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Original Article

# Comparison of Amine-Modified Polymeric Stationary Phases for Polar Metabolomic Analysis Based on Unified-Hydrophilic Interaction/Anion Exchange Liquid Chromatography/High-Resolution Mass Spectrometry (Unified-HILIC/AEX/HRMS)

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In metabolomic analysis, one of the most commonly used techniques to support the detection sensitivity and quantitation of mass spectrometry is combining it with liquid chromatography. Recently, we developed a method that enables comprehensive single-run measurement of hydrophilic metabolites using unified-hydrophilic interaction/anion exchange liquid chromatography/high-resolution mass spectrometry (unified-HILIC/AEX/HRMS) with a polymer-based mixed amines column (Gelpack GL-HilicAex). However, the importance of stationary phase functional groups and mobile phase conditions for the separation mechanisms and sensitive detection in unified-HILIC/AEX/HRMS is not yet fully understood. This study aimed to understand the importance of the mobile and stationary phases in unified-HILIC/AEX/HRMS. Two different alkali-resistant polymer-based amines-modified columns (Gelpack GL-HilicAex, primary, secondary, tertiary, and quaternary amine-modified polyglycerol dimethacrylate gel; Asahipak NH2P-50 2D, secondary amine-modified polyvinyl alcohol gel) and two eluents (acetonitrile and ammonium bicarbonate solution, pH 9.8) were used for comparative validation. A comparison of mobile phase conditions using both columns confirmed that the two-step separation from HILIC to AEX characteristic of unified-HILIC/AEX requires a linear gradient condition from acetonitrile to nearly 50% water and AEX with up to 40 mM bicarbonate ions. We found that when alkali-resistant hydrophilic polymer packing materials are modified with amines, unified-HILIC/AEX separation can be reproduced if at least one secondary amine associated with the amine series is present in the stationary phase. Furthermore, the difference in sensitivity in the HILIC and AEX modes owing to the different columns indicates the need for further improvements in the mobile phase composition and stationary phase.



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## 1. INTRODUCTION

Metabolomics is the comprehensive study of small molecule metabolites in biological systems and is widely used as a powerful method to elucidate the metabolic mechanisms of living organisms.[1)] Mass spectrometry (MS) is widely used in metabolomics because of its high sensitivity and comprehensive detection.[2,3)] MS also has the advantage of being combined with various chromatographic techniques, such as gas chromatography and liquid chromatography (LC), to identify and quantify a wide range of compounds by suppressing matrix effects and improving detection sensitivity and quantitation.[4)]

A common view in the field of metabolomics is that reversed-phase liquid chromatography/mass spectrometry is suitable for the separation and detection of hydrophobic metabolites. In contrast, hydrophilic interaction liquid chromatography/mass spectrometry (HILIC/MS) is suitable for the comprehensive observation of hydrophilic metabolites.[5,6)] Other methods, such as ion chromatography/mass

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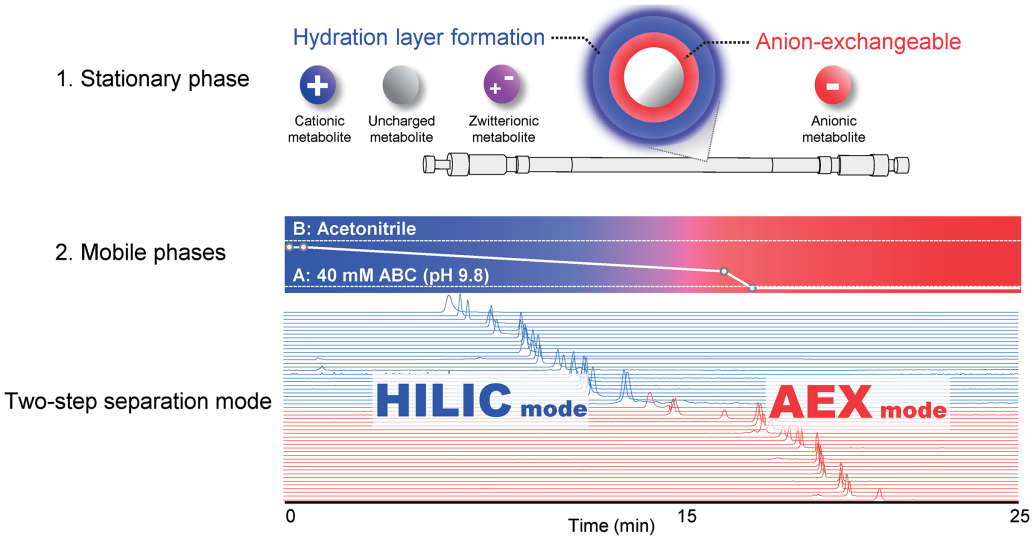


Fig. 1. Schematic of chromatographic separation and mass spectrometric detection of polar metabolites based on unified-HILIC/AEX/HRMS. ABC, ammonium bicarbonate; AEX, anion exchange; HILIC, hydrophilic interaction liquid chromatography; Unified-HILIC/AEX/HRMS, unified-hydrophilic interaction/anion exchange liquid chromatography/high-resolution mass spectrometry.

spectrometry (IC/MS) with an anion exchange (AEX) column and capillary electrophoresis-mass spectrometry (CE-MS) are excellent separation and detection methods for the analysis of highly polar metabolites.[7,8] However, IC/MS cannot, in principle, separate and detect both cationic and anionic polar metabolites simultaneously, and CE-MS has been shown to measure cations and anions simultaneously,[9] but there are no demonstrative examples of widely-targeted polar metabolomic analysis. The current reality of polar metabolomic analysis is that comprehensive detection and identification of polar metabolites requires the use of multiple separation modes, including HILIC/MS, CE-MS, and IC/MS, because of the widely varying physicochemical properties (log $P_{ow}$, charge characteristics, molecular distribution, *etc.*) of hydrophilic metabolites.[4,10] Comprehensive detection of the metabolome in a single assay will accelerate both large-scale metabolomics studies and research using rarely available samples.

