A Fast and Efficient Method to Assess 2D-HPLC Column and Method Combinations for Food Metabolomics Studies

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Overview

- 2D-LC coupled to MS/MS is a powerful analytical technique for food metabolomics and requires the combination of effective orthogonal LC methods to cover a wide polarity range.
- For method development, 1D-LC runs off relevant methods for both dimensions provided the data set to, simulate possible combinations and to assess them with statistical methods.

Introduction

Analytical requirements in food metabolomics and sensory science

Understanding metabolism of distinct food ingredients is an important pre-requisite in sensory science. Due to analyte diversity and general sample complexity, the related studies require analytical tools with outstanding separation and identification potential. Compound classes of interest exhibit a broad chemical variety, a wide relevant abundance range, and numerous additional matrix constituents. A powerful analytical approach is comprehensive 2D-(U)HPLC coupled to high resolution mass spectrometry with accurate mass determination. In spite of respective MS capabilities, it is important to account for an effective chromatographic separation, because ion suppression will otherwise prevent from getting a truly quantitative picture. The work presented describes a straight forward 2D-LC method development approach. It avoids full 2D-LC experiments and enables exploration of all possible combinations of fast and easy methods.

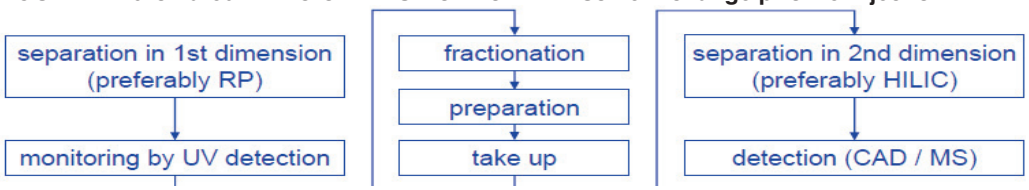
An automated 2D-LC approach for highest possible method combination flexibility

The instrumental set-up described in the experimental part was employed to generate all 1D-LC data used for the method development as described below. The same instrument can be used for the automated off-line 2D-LC process with fraction collection from the first dimension, solvent modification in these fractions and re-injection into a second dimension followed by MS/MS. The 2D combination of reversed phase and HILIC was primarily explored in this work, but special mixed mode mechanisms where considered in addition. The automated solvent modification by a partial evaporation followed by dosage of a solvent which is friendly for the 2nd LC dimension was a key pre-requisite for full freedom of method combination. Though outside the scope of this poster, the workflow for the 2D-LC is shown in Figure 1. Compound identification is by MS/MS, pre-quantification by Charged Aerosol Detection and exact quantification by MS with stable-isotope labeled internal standards.

The method development strategy

All reversed phase, HILIC and special mixed mode methods were run as generic gradients with a defined test sample set, covering typical compound classes of metabolomics studies, using UV and Charged Aerosol Detection as non-specific detection modes. Peak identification was accomplished by single injections. These experiments provided retention data from all methods without any tuning or optimization effort. The acquired data set was used to statistically characterize selectivities and to simulate possible 2D-LC pattern by graphically combining 1D data. This allowed for a quick assessment of orthogonality and population of the retention space prior to fine tuning eluting conditions and eventually setting up true automated 2D-LC workflows.

FIGURE 1. Automated 2D-LC/CAD/MS workflow with solvent change prior re-injection



Experimental

Instrumentation

- All experiments were performed on a Thermo Scientific™ Dionex™ UltiMate™ 3000 Dual RSLC system with fraction collection autosampler and charged aerosol detector (CAD). The system is capable of automated off-line 2D-LC with fraction solvent modification before re-injection.

