as the

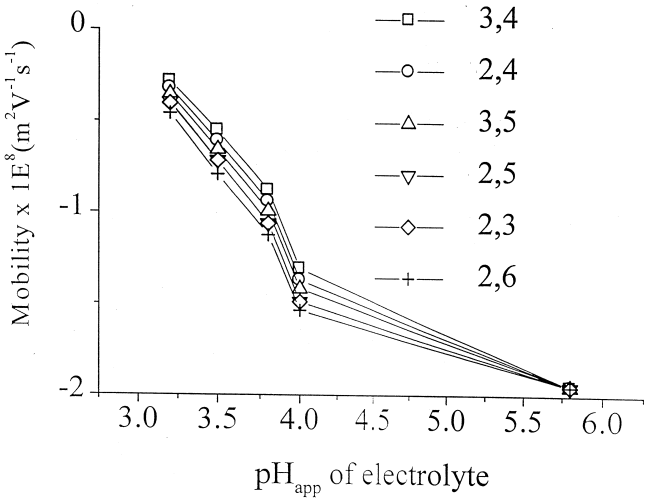

Fig. 4. Effect of pH_{app} on apparent mobilities (≈effective mobilities). PVA-coated capillary, 63 cm (56 cm effective length×50 μm I.D. BGE: 60 m*M* Tris–acetate buffer at selected pH values; applied voltage: 20 kV, UV detection at 200 nm; capillary temperature: 20°C.

concentration of the Tris–acetate increased. Although the band spacings between the peaks were not changed significantly, the resolution was improved noticeably due to peak shape enhancement. However, the resolution between 2,3- and 2,5-DFPAA isomers was still not sufficient.

3.4. Separation in RPLC mode

RPLC is a more mature and generally rugged technique than other modes of liquid chromatography. Separation by RPLC is primarily based on the differences of hydrophobicity of the solutes. RPLC

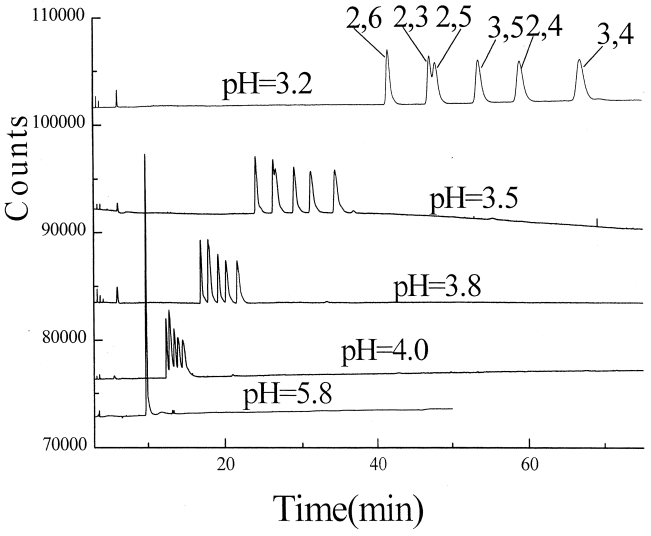

Fig. 5. Effect of pH_{app} on CZE separation of DFPAA isomers. Experimental conditions as in Fig. 7.

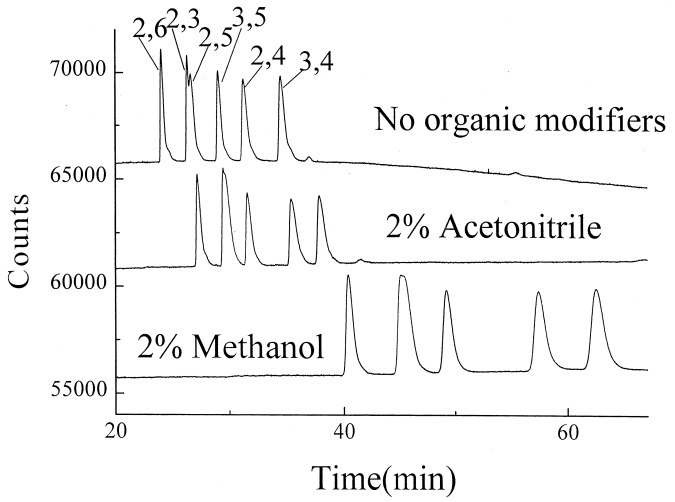

Fig. 6. Effect of organic modifiers on the CZE separation of DFPAA isomers. Experimental conditions as in Fig. 7, except the type of organic solvents was varied. Middle and bottom bands orders are the same as upper bands except 2,3- and 2,5-DFPAA co-eluted.

separations of ionic isomers tend to be more complicated. However, it is possible to optimize the resolution of the ionic isomers by selecting an appropriate pH value for the mobile phase and a suitable column.

