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Two-dimensional liquid chromatography with mixed mode stationary phases

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Abstract

Mixed mode stationary phases with ion-pairing reagent (acidic or basic) as integral part of hydrophobic chain offers unique selectivity, and hence, are ideal for multidimensional separations. The retention of hydrophobic components is a function of organic content, whereas that of charged species is a function of organic content, ionogenic modifier and its level in the mobile phase. Hence, by controlling the parameters influencing component retention (stationary phase and mobile phase), the selectivity of chemical components in the two-dimensional plane can be manipulated to improve the separation. A two-dimensional liquid chromatograph has been developed by coupling similar and dissimilar mixed mode stationary phases in the two dimensions. This technique has immense potential in resolving co-eluting components as the retention mechanism in the two-dimensions are complementary. However, with only part of the primary column eluent sampled into the secondary column, the technique is limited to qualitative analysis.

Keywords: Mixed mode interaction; Complementary separation; Two-dimensional liquid chromatography

1. Introduction

Coupled column chromatography can be quite powerful compared to its one-dimensional counterpart provided the retention mechanisms are complementary [1–3]. It results in either enhancing the resolution and or improving the analysis speed due to increased peak capacity [1–5]. Complementary separation in coupled column chromatography can be realized either by coupling dissimilar techniques [6–14] or by selectively tuning the operation parameters influencing retention like temperature [4,5,15–20], mobile phase [21,22], etc. However, coupling complementary techniques is challenging and often complicated, as the mode of operation and sampling criteria differ significantly with an increase in dissimilarities between techniques [22].

We have developed a 2D-LC system by modifying the plumbing of a commercial liquid chromatograph and by incorporating an electronically controlled, twelve-port, dual position switching valve between the two dimensions [21,22]. The primary column separation is comparable to conventional HPLC, whereas the secondary column separation is extremely fast lasting 10–20 s. The high-speed separation in the secondary dimension enables partial or complete transfer of primary column eluent to the secondary column. These, high-speed secondary chromatogram when plotted appropriately results in a two-dimensional contour plot.

In the earlier work, we discussed the importance of column selectivity and tuning operation parameters in developing 2D-LC [21,22]. In this paper, we have addressed the importance of mixed mode stationary phases in 2D-LC. For ionic components, 2D-LC involving mixed mode columns is superior to conventional reversed phase columns as their selectivity can be manipulated by changing the organic content and or the

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ionogenic modifier and its strength. However, the retention of hydrophobic components in the mixed mode column is comparable to conventional reversed phase columns. A test mixture of acidic, basic and neutral components when analyzed using mixed mode phases resolves into zones with the acidic and basic components on either side of neutral components. As, expected the 2D-LC separation using dissimilar mixed mode phases is superior to the one involving similar mixed mode phases of different dimensions. In addition, the presence of ion-pair reagents as integral part of the hydrophobic chain enables the use of very strong ionogenic reagents like sulfuric acid (200 mM or 1.25%) without the degradation of the bonded stationary phase. The 2D-LC system described in this article is simple and easy to build compared to other systems described in the literature. Most of the work described in literature use micro-LC in the primary dimension to address the sampling needs of the secondary dimension, usually the capillary zone electrophoresis [6–11]. Also, some systems require repetitive gradient in the secondary dimension to ensure complete elution of all sample components between successive sampling cycle [11,14]. The 2D-LC system described in this work uses a conventional HPLC system in both the dimension. Practically, any commercial HPLC system can be modified into a 2D-LC system with minimal modification. Similar or complementary phases could be used in the two-dimensions. A detailed comparison can be found in the earlier work and is not within the scope of this paper [22].

Like in conventional HPLC, a change in back pressure of either or both the secondary column (with time) will impact the two-dimensional separation. However, periodic measurement of the secondary column flow rates, replacement of the tubing and or the secondary columns (as and when required) eliminates these potential problems. We have found this system to be extremely reproducible with minimal back pressure problems over more than a year and a half of its use. The above technique has immense potential in chemical class separation and in resolving co-elution peaks.

