Optimization of a Comprehensive Two-Dimensional Normal-Phase and Reversed-Phase Liquid Chromatography System

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Abstract

The present investigation is based on the evaluation of the performance of a comprehensive two-dimensional liquid chromatography (LC×LC) system during method optimization. The LC×LC set-up, operated in normal phase (NP) mode (adsorption) in the first dimension (1D) and reversed-phase (RP) mode in the second dimension (2D), is equipped with a 1D microbore silica column and a 2D monolithic C_{18} column with a 10-port two position valve as the interface. A photodiode array detector is used after the 2D separation. A possible cause of peak distorsion because of the immiscibility of the mobile phases employed in the two dimensions is resolved. The optimization of the analytical run time and flow rate for both dimensions and the initial gradient in the 2D is carried out with various standard compounds. The potential and versatility of this LC×LC approach is demonstrated through the separation of 11 standard components, most of them allergens. The latter, which are characterized by a scattered distribution on the 2D space plane, underwent separation on both a hydrophobicity and polarity basis.

Introduction

Comprehensive multidimensional (MD) chromatography has been used in recent years to characterize and separate biomolecules, polymers, and other complex mixtures. It can be affirmed that for the analysis of such samples, the use of a single column generally proves to be an inadequate tool.

Comprehensive two-dimensional chromatography is achieved, essentially, through the union of standard chromatographic columns and a transfer device located between them. Such a device enables the passage or transfer of continuous fractions of one-dimensional (1D) effluent onto a two-dimension (2D) column as a series of sharp pulses (1–3). In this manner, the entire effluent from the first separation system is subjected to a second independent 2D separation. The amount of each fraction to be transferred from one separation dimension to the next is strictly dependent on 1D peak widths. In order to maintain the separation achieved in the 1D primary column, each 1D peak must be modulated at least three times (4).

The theoretical and practical aspects of comprehensive liquid chromatography (LC×LC) have been discussed in the literature (1,4,5). In contrast to comprehensive gas chromatography (GC×GC), there are far fewer LC×LC applications reported in the literature. It can be affirmed that LC×LC presents a greater flexibility when compared with GC×GC because the mobile phase composition can be adjusted in order to obtain enhanced resolution. Furthermore, LC methods present a wider variety of different separation modes, such as adsorption, partition, size-exclusion, ion-exchange, or affinity chromatography. This aspect allows the combination of a greater number of LC techniques with truly different selectivities. Some limitations are that the total peak capacities in LC×LC are lower than in GC×GC, detection systems are generally not as sensitive or as universal, and mobile phase incompatibilities can represent a serious obstacle. However, most separation modes can be easily interfaced when compatible mobile phases are used (6,7).

Primarily, 1D and 2D solvents should be miscible, otherwise a "plug" of solvent may travel down the 2D column, carrying a fraction of sample components that may cause band spreading or solute precipitation. Secondly, the 1D mobile phase must be weaker with respect to the 2D mobile phase, especially if large volumes are to be injected into the 2D column. If large volumes of such a solvent are used, the solvent will preferentially move the solutes down the 2D column until the strong solvent is diluted sufficiently for solutes to begin to be retained. In this type of situation, solute reconcentration seldomly occurs, and the resulting band spreading impairs the overall resolving power of the system (8).

The interfacing of normal-phase (NP) and reversed-phase (RP) systems is particularly difficult because of mobile phase immiscibility. The use of NP and RP modes in both dimensions can be

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useful in the separation of complex mixtures containing molecules, which vary in polarity and hydrophobicity. In comprehensive LC, totally orthogonal systems with no correlated selectiveness provide the highest number of separated peaks (9). NP and RP separation modes are partially correlated techniques. Murphy et al. (10) developed a comprehensive normal-phase liquid chromatography (NPLC) × reversed-phase liquid chromatography (RPLC) system for the analysis of alcohol ethoxylates. The employment of miscible solvents (aqueous solvent in the 1D) avoided the problem of incompatibility between mobile phases.

In cases where the 1D and 2D systems have incompatible solvents, the use of a microbore LC column in the 1D enables the injection of small volumes onto the secondary (2D) column, making the transfer of incompatible solvents possible without peak shape deterioration or resolution losses (6). In fact, it has been demonstrated that when using a 1D micro or capillary column coupled to a 2D conventional column working at a high flow rate, the dilution of 1D solvent occurs more rapidly through the 2D column, and therefore, band spreading is minimized (11,12).

