Poster Note 64420

Expanding the Coverage of Metabolome Using Multiple Liquid Chromatography Modes

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Overview

Purpose: To demonstrate a comprehensive comparison of different liquid chromatography approaches for the separation of 300 endogenous metabolites.

Methods: RPLC, HILIC, and mixed-mode LC were performed for the metabolite standards.

Results: Metabolites were generally divided into three categories based on the RPLC separation: **RP-Green**, **RP-Yellow** and **RP-Red**. The RP-Green category mainly contained nonpolar species that exhibited symmetric peak shapes and good retentions. The RP-Yellow category contained mainly polar compounds showing symmetric peaks but no retention. The third category, RP-Red was comprised of challenging to separate species showing all kinds of poor chromatographic results including splitting, tailing, broadening and very low response regardless of polarity. The three different LC modes demonstrated complementary separation for these metabolites.

Introduction

Mass spectrometry-based metabolomics is the comprehensive study of naturally occurring small molecules collectively known as the metabolome. Given the vast chemical and physical properties of endogenous metabolites and their wide concentration range, reducing the sample complexity using liquid chromatography separation as the frontend is widely used for expanding coverage in global metabolomics studies.

While reversed phase RPLC (C18-based) is the method most widely applied to metabolomics studies, alternatives such as hydrophilic interaction chromatography (HILIC) and mixed-mode chromatography have obtained increasing attention due to their improved retention for polar metabolites. However, a comprehensive comparison of these methods is lacking, especially using a relative large panel of compounds representing the vast diversity and complexity encountered in metabolomics studies.

Methods

Metabolite Standards Preparation

Chemical standards were validated with Human Metabolome Database (HMDB) to be endogenous metabolites. Every set of 24 standards were pooled into one vial we called a "batch." All metabolites were weighed and dissolved in 50%/50% MeOH/H_2O at the precise concentration of 0.5 mg/mL for HILIC and mixed-mode LC, and 20%/80% MeOH/H_2O for RPLC injection. Internal standard, stable-isotope-labeled d5-hippuric acid was spiked into all batches at 0.1 mg/mL. Samples were filtered with a 0.22 µm filter before injection.

Chromatography Methods

The LC methods were conducted on a Thermo Scientific™ Dionex™ UltiMate™ 3000 HPG (high pressure gradient) LC system. The Thermo Scientific™ Q Exactive™ hybrid quadrupoe-Orbitrap™ mass spectrometer was used for detection. Full scan MS data was acquired using positive/negative switching at resolution 70,000 (FWHM at *m/z* 200). Targeted MS/MS data was acquired to confirm identities at resolution 35,000 (FWHM at *m/z* 200).

For reverse phase LC, the following conditions were used:

- Column: Thermo Scientific™ Hypersil Gold™ C18, 150 x 2.1 mm, 1.9 µm
- Column Temperature: 55 °C;
- Injection volume: 2 µL
- Mobile phase: A = 0.1% formic acid in H_2O
  B = 0.1% formic acid in MeOH

	Retention [min]	Flow [ml/min]	%B
1	-3.0	0.45	0.5
2	0.0	0.45	0.5
3	5.5	0.45	50.0
4	6.0	0.45	98.0
5	12.0	0.45	98.0
6	13.0	0.45	0.5
7	15.0	0.45	0.5

For HILIC, the following conditions were used:

- Column: SeQuant® ZIC® p-HILIC 150 x 2.1 mm, 5 µm
- Column Temperature: 24 °C,
- Injection volume: 2 µL
- Mobile phase: A = 10 mM $AcONH_4$ in H_2O, pH = 9.8
  B = ACN

	Retention [min]	Flow [ml/min]	%B
1	0.0	0.25	90.0
2	15.0	0.25	30.0
3	18.0	0.25	30.0
4	19.0	0.25	90.0
5	27.0	0.25	90.0

For mix-mode LC, the following conditions were used:

- Column: Thermo Scientific™ Acclaim™ Trinity P1 100 x 2.1 mm, 3 um
- Column Temperature: 40° C
- Injection volume: 2 µL
- Mobile phase: A = 10 mM AcONH4 in H2O, pH = 5.0
  B = ACN:100mM $AcONH_4$ in H_2O (60%/40%) pH 5.0

	Retention [min]	Flow [ml/min]	%B
1	-5.0	0.35	0.0
2	0.0	0.35	0.0
3	20.0	0.35	100.0
4	22.0	0.35	100.0

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Results

Metabolite Categorization Based on RPLC (C18) Separation

Since RPLC is the most widely adopted separation method in the field of metabolomics, we first separated the 300 metabolites using C18 RPLC and observed their chromatographic retentions and MS responses using positive/negative switching. Observations were divided into several major types as coded from A to F in Table 1.

