Method Development in Comprehensive 2D-LC

Finding the Most Orthogonal Separation Systems for RPLC×RPLC Using Column and Solvent Screening

Suitable for Agilent
1290 Infinity III LC

Technical Overview

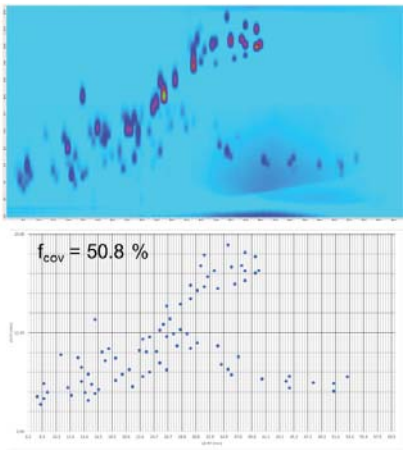
Author

Sonja Krieger
Agilent Technologies, Inc.
Waldbronn, Germany

Abstract

Comprehensive 2D-LC has high potential for the analysis of complex samples because of its increased separation power compared to 1D-LC. Most commonly, a combination of two reversed-phase separations (RPLC×RPLC) is used. It is important that orthogonal separation systems are used in the first and second dimension to fully exploit the achievable gain in separation power compared to 1D-LC.

This Technical Overview outlines an approach for developing a comprehensive 2D-LC method with reversed-phase LC in both dimensions for the analysis of a complex sample. The developed comprehensive 2D-LC method should provide maximum separation power by maximizing the fractional coverage of the two-dimensional separation space.



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Introduction

To achieve chromatographic separation of the large number of compounds contained in complex samples, chromatographic methods providing high separation power or peak capacity are required. Peak capacity is defined as the maximum number of peaks that can be separated in a given separation time with a certain resolution. The theoretical concept of peak capacity is used as a metric to characterize the separation power of chromatographic separations[1]. Comprehensive two-dimensional liquid chromatography (comprehensive 2D-LC or LC×LC) offers high potential for the analysis of complex samples because of its increased separation power compared to one-dimensional liquid chromatography (1D-LC)[2]. In a previous Application Note, it was shown that a three- to fourfold increase in practical peak capacity can be achieved using the Agilent 1290 Infinity 2D-LC solution compared to one-dimensional HPLC or UHPLC methods, while maintaining analysis time[3].

The increased separation power of comprehensive 2D-LC compared to 1D-LC can be reasoned by the peak capacity product rule[4]. Theoretically, the peak capacity of a comprehensive 2D-LC analysis (n_{2D}) is the product of the peak capacities of the first (^1n) and second (^2n) dimensions (Equation 1).

$$n_{2D} = {}^1n \times {}^2n$$

Equation 1.

This equation is only valid when the separations deployed in the first and second dimensions are completely independent of each other (orthogonal), and when the separation achieved in the first dimension is maintained upon transfer to the second dimension. In practice, the peak capacity of a comprehensive 2D-LC analysis can be calculated as follows (Equation 2)[1,2]:

$$n_{2D} = \frac{{}^1n \times {}^2n}{\beta} \times f$$

Equation 2.

In Equation 2, the factor β accounts for the partial remixing of compounds resolved in the first-dimension separation that occurs in the sample loops upon transfer from the first to the second dimension (undersampling). For a given first-dimension separation, undersampling becomes less severe with decreasing modulation time as the fractions transferred from the first to the second dimension become smaller.

The factor *f* corrects for limited orthogonality of the first and second-dimension separation or incomplete coverage of the two-dimensional separation space. As a metric for the correction factor *f*, the fraction of the two-dimensional separation space that can, in principle, be covered by peaks (fractional coverage, f_{cov}) can be used[2].

A more detailed description of the concept of peak capacity in comprehensive 2D-LC can be found in the *Agilent Technologies Primer*, "Two-Dimensional Liquid Chromatography; Principles, Practical Implementation and Applications"[5].

In method development for comprehensive 2D-LC, the first step is to choose the mode of separation for the first and second dimension. From Equation 2, the importance of using orthogonal separations in the first and second dimension can be seen. Highly orthogonal separation modes are, for example, the combinations of ion exchange chromatography (IEX) and reversed-phase LC (RP), size-exclusion chromatography (SEC) and RP, or normal-phase LC (NP) and RP[5]. Despite the correlation of the separation mechanisms that exists in the combination of two RP separations, this combination of separation modes is the most commonly used in comprehensive 2D-LC[2,6]. This can be attributed to the wide applicability and high efficiency of RP separations, the compatibility with mass spectrometric (MS) detection, the good compatibility between the first and second dimension, and the availability of stationary phases with different selectivity[2,5].

When developing a comprehensive 2D-LC method with RP in both dimensions (RPLC×RPLC), a pair of columns with different selectivity should be selected. One approach for classifying RP columns according to their selectivity is the Hydrophobic Subtraction Model (HSM). The HSM describes the interactions between RP columns and different analytes according to hydrophobicity, steric hindrance, acidity, basicity, and ion-exchange capacity. Using the HSM, a column selectivity factor can be calculated as a metric for the selectivity differences between two RP columns[1,2,5-8]. In this way, two RP columns that provide increased likelihood of achieving a high fractional coverage in a comprehensive 2D-LC method can be selected[5]. However, keep in mind that retention and selectivity depend not only on the column, but also on the mobile phase (organic solvent, pH) and the temperature[2,6]. In addition, both the characteristics of the separation systems (column and mobile phase) as well as the characteristics of the sample determine whether or not a high fractional coverage will be achieved in a comprehensive 2D-LC analysis. A combination of two separation systems might provide a high fractional coverage for one sample and a lower fractional coverage for another sample[2,5].

For a given sample, the relative selectivity of two columns or separation systems can, for example, be estimated from the correlation of the retention times on the two columns[1,7].