3.4.1. Selection of columns

Five common RPLC columns were selected for the study. With a mobile phase that consisted of a acetonitrile–phosphate buffer (pH_{app} 6.5) (95:5, v/v), all five columns gave different degrees of separation as shown in Fig. 7. The baseline resolution of all six isomers was achieved in less than 20 min on a Zorbax Rx C_8 column.

The elution orders were similar for all five columns selected. Among Zorbax C_8, Rx C_8 and XDB C_8 columns, the XDB C_8 column yielded the highest retention factor due to its ''extra-dense'' bonding. The retention factor decreased as the surface coverage decreased ($k_{C_8} < k_{Rx\ C_8} < k_{XDB\ C_8}$) [14]. Of note the band spacing between 3,5- and 3,4- peaks was increased significantly as the surface coverage increased. The isomers were less retentive on the phenyl and CN columns due to the increased polarity of these stationary phases.

The optimal separation was obtained on the Zorbax Rx C_8 column. We can speculate that the favorable separation selectivity of this column type was due to the large sterically protecting group (diisopropyl *n*-octyl) [14] which effectively shielded the deprotonated silanol sites. Repulsion between the negatively charged analytes and the silanol sites was minimized. Therefore, greater selectivity for the separation of the DFPAA isomers was achieved.

3.4.2. Effect of the mobile phase pH_{app}

The pH_{app} of the mobile phase is expected to influence the retention and selectivity for the separation of these acidic DFPPA isomers. Experiments were carried out on the Zorbax Rx C_8 column to determine the retention factors of these isomers by varying the pH_{app} of mobile phase (A) over a range from 2.0 to 7.0. As shown in Fig. 8, the retention factors of the isomers decreased dramatically as the pH_{app} of the mobile phase increased, with a rapid change in retention close to the pK_a of the solutes. At a low pH_{app}, as the isomers were in their neutral forms, the effect of the solute ionization on retention was essentially absent. Therefore, the separations under these conditions were controlled predominantly by a hydrophobic interaction between individual isomer and the Zorbax Rx C_8 stationary phase. Only partial separation was achieved under these con-

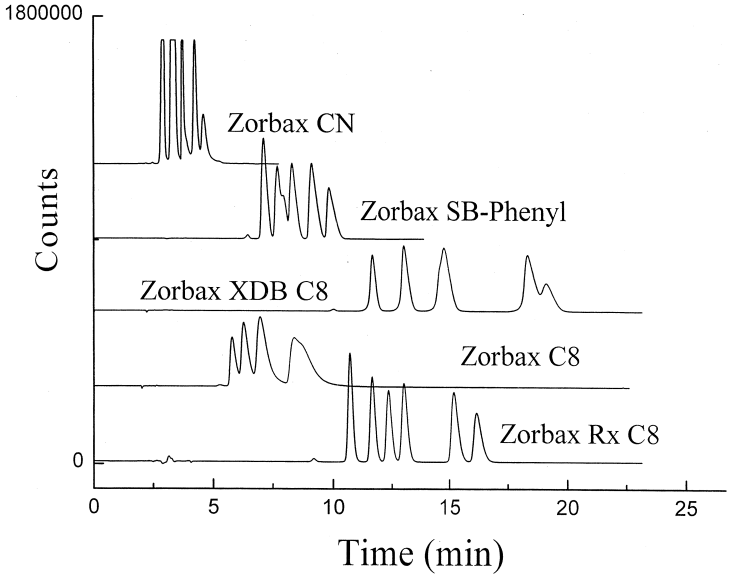

Fig. 7. Comparison of the chromatographic separation of DFPAA on selected columns. Mobile phase: acetonitrile–15 m*M* sodium phosphate buffer (pH_{app} 6.5) (5:95); flow-rate: 1.0 ml/min; UV detection: 210 nm, column temperature: 25°C. Bands orders (from left to right): 2,6-, 2,5-, 2,4-, 2,3-, 3,5- and 3,4-DFPAA.

ditions. As the pH_{app} value of the mobile phase increased, the selectivity was changed to the point where at a pH_{app} of 6.5, all six isomers were ionized, and they were distributed preferentially into the mobile phase. A favorable selectivity is due to differences in the polarity of each isomer as a consequence of their structural differences [23].