Recently, we have developed a single-run method for the comprehensive and simultaneous analysis of polar metabolites using unified-hydrophilic interaction/anion exchange liquid chromatography/high-resolution mass spectrometry (unified-HILIC/AEX/HRMS) with a polymer-based mixed amines column (Gelpack GL-HilicAex) (Fig. 1).[11] The unified-HILIC/AEX has unique chromatographic separation characteristics to separate and elute cationic, uncharged, zwitterionic, and anionic polar metabolites through a continuous transition of the separation modes from HILIC to AEX under analytical conditions of gradient elution using two mobile phases per column. Under unified-HILIC/AEX/HRMS mobile phase conditions, the analysis was performed starting with more than 95% acetonitrile (ACN) and gradually increasing the percentage of water ($H_2O$) containing 40 mM ammonium bicarbonate (ABC). In the HILIC mode, cationic, uncharged, and zwitterionic polar compounds are eluted by hydrophilic interactions, whereas in the AEX mode, anions that strongly interact with the stationary phase, such as adenosine 5'-triphosphate (ATP), are eluted based on the IC principle with ABC concentration gradients. A hydrophilic polymer-based stationary phase modified with primary, secondary, and tertiary amines together with quaternary ammonium was used to form a hydration layer that activated the HILIC mode. In addition, the pH-resistant polymer (pH 2–13) allows ion exchange with ABC under strongly basic conditions (pH 9.8). Thus, unified-HILIC/AEX/HRMS is a novel method for polar metabolomic analysis in which the two separation modes operate in a stepwise manner, and MS can be used as a detector. Further fundamental knowledge of chromatographic separation and detection is needed, that is, optimization of the stationary phase, functional groups, and mobile phase conditions, to improve the comprehensiveness and sensitivity of single-run metabolomics. For example, the need for primary, secondary, tertiary, and quaternary amines modifying the polymer-based stationary phase to achieve the unified-HILIC/AEX separation mode is not understood.

In this study, to better understand the importance of mobile and stationary phases in unified-HILIC/AEX/HRMS, we compared and validated the separation behavior and detection sensitivity of polar metabolites using two different alkali-resistant polymer-based amines-modified columns (Gelpack GL-HilicAex, primary, secondary, tertiary, and quaternary amines-modified poly[glycerol dimethacrylate] gel; Asahipak NH2P-50 2D, secondary amines-modified poly[vinyl alcohol] gel) under different and the same mobile phase conditions (Table 1). Fifty-two hydrophilic metabolites with a wide range of physicochemical properties detected in the HepG2 cell extracts were used as indicators to understand the unified-HILIC/AEX separation behavior under each condition. The performance of each column and elution condition was compared based on the retention time (RT), peak shape, and peak intensity of 52 hydrophilic metabolites, and the number of metabolic features detected by non-targeted analysis. Finally, improvements in the unified-HILIC/AEX/HRMS method are discussed.

## 2. EXPERIMENTAL

### 2.1. Chemicals and reagents

Phosphate-buffered saline (PBS), fetal bovine serum (FBS), penicillin–streptomycin solution (10,000 U/mL penicillin,

Table 1. Two column features of Gelpack GL-HilicAex and Asahipak NH2P-50 2D.

| Column name | Gelpack GL-HilicAex | Asahipak NH2P-50 2D |
|---|---|---|
| Base polymer particle | Poly(glycerol dimethacrylate) gel | Poly(vinyl alcohol) gel |
| Functional group | Mixed amines (primary, secondary, and tertiary amines and quaternary ammonium) | Secondary amines |
| Column dimension | 2.1 mm i.d.×150 mm | 2.0 mm i.d.×150 mm |
| Particle size (μmm) | 5 | 5 |
| pH availability | 2–13 | 2–13 |
| Pressure limit (MPa) | 40 | 10 |

i.d., inner diameter

10,000 μg/mL streptomycin), and trypsin–ethylene diamine tetraacetic acid (EDTA) solution (0.25% (w/v) trypsin, 1 mM EDTA) were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Dulbecco's modified Eagle's medium (DMEM) containing high glucose (4500 mg/mL) was purchased from Fujifilm Wako Pure Chemical Co., Ltd. (Osaka, Japan). LC–MS-grade $H_2O$, ACN, methanol (MeOH), and isopropanol (IPA) were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). LC–MS-grade ABC and 28% (v/v) ammonium hydroxide were purchased from Nacalai Tesque Inc. (Kyoto, Japan) and Fujifilm Wako Pure Chemical Co., Ltd., respectively. Authentic standards were obtained from Nacalai Tesque Inc., Fujifilm Wako Pure Chemical Co., Ltd., and Merck (Darmstadt, Germany). Table S1 lists the abbreviations used for the 52 polar metabolites.

## 2.2. Cell culture and sample preparation

HepG2 cells (Cellular Engineering Technologies Inc., Coralville, IA, USA) were cultured in high-glucose DMEM supplemented with 10% (v/v) FBS and 1% (v/v) penicillin–streptomycin solution in a 6-well plate (Corning Inc., NY, USA). The cultivation plates were incubated in a water-jacketed $CO_2$ incubator (WCI-165; ASTEC Co., Fukuoka, Japan) under a humidified atmosphere of 5% $CO_2$ at 37°C. After removing the medium, the HepG2 cells (approximately $1\times10^6$ cells) in each well were washed three times with 1 mL of ice-cold PBS and quenched with 1 mL of cold IPA/$H_2O$ (7:3, v/v) (−30°C). After scraping, the cell suspension (~1 mL) was transferred into a 2 mL Eppendorf tube. The samples were vigorously mixed by vortexing for 1 min, followed by sonication for 5 min. The samples were incubated on ice for 5 min, followed by centrifugation at 4°C and 16,000×*g* for 5 min to precipitate proteins. The collected supernatant (700 μL) was transferred to a 2 mL Eppendorf tube and was then evaporated under a vacuum using a centrifugal concentrator (UC-96R; TAITEC Co., Saitama, Japan). The samples were dissolved in 50 μL of IPA/$H_2O$ (7:3, v/v) and stored at −80°C until unified-HILIC/AEX/HRMS analysis.

## 2.3. LC/HRMS analysis

The liquid chromatography/high-resolution mass spectrometry (LC/HRMS) system comprised a wide-pH version of Nexera X2 UHPLC (Shimadzu Co., Kyoto, Japan) and an Orbitrap Exploris 120, a high-performance benchtop quadrupole Orbitrap mass spectrometer (Thermo Fisher Scientific) with a heated electrospray ionization source. The unified-HILIC/AEX/HRMS analytical method using a Gelpack GL-HilicAex column, 5 μm particle size, 2.1 mm inner diameter (i.d.)×150 mm (Resonac Techno Service Co., Ibaraki, Japan) was employed as described previously[11] with some modifications. The unified-HILIC/AEX conditions using a Gelpack GL-HilicAex (method A) were as follows: column temperature, 40°C; mobile phase (A), 40 mM ABC at pH 9.8 adjusted by adding 28% ammonium hydroxide; mobile phase (B), ACN; flow rate, 0.4 mL/min; and injection volume, 1 μL. The gradient conditions were as follows: $t$ = 0–0.5 min, 99% B; $t$ = 0.5–15.5 min, 99–40% B; $t$ = 15.5–16.5 min, 40–0% B; $t$ = 16.5–26.5 min, 0% B; $t$ = 26.5–27.5 min, 0–99% B; and $t$ = 27.5–35 min, 99% B. The full scanning HRMS analysis conditions were as follows: polarity, positive and negative ionization with positive/negative fast polarity switching function; sheath gas flow rate, 50 arb; auxiliary (aux) gas flow rate, 10 arb; spray voltage, 3.5 kV for positive ionization and −2.5 kV for negative ionization; ion transfer tube temperature, 325°C; S-lens level, 60; vaporizer temperature, 350°C; mass resolution, 60,000; automatic gain control (AGC) target (the number of ions to fill C-Trap), 1,000,000; maximum injection time, 200 ms; and scan range for two events, 50–200 (lower limit of $m/z$ set to 50 to observe bicarbonate ions) and 100–1000 ($m/z$).

