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Effects of first dimension eluent composition in two-dimensional liquid chromatography

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ABSTRACT

Comprehensive two-dimensional liquid chromatography (LC × LC) has received a great deal of attention during the past few years because of its extraordinary resolving power. The biggest advantage of this technique is that very high peak capacities can be generated in a relatively short time. Numerous approaches to maximize the peak capacity in LC × LC have been employed. In this work we investigate the impact of the first dimension mobile phase on selectivity. LC × LC has several potential advantages over one-dimensional LC (1DLC) in that unconventional solvents, at least in reversed-phase LC, can be used. For example, solvents which strongly adsorb in the UV in the first dimension are not problematic in LC × LC. This so because the UV detector is placed after the second dimensional column, as pulses of the first dimension eluent arrive at the second dimensional column, they elute well before the solutes of interest and therefore do not interfere at all with detection of solute peaks. So far, the most widely used solvents in reversed-phase 1DLC are methanol and acetonitrile. However, the "UV advantage" of 2DLC allows us to employ UV active solvents, such as acetone. We compare their differential selectivities to that of acetonitrile for the separation of 23 indole acetic acids of interest in plant biology. We also apply them to the separation of a maize seed extract, a very complex sample. In both sample sets, mobile phase composition can be an important parameter to increase the orthogonality of the two dimensions and thus, to increase the effective peak capacity of LC × LC.

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1. Introduction

Because of its high resolving power, two-dimensional liquid chromatography has been receiving a great deal of attention during the past few years, especially by those dealing with complex, usually biological samples [1,2]. Comprehensive 2DLC, which will be denoted as LC × LC as per the suggestion of Beens and co-workers [3], is done by sequentially transferring the entirety of the first dimension effluent, in many small aliquots, onto the second dimension column. In principle, LC × LC can greatly increase the peak capacity of LC over the most highly optimized one dimensional LC. As Karger et al. [4], later Giddings [5] and Guiochon et al. [6] have pointed out, under ideal circumstances the overall peak capacity should be equal, not to the sum, but rather to the product of the individual peak capacities of the first and second dimension separations.

However, there are three requirements to achieve the ideal peak capacity. (1) The two separation mechanisms have to be totally orthogonal. This requirement is usually a requirement imposed on the stationary phase chemistry. (2) The peaks must occupy the entire separation space. (3) The second dimension must be sufficiently fast so that none of the separation gained in the first dimension is sacrificed in the performance of the second dimension [7–12]. In accord with all three requirements, Stoll et al. defined an effective peak capacity for LC × LC which incorporated the first and second dimension peak capacities, a fractional "coverage factor" and an factor to correct for the undersampling of the first dimension when the second dimension is too slow [13]. This third requirement relates principally to the speed of the second dimension and is not considered here. The first and second requirements are somewhat related because only a totally orthogonal system will allow the peaks to occupy the entire separation space, and the extent to which the peaks occupy the separation space is a good indication of the orthogonality of the two dimensions. Achieving perfect orthogonality is extremely rare in practice, Giddings [5] has described in detail how peak capacity can effectively range from a maximum of the ideal peak capacity down to a minimum of a one dimensional peak capacity depending on the extent of correlation between the two retention mechanisms. If a two-dimensional separation is conducted in such a way that a high correlation exists between the retention times of analytes in the two separation dimensions, the full potential of the LC × LC will not be fully realized. Various metrics of orthogonality were critiqued in a recent book about LC × LC [1].

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It is unfortunate that the word "correlation" was used originally by Giddings [5] in this context because many workers have attempted to use the mathematical correlation coefficient as a means of measuring the degree of departure from the ideal 2D peak capacity [14]. In our view what really matters is not the correlation coefficient *per se* but in accord with Davis [1] the fraction of the 2D separation space (area), which is occupied by chromatographic peaks. Recognizing the inadequacy of the correlation coefficient in this context several groups have discussed other approaches to calculating the fraction of the 2D separation space that is occupied by peaks in LC × LC. Slonecker et al. [15] have used information theory to describe the predicted distribution of constituents across the separation spaces of a variety of projected 2D separations based on 1D separation data. More recently, Gilar et al. [16] hit upon a simple but very effective approach which amounts to casting a grid onto the separation space and then determining the fraction of the grid boxes that contain peaks. This fraction allows subsequent corrections to the ideal 2D peak capacity. Stoll et al. [17] introduced a correction factor, based on the Gilar approach, to the 2D peak capacity computed by the product rule that takes into account the fraction of the space occupied when one computes an effective 2D peak capacity so that it can be properly compared between different methods. This approach is used in this manuscript for all fraction coverage calculations.

The majority of studies concerned with the utilization of the 2D separation space have focused on the effect of different separation modes. There have been a number of successful mode combinations, such as SEC × RP [18,19], SEC × NP [20,21], NP × RP [22,23], and IEC × RP [24,25]. Additionally, there has been some work in which the same mode of separation was used in both dimensions (*e.g.* RPLC × RPLC [26,27]) but chemically dramatically different types of stationary phases were used (*e.g.* amide polar embedded groups *vs.* C18 in reversed-phase mode) [28]. In our own work we have paired a pentafluorophenyl first dimension phase with a carbon clad metal oxide second dimension [10–12,17,26]. While the mobile phase composition of the eluent is usually thought to be the most influential factor in controlling overall retention in RPLC, its impact on selectivity is considered to be rather less than the effect of changes in the stationary phase [29–35]. However, the effect of changing the mobile phase chemistry and pH have been shown to be very powerful for separating solutes in RPLC [36,37], especially for ionic solutes.