3.4.3. Effect of organic solvents

The effect of the ACN concentration was investigated at pH_{app} values equal to 2.2 and 6.5. At the low pH_{app} (2.2), all the isomers were in their neutral forms, the log *k* vs. % ACN of each isomer was linear. This is typical reversed-phased separation behavior. There was no satisfactory separation obtained from 0% to 30% of ACN at this pH_{app}. At the elevated pH_{app} value (6.5), all isomers were ionized and the retention factor of isomers decreased significantly. However, the separation process was still dominated by partitioning between the polar mobile phase and non-polar Rx C_8 stationary phase, and more hydrophobic compounds were retained more strongly. In order to confirm this explanation, we studied the retention behavior using phenylacetic acid. Phenylacetic acid lacks the difluoro atoms of DFPAA, and consequently should have a lower hydrophobicity and thus should elute earlier than DFPAA. Fig. 9 demonstrated that the retention factor of phenylacetic acid was smaller than that of DFPAA over the range of ACN concentration from 0% to 30%. The linear trend of the log *k* vs. % ACN also supports our explanation. The best separation of DFPAA was achieved at 5% of acetonitrile concentration. Utilizing methanol as the organic modifier, the peak efficiency of the isomers was unacceptable. We were unable to obtain a complete separation for each isomer in the concentration range from 0% to 50% of methanol.

3.4.4. Effect of temperature

The experimental results demonstrated the improvement in separation resulting from decreasing column temperature. However, the peak efficiency decreased as the temperature changed from 50°C to 5°C. An ambient temperature (25°C) was a good compromise in this case. Quantitatively, the fact that the increase in retention factors (*k*) with decreasing temperatures was linear (R^2 values>0.99) reflected the separation process to be enthalpically driven.

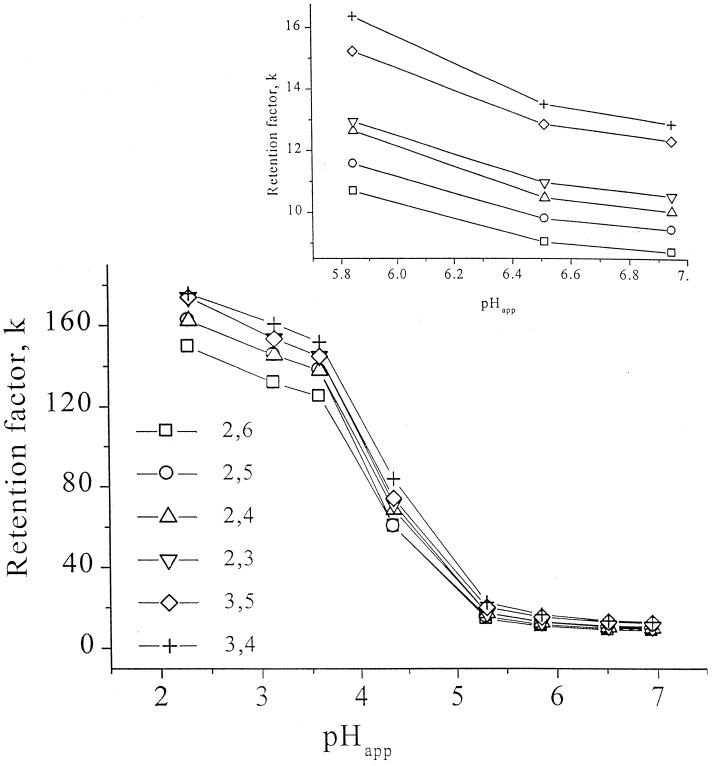

Fig. 8. Effect of pH_{app} of mobile phase A on the retention factor of DFPAA isomers. The chromatographic conditions as in Fig. 7, except utilizing only the Zorbax Rx C_8 column and pH_{app} of mobile phase A was varied.