2. Experimental

2.1. Chemicals and reagents

Most of the chemicals used in this study [benzylamine, dimethylbenzylamine, methylphenethylamine, methoxyphenamine, propranolol, tryptophan, 3-methyl-2-butanone, benzaldehyde, benzoic acid, malic acid, maleic acid, fumaric acid, benzonitrile and amitryptiline] were purchased from Sigma–Aldrich. Acetonitrile and water were purchased from Burdick and Jackson and sulfuric acid was purchased from Mallinckrodt Chemicals. Stock solutions of benzylamine, dimentylbenzylamine, methylphenethylamine, benzonitrile, benzaldehyde, methylbutanone and amitrytiline were prepared in acetonitrile. Stock solution of benzoic acid was made in a 1:1 mixture of acetonitrile–water mixture. Stock solution of malic acid, maleic acid, fumaric acid and salts of propranolol and methoxyphenamine were made in water. A 10-mL test mixture of these components was made by mixing different proportions of individual components in a 4:1 water–acetonitrile mixture. The final concentration of the individual components in the mixture was around 10–200 μg/mL. Fifty microliters of test mixture were injected into the HPLC. All the stock solutions and the mixtures were refrigerated and found to be stable throughout the study.

2.2. Chromatographic equipment

The instrumentation for orthogonal two-dimensional liquid chromatography is shown in Fig. 1. The key component of this instrument is an electronically controlled dual position valve (V). The twelve-port, dual position valve enables continuous, alternative sampling of the primary column (PC) eluent onto dual secondary columns (SC1 and SC2) through equivalent, dual sampling loops (L1 and L2). The mobile phase from the pump 1 flows through the injector onto the primary column (PC). The eluent from the primary column (PC) flows either through the sampling loops L1 or L2 to the waste (or detector). The mobile phase from pump 2 is spilt at the Valco union (U1), with part of the flow going through the secondary column (SC1) through the sampling loops (L1 or L0) and rest flowing through the secondary column (SC2) through the sampling loops (L0 or L2). Comparable volume of the sampling loops L0, L1 and L2 (40 μL each) results in constant flow through the secondary columns. The eluent from the secondary columns SC1 and SC2 flows into individual UV detectors 1 and 2. In experiments involving a secondary column, the position 2 in the twelve-port, dual position valve (V) is blocked (plugged). This results in partial sampling of the primary column eluent into a single secondary column SC1 for further separation.

In position 1 (Fig. 1, left hand side), the eluent from the primary column (PC) flows through the 40 microliter sample loop L1 before exiting to waste; whereas the contents of the loop L2 from the previous cycle is sampled onto the secondary column SC2. The flow through the loop L0 (valve port 3 to port 9) maintains undisrupted flow through the secondary column SC1. When the valve is switched to position 2, the eluent from primary column fills the other 40 microliter sample loop L2 before exiting to waste (Fig. 1, right hand side), whereas the contents of the loop L1 from previous cycle is sampled onto the secondary column SC1. The flow through loop L0 (valve port 9 to port 3) keeps undisrupted flow through the secondary column SC2. Thus, there is a continuous flow of mobile phase through the primary and secondary column throughout the separation. Depending upon the retention time range of the chemical components in the second dimension, either partial or complete transfer of the primary column eluent to the secondary column is realized. In the experiments described in this manuscript, approximately 20–40% of primary column eluent was sampled