2. Experimental

2.1. Materials and reagents

All solutes and solvents were of reagent grade or better and were used without further purification. Acetonitrile and methanol were obtained from Burdick and Jackson (Muskegon, MI). Acetone, sodium dihydrogen phosphate and phosphoric acid were from JT Baker (Philipsburg, NJ); sodium monohydrogen phosphate was obtained from Fisher Scientific (Fairlawn, NJ). HPLC grade water was obtained in-house from a Barnstead Nanopure deionizing system (Dubuque, IA). This water was boiled to remove carbon dioxide and cooled to room temperature before use. All aqueous eluents were prepared gravimetrically (0.01 g) and passed through a 0.45-μm nylon membrane filtration apparatus (Lida Manufacturing Inc., Kenosha, WI) immediately before use. None of the eluents used in this work were degassed prior to use.

2.1.1. Indolic metabolite standards

There are 23 indolic metabolite standard used in this research, including: 5-hydroxy-tryptophan, indole-3-acetyl-aspartic acid, indole-3-acetyl-glutamic acid, tryptophan, anthranilic acid, indole-3-acetyl-myo-inositol, indole-3-acetyl-glycine, 5-hydroxy-tryptamine, indole-3-acetyl-lysine, indole-3-acetyl-alanine, indole-3-acetic acid, indole-3-acetyl-glucose, indole-3-acetamide, indole-3-carboxylic acid, indole-3-acetyl-isoleucine, indole-3-acetyl-leucine, indole-3-propionic acid, indole-3-ethanol, tryptamine, indole-3-butryic acid, indole-3-acetyl-glutamine, indole, indole-3-acetonitrile. Analytical samples of these 23 indolic metabolite standards were prepared from stock solutions (∼1 mg/mL in 50/50 isopropanol/water) by dilution to ∼20 μg/mL with the aqueous component of 20 mM phosphate buffer at pH = 5.7. Anthranilic acid and indolic standards were obtained from Sigma–Aldrich except as noted. Indole-3-acetyl-glutamate and indole-3-acetyl-myo-inositol, mixed isomers, were prepared on a semi-preparative scale as previously described [38,39] for preparation of radio labeled compounds. Indole-3-acetyl-lysine, indole-3-acetyl- glutamine, indole-3-acetyl-isoleucine were gifts from Dr. Volker Magnus and indole-3-acetyl-β-d-glucose was prepared by Dr. Dina Keglevic (both from Ruder Boskovic Institute, Zagreb, Croatia).

2.1.2. Corn seed sample

The corn seed used for 1D- and LC × LC separations was Silver Queen (Burpee, Warminster, PA) and was extracted with the procedures published elsewhere [17].

2.2. Instrumentation and column

2.2.1. 1DLC separations

Conventional 1DLC gradient separations were performed using a standard Agilent 1100 liquid chromatograph controlled by version A.10.01 ChemStation software (Agilent Technologies; Wilmington, DE). This instrument was equipped with an in-line degasser, autosampler, ternary pumping system and a photodiode array detector (200–600 nm). The column thermostating compartment was a homemade water jacket to maintain temperature at 40 °C. Reversed phase separations were carried out with a homemade HC–OH column (200 mm × 1.0 mm, 5 μm particle) [40] using organic solvent gradients. The HC–OH phase is an ultra-stable, polar-embedded, hydrophilic silica stationary phase made by incorporating a hydroxyl methyl functionality into the hyper-crosslinked (HC) platform [41]. A solvent was either an aqueous buffer composed of 20 mM sodium phosphate at pH 5.7 or pH 2.7. The B solvent was pure acetonitrile or methanol or acetone. A flow rate of 0.10 mL/min was obtained from a simple flow-splitting apparatus constructed using different lengths of fused-silica capillary tubing (50 μm i.d., 360 μm o.d., Polymicro Technologies, Phoenix, AZ) and a low dead volume "tee" fitting. The total flow rate delivered by the Agilent pumping system was 1.0 mL/min, and thus, a split ratio of 10:1 was used to deliver a flow rate of 0.10 mL/min to the HPLC injector and column. The use of flow splitting in the first-dimension separation is to reduce the effective gradient delay time and improve gradient reproducibility [42]. The dwell volume of the first dimension system is 0.35 mL [26]. The column outlet was connected directly to the photodiode array detector using a 1.0-m length of 50 μm i.d. fused-silica tubing. Detection was at 220 nm.

2.2.2. LC × LC separations

The basic features of the LC × LC system used in this work were described in detail in a previous paper [26]. The first dimension of the LC × LC instrument was comprised of the same components described above for the 1DLC separations, including the HC–OH column as the first dimension column. The 10-port valve was actuated pneumatically using helium at 60 psi. The two sample loops used to alternately capture effluent fractions from the first-dimension separation or from the injector directly and deliver them to the second-dimension column were 67-cm lengths of 0.010-in.-i.d.