3.4.5. Effect of phosphate buffer concentration

The concentration of the phosphate buffer was varied from 10 m*M* to 45 m*M*. The retention factors of these isomers were increased slightly as the concentration increased with minor improvements in resolution. This behavior indicated strong hydrophobic interactions were dominant and that electrostatic interactions are negligible during the separation process.

3.4.6. Comparison of the elution order of 3,4-DFPAA in CZE and RPLC methods

Although the elution order of each isomer was different in each method, one interesting fact is observed. The desired isomer, 3,4-DFPAA is the last band in all five different techniques. This behavior is vital for the chromatographic analysis of a series of analytes, as it is typically desired to have the minor analytes elute in front of the major analyte, from practical standpoint. If the desired elution order is not achieved, the tail of the major enantiomer can cause interference towards the detection of minor amounts of the undesired analytes because of the large sample loading required to detect the minor analytes at the 0.1 area % level.

The detailed understanding of the elution order is not understood completely, but it appears that the same elution order of 3,4-DFPAA in CZE and RPLC methods is dictated by structural effects. When the separation occurred in RPLC modes, the major interaction is the hydrophobic interaction between the solute and the stationary phase. Since the 3,4-DFPAA has fluorine atoms at the *para* and *meta* positions, it was much less likely to form intramolecular hydrogen bonding between the fluorine and carboxylic acid functionalities in contrast to the other isomers. Therefore, it is plausible that the hydrophobic interactions between the fluorine atoms of 3,4-DFPAA and the stationary phase are stronger than for the other isomers thereby increasing the

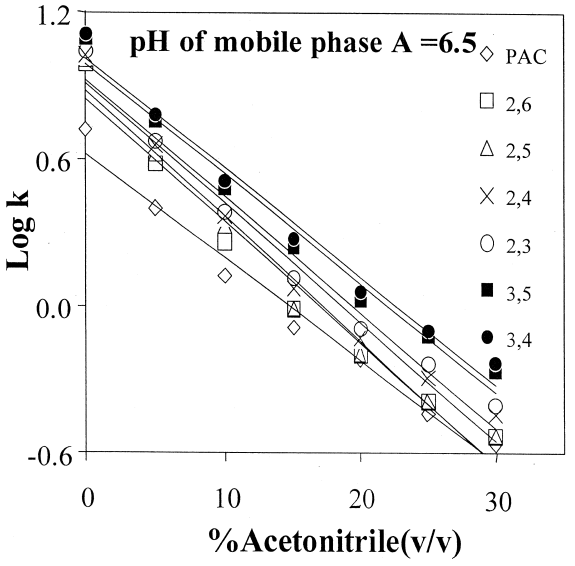

Fig. 9. Effect of acetonitrile concentration on retention (log k) of DFPAA isomers and phenylacetic acid at pH_{app} of mobile phase A=6.5. Other conditions as in Fig. 7, except utilizing only the Zorbax Rx C_8 column and the concentration of acetonitrile was varied.

retention and selectivity of the separation. In the case of CZE separation, the selectivity is due to pK_a differences between isomers. It is known that *para*-substituted electronegative atoms such as the halogens do increase the acidity of phenylalkanoic acids [21]. In fact, our investigation showed that the 3,4-DFPAA has the highest pK_a value of all the isomers (potentiometric titration). In the pH range studied, the degree of ionization of each isomer is different. Since the 3,4-DFPAA has the highest pK_a, it has the least charge when the pH_{app} is between 3.2 and 4.0. Therefore, it has the longest migration time.

Based on the results discussed above, it was concluded that the Zorbax Rx C_8 column with a 15 m*M* phosphate buffer (pH 6.5)–acetonitrile (95:5, v/v) as mobile phase was the optimum system. The optimized chromatogram is shown in Fig. 10. Note the peak efficiency of the last eluting component (3,4-DFPAA) was sufficient to provide the desired sensitivity, and as shown in Table 1, the (k) values for all components were in the range $0.5<k<20$. Also, the resolution was greater than 1.5 for the first five bands, which will be of very similar size in a real sample and the resolution between the main component (3,4-DFPAA) and its adjacent peak was greater than 2.0.