Type A: are compounds with good retention and thus good peak shape and MS responses in both positive and negative ion modes.

Type B & C: are similar to Type A showing good MS signal in only one polarity.

Type D: are compounds that are not well retained. They are mostly polar metabolites, eluting early with single symmetric peaks.

Type E-G: are compounds primarily presenting bad peaks and/or with poor MS signal.

Table 1. Several major types of separations base on RPLC and MS response through the survey of 300 endogenous metabolites.

Type	Good MS Response	Good Peak Shape	Good Retention	Positive	Negative	
A	√	√	√	√	√	RP-Green
B	√	√	√	√	–	RP-Green
C	√	√	√	–	√	RP-Green
D	√	√	–	√	√	RP-Yellow
E	√	–	√	√	√	RP-Red
F	√	–	–	√	√	RP-Red
G	–	–	–	√	√	RP-Red

Based primarily on peak shape and retention using RPLC, we arbitrarily divided the compounds into three categories color-coded in Table 1 and as shown in Figure 1.

RP-Green (mainly nonpolar metabolites: good retention, symmetric peaks).

RP-Yellow (mainly polar metabolites: early elution but single symmetric peaks).

RP-Red (polar or nonpolar: bad peaks including broadening, splitting, fronting, tailing, and low response).

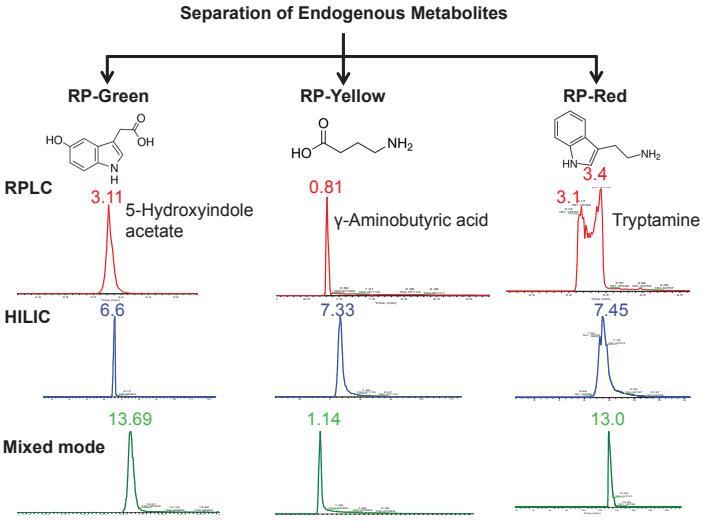


FIGURE 1. RP-Green: good retention with nice peak shape, e.g., 5-Hydroxyindole acetate. RP-Yellow: early elution with single symmetric peak, e.g. γ-Aminobutyric acid. RP-Red: bad chromatographic peak, e.g., splitting (Tryptamine). HILIC and mixed-mode LC could be applied to improve peak shapes.

Choose The Right Sample Solvent and Column Chemistry

The retention of compounds can be significantly affected by the sample solvent or column chemistry. However, many researchers attempt to use one "universal" sample solvent to perform sample injection onto multiple LC platforms, thus pursuing the benefit of an easy sample preparation protocol (e.g., direct injection of the supernatant). One popular solvent is MeOH/H_2Or in an 80/20 or 50/50 ratio. As shown in Figure 2, 50/50 MeOH/H_2O sample solvent results in a split peak on a Hypersil Gold (C18) column for Deoxyguanosine. The peak can be improved by using 10% methanol in water or a slightly different chemistry such as Hypersil Gold aQ, which is a polar endcapped column that is stable in a 100% aqueous mobile phase. However, the aQ column doesn't separate isomers such as leucine and isoleucine using 50%/50%:MeOH/H_2O; only 100% H_2O works. These examples indicate that the sample solvent needs to be carefully chosen for different LC columns and approaches, and is metabolites specific. Typical LC types, mobile phases and corresponding sample solvents are in Table 2.