The LC/HRMS analytical methods for comparison using an Asahipak NH2P-50 2D column, 5 μm particle size, 2.0 mm i.d. × 150 mm (Resonac Holdings Co., Ltd., Tokyo, Japan) were employed as described previously[12] with some modifications. The LC conditions for Asahipak NH2P-50 2D (method B) were as follows: column temperature, 40°C; mobile phase (A), 20 mM ABC (pH 9.8); mobile phase (B), ACN; flow rate, 0.2 mL/min; and injection volume, 1 μL. The gradient conditions (method B) were as follows: $t$ = 0–3.5 min, 95.3% B; $t$ = 3.5–8 min, 95.3–85.8% B; $t$ = 8–13 min, 85.8–76.3% B; $t$ = 13–14 min, 5% B; $t$ = 14–30 min, 5% B; $t$ = 30–31 min, 5–95.3% B; and $t$ = 31–41 min, 95.3% B. The LC and gradient conditions for the Asahipak NH2P-50 2D (method C) were the same as those for the unified-HILIC/AEX conditions using a Gelpack GL-HilicAex (method A), except for the flow rate (0.2 mL/min) because of the column pressure resistance of 10 MPa (Table 1). The full scanning HRMS analysis conditions were the same as those for unified-HILIC/AEX/HRMS.

## 2.4. Flow injection analysis

Flow injection-HRMS (FI-HRMS) analysis is a well-established and automated analytical technique performed without a column to confirm ionization efficiency. The FI conditions were as follows: analyte, ATP solutions prepared with 0, 1, 10, 20, and 40 mM ABC solutions (pH 9.8) to a final concentration of 10 μM; mobile phase solvent, 40 mM ABC (pH 9.8)/ACN (1:1, v/v); flow rate, 0.2 mL/min; and injection volume, 5 μL. The full scanning HRMS analysis

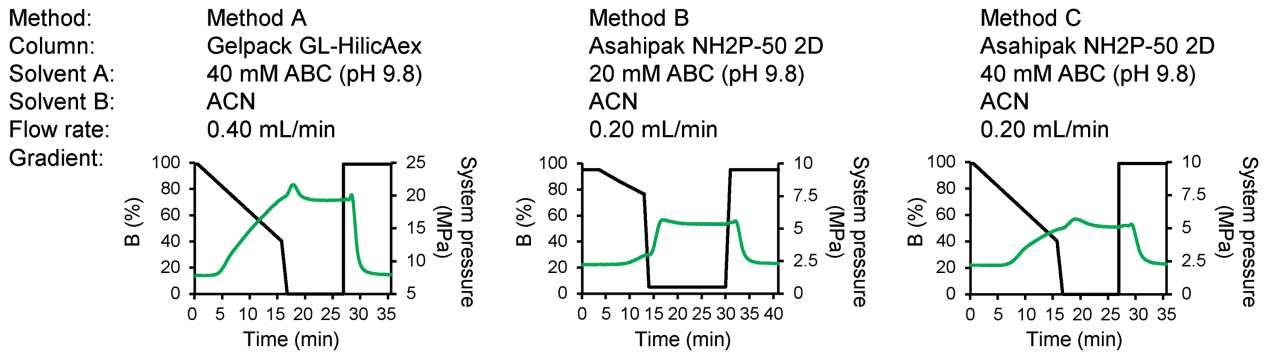


Fig. 2. Comparison of differences in specific parameters for each of the LC analysis conditions. ABC, ammonium bicarbonate; ACN, acetonitrile; LC, liquid chromatography.

conditions were identical to those of the unified-HILIC/AEX/HRMS method.

### 2.5. Peak alignment and detection

Compound Discoverer ver. 3.3 (Thermo Fisher Scientific) was used for data processing, including peak alignment, peak detection, data grouping, gap filling, and background subtraction.[13] Peak alignment for two datasets containing unified-HILIC/AEX/HRMS data (*i.e.*, procedure blank and HepG2 cell samples) was performed individually based on a non-parametric peak alignment algorithm using base peak chromatograms. The details of the peak alignment and detection procedures are listed in Table S2.

### 2.6. Metabolite identification

The peaks obtained after processing the LC/HRMS data of the HepG2 cell extracts by Compound Discoverer analysis were identified based on the "level 1-identified metabolites" criteria defined by the Metabolomics Standards Initiative.[14,15] Actual metabolite identification was performed by comparing the RT (RT tolerance, <0.2 min) and HRMS (precursor ion mass error tolerance of <10 ppm) of the samples with those of 52 authentic standards (Table S1) analyzed under identical conditions.

### 2.7. Data analysis

The chromatographic performance evaluation of each LC/HRMS method was based on RT, full widths at half maximum (FWHM), and their relative standard error values. The quantitative assessment of the identified metabolites was calculated from the peak areas and peak intensities of the HRMS precursor ions (mass error tolerance <10 ppm) using Cascade ver. 1.1 software (Reifycs Inc., Tokyo, Japan). Spearman's rank correlation coefficient ($r$) was calculated using the Pandas and NumPy libraries in Python. Statistical significance between control and test groups was determined using Two-way ANOVA followed by Dunnett's multiple comparisons test, which was performed using GraphPad Prism ver. 9 for Windows (GraphPad Software, La Jolla, CA, USA; https://www.graphpad.com).

