Comprehensive Polar Metabolite Profiling with HILIC-LC-MS

Rongrong Cheng[1], Jianwei You[1], Wen Jiang,[2] and Li Chen[1]
[1]Shanghai Key Laboratory of Metabolic Remodeling and Health, Institute of Metabolism & Integrative Biology, Fudan University,
[2]HILICON AB

METABOLIC PROFILING STUDIES using LC-MS technology have enabled the sensitive and reproducible detection of a wide range of metabolites in various biological samples, including biofluids, cells, tissues, and organisms. However, the analysis of polar/hydrophilic metabolites, such as small organic acids, amino acids, nucleotides, and sugars, meets challenges due to their poor retention in traditional reverse-phase LC-MS methods without using ion-pairing reagents in mobile phase or sample derivatization (1).

Hydrophilic interaction liquid chromatography (HILIC) offers a different retention mechanism that is advanced for straightforward separations of hydrophilic compounds (2). Although earlier works showed HILIC technology is effective for a small set of metabolites, it currently has achieved remarkable progress on improving chromatography separation and measurement repeatability, paving the way for comprehensive polar metabolite profiling.

In this application note, we describe a HILIC-LC-MS method using a polymeric iHILIC-(P) Classic column to accomplish a comprehensive polar metabolite profiling of several hundred metabolites in a single run. The results indicate remarkable chromatographic separation and repeatability of retention time. The importance of incorporating medronic acid in HILIC separations (3) and a straightforward sample extraction procedure are also touched.

Experimental

Sample preparation:

1) Metabolite standard samples were prepared in acetonitrile-methanol-water mixture (40:40:20, v/v%) and stored at -80 °C. Their final concentration was 10 µM.
2) 100 µL plasma or DMEM was mixed with 400 µL ice-cold methanol-acetonitrile solution (50:50, v/v%) by vortexing for 3-5s. The mixture was then incubated overnight at -80°C. Whereafter, samples were centrifuged at 15,000 rpm for 15 min at 4°C. Supernatant was transferred to a sample vial for LC-MS analysis.
3) The 293T cells after medium removal were extracted with 500 µL ice-cold acetonitrile-methanol-water solution (40:40:20, v/v %) for about 10 min. The cell extracts were transferred to 1.5 mL tubes and stored overnight at -80 °C. They were further cleaned by centrifuging to remove proteins at 15,000 rpm for 15 min at 4 °C. The supernatant was used for LC-MS analysis.

LC-MS/MS system:

A Shimadzu ExionLC AC HPLC system was connected to a TripleTOF 6600+ mass spectrometer from AB SCIEX. Electrospray ionization (ESI) in both positive and negative mode were used for detection. The ESI source parameters: source temperature at 500 °C, ion source gas 1 and 2 at 60 psi, curtain gas (CUR) at 35 psi, ion spray voltage floating (ISVF) at 5.5 kV or -4.5 kV for positive or negative modes. Mass spectrometer was set at TOF masses of 70–1200 Da.

HILIC separation:

Columns:

150 × 2.1 mm, 5 µm/200Å, iHILIC®-(P) Classic (P/N 160.152.0520, Sorbent Lot: 160-0520-10117 HILICON); Flow rate: 0.2 mL/min

Column temperature: 30 °C

Eluents:

A) 95:5 20 mM ammonium acetate and 0.1% ammonium hydroxide (v/v%) in water/ACN with 2.5 µM medronic acid.
B) Acetonitrile

TABLE 1: Gradient programs for separation with iHILIC-(P) Classic

time [min]	% B
0	85
2	85
7	60
12	35
12.1	20
15.9	20
16	85
23	85

Results and Discussion

Polymeric iHILIC-(P) Classic columns signify an outstanding advancement in HILIC separation under basic pH conditions, which enables successful separation of several hundred hydrophilic metabolites in a single run. This makes them a valuable tool for untargeted metabolomics studies in biological samples, especially when combined with a simplified extraction technique and ESI-MS detection.

Figure 1 demonstrates the untargeted metabolomics measurements for real biological samples. After excluding metabolites belonging to "lipid or lipid-like" classes, a dataset containing 699 unique hydrophilic metabolites was obtained. Among them, 286 were found exclusively in the 293T cells, 300 in plasma, and 113 in both. We were able to use our dataset of 154 metabolite standards to identify 61 metabolites exclusively in the 293T cells, 34 in plasma, and 59 in both. Our method is highly effective for a broad spectrum of metabolites, particularly for those belonging to the class of organic acids and their derivatives and nucleotides.

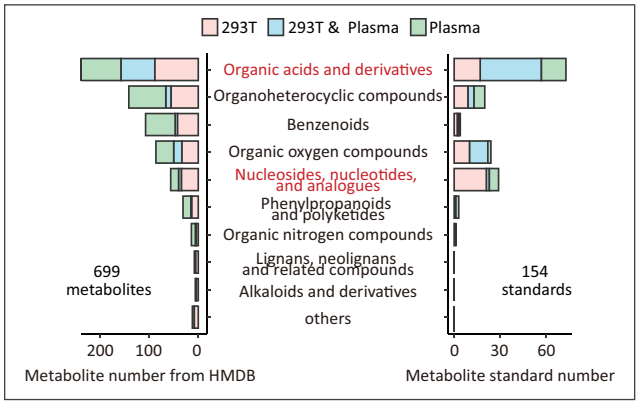

FIGURE 1 Untargeted profiling of hydrophilic metabolites in the 293T cells and plasma with HILIC-MS method in ESI- mode.