This Technical Overview outlines an approach for developing a comprehensive 2D-LC method with RP LC in both dimensions for the analysis of a complex sample.

The Agilent 1290 Infinity II 2D-LC solution is set up with additional solvent and column selection valves to enable column and solvent screening analyses without any hardware changes. Six different RP stationary phases are selected, taking into account selectivity differences according to the HSM. In the first step, a one-dimensional column and solvent screening is performed with the selected stationary phases, as well as different

organic solvents (acetonitrile, methanol) and mobile phase pH values (acidic, buffered at pH 5 and pH 8). To select suitable separation systems (stationary and mobile phase) for combination in a comprehensive 2D-LC method, the correlation of retention factors of selected compounds is calculated for all possible combinations. The combinations that provide the lowest correlations are tested in a comprehensive 2D-LC setup with full gradient in the second dimension, and the fractional coverage of the resulting 2D-LC chromatograms is determined. For the combination that provides the highest fractional coverage, a shifted gradient is designed for the second-dimension separation to further increase the fractional coverage.

Experimental

Instrumentation

An Agilent 1290 Infinity II 2D-LC solution was set up for two-dimensional column and solvent screening, and comprised the following modules:

- Agilent 1290 Infinity II High-Speed Pumps (2x G7120A)
- Agilent 1290 Infinity II Multisampler (G7167B) with cooler (option #100)
- Agilent 1290 Infinity II Multicolumn Thermostats (2x G7116B) with 8-column selection valve (G4239C) and capillary kit (G4239C option #005)
- Agilent 1290 Infinity II Diode Array Detectors (2x G7117B) with Max-Light Cartridge Cell 10-mm (G4212-60008)
- Agilent 1290 Infinity valve drives (4x G1170A) with solvent selection valve (G4235A)
- Agilent 1290 Infinity valve drive (G1170A) with 2-position/4-port duo-valve (2D-LC valve head, 1,200 bar (p/n 5067-4214)) equipped with two 80-µL loops

To enable column and solvent screening in the first and second dimension without the necessity of any hardware change, the first and second dimension Agilent 1290 Infinity II High-Speed Pumps were equipped with two solvent selection valves. Including the pumps' built-in solvent selection valves, switching between 13 solvents was possible for each pump channel. In the first and second dimension, an Agilent 1290 Infinity II Multicolumn Thermostat equipped with an 8-column selection valve was used. This enabled switching between six columns with an additional bypass and waste position.

Mass spectrometric (MS) detection was performed during the one-dimensional column, and solvent screening using an Agilent 6530 Accurate-Mass Q-TOF LC/MS system equipped with a dual Agilent Jet Stream ESI source (G1958-65268). The Q-TOF LC/MS system was operated in MS mode, therefore, the use of a TOF LC/MS system would be possible in the same manner. During the one-dimensional column and solvent screening, the outlet of the first dimension DAD was connected to the MS.

Software

- Agilent OpenLAB CDS ChemStation Edition software, rev. C.01.07 [27] with Agilent 1290 Infinity 2D-LC acquisition software product version A.01.02 SP1
- Agilent MassHunter Workstation software, LC/MS data acquisition for Agilent 6200 series TOF and Agilent 6500 series Q-TOF, version B.05.01, qualitative analysis version B.06.00
- GC Image LCxLC-HRMS Edition software version 2.5b0 for 2D-LC data analysis from GC Image LLC., Lincoln, NE, USA

Columns

One-dimensional screening and first dimension of 2D-LC:

- Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 × 100 mm, 1.8 µm (p/n 959758-902)
- Agilent ZORBAX RRHD Eclipse Plus C8, 2.1 × 100 mm, 1.8 µm (p/n 959758-906)
- Agilent ZORBAX RRHD Eclipse Plus Phenyl-Hexyl, 2.1 × 100 mm, 1.8 µm (p/n 959758-912)
- Agilent ZORBAX RRHD Bonus-RP, 2.1 × 100 mm, 1.8 µm (p/n 858768-901)
- Agilent ZORBAX RRHD SB-CN, 2.1 × 100 mm, 1.8 µm (p/n 858700-905)
- Agilent ZORBAX RRHT Eclipse PAH, 2.1 × 100 mm, 1.8 µm (p/n 959764-918)

Second dimension of 2D-LC:

- Agilent ZORBAX RRHD Eclipse Plus C18, 3.0 × 50 mm, 1.8 µm (p/n 959757-302)
- Agilent ZORBAX RRHD Eclipse Plus C8, 3.0 × 50 mm, 1.8 µm (p/n 959757-306)
- Agilent ZORBAX RRHD Eclipse Plus Phenyl-Hexyl, 3.0 × 50 mm, 1.8 µm (p/n 959757-312)
- Agilent ZORBAX RRHD Eclipse PAH, 3.0 × 50 mm, 1.8 µm (p/n 959757-318)
- Agilent ZORBAX RRHD SB-CN, 3.0 × 50 mm, 1.8 µm (p/n 857700-305)
- Agilent ZORBAX RRHD Bonus-RP, 3.0 × 50 mm, 1.8 µm (custom)

Chemicals

All solvents were LC grade. Acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22-µm membrane point-of-use cartridge (Millipak, EMD Millipore, Billerica, MA, USA). Ammonium acetate, ammonium bicarbonate, formic acid, acetic acid, and ammonia solution were obtained from Sigma-Aldrich (Steinheim, Germany).

Samples

The sample used throughout the study contained 70 compounds, and was a mixture of four submixtures comprising polycyclic aromatic hydrocarbons, phthalate esters, phenones, pesticides, and uracil as void marker.