Modules

- DGP-3000RS Dual-Gradient Pump
- WPS-3000TFC Thermostatted Fraction Collection Well Plate Sampler (modified)
- TCC-3000RS Thermostatted Column Compartment with 2-pos., 6-port and 2-pos.,10-port valve
- UltiMate 3000 RS Variable Wavelength Detector with 2.5 μm semi micro flow cell
- Corona *ultra* RS Charged Aerosol Detector

Columns

The list of columns is shown in Table 1. Columns are categorized by column code into reversed phase (RP), HILIC and mixed mode (MM). Note that some of the RP and HILIC columns may also exhibit mixed mode properties, but are not declared by the respective manufacturer as such.

TABLE 1. Category, name, manufacturer, and specifications of evaluated columns

Column Code	Full Column Name	Manufacturer	Length (mm)	Diameter (mm)	Particle Size (μm)
RP-1	Gemini NX C18	Phenomenex	50	2	3
RP-2	Gemini NX C18	Phenomenex	100	2	3
RP-3	Luna C18 (2)	Phenomenex	150	2	5
RP-4	Kinetex PFP	Phenomenex	100	2.1	2.6
RP-5	Luna PFP (2)	Phenomenex	150	2	3
RP-6	Luna Phenyl-Hexyl	Phenomenex	150	2	5
RP-7	Synergi Hydro-RP	Phenomenex	150	2	4
RP-8	Synergi Polar-RP	Phenomenex	150	2	4
RP-9	Synergi Polar-RP	Phenomenex	50	2	2.5
RP-10	Synergi Fusion-RP	Phenomenex	150	2	4
RP-11	Hypersil GOLD	Thermo Fisher Scientific	50	2.1	1.9
RP-12	Hypersil GOLD PFP	Thermo Fisher Scientific	50	2.1	1.9
RP-13	Hypersil GOLD aQ	Thermo Fisher Scientific	50	2.1	1.9
RP-14	Accucore aQ	Thermo Fisher Scientific	50	2.1	2.6
RP-15	Acquity UPLC HSS T3	Waters	100	2.1	1.8
RP-16	Acquity UPLC BEH Shield RP18	Waters	150	2.1	1.7
HILIC-1	Kinetex HILIC	Phenomenex	100	2.1	2.6
HILIC-2	Kinetex HILIC	Phenomenex	50	2.1	2.6
HILIC-3	TSK-Gel Amide-80	Tosoh Bioscience	50	2	3
HILIC-4	Acclaim HILIC-10	Thermo Fisher Scientific	150	2.1	3
HILIC-5	Accucore HILIC	Thermo Fisher Scientific	50	2.1	2.6
HILIC-6	Accucore-150-Amide-HILIC	Thermo Fisher Scientific	150	2.1	2.6
HILIC-7	Hypersil GOLD HILIC	Thermo Fisher Scientific	50	2.1	1.9
HILIC-8	Syncronis HILIC	Thermo Fisher Scientific	50	2.1	1.7
HILIC-9	Acquity BEH Amide	Waters	150	2.1	1.7
HILIC-10	Acquity BEH HILIC	Waters	150	2.1	1.7
HILIC-11	SeQuant ZIC-HILIC	Merck Millipore	150	2.1	3
HILIC-12	SeQuant ZIC-cHILIC	Merck Millipore	150	2.1	3
MM-1	Acclaim Mixed-Mode HILIC-1	Thermo Fisher Scientific	50	3	3
MM-2	Acclaim Trinity P1	Thermo Fisher Scientific	50	2.1	3

Methods

The set of test compounds is listed in Table 2 with partition coefficients logP as predicted by ACD Labs software. Individual solutions of all 31 compounds were prepared in ACN/water 1/1 (v/v) at a concentration of 100 mg/L. They were injected separately onto each of the 30 columns shown in Table 1 running the respective methods.

General method parameters

Injection volume: 3 µL; column temperature: 40 °C; flow rate: 0.2-0.5 mL/min (depending on column ID and particle diameter); detection: Charged Aerosol Deteciton and UV at 254 nm, data rate of 10 and 2.5 Hz, respectively.