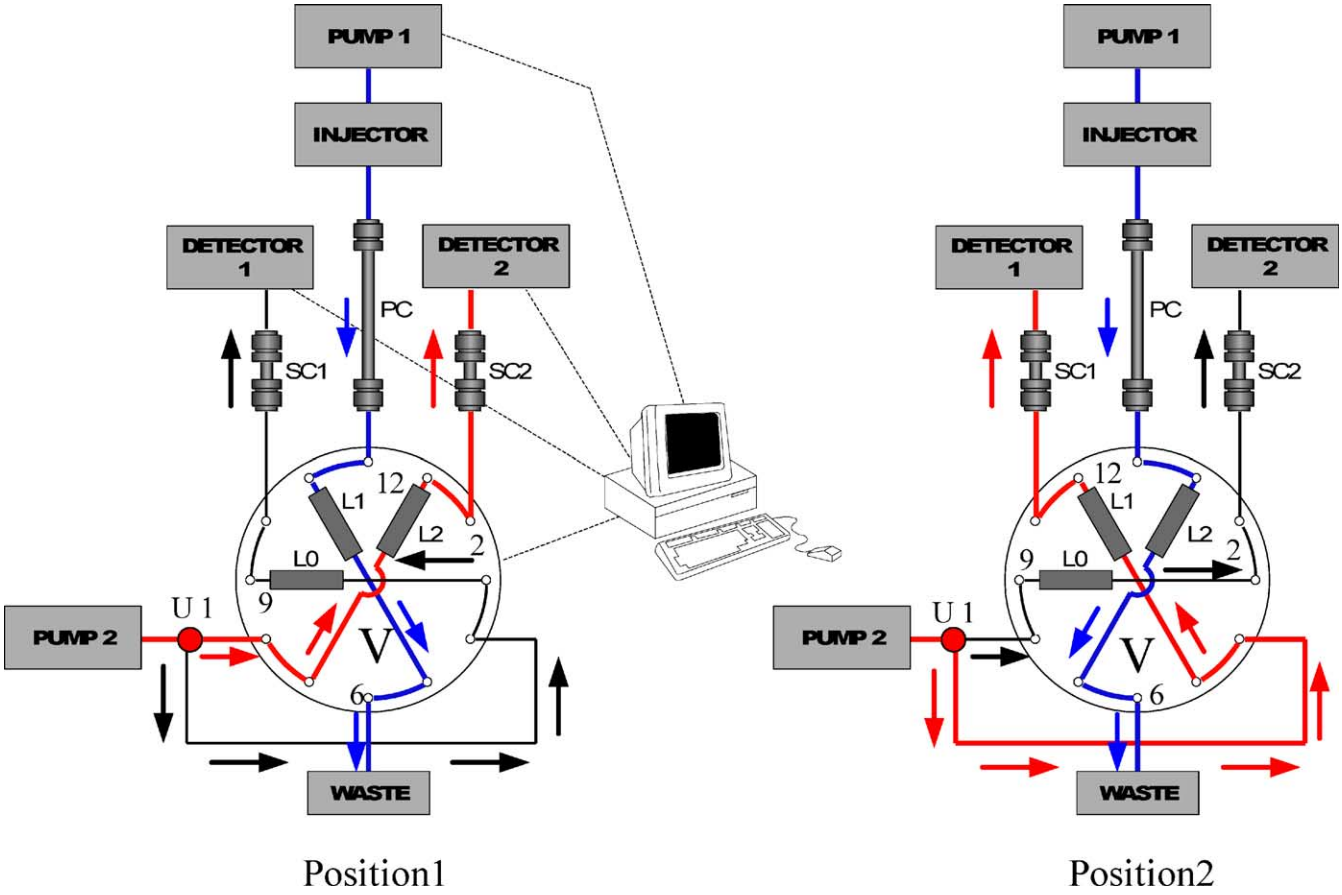

Fig. 1. Schematic of a 2D-LC system showing the flow of mobile phase through the two dimensions. In position 1, the primary column (PC) eluent flows through the sampling loop L1 to the waste. The mobile phase from pump 2 flows through secondary columns SC1 and SC2 flowing through sampling loops L0 and L2, respectively. When the valve position is switched (right hand side), the primary column eluent flows through sample loop L2 to waste. The mobile phase from pump 2 flows through secondary columns SC1 and SC2 flowing through sampling loops L1 and L0, respectively. The flow through the columns is uninterrupted throughout the chromatographic run with primary column eluent alternatively sampled into the secondary columns SC1 and SC2.