Polycyclic aromatic hydrocarbons (PAHs), mix 25, US EPA 16 (YA20950025AB, Dr. Ehrenstorfer, Augsburg, Germany). 16 compounds, 2,000 µg/mL in acetone/benzene:

Acenaphthene
Acenaphthylene
Anthracene
Benzo[a]anthracene
Benzo[a]pyrene
Benzo[b]fluoranthene
Benzo[k]fluoranthene
Benzo[ghi]perylene
Chrysene
Dibenzo[ah]anthracene
Fluoranthene
Fluorene
Indeno[1,2,3-cd]pyrene
Naphthalene
Phenantrene
Pyrene

Phthalate esters, analytes mix 3 (YA08060300HE, Dr. Ehrenstorfer, Augsburg, Germany), 17 compounds, 1,000 µg/mL in hexane:

Benzyl benzoate
Bis(2-*n*-butoxyethyl)phthalate
Bis(2-ethoxyethyl)phthalate
Bis(2-ethylhexyl)phthalate
Bis(2-methoxyethyl)phthalate
Bis(4-methyl-2-pentyl)phthalate
Butylbenzylphthalate
Diamyl phthalate
Di-*n*-butyl phthalate
Dicyclohexyl phthalate
Diethyl phthalate
Dihexyl phthalate
Diisobutyl phthalate
Dimethylphthalate
Dinonyl phthalate
Di-*n*-octyl phthalate
Hexyl-2-ethylhexyl phthalate

Phenones, RRLC checkout sample (Agilent Technologies, Waldbronn, Germany), nine compounds, 100 µg/mL in water/acetonitrile:

Acetanilide
Acetophenone
Benzophenone
Butyrophenone
Heptanophenone
Hexanophenone
Octanophenone
Propiophenone
Valerophenone

Pesticides, mix 34 (L18000034AL, Dr. Ehrenstorfer, Augsburg, Germany), 27 compounds, 10 µg/mL in acetonitrile:

Atrazine
Atrazine-desethyl
Atrazine-desethyl desisopropyl
Chloroxuron
Chlorpropham
Chlortoluron
Crimidin
Cyanazine
Diuron
Fenuron
Isoproturon
Linuron
Metamitron
Metazachlor
Methabenzthiazuron
Metobromuron
Metolachlor
Metoxuron
Metribuzin
Monolinuron
Prometryn
Propazine
Propham
Sebuthylazine
Simazine
Terbuthylazine
Terbutryne

Uracil (Sigma-Aldrich, Steinheim, Germany), 1,000 µg/mL in water.

The sample was obtained by the combination of the following volumes of each submixture, and contained approximately 9 µg/mL of each compound: 5 µL PAH mix, 10 µL phthalate ester mix, 100 µL phenone mix, 1,000 µL pesticide mix, and 10 µL uracil solution. For one-dimensional column and solvent screening with mass spectrometric detection, the sample was diluted 1:10 with acetonitrile.

Methods

One-dimensional column and solvent screening	
Columns	Agilent ZORBAX RRHD Eclipse Plus C18 Agilent ZORBAX RRHD Eclipse Plus C8 Agilent ZORBAX RRHD Eclipse Plus Phenyl-Hexyl Agilent ZORBAX RRHD Bonus-RP Agilent ZORBAX RRHD SB-CN Agilent ZORBAX RRHT Eclipse PAH (each 2.1 × 100 mm, 1.8 µm)
Solvents	A) H_2O + 0.1 % formic acid; B) Acetonitrile + 0.1 % formic acid A) H_2O + 0.1 % formic acid; B) Methanol + 0.1 % formic acid A) 10 mM Ammonium acetate pH 5; B) Acetonitrile A) 10 mM Ammonium acetate pH 5; B) Methanol A) 10 mM Ammonium bicarbonate pH 8; B) Acetonitrile A) 10 mM Ammonium bicarbonate pH 8; B) Methanol
Gradient	0 minutes – 20 %B 10 minutes – 95 %B
Stop time	15 minutes
Post time	5 minutes
Flow rate	0.500 mL/min
Temperature	40 °C
Injection volume	1 µL; injection with 2 µL water plugs
DAD detection	220 nm/4 nm, reference 400/100 nm and 254/4 nm, reference 400/100 nm; data rate 40 Hz
MS detection	The Agilent 6530 Accurate-Mass Q-TOF LC/MS system was operated in positive ionization mode with an acquisition rate of 5 spectra/second and the following Agilent Jet Stream ESI source conditions: Gas temperature 300 °C Gas flow 8 L/min Nebulizer 50 psi Sheath gas temperature 300 °C Sheath gas flow 9 L/min Capillary 3,000 V Nozzle 500 V

Two-dimensional screening	
First dimension	
Columns	Agilent ZORBAX RRHD Eclipse Plus C18 Agilent ZORBAX RRHD Eclipse Plus C8 Agilent ZORBAX RRHD Eclipse Plus Phenyl-Hexyl Agilent ZORBAX RRHD Bonus-RP Agilent ZORBAX RRHT Eclipse PAH (each 2.1 × 100 mm, 1.8 µm)
Solvents	A) H_2O + 0.1 % formic acid; B) Methanol + 0.1 % formic acid A) 10 mM Ammonium acetate pH 5; B) Acetonitrile A) 10 mM Ammonium acetate pH 5; B) Methanol
Gradient	0 minutes – 20 %B at 0.100 mL/min 50 minutes – 100 %B at 0.100 mL/min 60 minutes – 100 %B at 0.100 mL/min 61 minutes – 20 %B at 0.400 mL/min
Stop time	70 minutes
Temperature	40 °C
Injection volume	5 µL; injection with 5 µL water plugs
DAD detection	220 nm/4 nm, reference 400/100 nm and 254/4 nm, reference 400/100 nm; data rate 20 Hz; stop time 60 minutes
Second dimension	
Columns	Agilent ZORBAX RRHD Eclipse Plus C18 Agilent ZORBAX RRHD Eclipse Plus C8 Agilent ZORBAX RRHD Eclipse Plus Phenyl-Hexyl Agilent ZORBAX RRHD Bonus-RP Agilent ZORBAX RRHD Eclipse PAH (each 3.0 × 50 mm, 1.8 µm)
Solvents	A) H_2O + 0.1 % formic acid; B) Methanol + 0.1 % formic acid A) 10 mM Ammonium acetate pH 5; B) Acetonitrile A) 10 mM Ammonium acetate pH 5; B) Methanol
Flow rate	2.500 mL/min
Gradient	0.00 minutes – 20 %B 0.27 minutes – 100 %B
^{2}D Gradient stop time	0.40 minutes
Modulation time	0.50 minutes
Modulation	80 µL loops; cocurrent configuration; 0.50 minutes modulation time; modulation on: 0–60 minutes
Temperature	60 °C
DAD detection	220 nm/4 nm, reference 400/100 nm and 254/4 nm, reference 400/100 nm; data rate 80 Hz; stop time 60 minutes

