Expanding the Coverage of Metabolites Using Multiple Liquid Chromatography Separations

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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, to reduce the sample complexity using liquid chromatography separation as the frontend is widely used for expanding the coverage in global metabolomics studies.

While reversed phase RPLC (C18 based) being the most widely applied method in metabolomics study, a few alternatives like hydrophilic interaction chromatography (HILIC) and mix-mode have also obtained increasing attention recently owe to the improved retention for polar metabolites. However, a comprehensive comparison of these methods is still lacking, especially using a relative large panel of compounds representing the vast diversity of real complexity.

Methods

Metabolite Standards Preparation

Chemical standards were validated with Human Metabolome Database (HMDB) to be endogenous metabolites. Every 24 standards were pooled into one vial we called batch. All metabolites were weighted and dissolved in 50%/50% MeOH/H2O at the precise concentration of 0.5mg/mL for HILIC and mix mode LC, 20%/80% MeOH/H2O for RPLC injection. Internal standard, stable isotope labeled d5-hippuric acid was spiked into all batches at 0.1mg/mL. Sample were filtered with 0.22 µm filter before injection.

Chromatography Methods

All three LC methods were conducted on a Thermo Scientific™ Dionex™ Ultimate 3000™ HPG (high pressure gradient) system.

Thermo Scientific™ Q Exactive™ mass spectrometer was used for the detection. Full MS was acquired using positive/negative switching mode at resolution 70,000. Targeted MS/MS was acquired separately to confirm the identities at resolution 35,000.

For reverse phase LC, the following are used.

Column: Thermo Scientific™ Hypersil Gold™ C18, 150 x 2.1 mm, 1.9 µm

Column Temperature: 55 oC;

Injection volume: 2 µL

Mobile phase:

A = 0.1% formic acid in H2O;

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 are used.

Column: SeQuant® ZIC® - pHILIC 150 x 2.1 mm, 5 µm

Column Temperature: 24 oC,

Injection volume: 2 µL

Mobile phase:

A = 10 mM AcONH4 in H2O, 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 are 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 AcONH4 in H2O (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

Metabolites Categorization Based on RPLC (C18) Separation

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

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

Type B & C: similar to Type A but good MS signal with only one polarity.

Type D: are the compounds that could not be well retained. They are majorly polar metabolites, eluting early but showing single symmetric peak.

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

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

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

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

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

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

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

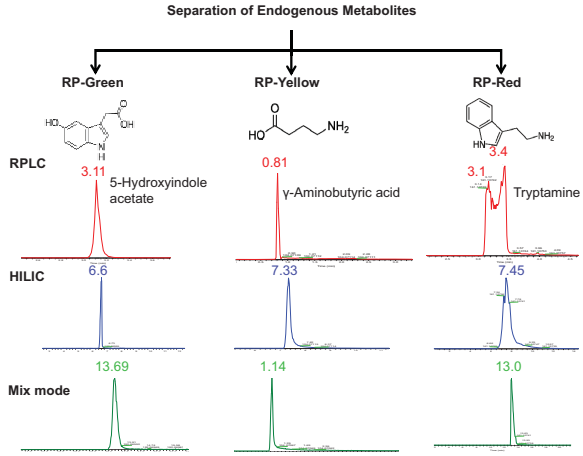


FIGURE 2. Choose The Right Sample Solvent and Column Chemistry. (A) Sample solvent (50/50:MeOH/H_2O) results in slitting peak on Hypersil Gold C18 column for Deoxyguanosine. (B) The peak can be improved by using 10% methanol in water, or (C) Hypersil Gold C18 aQ.

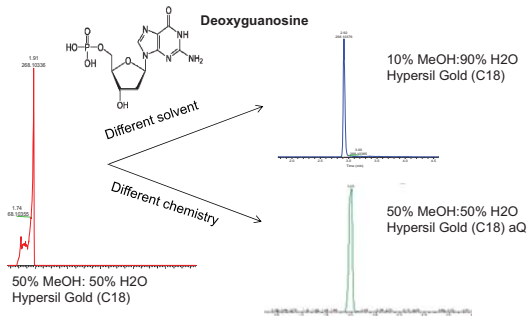


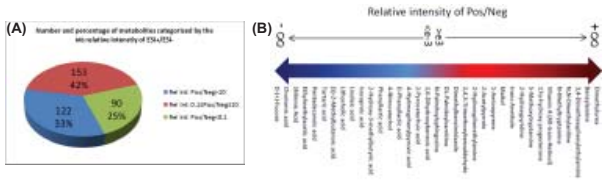
TABLE 2. Typical LC types, mobile phases and corresponding sample solvents.