Takeuchi et al. (12) used an LC capillary amino column operated in NP mode in the 1D and a secondary conventional ODS column in a heart cutting MD method. Hexane and acetonitrile–water mobile phases were employed in the 1D and 2D, respectively (13).

Recently, an NPLC×RPLC method has been developed and applied to the analysis of coumarins and psoralens in lemon essential oil (14). The use of a 1D microbore column in NP mode (adsorption) operated at a slow flow rate and a 2D C_{18} monolithic column operated at a much higher flow rate effectively eliminated problems connected with solvent incompatibility (14). A similar system has also been developed for the analysis of triglycerides in vegetable oils using microsilver ion LC in 1D and a C_{18} monolithic column in 2D (15).

Monolithic columns posses several favourable properties suitable for rapid 2D separations (16). The successful employment of monolithic columns in LC×LC systems has been reported in the literature (17,18).

The aim of the present study was to illustrate the chromatographic features of a comprehensive 2D NPLC×RPLC system using a microbore column in the 1D and a monolithic column in the 2D, respectively. Toluene and hexyl benzene were used as test analytes in the preliminary applications. The effectiveness of the LC×LC system was then evaluated further through the separation of a mixture of aromatic components (alcohols, aldehydes, and esters), characterized by different degrees of hydrophobicity and polarity.

Experimental

Instrumentation and chromatographic conditions

The analyses were carried out with the previously described MDLC system (14). The 1D, operated in the isocratic mode, consisted of a Shimadzu LC-10AD vp solvent-delivery unit (Shimadzu, Milan, Italy) and a Rheodyne two-position 6-port injection valve model 7725i equipped with a 2-µL loop (Rheodyne, Rohnert Park, CA). A Supelcosil LC-SI column (300 × 1 mm, 5-µm particle diameter) was used (Supelco, Milan, Italy) at a flow rate of 15.4 µL/min of *n*-hexane–ethanol (98:2). Pressure was 16 bar. A flow splitter device (Acurate, LC packings, Amsterdam, the Netherlands) was used between the pump and the injector to reduce the flow rate and maintain its stability.

The 2D, operated in the gradient mode, consisted of two Shimadzu LC-10AD vp delivery units connected in parallel to a gradient mixer, a Shimadzu SPD-M10A vp photodiode array detector, and a Shimadzu SCL 10A vp controller. The 2D column was a Merck Chromolith Flash (Merck KgaA, Darmstadt, Germany) (25 × 4.6-mm i.d.) equipped with a Merck Chromolith guard column (5 × 4.6-mm i.d.). The mobile phase used was water and acetonitrile. The gradient run was changed according to the requirements of the analysis. The flow rate was 4 mL/min. Pressure was 120 bar with H_2O–acetonitrile (70:30). The UV spectra of eluting peaks were monitored in the 190–360 nm range, and the chromatograms were acquired at 212 nm; sampling frequency was 12.5 Hz; time constant was 0.32 s. Data acquisition of the photodiode array detector was by Shimadzu Class vp 5.0 software.

Column switching was performed using an electronically controlled 10-port, two position Supelpro valve (Supelco, Milan, Italy) and controlled by a method editor software (Shimadzu). The valve was operated with two 20-µL injection loops. The valve was switched every 78 s by the Class vp programmed external events, allowing continuos, alternate sampling of the 1D eluent onto the 2D column.

The use of the export function of the Class vp software enabled the conversion of the ASCII data into a matrix with rows corresponding to a 78 s duration and data columns covering all successive 2D 78 s chromatograms using the laboratory-made LC×LC data managing software. Contour representation of the 2D chromatograms was through the same software.

Reagents

The pure standard components used in this study were purchased from Sigma Aldrich (St. Louis, MO). Stock solutions of toluene and hexyl benzene were prepared in hexane and in acetonitrile, each at 1000 ppm. Secondary solutions at 100 ppm were prepared in hexane (from stock solution in hexane) and in acetonitrile–water (30:70) (from stock solution in acetonitrile). From the 100 ppm solutions, 10 and 25 ppm solutions were prepared.

A mixture containing amyl cinnamic aldehyde, amyl cinnamic alcohol, eugenol, isoeugenol, methyl benzoate, cinnamic aldehyde, coumarin, 2-phenyl ethanol, benzyl alcohol, cinnamic alcohol, and anysil alcohol was prepared and diluted to obtain a solution of 100 and 25 ppm of each compound in hexane.

Results and Discussion

Optimization of 1D and 2D mobile phases

When coupling NP and RP modes, the main problem is mobile phase immiscibility, and in particular, the mobile phase used in

the NP mode (1D) is always stronger than the mobile phase at the head of the RP column (2D).

