SHIMADZU
Excellence in Science

High Performance Liquid Chromatograph Mass Spectrometer LCMS-8060NX

Application News

Comprehensive Analysis of Hydrophilic Metabolites Using Shim-pack™ Mix-HILIC

Yutaka Umakoshi

User Benefits

- The Shim-pack Mix-HILIC provides a comprehensive analysis of hydrophilic metabolites.
- It is applicable to biological samples such as *E. coli* and plasma.

■ Introduction

Metabolomics is a technology to comprehensively analyze metabolites in living organisms. Since metabolites have diverse physicochemical properties, analyzing them with a single method is difficult. Reversed-phase columns with C18 and PFPP (Pentafluorophenylpropyl) groups on the stationary phase and HILIC columns are often used for measuring metabolites in LC/MS. However, it is difficult to simultaneously analyze amino acids, nucleobases, nucleosides, nucleotides, coenzymes, and organic acids that are important for bioanalysis. Ion-pair reagents can be used to retain and separate highly polar compounds on a C18 column. However, simultaneous analysis of cations and anions is difficult in principle, and high concentrations of ion-pair reagents can cause equipment contamination.

This application introduces a comprehensive analysis of hydrophilic metabolites by the Shim-pack Mix-HILIC. Using this column enables the analysis of 49 components, including amino acids, nucleobases, nucleosides, nucleotides, coenzymes, and organic acids (Fig. 1). Examples of the analysis of *Escherichia coli* (*E. coli*) and human plasma are shown.

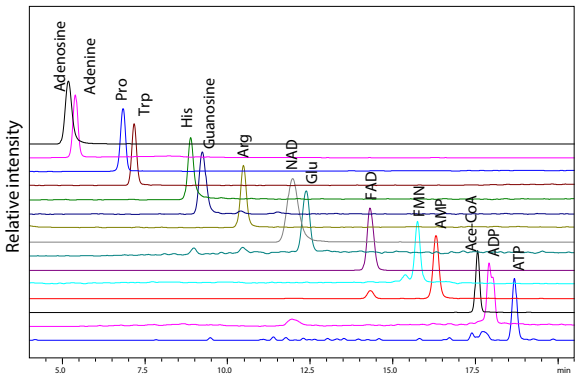

Fig. 1 MRM Chromatogram
(Standard Solution, Excerpts of Some Metabolites, Magnification Changes for Each Peak)

■ Shim-pack Mix-HILIC

Shim-pack Mix-HILIC is a polymethacrylate polymer-based column in which primary, secondary, tertiary amines and quaternary ammonium groups are modified on the surface. The column exhibits characteristic hydrophilic and ionic interactions by introducing amine and ammonium groups to highly cross-linked polymer particles. The upper pressure limit is 35 MPa, and the usable pH range is 2-13, allowing the column to be used even under basic mobile phase conditions.

■ Analytical Conditions

The instruments used were a Nexera™ X3 and an LCMS-8060NX. Table 1 shows the analysis conditions. Mobile phase A was prepared by adding 1 L of ultrapure water to 3162 mg of ammonium bicarbonate and 10 mL of 28% ammonia solution.

Under the gradient conditions shown, a separation mode dominated by hydrophilic interactions and a separation mode dominated by anion exchange act sequentially. Two types of continuous separation mode can achieve the analysis of compounds with various physicochemical properties.

Table 1 Analytical Conditions

[HPLC] Nexera X3	
Column:	Shim-pack Mix-HILIC (150 mm × 2.1 mm I.D., 5 µm) P/N: 227-32751-01
Column Oven:	40 °C
Solvent A:	Water + 40 mmol/L ammonium bicarbonate (pH 9.7)
Solvent B:	Acetonitrile
Rinse:	Methanol
Gradient:	B conc. 95% (0-0.5 min) → 40% (15.5 min) → 0% (16.5-26.5 min) → 95% (27.5-35 min)
Flowrate:	0.4 mL/min
Injection Volume:	5 µL
[MS] LCMS-8060NX	
Ionization:	ESI (IonFocus™)
Mode:	MRM
Nebulizing Gas:	2.0 L/min
Drying Gas:	10.0 L/min
Heating Gas:	10.0 L/min
DL Temp.:	250 °C
Heat Block Temp.:	400 °C
Interface Temp.:	300 °C
CID Gas Pressure:	270 kPa

Precautions when Performing the Analysis

Depending on the type of LC, the available pH range may be less than 9.7. Before analysis, check the available pH range of the instruments. At the end of the analysis, replace the solvent in the column with Solvent B 80% to prevent salt deposition. For long-term storage, follow the instruction manual and replace with the original packaging solvent (Acetonitrile/ (Water + 40 mmol/L ammonium bicarbonate + 6 mmol/L formic acid) = 40/60 (v/v)). After removing the column, replace Solvent A with ultrapure water and clean the entire system.