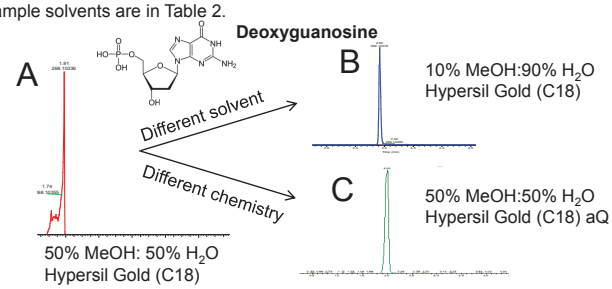


FIGURE 2. (A) Sample solvent (50/50:MeOH/H_2O) results in a split peak on Hypersil Gold C18 column for Deoxyguanosine. (B) The peak shape can be improved by using 10% methanol in water, or (C) a Hypersil Gold C18 aQ.

Table 2. Typical LC types, mobile phases and corresponding sample solvents.

LC type	Mobile phase (A/B)	Sample solvent (B%)	Injection volume
RPLC (C18)	H_2O/MeOH, both with 0.1% FA	<20%	2-10 uL
HILIC	10 mM AcONH4 in H_2O/ACN	>50%	2-5 uL
Mixed mode	$AcONH_4$ in H_2O/$AcONH_4$ in ACN	5%-95%	2-10 uL

Detect Endogenous Metabolites Using Both Polarities.

Chemicals (including metabolites) ionize differently under positive (+) and negative (-) electrospray ionization (ESI) modes. As shown in Figure 3, this difference is also important in MS-based metabolomics studies.

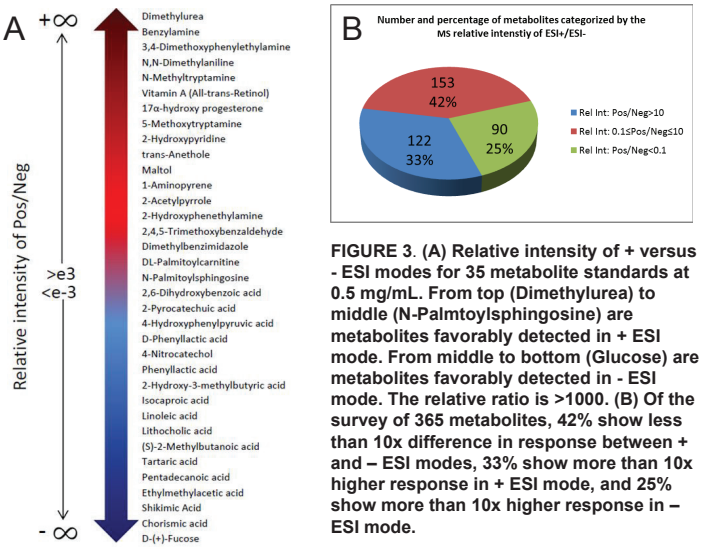
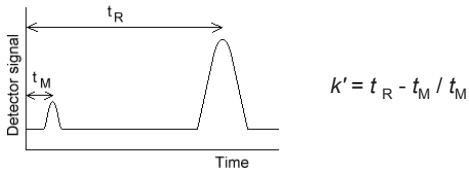


FIGURE 3. (A) Relative intensity of + versus - ESI modes for 35 metabolite standards at 0.5 mg/mL. From top (Dimethylurea) to middle (N-Palmtoylsphingosine) are metabolites favorably detected in + ESI mode. From middle to bottom (Glucose) are metabolites favorably detected in - ESI mode. The relative ratio is >1000. (B) Of the survey of 365 metabolites, 42% show less than 10x difference in response between + and – ESI modes, 33% show more than 10x higher response in + ESI mode, and 25% show more than 10x higher response in – ESI mode.

Complementarity of Multiple Separation Chromatography For Metabolites

Figures 4, 5 and 6 show how the three different LC modes\demonstrated complementary separation for the metabolites studied.



Retention Factor t_R is the retention time, and t_M is the column dead-time. If $k' < 1$, there will be less stable separations and a higher chance of chromatographic interferences at the beginning of the chromatogram.

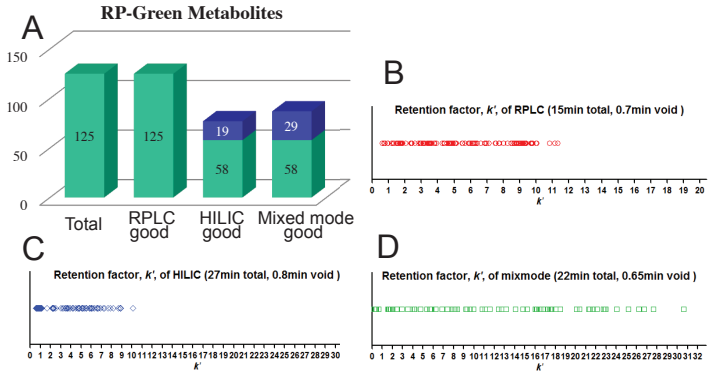


FIGURE 4. (A) The number of "good" peaks in the "RP-Green" category by RPLC, HILIC and mixed-mode LC. In this category, the peaks are symmetric and well retained using RPLC. (B) *k'* of RPLC. (C) *k'* of HILIC. (D) *k'* of mixed-mode LC. The X-axis ends with the maximal *k'* value in each mode. RPLC detected the largest number of peaks. Mixed-mode LC has the best retention time distribution for this category.