It has been demonstrated that peak distortion and splitting may occur when an analyte is dissolved in a solvent that is significantly stronger than the mobile phase into which the same analyte is injected. This effect is also dependent on the volume injected and is mainly linked to the initial conditions, before the injection plug becomes highly diluted by the mobile phase (11). In the LC×LC system, the sample solvent injected onto the secondary 2D column is, necessarily, the mobile phase used in the 1D separation, though the sample volume was linked to the analysis time in the 2D and to the flow rate in the 1D.

A study was carried out in order to verify the variation of peak shape and peak width ($w_{1/2}$) as a function of the initial mobile phase composition for the 2D column using two aromatic hydrocarbons (toluene and hexyl benzene). These two compounds are characterized by different hydrophobicity. Figure 1 shows the results obtained on transferring these two components onto the secondary 2D column using different first-step gradient compositions of the 2D mobile phase. It should be noted that these two compounds coeluted under the 1D conditions because they differ only in the length of the aliphatic chain and their alkyl hydrocarbon substituents contribute little to sample separation in NPLC, which uses a polarity adsorption mechanism. As can be seen, peak widths are narrower when the amount of 2D organic solvent in the mobile phase is reduced, confirming satisfactory peak focusing for both components. Toluene (less retained under RP conditions) showed an increasing value of $w_{1/2}$, as the initial amount of acetonitrile was increased from 0% to 20% and again from 20% to 40%. However, when 60% of acetonitrile was used, the $w_{1/2}$ value decreases because, under these conditions, toluene was scarcely retained and elutes very early. However, for hexyl benzene, both the $w_{1/2}$ and retention times remain practically unchanged when acetonitrile was increased from 0% to 20% and then to 40%. The $w_{1/2}$ value increased, and the band compression was less effective when a 60% initial amount of acetonitrile was used. The conclusions made from this study were that the use of a microcolumn in the 1D enabled the transfer of incompatible solvents and that peak focusing could be greatly improved by reducing the mobile phase strength at the head of the 2D column.

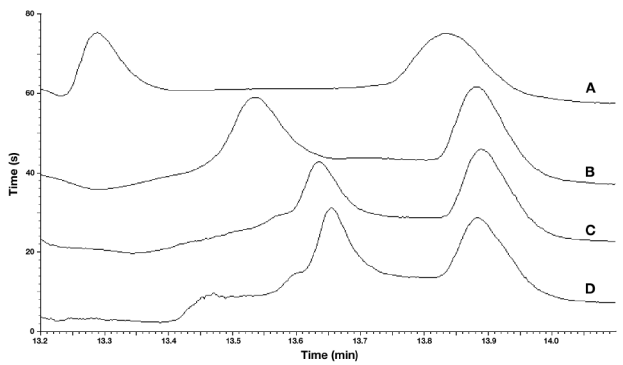

Figure 1. Untransformed 2D chromatograms of toluene and hexyl benzene (25 ppm in hexane) as a function of the 2D gradient initial step. Peak width variation for toluene ($w_{1/2}$T) and hexyl benzene ($w_{1/2}$H) as the percent of water changes in the first step gradient of the second dimension: 100% water: $w_{1/2}$T, 3.0 s; $w_{1/2}$H 3.9 s (A); 80% water: $w_{1/2}$T, 3.6 s; $w_{1/2}$H 3.9 s (B); 60% water: $w_{1/2}$T, 5.4 s; $w_{1/2}$H, 3.9 s (C); 40% water: $w_{1/2}$T, 4.2 s; $w_{1/2}$H, 6.0 s (D).