■ Samples and Pretreatments

E. coli grown in a liquid LB medium was diluted to an OD_{600} of 1, and 2 mL was used for analysis. The culture supernatant was removed by centrifugation (3000 g, 4 °C, 5 min). The following pretreatment was performed after washing twice with 1 mL of PBS and removing the PBS.

- *E. coli*
- Methanol 1000 µL
- Chloroform 400 µL

↓ Vortex 30 sec, Sonication 5 min
↓ Centrifugation (13000 rpm, 4 °C, 5 min)

- Supernatant 700 µL
- Chloroform 300 µL
- Ultrapure water 400 µL

↓ Vortex 30 sec
↓ Centrifugation (13000 rpm, 4 °C, 5 min)
↓ Centrifugal concentration of 500 µL of supernatant
↓ Redissolved in 50 µL of water/methanol (1/1)

LC/MS analysis

Human plasma was purchased from Kohjin Bio Co., Ltd., and the following pretreatment was performed.

- Plasma 50 µL
- Methanol 550 µL

↓ Vortex 30 sec, Sonication 5 min
↓ Centrifugation (13000 rpm, 4 °C , 5 min)

- Supernatant 500 µL
- Chloroform 500 µL
- Ultrapure water 400 µL

↓ Vortex 30 sec
↓ Centrifugation (13000 rpm, 4 °C , 5 min)
↓ Centrifugal concentration of 500 µL of supernatant
↓ Redissolved in 50 µL of water/methanol (1/1)

LC/MS analysis

■ Targeted Compounds

Table 2 shows the compounds to be targeted. Metabolites detected in *E. coli* and plasma are also shown.

Table 2 Targeted Compounds and Metabolites Detected in *E. coli* and Plasma

Abbreviation	Metabolite Name	Polarity	MRM	Retention time (min)	Collision Energy (V)	Classification	*E. coli*	Plasma
Ala	Alanine	+	90.1 > 44.1	8.1	-14	Amino acid	✓	✓
Arg	Arginine	+	174.9 > 70.2	10.5	-24	Amino acid	✓	✓
Asn	Asparagine	+	133.0 > 74.1	9.1	-17	Amino acid	✓	✓
Asp	Aspartic acid	+	134.0 > 74.0	12.2	-16	Amino acid	✓	✓
Gln	Glutamine	+	147.0 > 84.1	9.0	-19	Amino acid	✓	✓
Glu	Glutamic acid	+	147.8 > 84.1	12.4	-17	Amino acid	✓	✓
His	Histidine	+	155.9 > 110.1	8.9	-16	Amino acid	✓	✓
Ile	Isoleucine	+	132.1 > 86.2	6.6	-13	Amino acid	✓	✓
Leu	Leucine	+	131.9 > 86.2	6.4	-13	Amino acid	✓	✓
Lys	Lysine	+	146.9 > 84.2	10.5	-19	Amino acid	✓	✓
Met	Methionine	+	149.8 > 56.2	6.9	-18	Amino acid	✓	✓
Phe	Phenylalanine	+	166.1 > 120.1	6.4	-15	Amino acid	✓	✓
Pro	Proline	+	116.2 > 70.2	6.8	-17	Amino acid	✓	✓
Ser	Serine	+	105.8 > 60.2	9.4	-13	Amino acid	✓	✓
Thr	Threonine	+	120.2 > 74.2	8.6	-13	Amino acid	✓	✓
Trp	Tryptophan	+	205.2 > 188.1	7.2	-11	Amino acid	✓	✓
Tyr	Tyrosine	+	182.2 > 91.2	8.5	-29	Amino acid	✓	✓
Val	Valine	+	117.9 > 72.2	7.1	-13	Amino acid	✓	✓
Ace-CoA	Acetyl-Coenzyme A	+	810.1 > 303.2	17.6	-50	Coenzyme	✓	
CoA	Coenzyme A	+	768.0 > 261.1	17.7	-32	Coenzyme	✓	✓
FAD	Flavin adenine dinucleotide	-	784.0 > 437.1	14.3	28	Coenzyme	✓	✓
FMN	Flavin mononucleotide	+	456.9 > 439.1	15.8	-18	Coenzyme	✓	
NAD	Nicotinamide adenine dinucleotide	+	664.0 > 136.1	12.0	-52	Coenzyme	✓	
NADP	Nicotinamide adenine dinucleotide phosphate	-	741.9 > 620.0	17.4	18	Coenzyme	✓	
Adenine	Adenine	+	136.2 > 119.0	5.4	-26	Nucleobase	✓	
Cytosine	Cytosine	+	111.9 > 95.1	5.2	-21	Nucleobase	✓	✓
Guanine	Guanine	+	152.0 > 135.0	7.9	-22	Nucleobase	✓	
Uracil	Uracil	-	111.3 > 42.1	4.3	15	Nucleobase	✓	✓
Adenosine	Adenosine	+	268.0 > 136.0	5.2	-19	Nucleoside	✓	
Cytidine	Cytidine	+	243.8 > 112.1	6.7	-12	Nucleoside	✓	
Guanosine	Guanosine	+	283.9 > 152.0	9.2	-15	Nucleoside	✓	
Uridine	Uridine	+	245.0 > 113.1	6.7	-11	Nucleoside		
AMP	Adenosine 5'-monophosphate	+	348.0 > 136.1	16.3	-21	Nucleotide	✓	✓
ADP	Adenosine 5'-diphosphate	+	428.0 > 136.1	17.9	-25	Nucleotide	✓	
ATP	Adenosine 5'-triphosphate	+	508.0 > 410.0	18.7	-19	Nucleotide	✓	
CMP	Cytidine 5'-monophosphate	+	324.0 > 112.1	16.6	-14	Nucleotide	✓	
CDP	Cytidine 5'-diphosphate	+	404.0 > 112.1	17.8	-22	Nucleotide	✓	
CTP	Cytidine 5'-triphosphate	+	484.1 > 112.1	18.3	-24	Nucleotide	✓	
dTMP	Deoxythymidine 5'-monophosphate	+	322.8 > 81.0	15.8	-17	Nucleotide	✓	
dTDP	Deoxythymidine 5'-diphosphate	-	400.9 > 159.1	17.7	23	Nucleotide	✓	
dTTP	Deoxythymidine 5'-triphosphate	+	483.0 > 81.0	18.3	-38	Nucleotide		
GMP	Guanosine 5'-monophosphate	+	363.8 > 152.1	17.7	-17	Nucleotide	✓	
GDP	Guanosine 5'-diphosphate	+	444.0 > 152.0	18.4	-20	Nucleotide	✓	
GTP	Guanosine 5'-triphosphate	+	524.0 > 152.0	19.8	-31	Nucleotide	✓	
UMP	Uridine 5'-monophosphate	+	325.1 > 97.2	17.0	-17	Nucleotide		
UDP	Uridine 5'-diphosphate	+	405.0 > 97.2	18.0	-21	Nucleotide		
UTP	Uridine 5'-triphosphate	+	484.9 > 97.1	18.5	-28	Nucleotide		
Cit	Citric acid	-	191.2 > 111.0	17.8	11	Organic acid	✓	✓
Mal	Malic acid	-	133.2 > 115.0	15.6	15	Organic acid	✓	✓