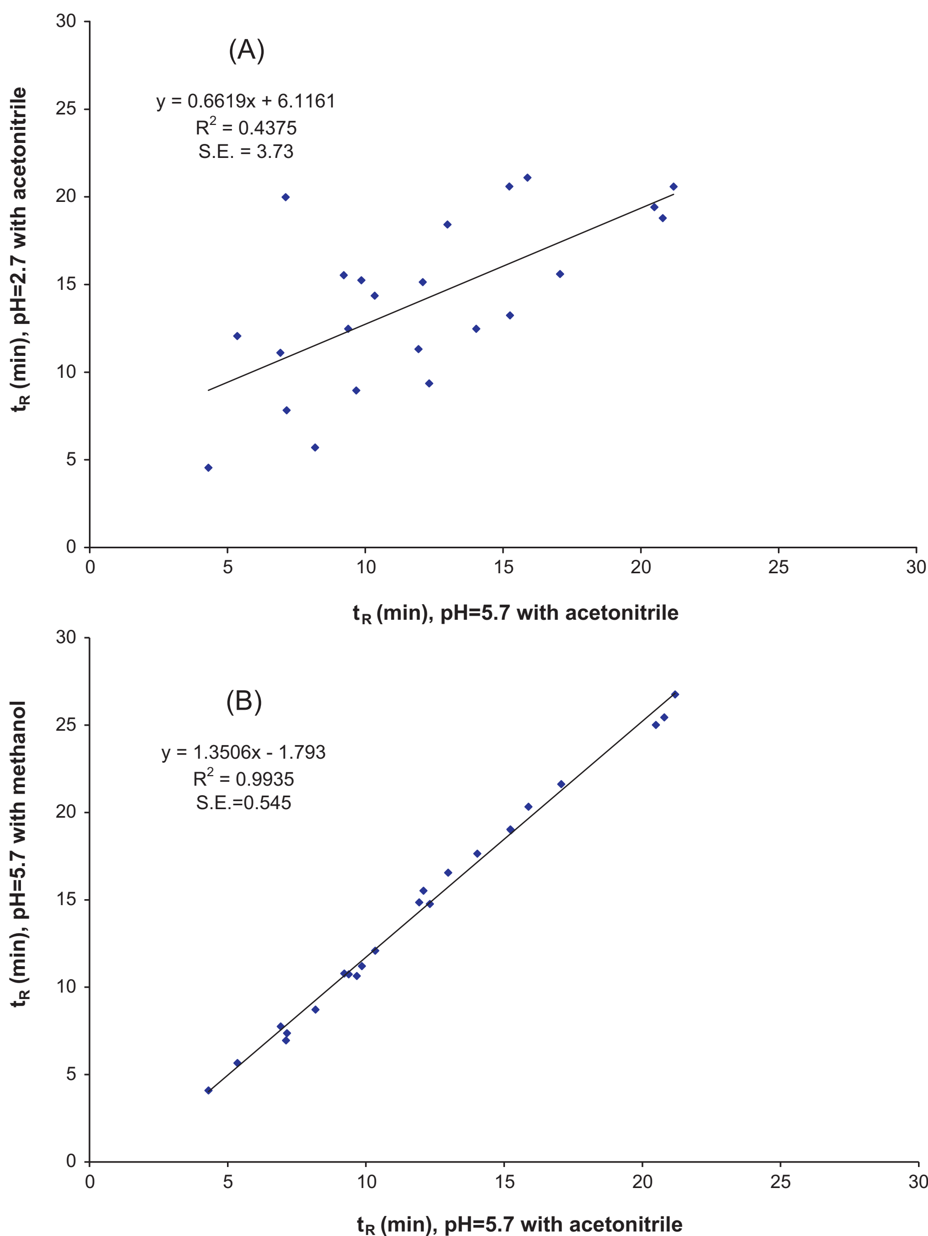

Fig. 1. Effect of mobile phase composition and pH on retention. Stationary phase is the HC–OH material described in the text. Solutes are the twenty-three indole acetic acid. Temperature is 40 °C. Flow rate is 0.1 mL/min. (A) plot of retention times with Solvent A (phosphorous buffer) at pH 5.7 *vs.* at pH 2.7, in both cases, solvent B (organic modifier) is acetonitrile; (B) plot of retention times with solvent B is acetonitrile versus methanol, in both cases, solvent A (phosphorous buffer) is at pH 5.7.

PEEK tubing, such that the volume of each loop was 34 μL. A Metalox column oven (Model 200-C, Anoka. MN) was used to preheat the mobile phase delivered to the second-dimension column and maintain the column at 100.0 (±0.1 °C).

The column used in the second dimension was packed in-house with a prototype carbon-clad zirconia reversed-phase material (8% carbon, ZirChrom Separations, Inc.; Anoka, MN). The extra-column volume is 0.05 mL and the hold-up volume is 0.075 mL. Absorbance spectra were collected at a rate of 80 Hz over the range of 200–600 nm at all time points of each LC × LC chromatogram. Each second-dimension separation in the LC × LC separations consisted of a reversed-phase gradient from 0 to 100% B, where the A solvent was 10 mM phosphoric acid in water (pH = 2) and the B solvent was pure acetonitrile. The total gradient cycle time was 21 s, with a second dimension gradient time (^2t_g) of 17 and 4 s for re-equilibration of the HPLC column. The 4-s re-equilibration time corresponds to roughly two column volumes of solvent; one column volume is required to flush the strong (ACN-rich) solvent from the system, and the second column volume is required to actually re-equilibrate the HPLC column to the extent that the repeatability of retention time in the second dimension is satisfactory (0.002 min standard deviation). LabVIEW 6.0 software and a 6024E data acquisition board (National Instruments Inc., Austin, TX) were used to control the coordination of the first-dimension HP 1100 system, the 10-port valves, second-dimension pumping systems, and photodiode array detector using simple programs written in-house.