Final 2D-LC method	
First dimension	
Column	Agilent ZORBAX RRHD Eclipse Plus C8, 2.1 × 100 mm, 1.8 µm
Solvents	A) 10 mM Ammonium acetate pH 5 B) Acetonitrile
Gradient	0 minutes – 20 %B at 0.100 mL/min 50 minutes – 100 %B at 0.100 mL/min 60 minutes – 100 %B at 0.100 mL/min 61 minutes – 20 %B at 0.400 mL/min
Stop time	70 minutes
Temperature	40 °C
Injection volume	5 µL; injection with 5 µL water plugs
DAD detection	220 nm/4 nm, reference 400/100 nm and 254/4 nm, reference 400/100 nm; data rate 20 Hz; stop time 60 minutes
Second dimension	
Column	Agilent ZORBAX RRHD Eclipse PAH, 3.0 × 50 mm, 1.8 µm
Solvents	A) 10 mM Ammonium acetate pH 5 B) Methanol
Flow rate	2.500 mL/min
Gradient	0.00 minutes – 30 %B 0.27 minutes – 35 %B
^{2}D Gradient stop time	0.40 minutes
Modulation time	0.50 minutes
Gradient modulation	30 %B at 0.00 minutes to 100 %B at 60 minutes 35 %B at 0.27 minutes to 70 %B at 30 minutes to 100 % B at 40 minutes
Modulation	80 µL loops; cocurrent configuration; 0.50 minutes modulation time; modulation on: 0–60 minutes
Temperature	60 °C
DAD detection	220 nm/4 nm, reference 400/100 nm and 254/4 nm, reference 400/100 nm; data rate 80 Hz; stop time 60 minutes

Results and Discussion

To outline an approach for method development for comprehensive 2D-LC, a complex mixture comprising polycyclic aromatic hydrocarbons, phthalate esters, phenones, and pesticides was used. The complete sample contained 70 compounds, including uracil used as void marker.

As the first step, a one-dimensional column and solvent screening was performed to identify RP separation systems that provide differences in selectivity for the analyzed sample. For the one-dimensional column and solvent screening, six RP materials were selected, taking into account the classification of chromatographic selectivity according to the HSM. The selected RP materials included Agilent ZORBAX RRHD Eclipse Plus C18, Agilent ZORBAX RRHD Eclipse Plus C8, Agilent ZORBAX RRHD Eclipse Plus Phenyl-Hexyl, Agilent ZORBAX RRHD Bonus-RP, Agilent ZORBAX RRHD SB-CN and Agilent ZORBAX RRHT Eclipse PAH. The characteristics of more than 600 RP columns are available as a web-based database (www.hplccolumns.org) that allows comparing columns according to a column selectivity factor. It can be found here, for example, that Bonus RP and Eclipse PAH provide different selectivity compared to Eclipse Plus C18 or Eclipse Plus C8 columns. The selected mobile phases include the use of different organic solvents (acetonitrile and methanol) and different pH values (acidic, buffered at pH 5 and pH 8). During the one-dimensional column and solvent screening, mass spectrometric (MS) detection was applied to allow tracking of selected compounds between different chromatograms.

Figure 1 shows chromatograms from the one-dimensional analyses using the Eclipse Plus C8 column with 10 mM ammonium acetate pH 5/acetonitrile (A) and the Eclipse PAH column with 10 mM ammonium acetate pH 5/methanol (B).

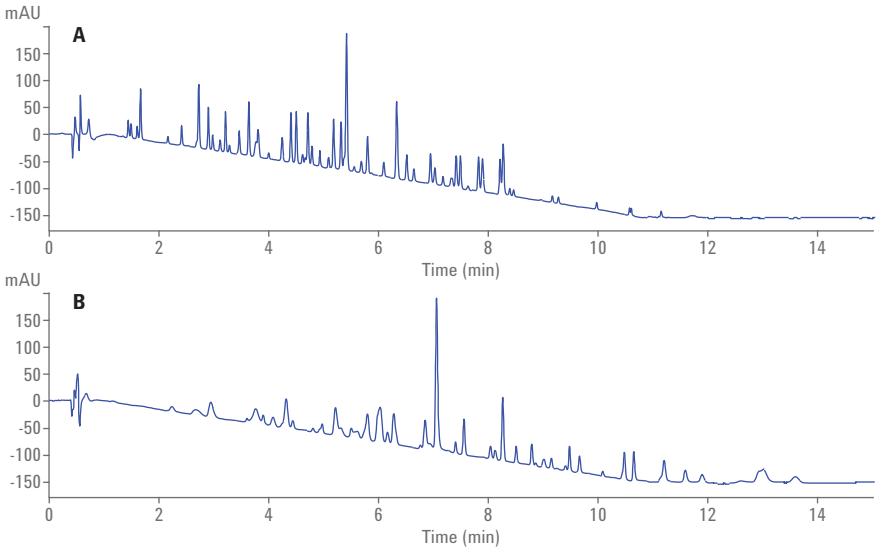

Figure 1. One-dimensional analysis of the sample at a concentration of 9 µg/mL with UV detection at 220 nm; (A) Agilent ZORBAX RRHD Eclipse Plus C8 with 10 mM ammonium acetate pH 5/acetonitrile; (B) Agilent ZORBAX RRHT Eclipse PAH with 10 mM ammonium acetate pH 5/methanol.