## 3. RESULTS AND DISCUSSION

### 3.1. Comparison of stationary phase properties between a Gelpack GL-HilicAex column and an Asahipak NH2P-50 2D column

The base material of the stationary phase used in Gelpack GL-HilicAex is based on a highly hydrophilic glycerol dimethacrylate-based bare polymer prepared by radical polymerization of glycerol dimethacrylate (Table 1). Cation-dense mixed amines polymer stationary phases with a branched chain structure of primary, secondary, and tertiary amines along with quaternary ammonium were prepared by treating a glycerol dimethacrylate bear polymer with epichlorohydrin followed by amination with polyethyleneimine. The stationary phase of Asahipak NH2P-50 2D was prepared by modifying a poly(vinyl alcohol) resin with only secondary amines as functional groups (Table 1). Both Gelpack GL-HilicAex and Asahipak NH2P-50 2D are less adsorptive to hydrophobic substances and prevent column contamination.[16] In addition, these polymer-based amines-modified columns solve the problem of poor RT repeatability due to the aging degradation of silica-based amino columns,[17] are resistant to stationary phase swelling upon complete displacement of ACN in aqueous solvents,[11] and allow robust analysis in the HILIC mode. Both Gelpack GL-HilicAex and Asahipak NH2P-50 2D can be used at pH 2–13 and are stable under the basic conditions required for AEX mode. Ion exchange in AEX mode requires the AEX capacity of the packing material. The percentages of primary, secondary, and tertiary protonated amines and quaternary ammonium cations in Gelpack GL-HilicAex at pH 9.8 are 66%, 84%, 37%, and 100%, respectively. In comparison, the percentage of secondary protonated amines in Asahipak NH2P-50 2D at pH 9.8 is more than 93%, indicating that it has sufficient AEX properties.[11,18] Conversely, poly(glycerol dimethacrylate) particles have higher mechanical strength than poly(vinyl alcohol) packing materials,[19] so the column pressure resistance of Gelpack GL-HilicAex (pressure limit of 40 MPa) is higher than that of Asahipak NH2P-50 2D (pressure limit of 10 MPa), as shown in Table 1. Therefore, LC performance comparisons of polar metabolomic analysis using both columns were performed under optimal flow rate conditions within their respective column pressure limits (Table 1 and Fig. 2).

### 3.2. Retention behavior of polar metabolites on Asahipak NH2P-50 2D column using previously published LC separation conditions

Unified-HILIC/AEX has unique chromatographic separation properties that separate and elute cationic, uncharged, zwitterionic, and anionic polar metabolites by a continuous transition of separation mode from HILIC to AEX under analytical gradient elution conditions using a Gelpack

GL-HilicAex column and two mobile phases, ACN and 40 mM ABC solution (pH 9.8).[11] In contrast, a single-run metabolomic analysis method using an Asahipak NH2P-50 2D column has been reported.[12] Therefore, we first evaluated the separation behavior of polar metabolites under the previously reported LC/HRMS analysis conditions using the Asahipak NH2P-50 2D column. The LC/HRMS conditions (method B) using the Asahipak NH2P-50 2D column, set up according to a previous report,[12] were matched to our unified-HILIC/AEX/HRMS analytical condition (method A) using a Gelpack GL-HilicAex column, except for the ABC concentration, flow rate, and gradient conditions (Fig. 2). The separation behavior was evaluated using 52 hydrophilic metabolites with a wide range of physicochemical properties detected by LC/HRMS analysis of the HepG2 cell extracts as indicators (Table S1).

Unified-HILIC/AEX/HRMS analysis of 52 polar metabolites using the Gelpack GL-HilicAex column (method A) showed good retention, separation, and peak shape with good reproducibility (Figs. 3A, B) as previously reported.[11] In contrast, LC/HRMS conditions using the Asahipak NH2P-50 2D column (method B) retained 26 polar metabolites, including 2 cationic, 7 uncharged, 14 zwitterionic, and 3 anionic metabolites, ranging from adenosine to glutamic acid (Glu). Still, LC separation was poor, eluting in 1.8 min with RT ranging from 15.3 to 17.1 min (Fig. 3A). Similarly, 18 of the 23 anionic metabolites ranging from Flavin adenine dinucleotide (FAD) to guanosine 5'-triphosphate (GTP) were eluted in 3.3 min with RT ranging from 18.4 to 21.7 min (Fig. 3A). The leucine (Leu) and isoleucine (Ile) isomers could not be separated under LC/HRMS conditions (method B) using the Asahipak NH2P-50 2D column (Fig. 3C). In addition, dramatic peak tailing was observed for multivalent nucleotides such as ATP, uridine 5'-triphosphate (UTP), and GTP (Fig. 3D), resulting in larger FWHM values (Fig. 3B). This phenomenon is consistent with a previous study[12] and may be due to the use of a 20 mM ABC solution, which did not allow sufficient AEX with bicarbonate ions.[11] These results suggest that the stepwise separation mechanism and elution capabilities of the HILIC and AEX modes do not work well under LC/HRMS conditions (method B) using the Asahipak NH2P-50 2D column. Therefore, the use of a linear gradient until the percentage of aqueous solution reaches 50%, followed by isocratic elution using a concentration gradient of 40 mM ABC, may be essential for a smooth transition of the separation modes from HILIC to AEX.

### 3.3. Understanding stationary phase conditions to achieve unified-HILIC/AEX separation

Next, we evaluated the differences in LC separation behavior for the two stationary phases using two LC/HRMS analytical methods with matching conditions other than the flow rate, that is, method A using the Gelpack GL-HilicAex column and method C using the Asahipak NH2P-50 2D column (Fig. 2). The RTs and FWHMs of 52 hydrophilic metabolites detected in the two LC/HRMS analyses of the HepG2 cell extracts were used to evaluate the differences between the two analytical methods (Fig. 4). Surprisingly, the LC/HRMS retention behavior using the Gelpack GL-HilicAex and Asahipak NH2P-50 2D columns did not differ significantly in the elution order or FWHM, except for propylamine (Figs. 4A, B). Indeed, drawing a scatter plot with RTs of hydrophilic metabolites detected by both methods yielded a good $r$ of 0.9966 (Fig. 4C). The separation of the Leu and Ile isomers, which was not achieved with method B using the Asahipak NH2P-50 2D column (Fig. 3C), was also achieved with method C (Fig. 4D). Nucleotides such as ATP, UTP, and GTP, which showed significant peak tailing in method B (Fig. 3D), also showed good peak shape in method C (Fig. 4E). These results suggest that Gelpack GL-HilicAex and Asahipak NH2P-50 2D are stationary phases with similar retention behavior and separation performance. In method B using the Asahipak NH2P-50 2D column, the delayed elution of polar metabolites in the first half of the HILIC mode and the slightly wider peak width of anionic metabolites in the second half of the AEX mode have been suggested to be due to the lower flow rate.[20] In summary, the experimental results indicate that hydrophilic interactions and AEX capacity are necessary for the unified-HILIC/AEX separation and that amine(s)-modified alkali-resistant hydrophilic polymer packing material with a pressure resistance of approximately 40 MPa is the necessary stationary phase condition.