Gradient conditions for RP columns

A: 1% formic acid in H_2O, B: CH_3CN; linear from 0% B to 100% B, timing was column dependent

Gradient conditions for HILIC columns

A: CH_3CN, B: H_2O, C: aqueous 100 mM NH_4OAc of different pH, constant 5 or 10% C, linear from max. 95% A to min. 20% A, conditions were column dependent

Gradient conditions for mixed mode columns

Mixed Mode – 1
A: CH_3CN, B: H_2O, C: aqueous 100 mM NH_4OAc of different pH, in RP mode linear from 10% A and 5% C to 95% A and 5% C, in HILIC mode linear from 0% B and 10% C to 40% B and 10% C

Mixed-Mode-2
A: CH_3CN, B: H_2O, C: aqueous 100 or 200 mM NH_4OAc pH 3,5, simultaneous linear ionic strength and solvent gradients, in RP mode 0% to 75% A and 2,5% to 40% C, in HILIC mode 5% to 40% B and 5% to 40% C

Control Software and Data Analysis

Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System 6.8 SR 11d was used to control the UHPLC instruments and perform the primary integration for retention time determination.

The normalized retention times were calculated by the following formula: $$Rt_{i(norm)} = \frac{Rt_i - Rt_{\min}}{Rt_{\max} - Rt_{\min}}$$

Statistical evaluation of method orthogonality as well as data visualization was performed within the data analysis and visualization platform R (version 3.0.2) using the pheatmap package (version 0.7.7) for heatmap analysis, and the ggplot2 package (version 0.9.3.1) for calculation of simulated 2D-LC maps. For cluster analysis in the heatmap, squared Euclidean distances were applied as distance measure on the normalized retention times, while clusters were formed according to Ward´s minimum variance method. Orthogonality parameters were computed using user-written code.

TABLE 2. Food relevant test compounds ordered by logP (predicted by ACD Labs software)

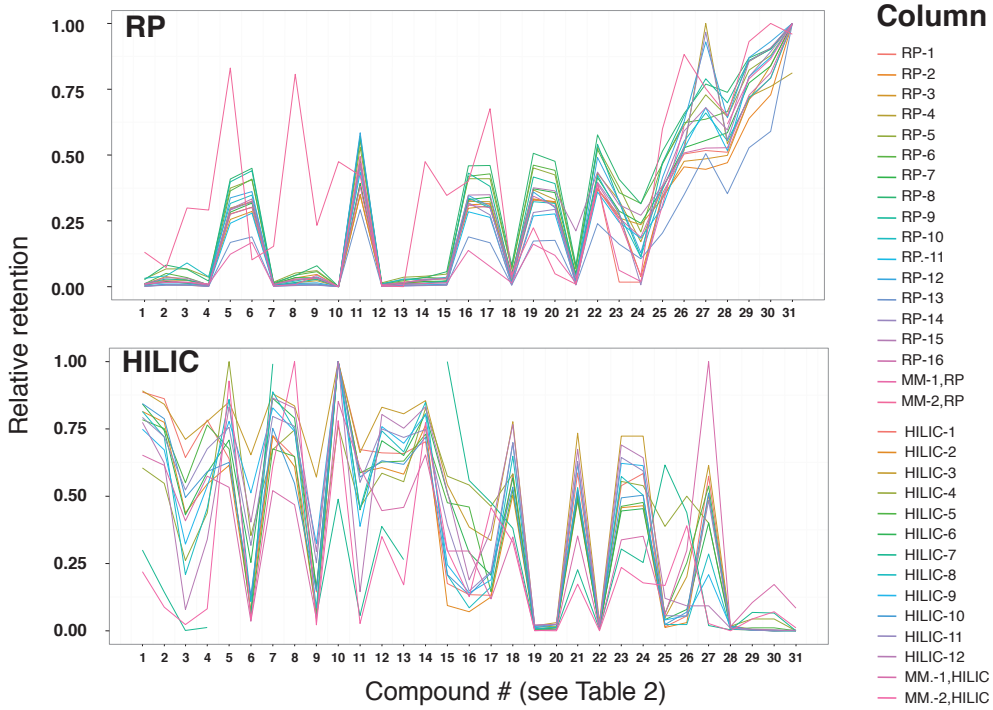
Compound #	Compound name	logP	Compound #	Compound name	logP
1	Carnitine	-4.52	17	Hippuric acid	0.31
2	Acetylcarnitine	-4.149	18	Tyrosine	0.38
3	Acetylcholine	-3.9	19	p-Hydroxyphenyl pyruvic acid	0.445
4	Choline	-3.7	20	α-Phenylacetamide	0.45
5	Folic acid	-2.87	21	Leucine	0.73
6	Riboflavin	-2.02	22	Melatonin	0.96
7	Glutamic acid	-1.43	23	Tryptophan	1.04
8	Malic acid	-1.26	24	Phenylalanine	1.11
9	Adenosine	-1.02	25	p-Coumaric acid	1.88
10	Ornithine	-0.96	26	5β-Cholic acid	2.62
11	Octanoylcarnitine	-0.96	27	palmitoylcarnitine	3.29
12	Alanine	-0.68	28	Testosterone	3.47
13	Proline	-0.57	29	9Z,12Z-Linoleic acid	7.18
14	Adenosine 5'-monophosphate	-0.22	30	Stearic acid	8.22
15	Urocanic acid	0.01	31	Cholesterol	9.85
16	(+)-Biotin	0.12			