3. Results and discussions

3.1. pH effect in 1DLC

Mobile phase pH is an important factor that can be optimized in liquid phase separations and in many cases such changes will greatly change the separation selectivity. The 23 indole acetic

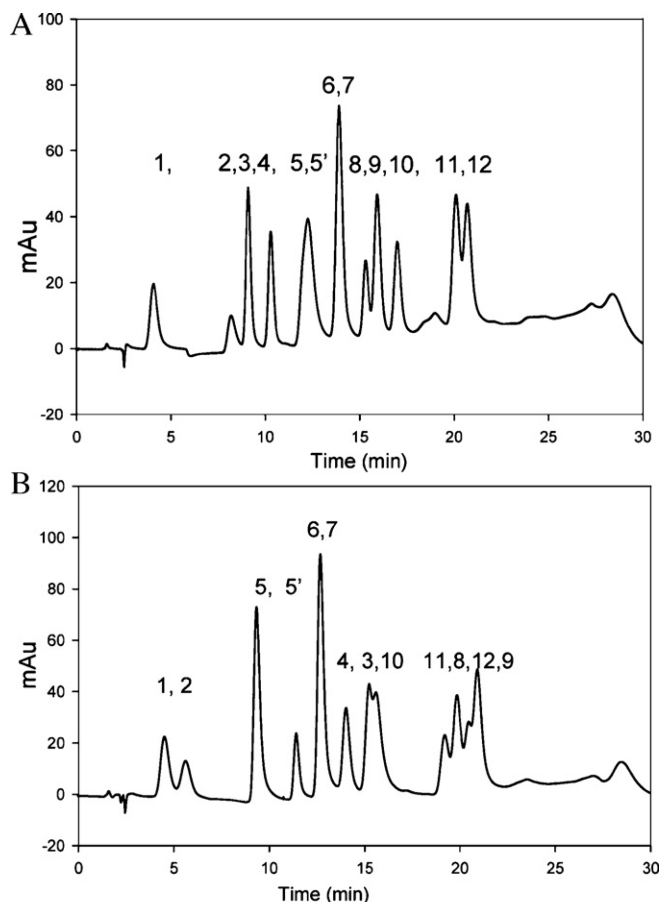

Fig. 2. Chromatogram of pH effect on selectivity for selected 12 indole acetic acids. Stationary phase is HC–OH. Temperature is 40 °C. Flow rate is 0.1 mL/min. (A) Solvent A is phosphate buffer at pH 5.7, Solvent B is acetonitrile, gradient condition is 0-40-0-0%B in 0-23-23.01-30 min. (B) Solvent A is phosphate buffer at pH 2.7, Solvent B is acetonitrile, gradient condition is 0-42-0-0%B in 0-23-23-23.01-30 min. 12 Indole acetic acids are: (1) 5-hydroxytryptophan, (2) 5-hydroxytryptamine, (3) indole-3-acetic acid, (4) indole-3-acetyl-alanine, (5) indole-3-acetyl-lysine, (6) indole-3-acetamide, (7) indole-3-acetyl-isoleucine, (8) tryptamine, (9) indole-3-ethanol, (10) indole-3-butyric acid, (11) indole-3-acetyl-acetonitrile, (12) indole.

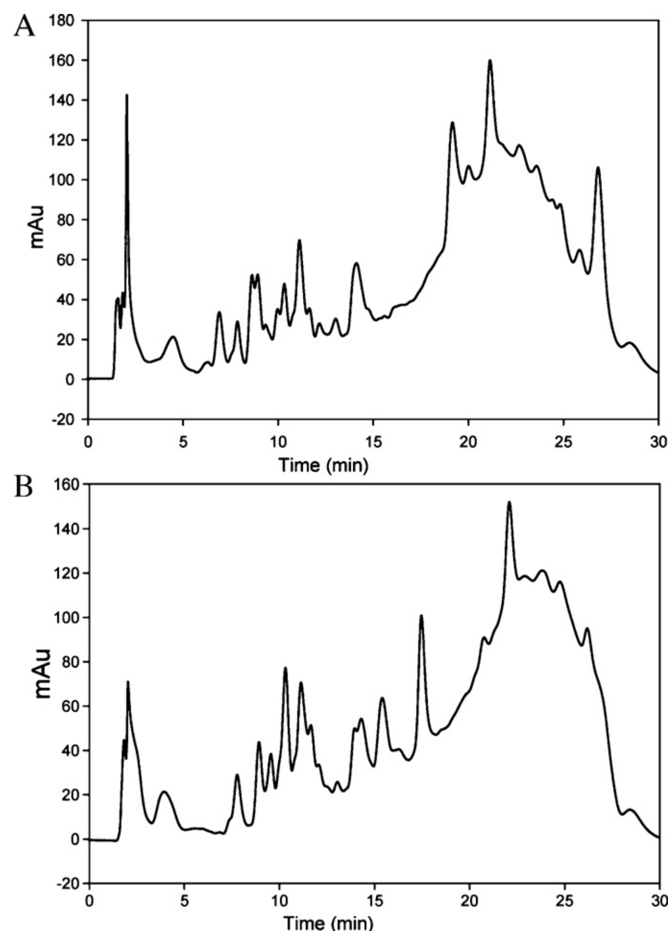

Fig. 3. Effect of pH on the maize extract sample in one-dimensional separation with HC–OH phase. Gradient condition: 5-40-40-70-5-5% ACN in 0-20-22-23-23-30 min, temperature is 40 °C, flow rate is 0.1 mL/min. (A) Eluent A is 20 mM phosphate buffer at pH = 5.7; (B) eluent A is 20 mM phosphate buffer at pH = 2.7.

acids represent a structurally related yet highly variegated sample set containing acidic, basic, zwitterionic as well as neutral compounds which makes this set ideal for the investigation of pH effects on selectivity. Note these solutes are prototypical analytes for an important class of constituents in extracts from maize seed [26]. We have used them extensively in basic studies of LC × LC [10,11,17,26].