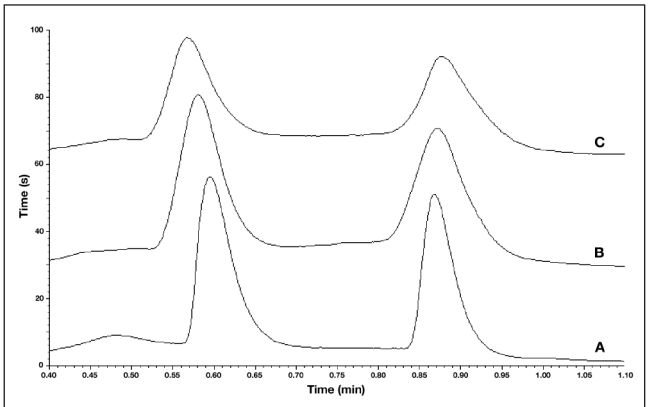

Figure 2. Peak width variation for toluene ($w_{1/2}$T) and hexyl benzene ($w_{1/2}$H) as a function of 2D solvent composition injection at a constant 2D mobile phase gradient. Untransformed RP chromatogram of toluene and hexyl benzene diluted in H_2O–acetonitrile (70:30 v/v), $w_{1/2}$T, 2.4 s; $w_{1/2}$H, 2.4 s (A). Untransformed RP chromatogram of toluene and hexyl benzene diluted in hexane, $w_{1/2}$T, 3.0 s; $w_{1/2}$H, 4.2 s (B). Untransformed LC×LC chromatogram of toluene and hexyl benzene in hexane: $w_{1/2}$T, 3.6 s; $w_{1/2}$H, 4.2 s (C).

In order to further evaluate the effects of peak focusing achieved under these conditions, another series of analyses was performed to study the effect of solvent composition of a 1D column transferred onto a 2D column. To simulate the condition, toluene and hexyl benzene were diluted in different solvents: hexane (to simulate the LC×LC conditions) and a mixture of water–acetonitrile (solvent composition equal or very similar in eluent strength to the mobile phase used in the 2D separation), and then they were directly injected into the 2D column. The solvent amount and volume was the same as that which would be transferred in the LC×LC comprehensive analysis (300 ng/20 µL). Figures 2A–2C indicate that peak widths are narrower when the analytes injected are dissolved in the same solvent used as the 2D mobile phase. However, peak widths increased when an *n*-hexane-based solvent was used as the simulated transfer solvent. The results were comparable with those obtained through an actual MD comprehensive analysis (Figure 2C). However, even though peak widths increased, the overall peak shapes were acceptable.

Optimization of 2D monolith column

The use of the monolithic column in the fast 2D permits (because of its higher permeability) the performance of successive gradient cycles with a very brief equilibration time when compared with conventional particulate columns (15). For example, under the experimental conditions used for the separation of toluene and hexyl benzene, the use of a gradient program permits the reduction of the analysis time to below 1 min. If the same analysis were carried out under isocratic conditions using an adequate mobile phase mixture for effective peak focusing (e.g., water–acetonitrile, 70:30), hexyl benzene would not elute from the column. Moreover, it has been demonstrated that monolithic columns can work at high flow rates without loss in

terms of resolution because of the improved mass transfer properties of the monolithic skeleton versus particle-packed columns (15). To study the effect of flow rate, five single RP analyses of toluene and hexyl benzene injected directly into the monolithic column at different flow-rates were performed. Solutions (20 µL) of these two hydrocarbons in *n*-hexane were injected, and the same gradient was maintained. The flow rate was increased from 4 up to 8 mL/min. The results from to these analyses are summarized in Table I. As can be seen from Table I, peak widths gradually decrease as the flow rate through the column increased. This effect was because of the faster dilution of the solute solvent in the mobile phase. Furthermore, peak resolution increased with increasing column flow rate. The column back pressure, measured at the initial conditions when the amount of water was highest, showed acceptable values even at 8 mL/min.

Table I. Monolithic Column Back Pressure, Peak Widths, and Resolution Values at Different Flow Rates in RP Mode for a Mixture of Toluene and Hexyl Benzene in Hexane

Flow rate (mL/min)	Pump Pressure (bar)	W½ Toluene (s)	W½ Hexyl benzene (s)	t_R* Toluene (s)	t_R Hexyl benzene (s)	Resolution
4	74	3.6	4.8	40.2	52.2	1.6
5	97	3.0	4.2	34.2	48.0	1.9
6	119	2.4	3.9	31.2	45.0	2.2
7	139	2.1	3.6	28.2	42.0	2.4
8	166	1.8	3.6	25.8	40.2	2.5

* t_R = retention time.