Results

Relative retention pattern of columns in RP and HILIC mode

Figure 2 shows the retention behavior of the evaluated columns in the 2 different modes. While quite similar pattern were obtained in RP, the behavior in HILIC was more diverse and the correlation with logP was very weak in both modes. The 2 mixed mode columns were studied in both modes and plotted accordingly. Relative to all RP columns, the Acclaim Trinity P1 showed a significantly different retention pattern. In HILIC mode, the Thermo Scientific™ Acclaim™ Mixed Mode HILIC-1 column acted more differently to the HILIC phases than the Thermo Scientific™ Acclaim™ Trinity™ P1. The plots in Figure 2 are helpful to select columns for the 2D-LC combinations.

FIGURE 2. Relative retention of 31 test compounds on all RP and HILIC columns, and on the two mixed mode columns (MM) in both RP and HILIC mode.

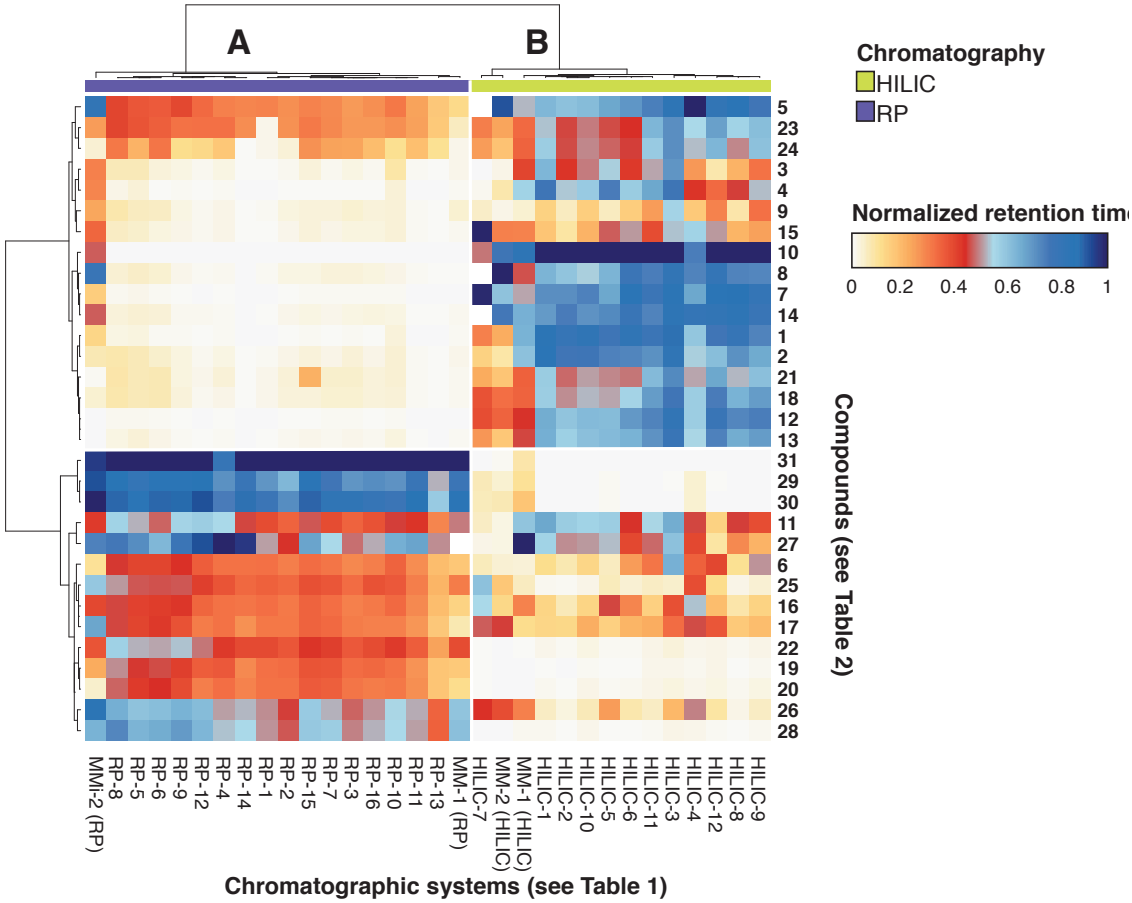


Heatmap and Hierarchical Cluster Analysis

While aimed at visualizing the retention behavior of the target analytes on all stationary phases, hierarchical agglomerative clustering was applied on the normalized retention data and a heatmap containing the analytes in the rows and the stationary phases in the columns with the individual values represented as colors was calculated (Figure 3). The hierarchical analysis arranged the chromatographic systems into the two large clusters **A** and **B**. Cluster **A** comprised the reversed-phase stationary phases showing only low retention for most of the analytes with low partition coefficient logP. Interestingly, the two mixed-mode columns change their cluster membership according to the elution gradient demonstrating their ability to work in either RP or HILIC mode.