### 3.4. Effect of mobile phase and stationary phase conditions on MS detection sensitivity

The detection sensitivity of amino acids and anionic polar metabolites by unified-HILIC/AEX/MS tends to be lower than other analytical methods in previous studies,[9,11,21–26] although absolute comparisons cannot be made because of the different types and performance of the mass spectrometers used (Tables S3 and S4). Thus, it is important to understand the impact of stationary and mobile phases of the methods used on detection sensitivity to improve the comprehensiveness of single-run metabolomic measurements using unified-HILIC/AEX/HRMS. Here, we compared three LC/HRMS methods (methods A–C) (Fig. 2) based on the peak areas of 52 hydrophilic metabolites identified from HepG2 cells and the number of metabolic features detected by non-targeted analysis (Fig. 5). The peak area and peak height values of the unified-HILIC/AEX/HRMS using the Gelpack GL-HilicAex column (method A) were larger than those of methods B and C using the Asahipak NH2P-50 2D column (RTs 6–10 min), which were mainly separated and eluted in the HILIC mode (Figs. 5A, B). In method A, the RT in HILIC mode ranged from 6.2 min for thymidine to 13.4 min for Glu, a difference of 7.2 min, while in method C, the RT ranged from 10.6 min for thymidine to 16.2 min for Glu, a difference of 5.6 min. Thus, the HILIC separation efficiency of the Asahipack NH2P-50 2D column was lower than that of the Gelpack GL-HilicAex column, suggesting that the biological matrix in the HepG2 extract affected the peak area and height values. A possible reason for the lower HILIC separation efficiency of the Asahipak NH2P-50 2D column is that Gelpack GL-HilicAex, which has primary amine functional groups with strong hydrogen bonding, has a higher hydration layer formation capacity than Asahipak NH2P-50 2D, which has only secondary amine functional groups.[27]

In contrast, peak tailing of nucleotides such as ATP, UTP, and GTP was observed in method B using Asahipak NH2P-50 2D; however, the peak area values were higher than those in method A (Fig. 5A). This may be due to the fact that the bicarbonate ion concentration in method B

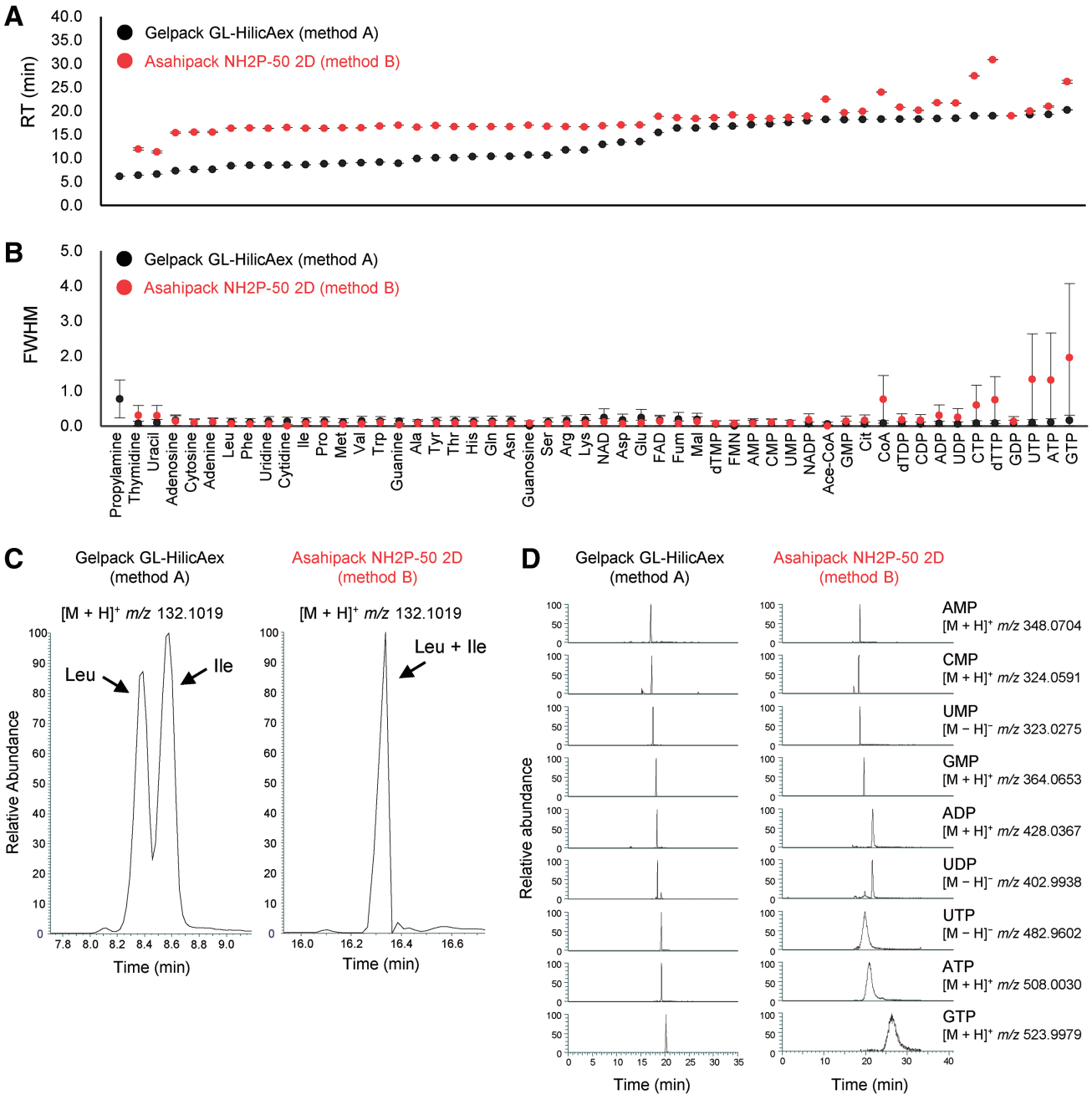


Fig. 3. Comparison of LC separation behavior of two LC/HRMS analytical methods (method A *vs.* method B), with 52 polar metabolites detected in HepG2 cells. RTs (A) and FWHMs (B) for identified metabolites, isomer separation of Leu and Ile (C), and LC/HRMS chromatograms of nucleotide-related metabolites (D). Values are presented as the mean ± standard error ($n$ = 5). See Table S1 for abbreviations of the 52 polar metabolites. FWHM, full widths at half maximum; HRMS, high-resolution mass spectrometry; LC, liquid chromatography; RT, retention time.

was approximately half that of method A, which reduced the background noise of bicarbonate ions. In fact, the total area values per LC/HRMS analysis of bicarbonate ion at $m/z$ 60.9926 ± 10 ppm $[M–H]^-$, observed for the three analytical methods (methods A, B, and C) were $1.0 \times 10^{12}$, $7.2 \times 10^{11}$, and $8.3 \times 10^{11}$, respectively, with the higher ABC concentration used and faster flow rate of method A was the highest (Fig. 6). In addition, FI-HRMS analysis of 10 μM ATP diluted with 0, 1, 10, 20, and 40 mM ABC showed a decrease in ATP peak area with increasing ABC concentration in both positive and negative ion modes (Fig. 7).