into the secondary column. With peaks from the primary column lasting several seconds, each peak eluting from the primary column is sampled at least once into the secondary column for further separation. Ideally, the primary column eluent has to be sampled at least four times into the secondary dimension to preserve the primary column separation [23]. However, this can not be always realized as the frequency of generating secondary chromatograms is a function of retention time range of most retained and least retained components in the secondary dimension and not on their absolute secondary column retention time. Sampling faster than the retention time range will result in aliasing problem, with components appearing at inappropriate locations of the two-dimensional plane. Usually, the strongly retained component in the secondary dimension will appear at low retention times.

All instruments used in this study are commercially available. A HP1100 series from Agilent Technologies equipped with a degasser (G1322A0), quaternary pump (G1311A), auto-sampler (G1313A) and diode array detector (G1315A) were used to generate the two-dimensional chromatogram. All the instruments were controlled by Agilent Chemstation software. An electronically activated twelve-port, dual position valve purchased from Valco Instruments Co. Inc. (Houston, Texas) was controlled by a multi-mode, programmable timer (ICM 500 purchased from ICM Corp., Cicero, New York). The external contact closure from the pump was used to synchronize the ICM timer between successive runs. This enables effective subtraction of system peaks resulting from differences between the mobile phase strength at the head of secondary column and the eluent sampled into the secondary column. Details pertaining to the system peak and its elimination are discussed in our earlier work [22]. The data from Chemstation (primary column retention time and detector response) were exported into Microsoft Excel and were updated with second dimension retention times prior to re-processing using PSI-Plot. The PSI-Plot purchased from Poly Software International (Pearl River, New York) enabled display of the two-dimensional data either as a contour plot or a three-dimensional plot.

2.3. Chromatographic conditions

The chromatographic conditions for different studies are summarized below.

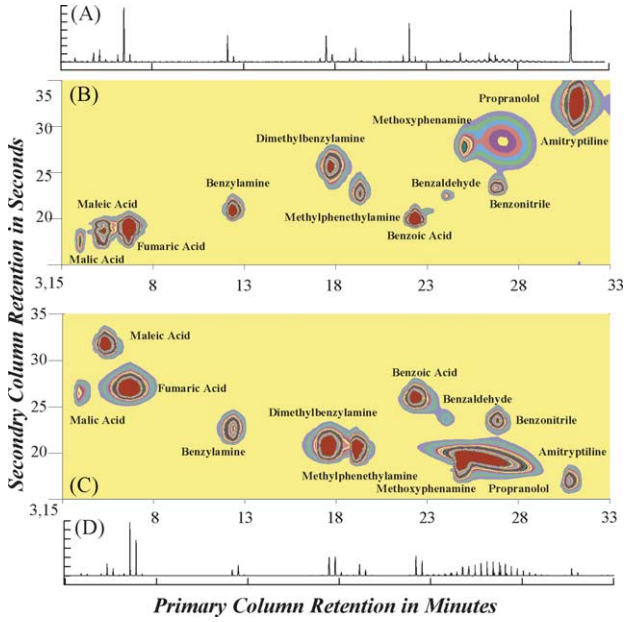

Fig. 2. A complementary 2D-LC separation of a test mixture on a Primesep-100 (15 cm × 4.6 mm × 10 μm) in the primary dimension and Primesep-100 ((B), 2 cm × 4.0 mm × 5 μm) and Primesep-B ((C), 2 cm × 4.0 mm × 5.0 μm) columns in parallel in the secondary dimension is shown above. The one-dimensional chromatograms in (A) and (D) were used in the generation of the two-dimensional contour plots. The primary column flow rate was 0.5 mL/min, and the secondary column flow rates were 2.8 mL/min on Primesep-100 and 2.2 mL/min Primesep-B in the secondary dimension. Approximately 20% of primary column eluent were sampled alternatively into each of the secondary columns. The UV detection was at 215 nm.