Identification of sample components and tracking of peaks between chromatograms was enabled by the detection of accurate masses using TOF-MS. As expected, polycyclic aromatic hydrocarbons and phenones could not be ionized with the chosen conditions. Additionally, certain compounds contained in the sample are isomers (for example, prometryn and terbutryne) and, therefore, could not be distinguished based on TOF-MS detection. Overall, 30 pesticides and phthalate esters could be confidently identified in most chromatograms. Figure 2 shows the chromatograms of the compounds identified during the one-dimensional analyses using the Eclipse Plus C8 column with 10 mM ammonium acetate pH 5/acetonitrile (A) and the Eclipse PAH column with 10 mM ammonium acetate pH 5/methanol (B).

For the identified sample components, the compound information was exported from MassHunter. From the retention times of the identified compounds and the void time determined from the retention time of uracil, retention factors (k) were calculated for the identified compounds for all column and solvent combinations using Excel. To evaluate which combination of two separation systems (column and solvent combination) provided the most selectivity differences for the sample analyzed, the correlation of retention factors was calculated for all possible combinations of separation systems.

Generally, it could be seen that the combinations of separation systems using acetonitrile in one separation system and methanol in the other showed lower correlations compared to combinations using the same organic solvent in both separation systems. Accordingly, in comprehensive 2D-LC with RP in both dimensions, it is common practice to use acetonitrile in one dimension and methanol in the other[2].

For the sample analyzed, the mobile phase pH value did not have a strong influence on selectivity. However, it should be noted that for samples containing compounds with a wide range of acidity/basicity, differences in selectivity can be achieved even with similar columns used at different pH values[2].

The lowest correlation between retention factors was calculated for the following combinations of separation systems; for comparison purposes, also a combination with high correlation between retention factors is shown.

- Eclipse PAH with 10 mM ammonium acetate pH 5/methanol and Eclipse Plus C8 with 10 mM ammonium acetate pH 5/acetonitrile (R^2 = 0.957)
- Eclipse PAH with 10 mM ammonium acetate pH 5/acetonitrile and Bonus RP with H_2O + 0.1 % formic acid/methanol + 0.1 % formic acid (R^2 = 0.957)
- Eclipse PAH with 10 mM ammonium acetate pH 5/acetonitrile and Eclipse Plus Phenyl-Hexyl with H_2O + 0.1 % formic acid/methanol + 0.1 % formic acid (R^2 = 0.960)
- Eclipse Plus C18 with 10 mM ammonium acetate pH 5/acetonitrile and Eclipse Plus Phenyl-Hexyl with 10 mM ammonium acetate pH 5/acetonitrile (R^2 = 0.998)

The above mentioned combinations of separation systems were tested in a comprehensive 2D-LC setup with full gradient used in the second dimension to identify the combination that provides the highest fractional coverage of the 2D chromatogram for the analyzed sample.

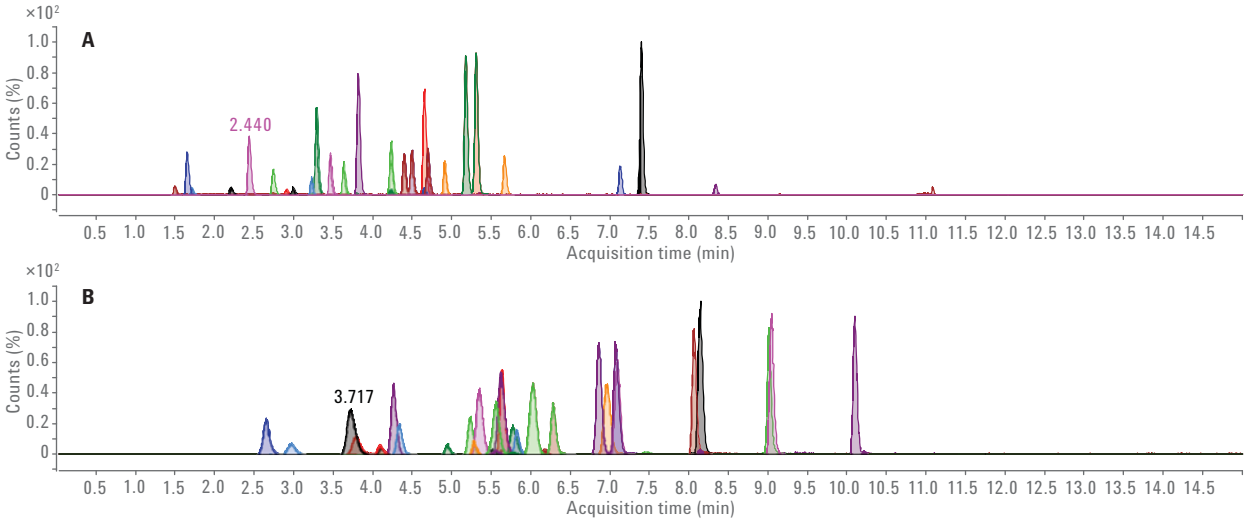

Figure 2. One-dimensional analysis of the sample at a concentration of 0.9 µg/mL with MS detection; the EICs of identified compounds are shown; (A) Agilent ZORBAX RRHD Eclipse Plus C8 with 10 mM ammonium acetate pH 5/acetonitrile; (B) Agilent ZORBAX RRHT Eclipse PAH with 10 mM ammonium acetate pH 5/methanol.