The impact of LC/HRMS detection sensitivity was also assessed based on the number of metabolic features detected from HepG2 cells using non-targeted analysis. The RTs for each condition were divided using the RT of Glu, the boundary between the HILIC and AEX modes validated in the previous unified-HILIC/AEX/HRMS analysis,[11] as an index to compare the number of metabolic features (method A, RT 0–13.5 min and 13.5–28.0 min; method B, RT 0–17.1 min and 17.1–30.0 min; and method C, RT 0–16.2 min and 16.2–28.0 min) (Fig. 5C). Unified-HILIC/AEX/HRMS (method A) using the Gelpack GL-HilicAex column detected 3697

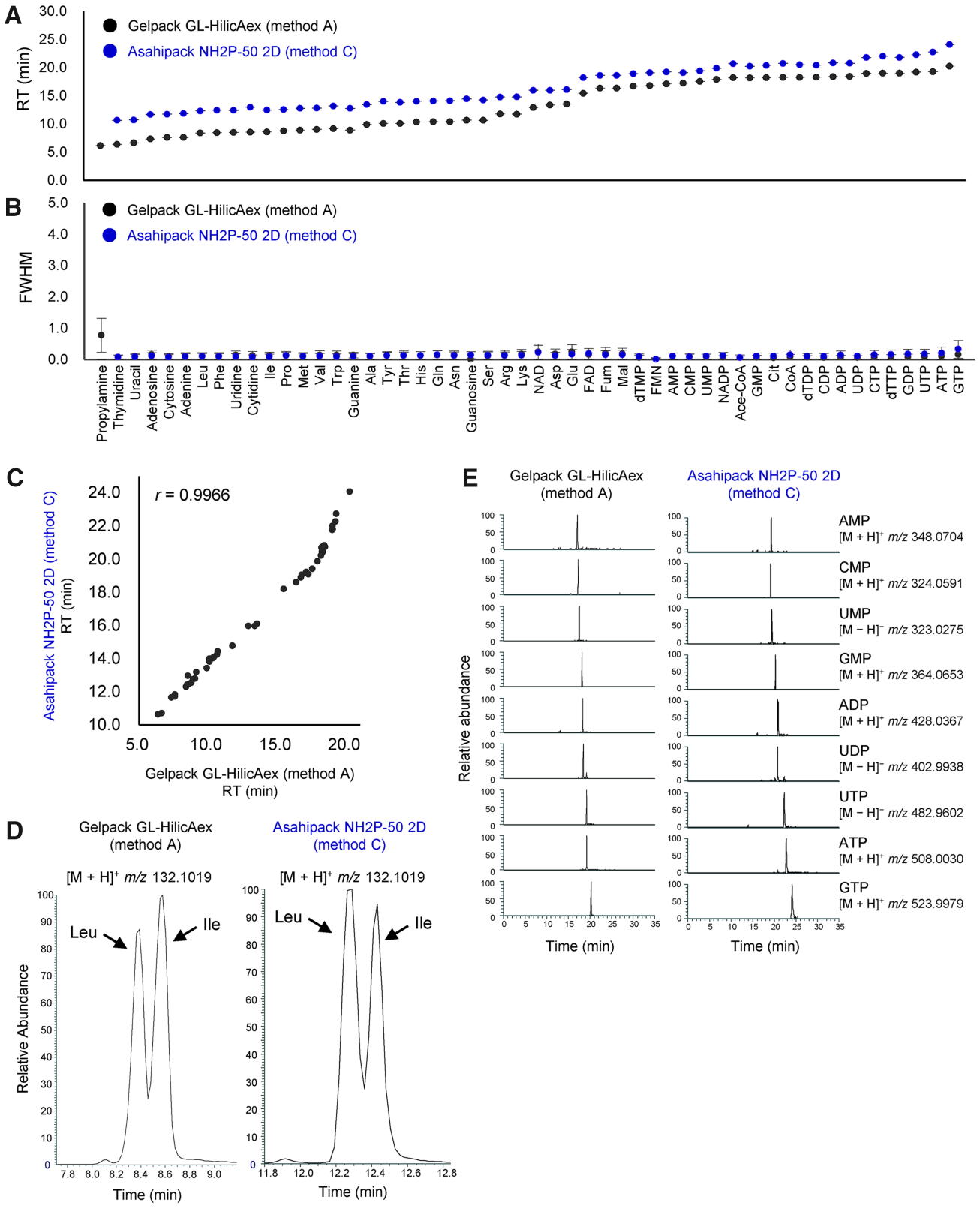


Fig. 4. Comparison of LC separation behavior of unified-HILIC/AEX/HRMS analytical methods (method A *vs.* method C) using two different columns with 52 polar metabolites detected in HepG2 cells. RTs (A) and FWHMs (B) for identified metabolites, scatter plots based on RTs of hydrophilic metabolites detected by both methods (C), isomer separation of Leu and Ile (D), and LC/HRMS chromatograms of nucleotide-related metabolites (E). Values are presented as the mean ± standard error ($n$ = 5). Spearman's rank correlation coefficient ($r$) was calculated using the Pandas and NumPy libraries in Python. See Table S1 for abbreviations of the 52 polar metabolites. FWHM, full widths at half maximum; RT, retention time; Unified-HILIC/AEX/HRMS, unified-hydrophilic interaction/anion exchange liquid chromatography/high-resolution mass spectrometry.