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
Mix mode	$AcONH_4$ in H_2O/$AcONH_4$ in ACN	5%-95%	2-10 uL

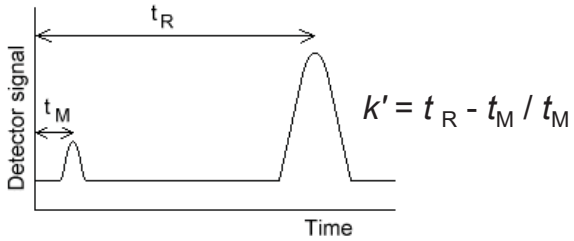
Detect Endogenous Metabolites Using Both Polarities

Chemicals (including metabolites) ionize differently under ESI (+) and ESI (-). Positive and negative mode detection might be equally important in MS-based metabolomics study, as shown below.

FIGURE 3. (A) Through the survey of 365 metabolites, 42% are within 10x different between positive and negative mode, 33% are more than 10x higher using positive and 25% are more than 10x higher using negative mode. (B) Relative intensity of positive/negative mode for 35 metabolite standards at 0.5 mg/mL by LC/MS.

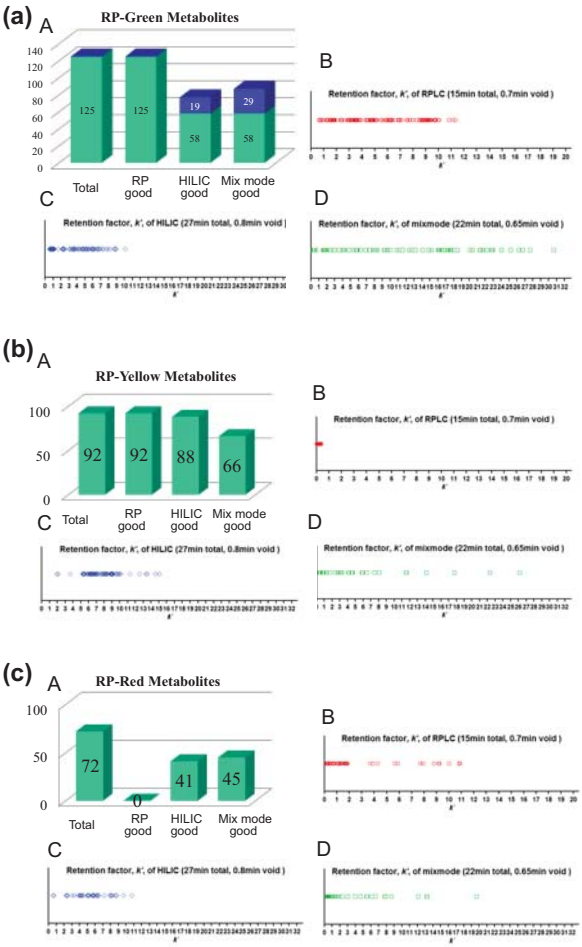


Complementarity of Multiple Separation Chromatography For Metabolites



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. The numbers of good peaks by RP, HILIC and Mix mode from "RP-Green" (a), from "RP-Yellow" (b), and from "RP-Red" (c).



Conclusion

The high diversity of endogenous metabolites' chemical and physical properties requires complementary separation technologies.

RPLC is the most widely applied separation method in metabolomics field. Out of ~300 endogenous metabolites, our RPLC method detected the largest number (217, 72%) of good-shape peaks. However, only 145 (60%) of the 217 eluted after the void volume. The peaks in void (k'<1) may subject to severe chromatographic interferences.

Our HILIC method detected 77 good peaks out of 125 less polar compounds that appear to be easy for RP (Figure 4A), but it detected extra 129 good peaks including metabolites that elute in the void volume or bad peaks in RPLC (Figures 5 and 6). So the number of more meaningful compounds, i.e., having a good retention factor (k'>1), is 200. It outperformed the RP and mix mode approaches.

Mix mode separation appears to be a promising technology. It spreads the retention across the entire separation and improves some peak shape 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 RP method (145). This indicates that the mix mode chromatographic conditions might need further optimization.

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