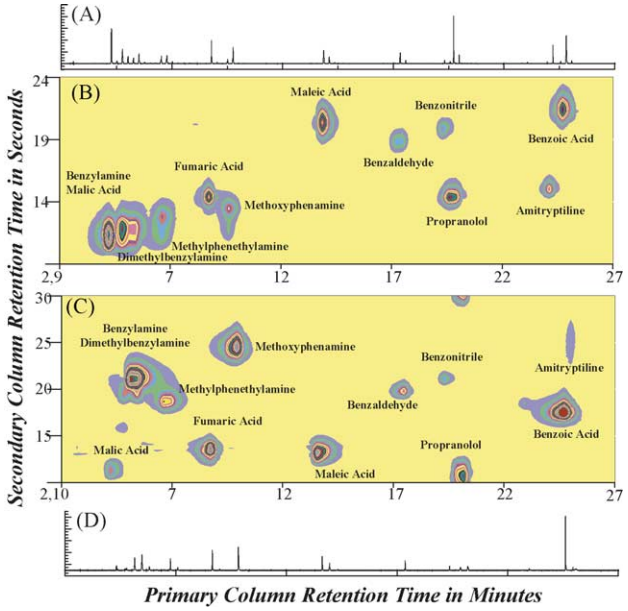

Fig. 3. A complementary 2D-LC separation of a test mixture on a Primesep-B column (15 cm × 4.6 mm × 5 μm) in the primary dimension and Primesep-B ((B), 2 cm × 4.0 mm × 5 μm) and Primesep-100 ((C), 2 cm × 4.0 mm × 5.0 μm) columns in the secondary dimension is shown above. The one-dimensional chromatograms (A) and (D) were used in the generation of two-dimensional contour plots. The primary column flow rate was 0.5 mL/min, and the secondary column flow rates were 2.25 mL/min on Primesep-B and 2.50 mL/min on Primesep-100 in the secondary dimension. The experiments (Primesep-B/Primesep-B and Primesep-B/Primesep-100) were run individually. Approximately 20% of primary column eluent were sampled into the secondary columns. The UV detection was at 215 nm.

2.4. Experimental conditions for the primary dimension

For all the studies, either a 15 cm × 4.6 mm × 5 μm Primesep-B column or a 15 cm × 4.6 mm × 10 μm Primesep-100 column from SIELC were used in the primary dimension. For the study in Fig. 2, a Primesep-100 column was used in the primary dimension and for the studies in Figs. 3 and 4, a Primesep-B column was used. The starting HPLC condition was 50:5:45 percent of 1.25% sulfuric acid (200 mM):acetonitrile:water which was programmed to 50:50 percent of 1.25% sulfuric acid (200 mM):acetonitrile over 30 min. The flow rate through the primary column was 0.5-mL/min till 30 min and was increased to 1.5-mL/min during the re-equilibration lasting 15 min.

2.5. Experimental conditions for dual, parallel columns in the secondary dimension

For the results discussed in Fig. 2, a dual 2 cm × 4.0 mm × 5 μm Primesep-B and Primesep-100 guard columns from SIELC were used in parallel in the secondary dimension. An isocratic mobile phase made of 20:30:50 percent of 1.25% sulfuric acid (200 mM):water:acetonitrile was used in the secondary dimension. The primary column eluent was sampled every 10 seconds into each of the secondary columns. Approximately 20% of the primary column eluent was sampled into each of the secondary column (sample loop volume × number of secondary chromatograms generated in a column per min × 100/primary column flow rate; 40 μL × 3 × 100/500 μL). The UV detection was at 215 nm.