As a means of determining the fractional coverage of the 2D chromatograms, the Gilar-Stoll approach was chosen. Gilar *et al.* geometrically described the orthogonality of two-dimensional systems by plotting the normalized retention data into a 2D separation space divided into "bins" with the size of the peak width in both dimensions. The orthogonality was then determined from the percentage of bins occupied by peaks[9]. In a modification of the Gilar approach proposed by Stoll, a user-selectable bin size is used, and empty bins within the perimeter of the occupied bins are included. This is reasoned by the idea that the separations employed allow a compound to elute in those empty bins within the perimeter of the occupied bins, even if this is not the case for the actual sample analyzed[1,10].

To determine the fractional coverage for the combinations of separation systems tested in a comprehensive 2D-LC setup, the retention times of peaks detected after the two-dimensional separation were plotted onto a grid with a bin size according to the average peak width at 5σ in the first (0.34 minutes) and second (0.6 seconds) dimension. The bins within the perimeter of the bins occupied by peaks were counted. Then, the fractional coverage was calculated as the percentage of those included bins compared to the bins inside a useful separation window. The useful separation window was chosen from 6 minutes (k = 2) to the end of the gradient in the first dimension, and from the start to the end of the gradient at 24 seconds in the second dimension. Figure 3 shows the chromatograms and the retention time plots used for the determination of the fractional coverage obtained from the comprehensive 2D-LC setup with the Eclipse PAH column with 10 mM ammonium acetate pH 5/methanol in the first dimension combined with the Eclipse Plus C8 column with 10 mM ammonium acetate pH 5/acetonitrile in the second dimension and *vice versa*. For comparison purposes, the chromatogram and the retention time plot from the comprehensive 2D-LC setup with the Eclipse Plus C18 column with 10 mM ammonium acetate pH 5/acetonitrile in the first dimension and the Eclipse Plus Phenyl-Hexyl column with 10 mM ammonium acetate pH 5/acetonitrile in the second dimension are also shown. Table 1 summarizes the fractional coverage determined for all combinations of separation systems tested in a comprehensive 2D-LC setup.

As can be seen from Table 1, the highest fractional coverage was obtained for the combination of the ZORBAX Eclipse PAH column with 10 mM ammonium acetate pH 5/methanol and the Eclipse Plus C8 column with 10 mM ammonium acetate pH 5/acetonitrile. As expected, the determined fractional coverages correlate to a certain extent with the correlation coefficients calculated from the one-dimensional column and solvent screening. However, note that only certain components of the sample (pesticides and phthalate esters) were taken into account in the calculation of the correlation coefficients in the one-dimensional screening.

During the two-dimensional testing of the different combinations of separation systems, a full gradient ranging from 20 to 100 %B was used in the second dimension. This means that every fraction injected to the second dimension experienced the same gradient from 20 to 100 %B. A full gradient offers a high bandwidth suppression effect upon transfer from the first to the second dimension leading to a very narrow peak width in the second dimension. Conversely, the fractional coverage is generally lower, and compounds tend to be distributed around a diagonal line

Table 1. Fractional coverage of the useful 2D separation space for the combinations of separation systems tested in a comprehensive 2D-LC setup.

1D column and mobile phase	2D column and mobile phase	Fractional coverage (%)
Agilent ZORBAX RRHT Eclipse PAH with 10 mM ammonium acetate pH 5/methanol	Agilent ZORBAX RRHD Eclipse Plus C8 with 10 mM ammonium acetate pH 5/acetonitrile	17.2
Agilent ZORBAX RRHD Eclipse Plus C8 with 10 mM ammonium acetate pH 5/acetonitrile	Agilent ZORBAX RRHD Eclipse PAH with 10 mM ammonium acetate pH 5/methanol	18.4
Agilent ZORBAX RRHT Eclipse PAH with 10 mM ammonium acetate pH 5/acetonitrile	Agilent ZORBAX RRHD Bonus RP with H_2O + 0.1 % formic acid/methanol + 0.1 % formic acid	13.0
Agilent ZORBAX RRHD Bonus RP with H_2O + 0.1 % formic acid/methanol + 0.1 % formic acid	Agilent ZORBAX RRHD Eclipse PAH with 10 mM ammonium acetate pH 5/acetonitrile	14.6
Agilent ZORBAX RRHT Eclipse PAH with 10 mM ammonium acetate pH 5/acetonitrile	Agilent ZORBAX RRHD Eclipse Plus Phenyl-Hexyl with H_2O + 0.1 % formic acid/methanol + 0.1 % formic acid	10.2
Agilent ZORBAX RRHD Eclipse Plus Phenyl-Hexyl with H_2O + 0.1 % formic acid/methanol + 0.1 % formic acid	Agilent ZORBAX RRHD Eclipse PAH with 10 mM ammonium acetate pH 5/acetonitrile	14.9
Agilent ZORBAX RRHD Eclipse Plus C18 with 10 mM ammonium acetate pH 5/acetonitrile	Agilent ZORBAX RRHD Eclipse Plus Phenyl-Hexyl with 10 mM ammonium acetate pH 5/acetonitrile	9.6
Agilent ZORBAX RRHD Eclipse Plus Phenyl-Hexyl with 10 mM ammonium acetate pH 5/acetonitrile	Agilent ZORBAX RRHD Eclipse Plus C18 with 10 mM ammonium acetate pH 5/acetonitrile	8.0