The retention time depends on your system and column lot, so check with the reference standards.

■ Results

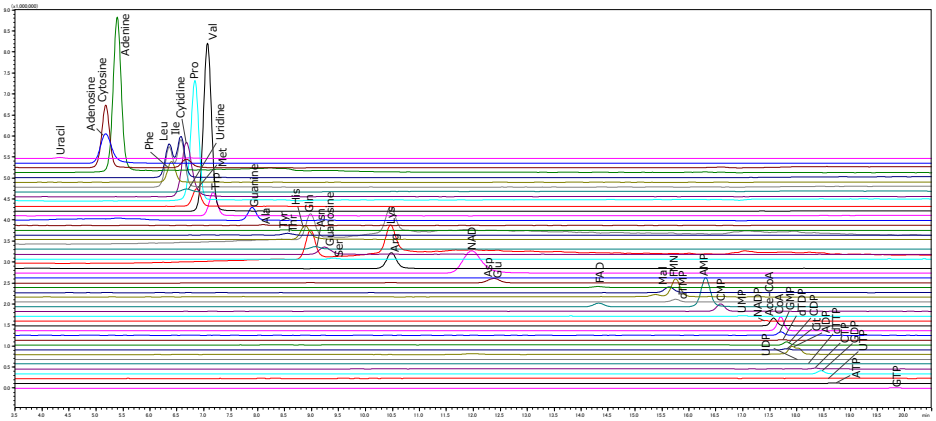

Fig. 2 MRM Chromatogram (Standard Solution: 1 µmol/L)

The chromatogram obtained from the analysis of the standard is shown in Fig. 2. Amino acids, nucleobases, and nucleosides, which are uncharged, cationic, and zwitterionic compounds, were eluted in the aqueous solvent range up to 50% (0-12.8 min). Anionic compounds such as nucleotides, coenzymes, and organic acids were eluted in the aqueous solvent above 50% (after 12.8 min).