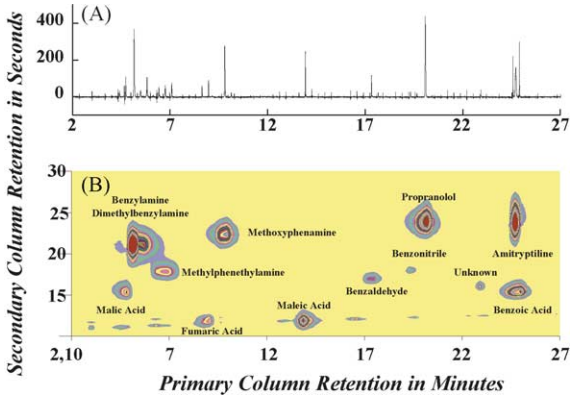

Fig. 4. A complementary 2D-LC separation of a test mixture on a Primesep-B column (15 cm × 4.6 mm × 5 μm) in the primary dimension and Primesep-100 column (2 cm × 4.0 mm × 5 μm) in the secondary dimension is shown above. The one-dimensional chromatogram in (A) was used in the generation of two-dimensional contour plot. The primary column flow rate was 0.5 mL/min and the secondary column flow rate was 3.25 mL/min. Approximately 20% of primary column eluent was sampled into the secondary column. The UV detection was at 215 nm.

2.6. Experimental conditions for single column in the secondary dimension

For the 2D-LC separation discussed in Fig. 3B, a 2 cm × 4.0 mm × 5.0 μm Primesep-B guard column from SIELC was used in the secondary dimension. For the 2D-LC separation discussed in Fig. 3C, a 2 cm × 4.0 mm × 5.0 μm Primesep-100 guard column from SIELC was used in the secondary dimension. An isocratic mobile phase made of 20:30:50 percent of 1.25% sulfuric acid (200 mM):water:acetonitrile was used in the secondary dimension. The flow rate through Primesep-B and Primesep-100 columns were 2.25 mL/min and 2.50 mL/min, respectively. The experiments were run individually (Primesep-B/Primesep-B and Primesep-B/Primesep-100). Approximately 20% of the primary column eluent was sampled onto the secondary column. For these studies, one of the two sampling loop in the twelve-port, dual position valve was blocked (plugged) at position 2. This enabled the use of a single column in the secondary dimension.

The experimental conditions for the results discussed in Fig. 4, is similar to the one discussed above (Primesep-B in the primary and a Primesep-100 guard column in the secondary) with the exception of a higher flow rate of 3.25 mL/min in the secondary dimension. The higher flow rate enables to address the aliasing or wrap around problem. The UV detection was at 215 nm.

3. Results and discussion

3.1. Complementary 2D-LC separation with Primesep-100 in the primary and dual, mixed mode stationary phases in parallel (Primesep-100 and Primensep-B) in the secondary dimension

A 2D-LC separation of some acidic, basic and neutral components using a Primesep-100 column (15 cm × 4.6 mm × 10 μm) in the primary dimension and dual Primesep-100 and Primesep-B guard columns (2 cm × 4.6 mm × 5.0 μm) in parallel in the secondary dimension is shown in Fig. 2. The two-dimensional plot in Fig. 2B was generated using similar phases in both dimensions (Primesep-100/100) and the two-dimensional plot in Fig. 2C was generated using complementary, mixed mode phases in the two dimensions (Primesep-100/B). The one-dimensional trace in Fig. 2A and D are the detector response for each of the two secondary columns as a function of time. The chromatographic data (detector response as a function of primary column retention time) was exported into a spreadsheet program and was updating with secondary column retention time. The resultant data was processed using graphics software into a two-dimensional contour plot by stacking the high-speed chromatograms followed by linear interpolating of data. The details of generating two-dimensional contour plots from high-speed secondary chromatograms, computing secondary column retention time can be found in the earlier work [22].