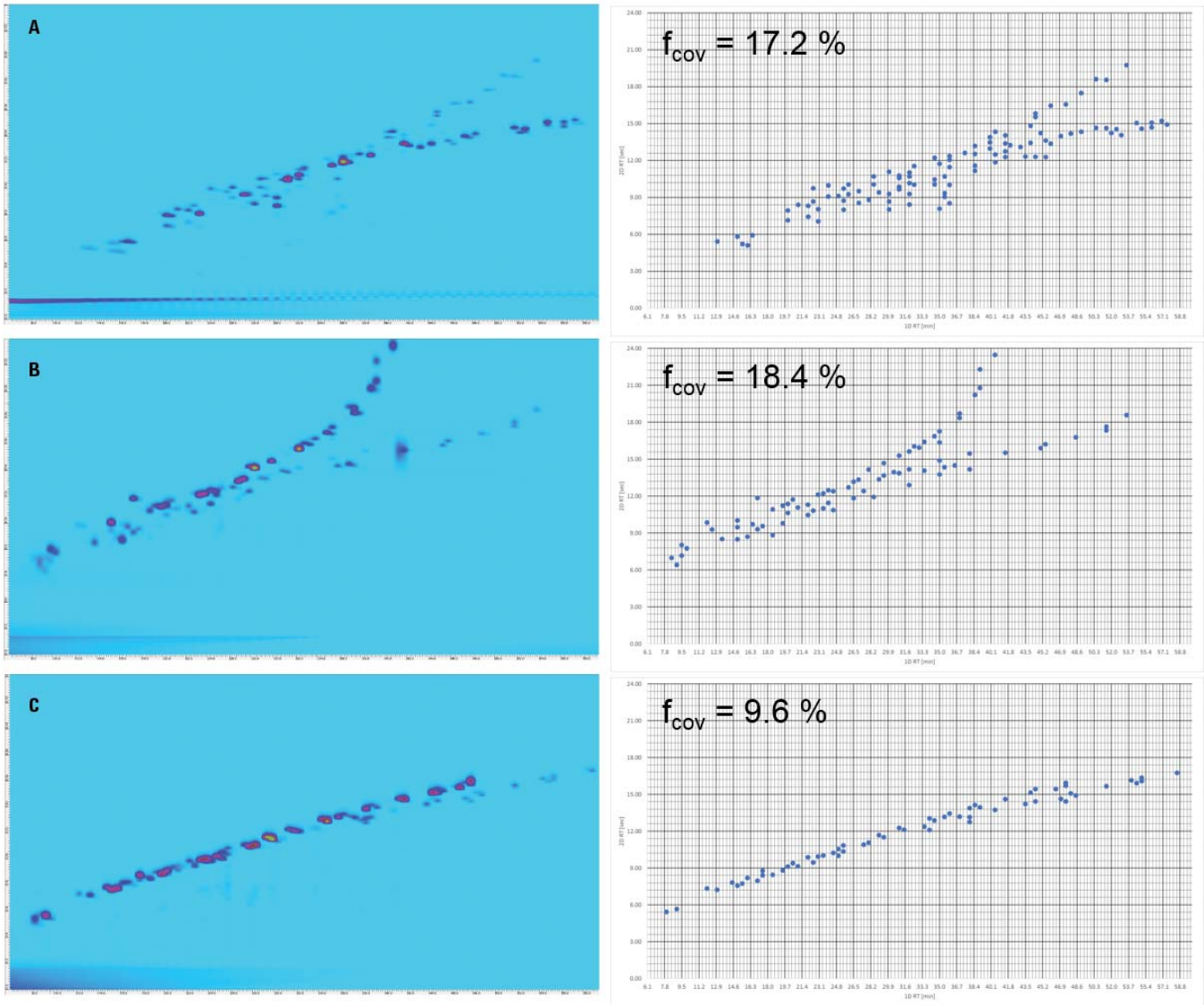

Figure 3. 2D-LC chromatograms with UV detection at 220 nm and retention time plots of certain combinations of separation systems tested in a comprehensive 2D-LC setup; (A) 1D: Agilent ZORBAX RRHT Eclipse PAH with 10 mM ammonium acetate pH 5/methanol, 2D: Agilent ZORBAX RRHD Eclipse Plus C8 with 10 mM ammonium acetate pH 5/acetonitrile; (B) 1D: Agilent ZORBAX RRHD Eclipse Plus C8 with 10 mM ammonium acetate pH 5/acetonitrile, 2D: Agilent ZORBAX RRHD Eclipse PAH with 10 mM ammonium acetate pH 5/methanol; (C) 1D: Agilent ZORBAX RRHD Eclipse Plus C18 with 10 mM ammonium acetate pH 5/acetonitrile, 2D: Agilent ZORBAX RRHD Eclipse Plus Phenyl-Hexyl with 10 mM ammonium acetate pH 5/acetonitrile.

since compounds with weak retention on the first dimension RP column will tend to show weak retention also on the second dimension RP column. For a given combination of separation systems, the fractional coverage can be increased by using a shifted gradient in the second dimension[2,11]. Using a shifted gradient means that the second-dimension gradient is continuously changed with increasing first-dimension retention time so that every fraction injected to the second dimension column experiences a shallower and slightly different gradient.

When a shifted gradient was developed for the combination of the Eclipse PAH column with 10 mM ammonium acetate pH 5/methanol in the first dimension and the Eclipse Plus C8 column with 10 mM ammonium acetate pH 5/acetonitrile in the second dimension, the fractional coverage could be increased. But then, the decreased bandwidth suppression effect due to the increased percentage of organic at the start of the second-dimension gradient experienced by the polycyclic aromatic hydrocarbons eluting late from the first-dimension Eclipse PAH column led to broad second-dimension peaks for those compounds (data not shown). Generally, it is beneficial to place the more hydrophobic column in the second dimension to achieve a better bandwidth suppression effect[2].

For the combination of the Eclipse Plus C8 column with 10 mM ammonium acetate pH 5/acetonitrile in the first dimension with the Eclipse PAH column with 10 mM ammonium acetate pH 5/methanol in the second dimension, the shifted gradient shown in Figure 4 was developed to increase the fractional coverage.