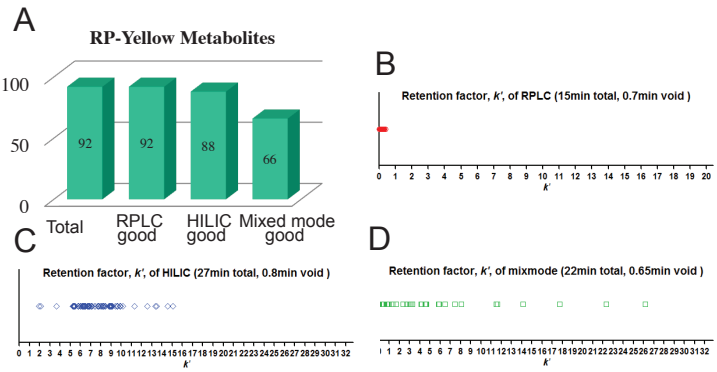


FIGURE 5. (A) The number of "good peaks in the "RP-Yellow" category by RPLC, HILIC and mixed-mode LC. In this category, the peaks are symmetric but not well retained in RPLC. (B) *k'* of RPLC. (C) *k'* of HILIC. (D) *k'* of Mix mode. The X-axis ends with the maximal *k'* value in each mode. All peaks in RPLC elute in void volume. Most of HILIC's *k'* values are in a good range.

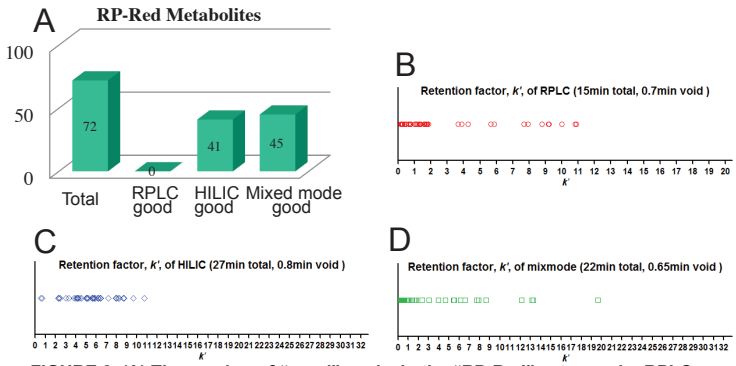


FIGURE 6. (A) The number of "good" peaks in the "RP-Red" category by RPLC, HILIC and mixed-mode LC. In this category, the peak shapes are poor in RPLC. (B) *k'* of RPLC. (C) *k'* of HILIC. (D) *k'* of mixed-mode LC. The X-axis ends with the maximal *k'* value in each mode. RPLC detected no good peaks. HILIC and mixed- mode LC improved the peak shape significantly. Most of HILIC's *k'* values are in a good range.

Conclusion

- The high diversity of endogenous metabolites' chemical and physical properties requires the use of complementary separation technologies.
- RPLC is the most widely applied separation method in the metabolomics field. Out of ~300 endogenous metabolites, the RPLC-MS method used here detected the largest number (217, 72%) of "good-shaped" peaks. However, only 145 (60%) of the 217 eluted after the void volume. The peaks in the void (k'<1) may be subject to severe chromatographic interferences.
- The HILIC-MS method detected 77 "good-shaped" peaks out of 125 less polar compounds that appear to be easy for RPLC to separate, but it detected an additional 129 "good-shaped" peaks including metabolites that elute in the void volume or "bad" peaks in RPLC. So the number of more meaningful compounds, i.e., having a good retention factor (k'>1), is 200. HILIC outperformed the RP and mix mode approaches.
- Mixed mode separation appears to be promising. It spreads the retention across the entire separation and improves peak shapes of metabolites that are challenging to RPLC. However, the total number of "good" peaks with good retention (k'>1) is 135, which is lower than HILIC (200) and comparable to RPLC (145). This indicates that the mixed-mode chromatographic conditions may need further optimization.

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