A 2D-LC separation of test mixture with similar phases in both the dimension (Primesep-100 and Primesep-100) is shown in Fig. 2B. Ideally, with similar phases in both the dimension, one would expect strong cross-correlation in the two-dimensional retention. However, operating the primary column to maximize hydrophobic interaction (and minimize ionogenic interaction) and the secondary column to maximize ionogenic interaction (and minimize hydrophobic interaction) results in some peak scattering. Under these conditions, different modes of interactions are observed for different chemical classes present in the sample (acidic, basic and neutrals). Retention of neutral components (benzaldehyde and benzonitrile) in both the dimension is hydrophobic as there is no ionic interaction. Thus, chemical components strongly retained in the primary column are also strongly retained in the secondary (along the diagonal in the two-dimensional plane). Retention of acidic components in the primary dimension is predominantly hydrophobic as ionic interaction is minimal at low pH. With the exception of benzoic acid, all the short chain acids in the sample mixture (malic, maleic and fumaric acid) have comparable, low primary column retention. On the contrary, in the secondary dimension, all the acidic components have comparable, low retention as both hydrophobic and ionogenic interactions are minimal at high organic content and low pH. Thus, benzoic acid has similar secondary column retention as other short chain dibasic acids in the sample. The retention of basic components in the two-dimensions is quite complex as they exhibit mixed mode of interaction (ionic and hydrophobic), the levels of which vary for different components. In the primary column, the retention is mixed mode. Under acidic condition, the protonated amines interact strongly with the acidic ion pair reagent in the stationary phase, in addition to hydrophobic interaction. Although, the retention order of basic components in the secondary column seems comparable to primary dimension, the interaction mechanism is different. For the early eluting amines (benzyl amine, dimethylbenzyl amine and methylphenethylamine), the retention in the secondary column is predominantly ionogenic as hydrophobic interaction is low at high organic content in the secondary column mobile phase. The retention order of dimethlbenzylamine and methylphenethylamine are reversed in the secondary dimension. However, for later eluting, bulky amines (propranolol and amitryptiline), the organic content in the two dimensions are comparable, and hence, have similar retention characteristics in the two-dimension. The scattering of sample components observed in the two-dimensional plane results from difference in the interaction of sample components in the similar mixed mode phases in the two-dimension.

The 2D-LC separation shown in Fig. 2C with different mixed mode phases in the two dimensions (Primesep-100 and Primesep-B, bottom) is complementary to the one discussed above. Components strongly retained in the primary column are weakly retained in the secondary column and vice versa.

Under the experimental conditions (Fig. 2C), the short chain, dibasic acids (malic, maleic and fumaric acid) exhibiting low retention in the primary column are strongly retained in the secondary column. This results from strong ionic interaction of the acidic components and protonated basic phase in the secondary dimension (Primesep-B). Among the dibasic acids, the *cis* form (maleic acid) is strongly retained in the secondary dimension relative to *trans* form (fumaric acid). The retention of malic acid is comparable to fumaric acid. The secondary column retention of late eluting benzoic acid is comparable to fumaric and malic acid as hydrophobic interaction is low at high organic content in the secondary dimension. On the contrary, the basic hydrophobic components exhibiting strong retention in the primary column (hydrophobic and ionogenic) exhibit low retention in the secondary column. This is partially due to high organic content minimizing the hydrophobic interactions and repulsion or exclusion of protonated basic components from the protonated basic stationary phase (minimizes ionogenic interactions). The neutral components (benzaldehyde and benzonitile) have intermediate retention relative to acidic and basic components. The relative locations of charged sample components in the two plots in Fig. 2B and C are complementary.

Relative intensities of sample components vary in the two, two-dimensional plot shown in Fig. 2 depending upon the fraction of the peak (front, center or the tail) sampled from the primary column to secondary column. Transfer of sample component from the center of a peak will produce an intense spot relative to a transfer originating from the front or tail of a peak. In the chromatograms in Fig. 2, the eluent from the primary column is alternatively sampled into each of the two secondary columns.