Fig. 1A shows a plot of the retention times at pH 5.7 (ordinate) *vs.* pH 2.7 (abscissa) using acetonitrile as the eluent, whereas Fig. 1B shows the effect of methanol *vs.* acetonitrile both at pH 5.7. Thus any solute lying above the line in Fig. 1A will be more retained at pH 2.7 than at 5.7 and *vice versa* for solutes below the line. There are clearly many changes in elution order when the pH is changed. Most of our compounds are amino-acid type molecules containing both acidic and basic functional groups. The neutral compounds are not sensitive to pH and thus their retentions do not change. However, the acids with pK_a less than 2.7 are neutral at low pH but become ionized at high pH and so increasing the pH from 2.7 to 5.7 decreases their retention. For the basic compounds in our sets, where the pK_a are all above 9.5, their retentions are not strongly affected when changing pH from 2.7 to 5.7. As a result, the elution order and selectivity of the twenty three indole acetic acid derivatives change dramatically upon changing the pH. We selected a smaller set of twelve indole acidic acids out of the twenty three to illustrate the changes in elution order; the results are given in the chromatograms in Fig. 2.

The eluent pH will also change the elution profiles for complex samples such as maize. Fig. 3 shows a comparison of chromatograms at pH 2.7 and 5.7. Since the maize sample contains hundreds of compounds at various concentrations, there is no easy way to label any peaks. Any single peak in the first dimension could contain quite a few compounds [26]. However, we still observe significant differences in the elution pattern. For example, a distinctive peak eluted at 27 min with the pH = 5.7 buffer is not present at the same position in the pH = 2.7 buffer.

3.2. *pH effect in LC × LC*

The fact that the mobile phase pH can be used to change selectivity is an interesting point that can benefit LC × LC. In LC × LC there is an absolute need for maximally different selectivities in the two dimensions to produce the highest effective peak capacity. The most direct approach to achieving this is to use radically different modes of separation as discussed above; nonetheless, we and others have used RPLC in both dimensions [26,43–45] but do so using pairs of reversed phases with radically different selectivity [46,47] or different mobile phase [48,49]. Here, we explore the possibility of changing selectivity by applying different pHs in the mobile phase of the first dimension. Gilar's work indicates that even if similar columns are used in the two dimensions, as long as the pH effect is big enough one can still achieve a high degree of orthogonality

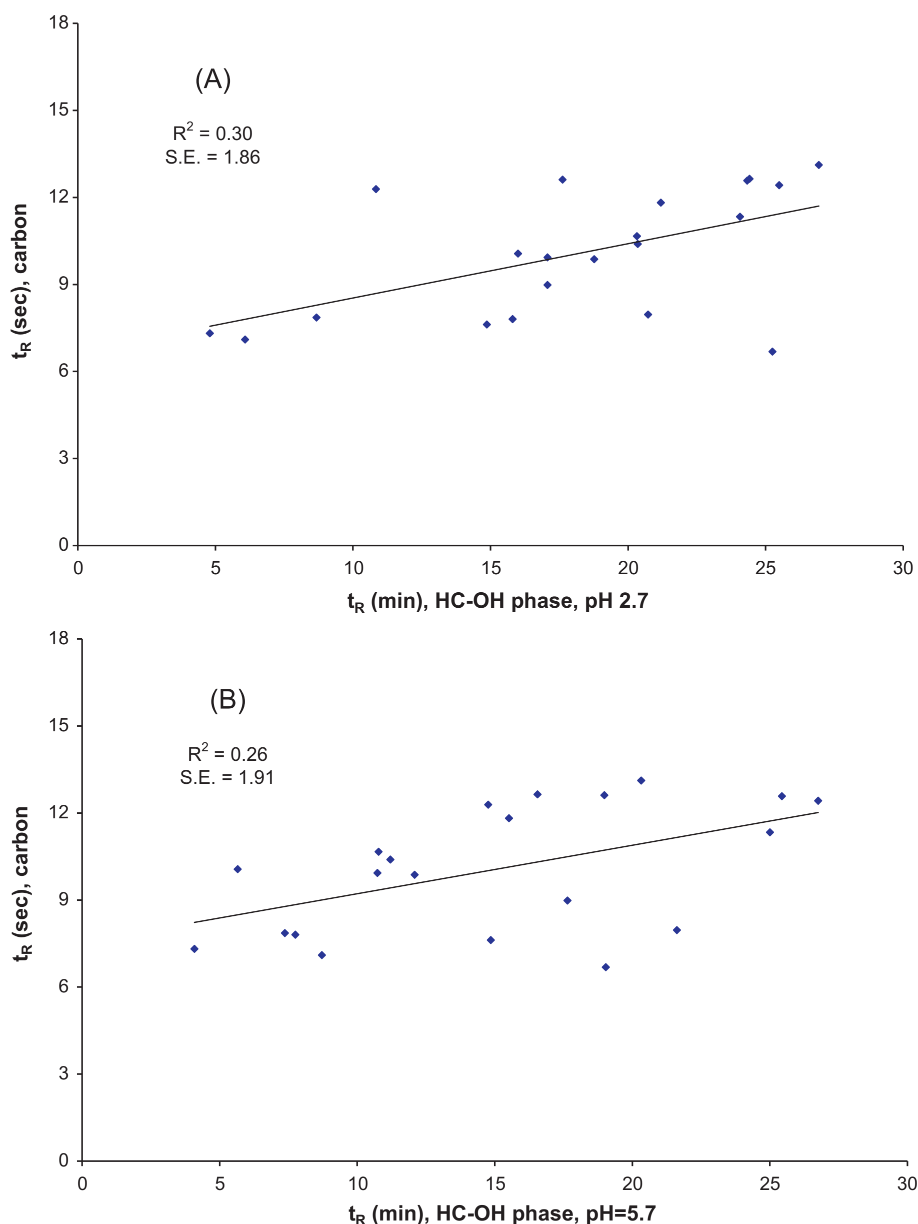

Fig. 4. Correlation between retention times between HC–OH phase and ZirChrom carbon phase. Gradient condition on carbon phase: Eluent A is 10 mM phosphoric acid, Eluent B is acetonitrile, temperature is 110 °C, flow rate is 3 mL/min.