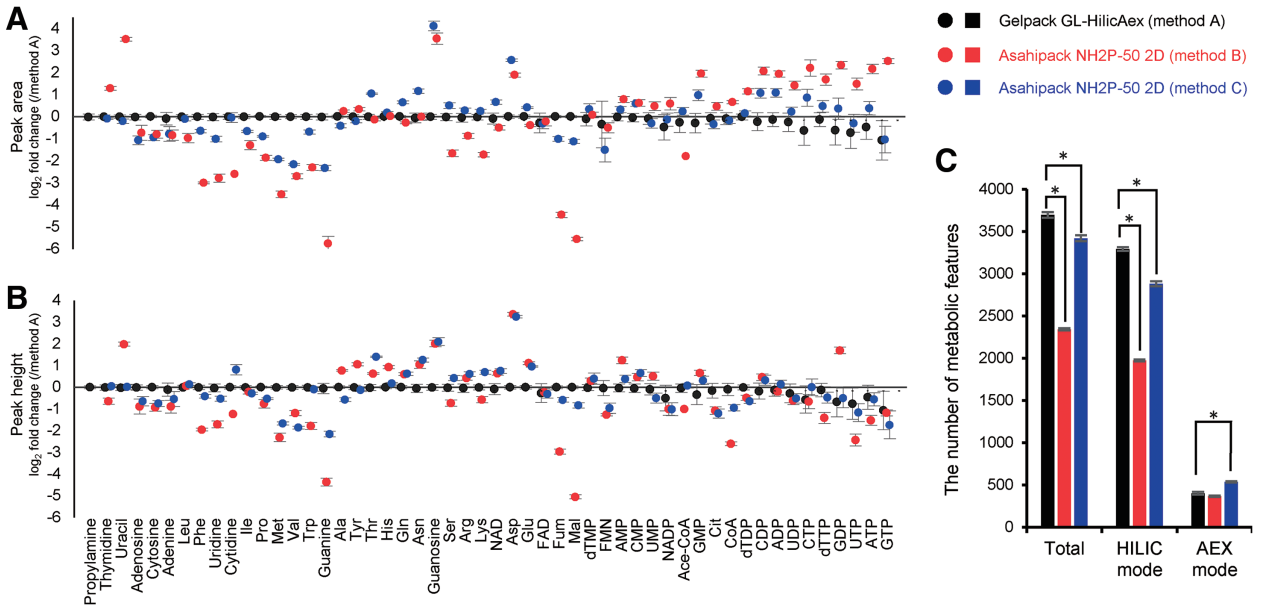


Fig. 5. Sensitivity evaluation among three LC/HRMS analytical methods. Peak areas (A) and peak intensities (B) of identified metabolites and the number of metabolic features detected in non-targeted analysis from HepG2 cell extracts (C). Values are presented as the mean ± standard error ($n$ = 5). Statistical significance was determined using Two-way ANOVA followed by Dunnett's multiple comparisons test (*$p$ <0.0001). See Table S1 for abbreviations of the 52 polar metabolites. AEX, anion exchange; ANOVA, analysis of variance; HILIC, hydrophilic interaction liquid chromatography; HRMS, high-resolution mass spectrometry; LC, liquid chromatography.

metabolic features at RT 0–28.0 min, of which 3294 (89.1%) were detected in HILIC mode and 403 (10.9%) in AEX mode. Similarly, method B using the Asahipak NH2P-50 2D column detected 2343 metabolic features at RT 0–30.0 min, 1975 (84.3%) at RT 0–17.1 min and 368 (15.7%) at RT 17.1–30.0 min. In addition, method C using the Asahipak NH2P-50 2D column detected 3420 metabolic features at RT 0–28.0 min, of which 2882 (84.3%) were detected in HILIC mode and 538 (15.7%) in AEX mode (Fig. 5C). In the HILIC mode region, method A using the Gelpack GL-HilicAex column had a higher number of metabolic features, while method C using the Asahipak NH2P-50 2D column had a higher number of metabolic features in the AEX mode region. This was similar to the results of the 52 polar targeted metabolite analysis (Figs. 5A, B). One of the reasons for the relatively higher number of metabolic features in the AEX mode region in method C compared to method A has been suggested to be the effect of increased sensitivity due to lower flow rates.[20] However, the present experimental results alone do not provide a definitive explanation for the improved detection sensitivity of Asahipak NH2P-50 2D in AEX mode compared with Gelpack GL-HilicAex. Further improvement of the column bleed problem, development of a stationary phase with reduced ion exchange capacity that can reduce ABC concentration while maintaining separation performance, and mobile phase conditions for high-sensitivity analysis are needed. In addition, the pH of the ABC solution used as the mobile phase in unified-HILIC/AEX/HRMS is 9.8, so caution must be exercised in the results for metabolites such as nicotinamide adenine dinucleotide, which are generally unstable under basic conditions.[28]

In summary, by evaluating the mobile phase conditions of unified-HILIC/AEX/HRMS and the stationary phase conditions of different series of amine-modified hydrophilic polymers, we identified the key factors for the two-step separation and retention mechanism of HILIC and AEX. In particular, we reconfirmed that the mobile phase conditions of the linear gradient from ACN to $H_2O$ and AEX by isocratic elution with 40 mM ABC were essential for the separation mechanism of unified-HILIC/AEX. Furthermore, the stationary phase features required to achieve unified-HILIC/AEX separation were found to be amine(s)-modified alkali-resistant hydrophilic polymer packing material with a pressure resistance of approximately 40 MPa, which is required for hydrophilic interactions and AEX capacity. Although at least one secondary amine in the stationary phase reproduces the unified-HILIC/AEX separation, further verification is required, including the need for a primary amine in terms of separation performance and sensitivity. In addition, several improvements were found in the AEX mode region of the unified-HILIC/AEX/HRMS that improve detection sensitivity compared to the HILIC mode region. Specifically, i) reducing the additive concentration (*i.e.*, ABC and ammonium hydroxide) by reducing the AEX capacity while maintaining the hydration layer formation in HILIC, ii) evaluating the effect of column bleed, iii) evaluation of sensitivity improvement by adding an organic solvent after column separation, and iv) designing new stationary phases using different materials. These advances are expected to improve the coverage and detection sensitivity of single-run hydrophilic metabolomics analysis and contribute to the analysis of rare samples and large-scale metabolomics.