E. coli and plasma samples were analyzed as examples for this application. As shown in Table 2, of the 49 metabolites targeted, 44 were detected in *E. coli* and 25 in plasma. Figs. 3 and 4 show the respective MRM chromatograms.

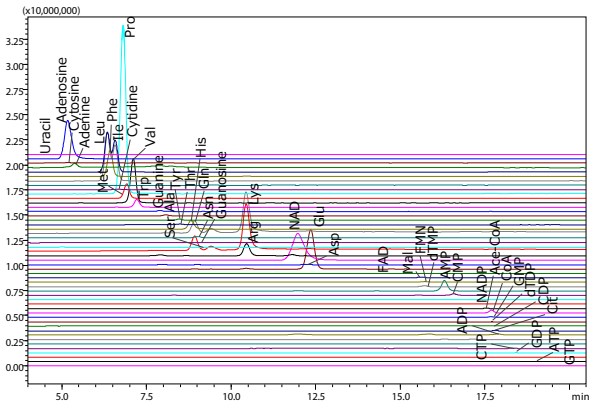

Fig. 3 MRM Chromatogram (*E. coli*)

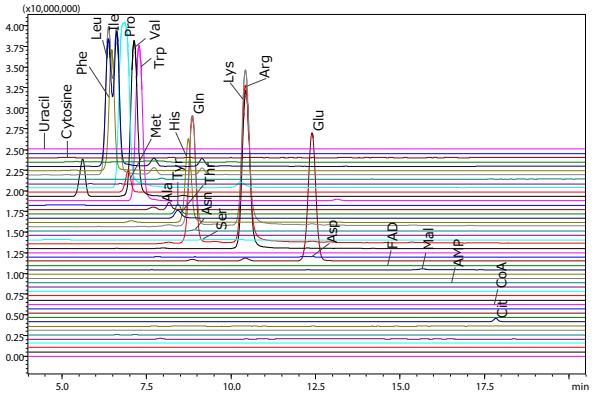

Fig. 4 MRM Chromatogram (Plasma)

Some of the metabolites detected in *E. coli* are shown in Fig. 5. A wide range of compounds were detected in *E. coli*, including amino acids, nucleobases, nucleosides, nucleotides, coenzymes, and organic acids.

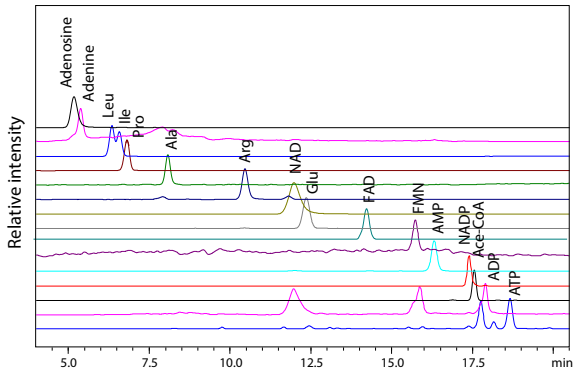

Fig. 5 MRM Chromatogram
(*E. coli*, Excerpts of Some Metabolites, Magnification Changes for Each Peak)

■ Conclusion

The Shim-pack Mix-HILIC can be used to comprehensively analyze hydrophilic metabolites such as amino acids, nucleobases, nucleosides, nucleotides, coenzymes, and organic acids. Since the method can be applied to biological samples such as *E. coli* and plasma, it is expected to be an effective tool for metabolome analysis.

■ Acknowledgment

We appreciate Dr. Takeshi Bamba and Dr. Kota Nakatani of the Medical Institute of Bioregulation, Kyushu University for their contributions to the development of this application.

Shim-pack, Nexera and IonFocus are trademarks of Shimadzu Corporation or its affiliated companies in Japan and/or other countries.

01-00679-EN First Edition: Feb. 2024

SHIMADZU
Shimadzu Corporation
www.shimadzu.com/an/

For Research Use Only. Not for use in diagnostic procedures.
This publication may contain references to products that are not available in your country. Please contact us to check the availability of these products in your country.
The content of this publication shall not be reproduced, altered or sold for any commercial purpose without the written approval of Shimadzu.
See http://www.shimadzu.com/about/trademarks/index.html for details.
Third party trademarks and trade names may be used in this publication to refer to either the entities or their products/services, whether or not they are used with trademark symbol "TM" or "®".
Shimadzu disclaims any proprietary interest in trademarks and trade names other than its own.
The information contained herein is provided to you "as is" without warranty of any kind including without limitation warranties as to its accuracy or completeness. Shimadzu does not assume any responsibility or liability for any damage, whether direct or indirect, relating to the use of this publication. This publication is based upon the information available to Shimadzu on or before the date of publication, and subject to change without notice.

© Shimadzu Corporation, 2024