Despite the broad diversity of RP columns, a highly correlated retention pattern (mean Spearman´s ρ = 0.935) was observed. The large bright upper left part of the heatmap indicates, that 14 out of the 31 analytes show virtually no retention on the RP-type materials (cluster **A**), while the Trinity P1 column (MM-2) forms a separate sub-cluster due to its unique retention behavior.
Cluster **B** consisted of HILIC stationary phases. Retention behavior of the HILIC columns was less homogenous (mean Spearman´s ρ = 0.823). Both Mixed Mode columns and the Thermo Scientific™ Hypersil GOLD™ HILIC column (HILIC-7) showed the lowest retention.

FIGURE 3. Heatmap and Hierarchical Cluster Analysis of retention data from Figure 2



Constructed 2D-LC Retention Maps and Method Orthogonality Assessment

Figure 4 compares two 2D-LC retention maps as constructed from the 1D retention data. The bubble positions indicate the relative retention in both dimensions, the biggest bubble sizes represent the sharpest peaks and the darkest bubbles represent the most symmetric peaks. The left combination came out as most orthogonal one of all combinations, the example on the right shows a combination of medium orthogonality. The outstanding retention behavior of the Trinity P1 column in RP mode (see Figure 3) was the mainly attributed to the success of this respective combination. Other RPxHILIC combinations often proved as rather complementary instead of orthogonal.