3.5. Validation studies

3.5.1. Comparison of the limit of detection (LOD) for the five methods

One of the most important aspects for isomeric

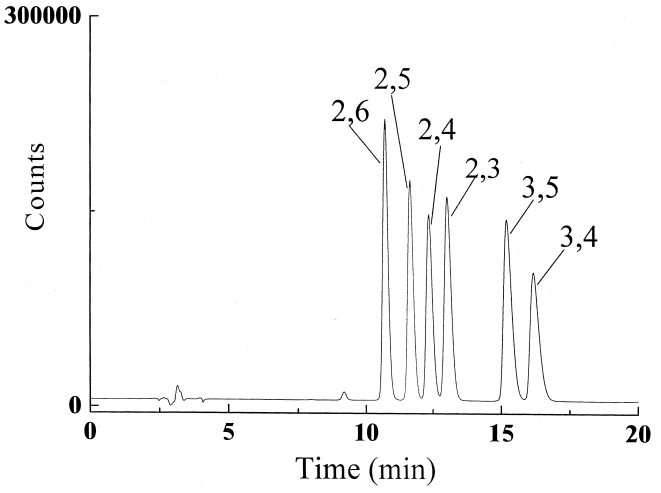

Fig. 10. Optimized chromatographic separations of DFPAA isomers in RPLC. Column: Zorbax Rx C_8, 250×4.6 mm, 5 μm particle size. Mobile phase: acetonitrile–15 m*M* sodium phosphate buffer (pH_{app} 6.5) (5:95); flow-rate: 1.0 ml/min; UV detection: 210 nm, column temperature: 25°C.

Table 1
The retention factor (k) and resolution (R_s) for the optimized RPLC method[a]

	2,6-DFPAA	2,5-DFPAA	2,4-DFPAA	2,3-DFPAA	3,5-DFPAA	3,4-DFPAA
k	4.0	4.5	4.8	5.1	6.1	6.6
	2,5-/2,6-DFPAA	2,4-/2,5-DFPAA	2,3-/2,4-DFPAA	3,5-/2,3-DFPAA	3,4-/3,5-DFPAA	
R_s	2.2	1.6	1.6	4.3	2.0	

[a] Chromatographic conditions as in Fig. 10.

impurity method validation is LOD. The LOD of all five techniques were determined through a linearity study (R^2 values>0.99) at the concentration of the solution injected producing a signal-to-noise ratio of 3 [24]. Since the desired isomer, 3,4-DFPAA was also the last band in all separations, it was a good candidate to choose for evaluation of the method LOD. Serial dilutions of a 3,4-DFPAA solution were prepared between 0.02% and 125% of the target concentration (0.5 mg/ml) for each method. As we can see from Table 2, the RPLC techniques showed superior LOD to other techniques in determination of isomeric impurity of 3,4-DFPAA.

3.5.2. Limits of quantitation (LOQs)

The LOQ was found to be 0.05% of target concentration (0.5 mg/ml) based on satisfaction of three criteria: (A) the S/N ratio of the LOQ (0.05%) solution was greater than 10; (B) the percent difference of response factor values at the LOQ and 5×LOQ was less than 20; (C) the relative standard deviation (RSD) of area counts for three injections at the LOQ level was less than 15%.

3.5.3. Precision and accuracy

A 0.5 mg/ml sample of 3,4-DFPAA was spiked with five other isomers at the 0.5% (w/w) level. The spiked solution was injected six times consecutively. The RSD of 3,4-DFPAA for six injections was less than 0.1% based on the area percent. The average recovery of each isomer was at least 95%.

Table 2
Comparison of method limit of detection[a]

	NPLC	SFC	GC	CZE	RPLC
LOD	0.03%	0.5%	0.3%	0.5%	0.01%

[a] Conditions as in Fig. 1 for NPLC; as in Table 1 for RPLC and as in Fig. 5 for CZE, except the pH value of the electrolyte was 3.8. The conditions of SFC and GC are the same as described in Experimental.

3.5.4. Robustness

A solution containing 0.5 mg/ml 3,4-DFPAA was prepared in the recommended diluent and stored at ambient temperature and light. The RSD of the 3,4-DFPAA peak was 0.1% based on area percent over a 24-h period. Three lots of Zorbax Rx C_8 columns were examined. The column-to-column variability was minimal in terms of retention times and peak efficiency.

4. Conclusion

The separations of DFPAA positional isomers could be achieved by RPLC, NPLC, SFC, GC and CZE techniques. For the separation of the isomeric impurities of DFPPA, RPLC was found to be superior to the other chromatographic techniques in terms of selectivity and sensitivity.

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