3.2. Complementary 2D-LC separation with Primesep-B in the primary and dual, mixed mode stationary phases in parallel (Primesep-100 and Primensep-B) in the secondary dimension

A 2D-LC separation of some acidic, basic and neutral components using a Primesep-B column (15 cm × 4.6 mm × 5 μm) in the primary dimension and dual Primesep-100 and Primesep-B guard columns (2 cm × 4.6 mm × 5 μm) in the secondary dimension is shown in Fig. 3. The two-dimensional plot in Fig. 3B was generated using similar phases in both dimensions (Primesep-B/B) and the two-dimensional plot in Fig. 3C was generated using complementary, mixed mode phases in the two dimensions (Primesep-B/100). The one-dimensional trace in Fig. 3A and D are the detector response for each of the two secondary columns as a function of time.

A 2D-LC separation of test mixture with similar phases in both the dimension (Primesep-B and Primensep-B) is shown Fig. 3B. With similar phases in both the dimension, one would expect strong correlation in the two-dimensional retention. However, as explained in earlier, some scattering is observed in the two-dimensional plane due to differences in the interaction of each chemical component in similar phase operated under different conditions of the columns (primary column operated to minimize ionic and maximize hydrophobic interaction and vice versa in the secondary). The retention of acidic components in the primary dimension is mixed mode (ionic and hydrophobic) and that of basic and neutral components is purely hydrophobic. In the secondary dimension (Fig. 3B), the retention of acidic components is predominantly ionic due to strong interaction of acidic components with the protonated basic stationary phase and suppression of hydrophobic interaction at high organic content. On the contrary, the basic components have low secondary column retention irrespective of their primary column, as these protonated basic components are repelled by the protonated basic stationary phase. Also, the hydrophobic interaction of basic components is minimized at higher organic content. The neutral components (benzaldehyde and benzonitrile) have intermediate retention relative to acidic and basic components. The 2D-LC separation shown in this chromatogram is similar to the one involving similar phases in Fig. 2B except that the retention of charged components is reversed. Maleic acid, a short chain, dibasic acid has very strong retention in both the dimension (ionic interaction). Fumaric acid, the *trans* form of maleic acid has comparatively lower retention in the secondary dimension. In this chromatogram, malic acid, a short chain, dibasic acid co-elutes with benzylamine. Thus, subtle differences in interaction of charged components in similar phases results in scattering of sample components in the two-dimensional plane.

The 2D-LC separation shown in Fig. 3C with dissimilar mixed mode phases (Primesep-B and Primesep-100) is complementary to the one discussed above. Under the experimental conditions (Fig. 3C), the acidic components have very low secondary column retention irrespective of primary column retention. This is due to reduced hydrophobic and ionic interaction for these components in the secondary dimension. However, the protonated basic components are strongly retained in the acidic, secondary dimension irrespective of their primary column retention. The neutral components (benzaldehyde and benzonitile) have intermediate retention relative to acidic and basic components. In this chromatogram, the relative location of propranolol is in disagreement with other amines present in the sample possibly due to wrap around problem. This results from the sampling of primary column eluent onto the secondary column at rates faster than the retention time range of most and least retained sample components in the secondary dimension. This problem is resolved in the 2D-LC separation in Fig. 4 where the secondary column is operated at a higher flow rate of 3.25 mL/min.

4. Conclusions

In this paper, we have successfully demonstrated the potential of mixed mode phase in generating complementary

2D-LC separation. Simple manipulation of organic content and ionogenic modifier in the mobile phase enables to modify the selectivity of charged sample components in the two-dimensional plane and resolve them into chemical class (acidic, basic and neutral zones). In fact all the separations in the secondary dimension were realized using 2 cm guard columns. Each chemical component has a unique pair of retention times enabling positive identification and their location a measure of its physical property. Commercial HPLC system can be readily modified into a 2D-LC system with minimal change in plumbing and by incorporating a twelve-port, dual position valve and a timer. Similar or complementary columns could be used in the secondary dimension. This technique has potential in resolving co-eluting peaks. However, as mentioned earlier, partial sampling of primary column eluent (hence, insufficient sampling) limits this technique to qualitative analysis with some loss in primary column resolution.

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