[37] if the analytes in the mixture have a wide range in acidity/basicity.

We selected the HC–OH phase as the first dimension column and a ZirChrom carbon phase as the second dimension column because of the dramatic difference in selectivity between carbon and almost any RPLC material [50]. We wanted to see if applying different pHs on the two dimensions whether the orthogonality could be changed. While the pH for the second dimension's mobile phase was held at 2, we changed the pH of the first dimension eluent from 2.7 to 5.7. The correlation between the retention times on the first dimension HC–OH phase at pH 2.7 or 5.7 and the retention times on the second dimension carbon phase at pH = 2 are shown in Fig. 4. The correlation coefficient and standard error changed from 0.30 and 1.86 for a pH of 2.7 to 0.26 and 1.91 for a pH of 5.7. The low correlation coefficient supports the dramatic difference in selectivity between the two phases. The lower correlation coefficient at pH 5.7 suggests a somewhat improved orthogonality when the two dimensions are run at different pHs.

We also separated a complex maize sample by the same LC × LC system described above. The second dimension still used 10 mM phosphoric acid (pH 2) as the buffer while the first dimension was run at both pH 2.7 and pH 5.7 with the appropriate buffers. Representative chromatograms are given in Fig. 5. As discussed previously, fractional coverage is a better metric of orthogonality as the effective peak capacity in LC × LC is directly related to the fractional coverage (see Fig. 6). For the maize sample, the coverage increased from 0.37 to 0.46 when the first dimension pH was changed from 2.7 to 5.7.

Another important parameter also related to the resolving power is the number of observable peaks. We counted the peaks at a signal to noise ratio larger than 15:1. When the mobile phase pHs of the two dimensions were similar, 110 peaks were counted but when the pHs in the two dimensions were different, there were 131 peaks. The increased fractional coverage thus the resulting increased effective peak capacity along with the increased number of observed peaks confirmed the improvement in the separation

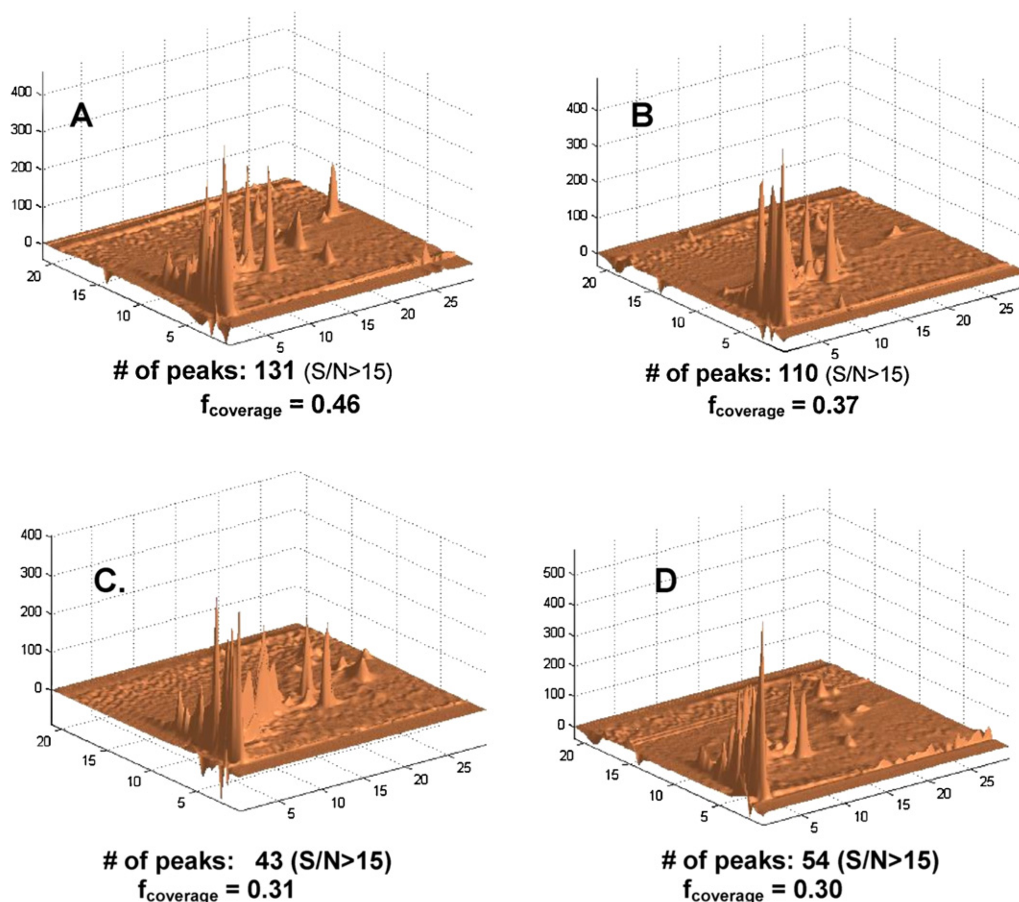

Fig. 5. 2DLC chromatogram with maize sample with different mobile phase composition in the first dimension. 2nd dimension condition is constant: ZirChrom carbon phase, Eluent A is 10 mM phosphoric acid, eluent B is ACN, 0-100-0% ACN in 0-0.29-0.30 min, temperature is 100 °C, flow rate is 3 mL/min; 1st dimension condition: HC–OH phase, temperature is 40 °C, flow rate is 0.1 mL/min, other conditions are varied as following description: (A) Eluent A is 20 mM phosphate buffer at pH = 5.7, eluent B is acetonitrile, gradient condition is 5-40-40-70-5-5% ACN in 0-20-22-23-23.01-30 min (B) Eluent A is 20 mM phosphate buffer at pH = 2.7, eluent B is acetonitrile, gradient condition is 5-40-40-70-5-5% ACN in 0-20-22-23-23.01–30 min (C) A is 20 mM phosphate buffer at pH = 5.7, eluent B is methanol, gradient condition is 5-50-50-80-5-5% methanol in 0-20-22-23-23.01-30 min (D) A is 20 mM phosphate buffer at pH = 5.7, eluent B is acetone, gradient condition is 5-35-35-65-5-5% methanol in 0-20-22-23-23.01-30 min.

when different pHs were used in the two dimensions. In the corresponding 1D separations (see Fig. 3) there were no more than 30 peaks.