Table 3 depicts five different ways to statistically assess the orthogonal behavior of the two shown combinations (see also references 1-3 for statistics). The most significance and best accordance with visual data assessment was obtained with the harmonic mean 3rd nearest neighbor method.

FIGURE 4. Constructed 2D-LC charts with peak quality indicators for 2 method combinations

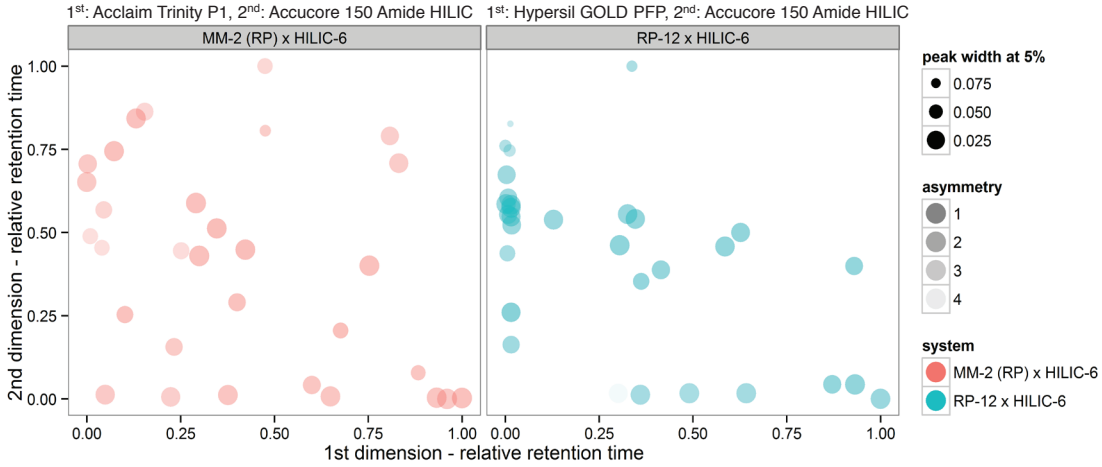


TABLE 3. Statistical evaluation of method orthogonality by different methods (high values of surface coverage and nearest neighbor methods as well as low absolute value of correlation coefficients account for better orthogonal behavior).

	System 1 (MM2-RP x HILIC-6)	System 2 (RP-12 x HILIC-6)
2D-binning surface coverage	0.556	0.444
Konvex hull surface coverage	0.273	0.225
Harmonic mean 3rd nearest neighbor	0.171	0.078
Pearson correlation coefficient	-0.359	-0.577
Spearman correlation coefficient	-0.381	-0.676

Conclusion

- 1D-LC experiments of a multitude of RP, HILIC and mixed mode methods provided the basis for constructing 2D-LC maps and statistically assess their orthogonal behavior. These constructions are valid as the 2D-LC instrument enables solvent change in fractions from 1st dimension (no limitations).
- Trinity P1 as a tri-modal mixed mode column provided a superior retention for the 1st dimension over all RP columns for the studied food metabolomics relevant compound set (Table 2).
- The harmonic mean 3rd nearest neighbor method (see Reference 3) proved to be the best statistical test for the orthogonal behavior assessment of method combinations.

References

1. Gilar et al., Orthogonality of Separation in Two-Dimensional Liquid Chromatography, Anal. Chem. 2005, 77, 6426-6434.
2. Gilar et al., Comparison of Orthogonality Estimation Methods for the Two-Dimensional Separations of Peptides, Anal Chem. 2012;84(20):8722-32
3. Nowik et al., Assessment of Two-Dimensional Separative Systems Using Nearest-Neighbor Distances Approach. Part 1: Orthogonality Aspects, Anal. Chem. 2013, 85, 9449−9458.

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