The described HILIC-LC-MS method is effective for several biologically important polar metabolites that were difficult to measure. As shown in Figure 2A, highly polar compounds with multiple phosphate groups, such as fructose 1, 6-bisphosphate and inositol hexaphosphate, are easily identified. Additionally, four biologically important metabolites and their isomeric forms were efficiently separated as well, shown in Figure 2B. Moreover, it's worth emphasizing that our sample extraction methods, which avoid drying and reconstitution, ensure the preservation of fragile metabolites, like nicotinamide adenine dinucleotide (NAD) and its phosphorylated form (NADP), as illustrated in Figure 2C.

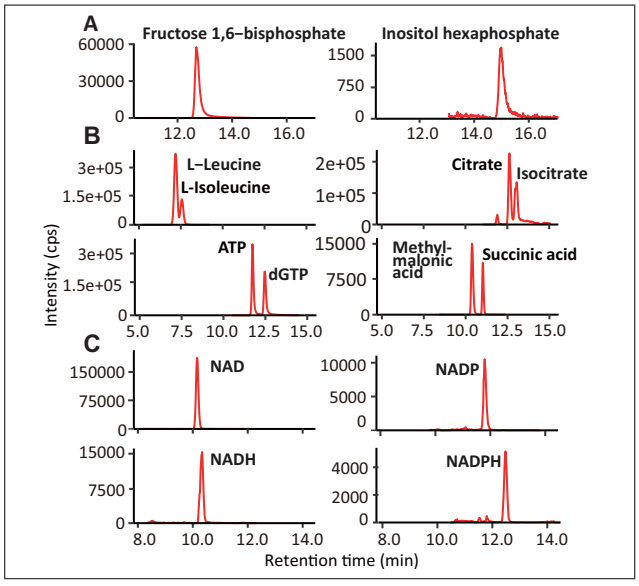

FIGURE 2 Extracted ion chromatograms of polar metabolites from standards or samples.

Figure 3 demonstrates the chromatographic repeatability of the polymeric iHILIC-(P) Classic columns. When analyzing identical amino acid mixture samples, we observed excellent peak shapes and retention time (RT) reproducibility across three different columns (serial number: #20308, #20504, and #40174), marked in green, blue, and red, respectively. In addition, the column (#40174) showed consistent RTs with a median delta RT of less than 0.03 min for >1000 injections of real samples and standards over a four-month period between July and October 2024.

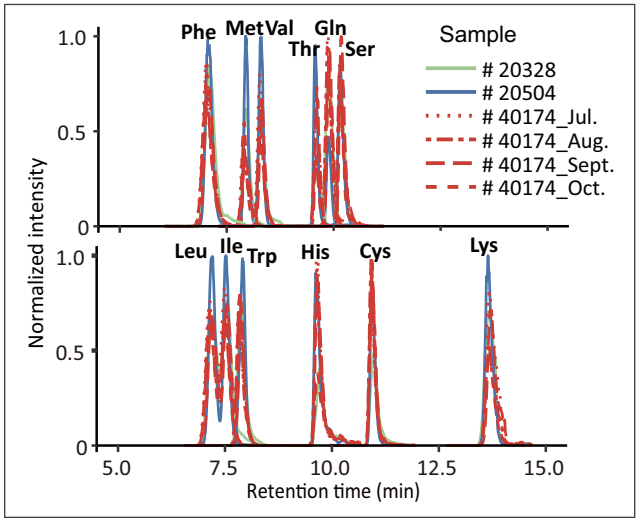

FIGURE 3 Extracted ion chromatograms of amino acids from Dulbecco's Modified Eagle Medium (DMEM) samples.

Conclusion

In summary, the current HILIC-LC-MS method profiles more than 500 polar metabolite standards across various categories in metabolomics studies. It revealed excellent chromatographic separation and retention time stability over several months. Along with straightforward sample preparation, the method enables comprehensive polar metabolite profiling of biological samples.

References

(1) Hu, Q., Tang, H., Wng,Y., Challenges in Analysis of Hydrophilic Metabolites Using Chromatography Coupled with Mass Spectrometry. J. Anal. Test. **2020**, 4, 140–162. https://doi.org/10.1007/s41664-020-00126-z

(2) Alpert A.J., Hydrophilic-interaction chromatography for the separation of peptides, nucleic acids and other polar compounds. J. Chromatogr. **1990**, 499, 177-96. https://doi.org/10.1016/S0021-9673(00)96972-3

(3) Hsiao, J.J., Potter , O.G., Chu, TW, Yin H., Improved LC/MS Methods for the Analysis of Metal-Sensitive Analytes Using Medronic Acid as a Mobile Phase Additive. Anal Chem. **2018,** 90, 9457-9464. https://doi.org/10.1021/acs.analchem.8b02100

HILICON AB
Tvistevägen 48 A, SE-90736 Umeå, Sweden
+46 (90) 193469
www.hilicon.com
info@hilicon.com