In Figure 5, the chromatogram resulting from the final comprehensive 2D-LC method using the Eclipse Plus C8 column with 10 mM ammonium acetate pH 5/acetonitrile in the first dimension and the Eclipse PAH column with 10 mM ammonium acetate pH 5/methanol and shifted gradient in the second dimension is shown. For determination of the fractional coverage, a grid with a bin size of 0.41 minutes in the first dimension and 2.5 seconds in the second dimension (average peak width at 5σ) was chosen. The increased second-dimension peak width can be explained by the shallower gradient that the individual fractions injected to the second-dimension column experience when a shifted gradient is used. By using a shifted second-dimension gradient, the fractional coverage could be greatly increased to more than 50 %, and sample components that were still partly or completely coeluting with the use of a full second-dimension gradient were now separated.

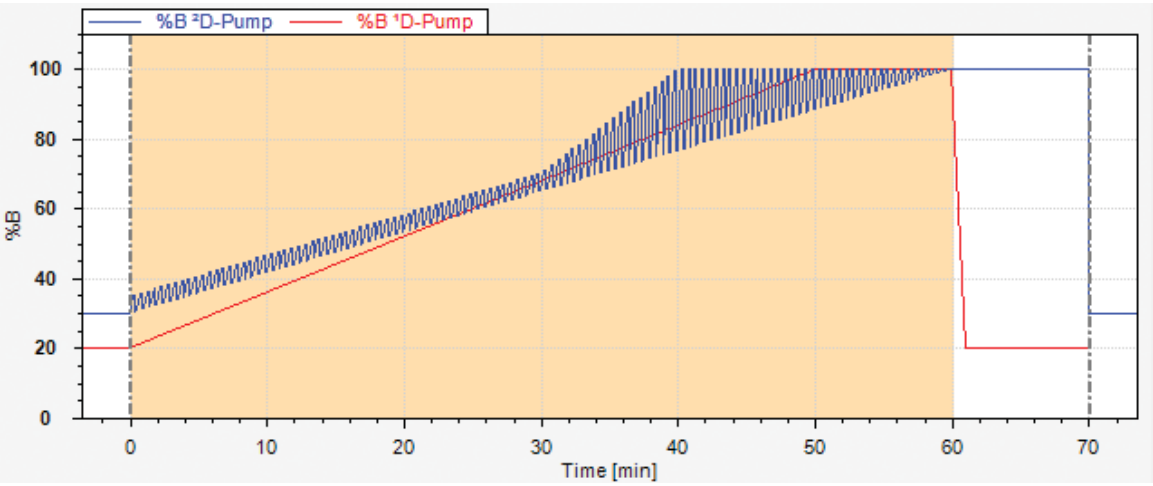

Figure 4. Shifted gradient developed for the second dimension separation of the comprehensive 2D-LC method with the Agilent ZORBAX RRHD Eclipse Plus C8 column with 10 mM ammonium acetate pH 5/acetonitrile in the first dimension and the Agilent ZORBAX RRHD Eclipse PAH column with 10 mM ammonium acetate pH 5/methanol in the second dimension (final 2D-LC method).

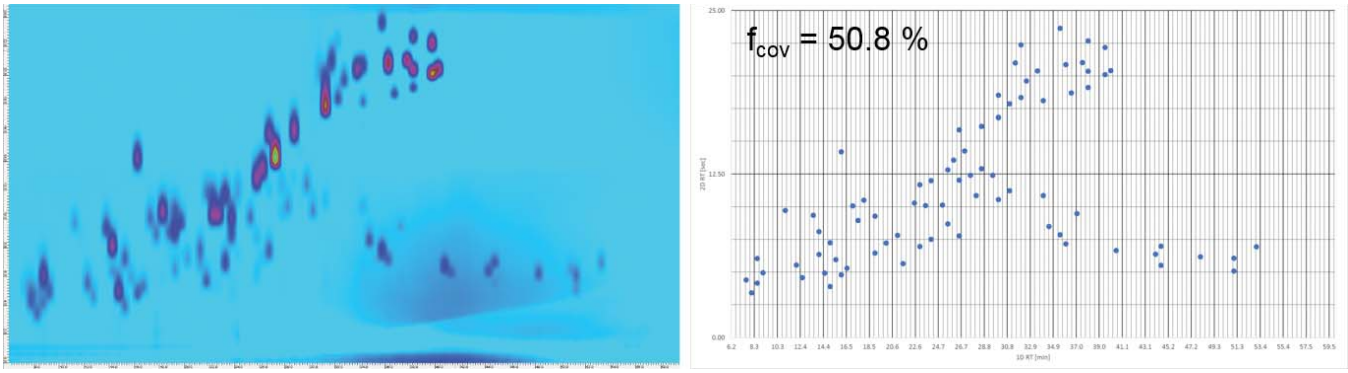

Figure 5. 2D-LC chromatogram with UV detection at 220 nm and retention time plot of the comprehensive 2D-LC method with the Agilent ZORBAX RRHD Eclipse Plus C8 column with 10 mM ammonium acetate pH 5/acetonitrile in the first dimension and the Agilent ZORBAX RRHD Eclipse PAH column with 10 mM ammonium acetate pH 5/methanol and shifted gradient in the second dimension (final 2D-LC method).

Conclusions

Comprehensive 2D-LC has high potential for the analysis of complex samples because of its increased separation power compared to 1D-LC. The most commonly used combination of separation modes in comprehensive 2D-LC is the combination of two RP separations (RPLC×RPLC). To exploit the achievable gain in separation power compared to 1D-LC, it is important that orthogonal separation systems or separation systems with different selectivity are used in the first and second dimension. The classification of RP columns according to their selectivity performed in the HSM offers a means of selecting RP columns with different selectivity. In a one-dimensional column and solvent screening, the relative selectivity of two separation systems can be assessed for a given sample from the correlation of the retention factors. To compare the orthogonality of different combinations of separation systems in a comprehensive 2D-LC setup, the fractional coverage of the resulting 2D-LC chromatogram can be used. For a given combination of separation systems, the fractional coverage can be greatly increased by using a shifted gradient in the second dimension.

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