3.3. Organic modifier effect (methanol vs. acetonitrile) in 1DLC and LC × LC

The organic modifier also plays an important role in establishing selectivity. Acetonitrile is the most widely used organic modifier in liquid chromatography due to its solubility properties, lower UV cut-off range, and low viscosity. Additionally, methanol is used very extensively in liquid chromatography. It is sometimes the preferred organic modifier with certain types of columns to promote specific types of interactions which acetonitrile cannot [51]. It can produce different selectivities compared to acetonitrile depending on the phases and the solutes of interest [52]. Consequently we measured the retention times of 23 indole acetic acid derivatives with methanol as the organic modifier on the HC–OH column. Mobile phase A uses 20 mM phosphate buffer at pH 5.7. The final eluent composition was 52% methanol (v/v) which elutes the most retained solutes at retention times similar to those obtained with the acetonitrile–water mixture. The correlation of the two solvents is given for the 23 indole acetic acids (see Fig. 1B). The correlation coefficient is 0.99 (standard error 0.545), indicating almost no changes in selectivity. Compared to the pH effect discussed above, the change in organic modifier had a much smaller effect on our model molecules. Obviously different organic modifiers do have the potential to change the selectivity but this is strongly dependent on the sample set as well as the stationary phases used.

The chromatogram generated with methanol in the first dimension in a LC × LC system with the maize sample is shown in Fig. 5C. The fractional coverage drops to 0.31, compared to 0.46 with acetonitrile as the organic modifier. This indicates that acetonitrile is the more suitable solvent in the first dimension for the maize sample. The number of visible peaks with S/N larger than 15 decreases to 43.

3.4. Unusual organic modifiers in LC × LC

The array of organic solvents used in RPLC is really quite limited and most commonly include methanol, acetonitrile, isopropanol and THF. Many solvents cannot be used or their use is very limited due to their high viscosity in mixtures with water. Even isopropanol and THF are problematic in this regard but solvents such as DMSO and DMF have even higher viscosities. Some solvents which are chemically very interesting and not problematic from the perspective of viscosity are not used due to their high UV cut-off wavelengths. Acetone is probably the best known such solvent [53]. However, LC × LC is quite immune to both the viscosity and the UV absorptivity problems. One must bear in mind that the amount of solution injected into the second dimension is related to the first dimension flow rate and the sampling time which is equal to the second dimensional analysis time [11]. In our case it is only 34 μL.

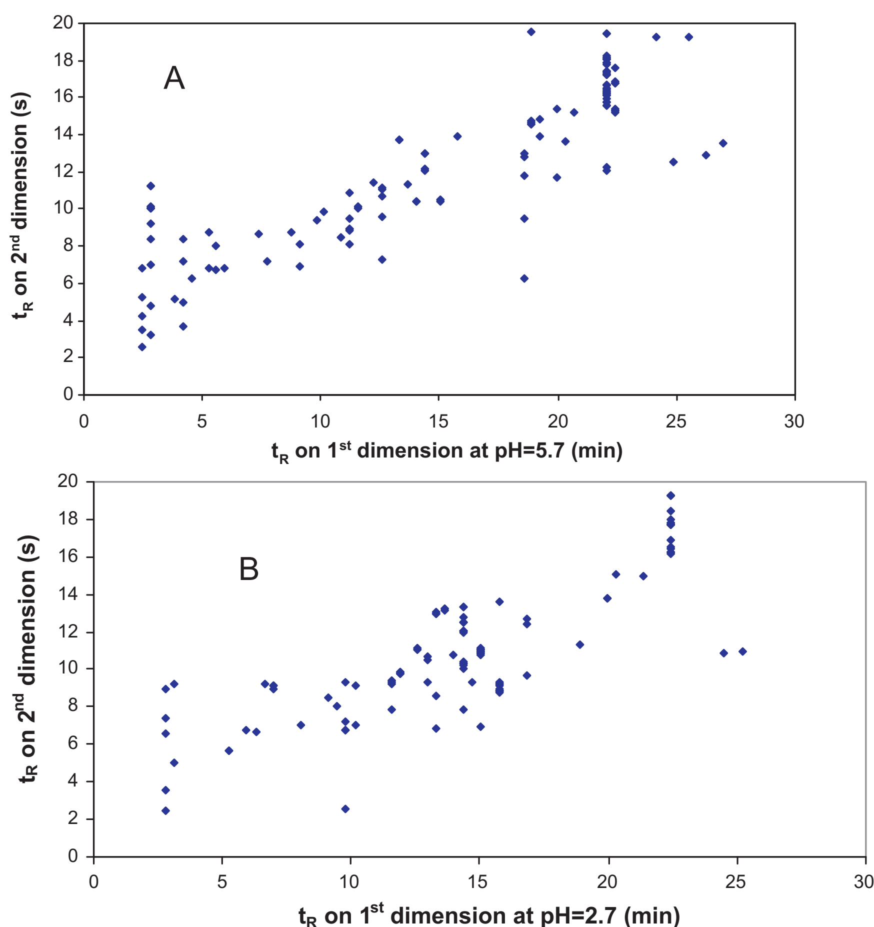

Fig. 6. Fraction coverage comparison with 2DLC separations of maize sample applying different pHs in the first dimension. Both dimension conditions see Fig. 5A and B.