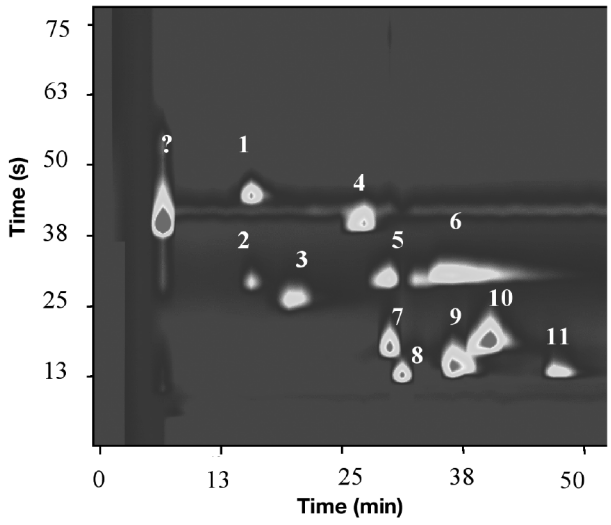

Figure 3 Bidimensional chromatogram of 11 standards (25 ppm) in hexane: amyl cinnamic aldehyde, 1; cinnamic aldehyde, 2; methyl benzoate 3; amyl cinnamic alcohol, 4; eugenol, 5; isoeugenol, 6; coumarin, 7; 2-phenyl ethanol, 8; benzyl alcohol, 9; cinnamic alcohol, 10; anisyl alcohol, 11. LC×LC operational conditions: 1D gradient, *n*-hexane–ethanol (98:2) in isocratic mode. Run time: (1D) 65 min, (2D) 78 s. 2D gradient: 0–6 s, 70% water; 6–12 s, from 70% water to 50% water; 12–24 s, from 50% to 30% water; 24–36 s, from 30% water to 0% water (hold for 30 s); and followed by 11 s for re-equilibration of the column. Wavelength: 212 nm.

An excellent example of the separation power of the optimized NPLC×RPLC system is shown in Figure 3, which illustrates a 2D chromatogram relative to a mixture of standard compounds of known allergens (18). The analysis was carried out under conditions that avoided problems of mobile phase incompatibility and allowed effective focusing of components at the head of the 2D column. In this application, the 1D microbore column was operated under isocratic conditions at a low flow rate (15.4 µL/min) and the 2D monolith column at a high flow rate (4 mL/min), performing a repetitive gradient program to achieve the separation of components with different hydrophobicity and polarity. Under these conditions, and in accordance with results previously reported (14), 11 s was sufficient for column reconditioning. An initial 2D mixture of water–acetonitrile (70:30) was selected for the first 6 s of the secondary analysis to avoid band broadening effects. The 11 peaks were distributed over a large part of the 2D-space, confirming that the NP and RP modes were only slightly correlated techniques (9). The 1D NPLC separation is shown along the *x*-axis with a time scale of 0–55 min, and the 2D RPLC separations are shown along the *y*-axis with a time scale of 0–78 s.

The conditions used for the comprehensive LC×LC analysis were also optimized according to the consideration that the number of modulations per 1D peak should be at least three in order to achieve a complete 2D characterization of the sample (4). The use of the microbore column in 1D (operated at a low flow rate) satisfied this requirement. The flow rate in the 1D separation was optimized in relation to the analysis time of the 2D separation [i.e., the elution time in 2D must be equal to or less than the time required to fill the 20 µL loop (78 s)]. When these conditions were met, a comprehensive separation was achieved with no loss of 1D effluent and, therefore, complete transfer from the 1D to the 2D system was achieved.

The following observation can be made from Figure 3: under both NP and RP monodimensional chromatographic conditions, some peaks are difficult or impossible to separate. In particular, under RP conditions, isomers such as eugenol (peak 5) and isoeugenol (peak 6) undergo coelution, though they are usually well-separated in NP adsorption chromatography (20). Under NP conditions, peaks 1 and 2, 5 and 7, or 6 and 9 underwent complete coelution because of their similar polarity, though they are well separated in RP mode. On the basis of the component structures, it was observed that components with increased hydrophobicity showed increased retention in RP mode: benzyl alcohol (peak 9) elutes before cinnamic alcohol (peak 10) and amyl cinnamic alcohol (peak 4) is more strongly retained because of the presence of the additional aliphatic chain. On the other hand, under NP conditions their elution is based on the degree of polarity. If the same three components were considered, amyl cinnamic alcohol elutes first, and benzyl alcohol and cinnamic alcohol eluted later.

Conclusion

The experimental results of systematic changes in flow rate

and first step gradient composition in the 2D, when using comprehensive NPLC×RPLC, have been illustrated. The employment of a microbore column in the 1D operated at a low flow rate and a monolith column in the second avoided problems derived from inmiscibility between mobile phases. Moreover, it has been shown that the use of a low percentage of strong solvent in the first step gradient of the 2D improved the chromatographic performance because of improved peak focusing.

The use of a monolithic column in the 2D enabled the necessary very fast analyses, operating at flow rates as high as 8 mL/min with low back-pressures and without loss of resolution.

The NPLC×RPLC combination described in this research is suitable for the analysis of complex mixtures containing compounds with a wide range of polarity and hydrophobicity. The system used in this study is completely automated and assembled using commercially available instrumentation.

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