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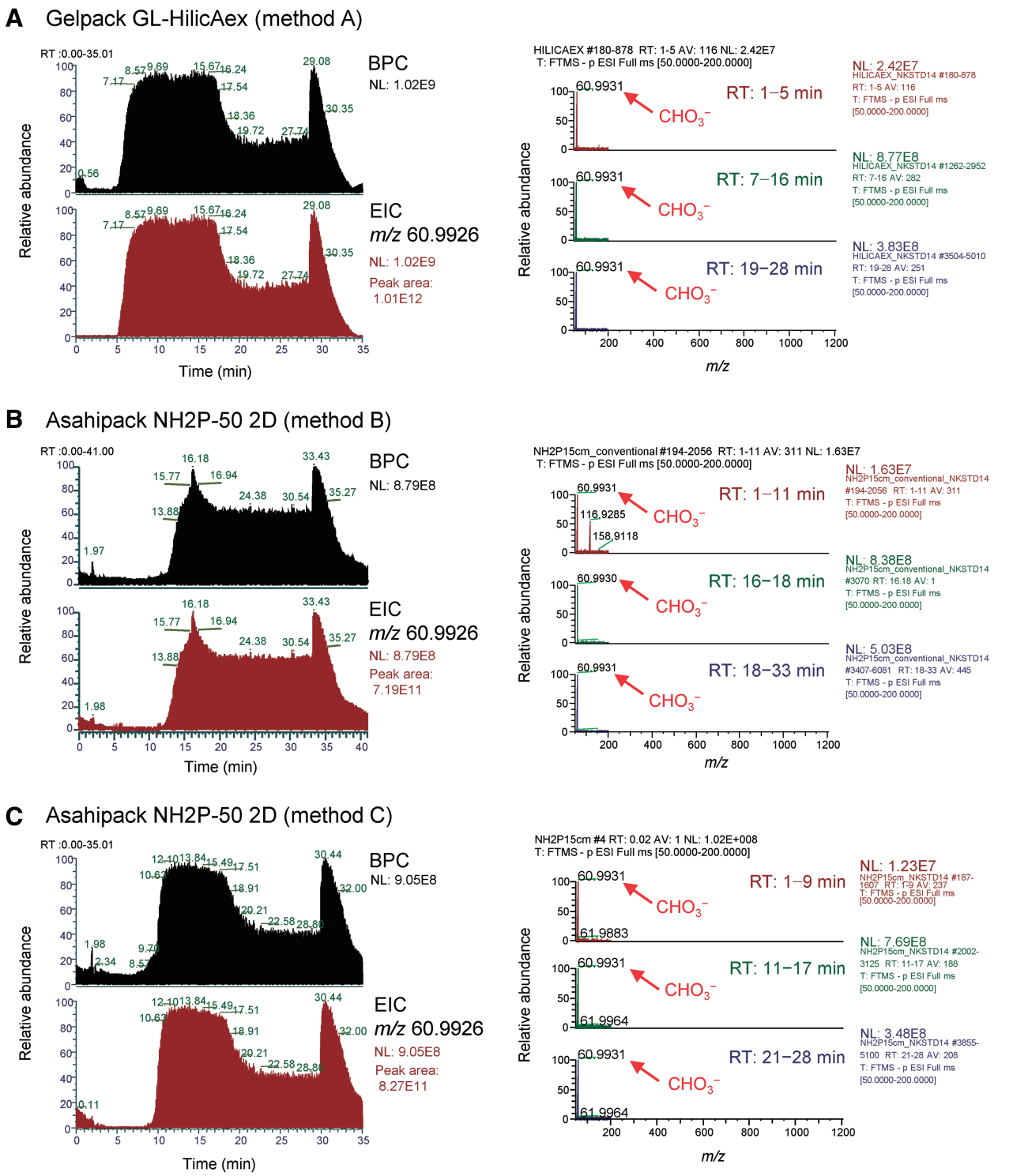


Fig. 6. Monitoring and comparison of bicarbonate ions in three LC/HRMS methods. BPCs, EICs of bicarbonate ions, and average mass spectra in the *m/z* 50–200 at each RT for negative ionization for methods A (A), B (B), and C (C). BPCs, base peak chromatograms; EICs, extracted ion chromatograms; HRMS, high-resolution mass spectrometry; LC, liquid chromatography; RT, retention time.

AMED-CREST (JP23gm1010010 for T.B.) from Japan Agency for Medical Research and Development (AMED); AMED-BINDS (JP22ama121055 for T.B.); AMED-P-PROMOTE (22ama221213h0001 for Y.I.); and KAKENHI (JP23KJ1688 for K.I., JP20K15101 for M.T., JP22H01883 for Y.I., JP22K18924 for Y.I., and JP22H05185 for Y.I.,) from Japan Society for the Promotion of Science (JSPS). This work was also partly performed in the Medical Research Center Initiative for High Depth Omics Program of the Medical Institute of Bioregulation (MIB) and the Cooperative Research Project Program of the MIB, Kyushu University.

## CONFLICTS OF INTEREST

The authors declare no competing financial interest.

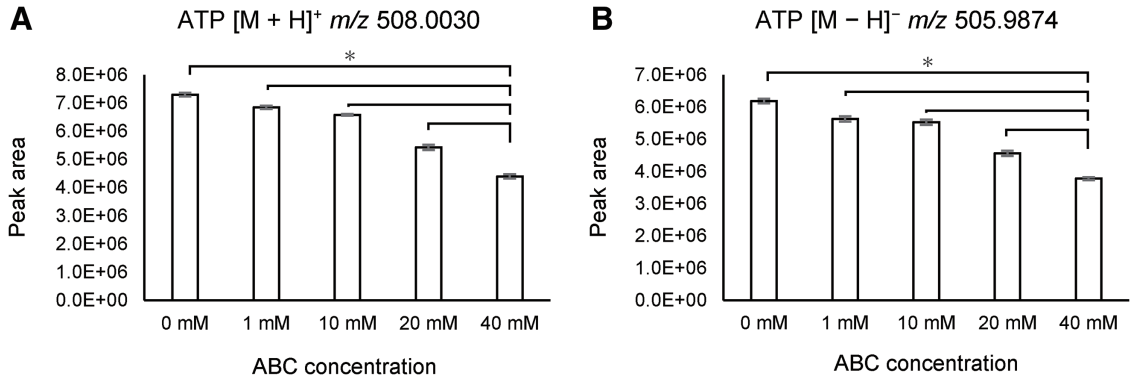


Fig. 7. FI-HRMS analysis of 10 μM ATP diluted in 0, 1, 10, 20, and 40 mM ABC solutions. Peak areas of ATP in positive ionization (A) and negative ionization (B). Values are expressed as mean ± standard error ($n$ = 3). Significant differences were calculated by Dunnett's test (*$p$ <0.0001 compared to 40 mM ABC condition). ABC, ammonium bicarbonate; ATP, adenosine 5'-triphosphate; FI-HRMS, flow injection-high-resolution mass spectrometry.

## NOTE

The study represents a portion of the dissertation submitted by K. Ikeda to Kyushu University in partial fulfillment of the requirement for his Ph.D. degree.

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