With regard to the viscosity problem the first dimension of on-line LC × LC is carried out at low velocity to avoid flooding the second dimension with large sample volumes [26], thus pressure drop is seldom a limiting issue for the first dimension separation. The UV absorptivity of the first dimension eluent is also of minimal impact as the organic component will be essentially unretained on the second dimension in an RP × RP separation.

Acetone, which has a UV cut-off at 330 nm [54], is not used in conventional RPLC with UV detectors. Here, we use acetone to demonstrate the possibility of using an unusual solvent in a LC × LC system. We tested the separations of both the 12 indole acetic acids and the maize sample by LC × LC. Fig. 7A shows the second dimension UV chromatogram with the indole acetic acids. An expanded time scale from 14 to 15 min is given in Fig. 7B; it shows three second dimension injections. The peaks eluting at 14.05, 14.4 and 14.75 min are acetone peaks; the peaks eluting at 14.2, 14.55 and 14.9 min are all the same indole acetic acid analyte. In the third injection, between 14.75 and 15.1 min, clearly another indole acetic acid begins to elute at 14.87 min. Their separation and detection are not impacted at all by using acetone in the first dimension. The chromatogram of the LC × LC separation of 12 indole acetic acids is shown in Fig. 7C. Compared to the chromatogram of the LC × LC separation using acetonitrile in the first dimension, the second dimension retention times for all 12 acids did not change. Although the first dimension retention times do not seem to change much with acetone, they could potentially change depending on the phase and sample set as discussed above.

The maize extract was also analyzed by LC × LC using acetone as the first dimension modifier (see Fig. 5D). The fractional coverage is only 0.30. The number of peaks with signal to noise ratio larger than 15 is 54. Both metrics suggest that use of acetone in the first dimension does not give an effective peak capacity as high as obtained with either acetonitrile or methanol. However, the specific effect on the fractional coverage is not the point at this juncture rather it is the ease with which LC × LC accommodates a strongly UV absorbing eluent.

4. Concluding remarks

We studied various mobile phase effects in several one and two dimensional LC systems. Although the most direct approach to increasing orthogonality is to apply two different chromatographic modes to the two dimensions, applying different pHs or organic modifiers in the two dimensions can be quite important. The chief conclusions of this study are:

(1) For our two sets of test samples (23 indole acetic acids and an extract of maize seeds), pH had a much larger effect in changing selectivity in a 1DLC separation. By using quite different pHs in the LC × LC separations we found that both the fractional coverage and the number of observed peaks were both substantially increased. This will result in an improved effective peak capacity. Since the effective peak capacity of LC × LC is directly related to the fractional coverage the separating power of LC × LC will be increased.

(2) In LC × LC, unusual organic modifiers such as acetone, that are not suitable for use in traditional LC–UV systems, can be used in the first dimension because they will elute in the dead time of the second dimension separation and thus not interfere with other solutes. This approach opens a new door for future

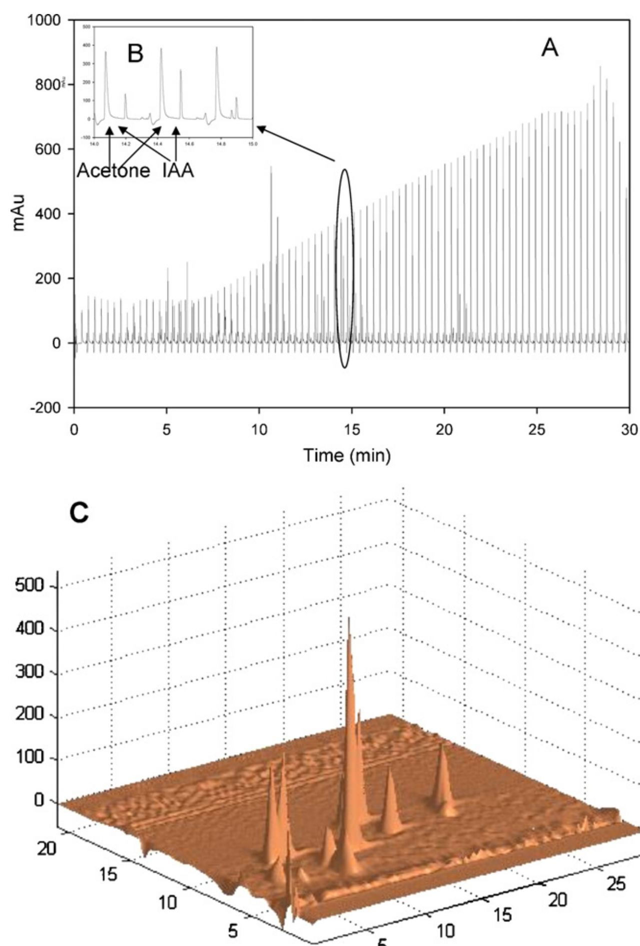

Fig. 7. Chromatogram applying acetone as unusual solvent in the first dimension in a 2DLC. See Fig. 5D for experimental conditions. (A) unprocessed chromatogram from 2DLC (B) blow up scale of 3 s dimension cycles (C) processed 2DLC chromatogram with selective 12 indole acetic acids.

LC × LC development to maximize peak capacity. It is however, necessary that the first and second dimension solvents be compatible and that the first dimension solvent not cause excessive band broadening or otherwise malformed peaks in the second dimension.

(3) Use of different organic modifiers in the two dimensions has the potential to change the fractional coverage and thus the effective peak capacity.

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