Serum Amine Profiling through Fluorine-Labeled ^{19}F NMR Analysis

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Abstract

We present a novel approach utilizing ^{19}F-nuclear magnetic resonance (NMR) spectroscopy for serum amine profiling. Our method introduces a highly efficient and reliable technique for fluorine labeling of amine metabolites via Schiff base formation. By employing this fluorine labeling, we successfully achieve accurate identification and quantification of amine metabolites in human serums, providing valuable insights for metabolomics research.

Main Text

Amino acids and biogenic amines play a crucial role in various physiological processes. Understanding the metabolic pathways and regulation of these amines through amine metabolite profiling is essential for unraveling the underlying mechanisms of diseases and disorders, including neurological and metabolic disorders, and cancer.[1, 2] Moreover, amine metabolite profiling has significant implications in drug discovery, toxicology, and personalized medicine.[3, 4] The current techniques for amino acid profiling predominantly rely on the use of mass spectrometry (MS) in conjunction with chromatographic methods like liquid chromatography (LC) or gas chromatography (GC).[5, 6] To enable the identification and quantification of amine metabolites, researchers have developed diverse derivatizing reagents, LC modes, and advanced MS detection methods.[7–9] These advancements contribute to more accurate and specific measurements of amino acids, enhancing the precision and reliability of amino acid profiling. Although MS is a highly sensitive technique that can detect a wide range of metabolites in complex mixtures, it can be limited by matrix effects and ion suppression.[10] These factors can affect the accuracy and reproducibility of the results.

Nuclear magnetic resonance (NMR) spectroscopy is employed as a valuable complementary analytical technique for metabolite analysis.[11,12] The utilization of NMR is driven by its quantitative and non-destructive nature, which enables a detailed examination of the structure and dynamics of metabolites in solution. In particular, ^{1}H NMR is the preferred approach in NMR-based metabolite analysis, owing to the prevalence of ^{1}H atoms in most metabolites. Nevertheless, the resulting spectra pose significant complexity due to the presence of overlapping peaks. Since nearly all metabolites, as well as water, contain ^{1}H atoms,

distinguishing and interpreting different metabolites becomes challenging. Various approaches can improve the signal resolution of ^{1}H NMR spectra. These methods include elevating the magnetic field strength (typically 600 MHz or higher), incorporating cryogenic probes, employing pulse sequences, and implementing spectral editing techniques.[13] However, the simultaneous detection of multiple metabolites still poses a challenge in ^{1}H NMR analysis.

In order to simplify the signaling outcomes in NMR spectra, heteronuclear NMR techniques, such as ^{13}C, ^{15}N, ^{19}F, ^{29}Si, ^{31}P, and others, can be employed. Among these, ^{19}F NMR has emerged as a valuable method for investigating metabolic pathways and the impact of fluorine substitution on drugs or other compounds.[14] ^{19}F NMR spectroscopy possesses advantageous properties, including 100% natural abundance, a relative receptibility of 0.834, and a wide chemical shift range of over 400 ppm. This technique offers a broader chemical shift range and simpler spectra compared to ^{1}H NMR, making it effective for analyzing fluorinated compounds. However, there is a significant challenge in utilizing ^{19}F NMR for metabolite profiling. To make metabolites detectable by ^{19}F NMR, fluorine-containing functional groups need to be incorporated into them. Therefore, the reliability, specificity, and generality of the F-labeling method are crucial for successful ^{19}F NMR-based metabolite analysis. Although several F-labeling protocols exist for amines and alcohols, they have not yet been optimized for metabolite analysis.[15–18] In our study, we have developed a highly efficient and reliable F-labeling group for primary amines, and we have demonstrated its effectiveness in providing a comprehensive analysis of amine metabolites in serum using ^{19}F NMR (Figure 1A).

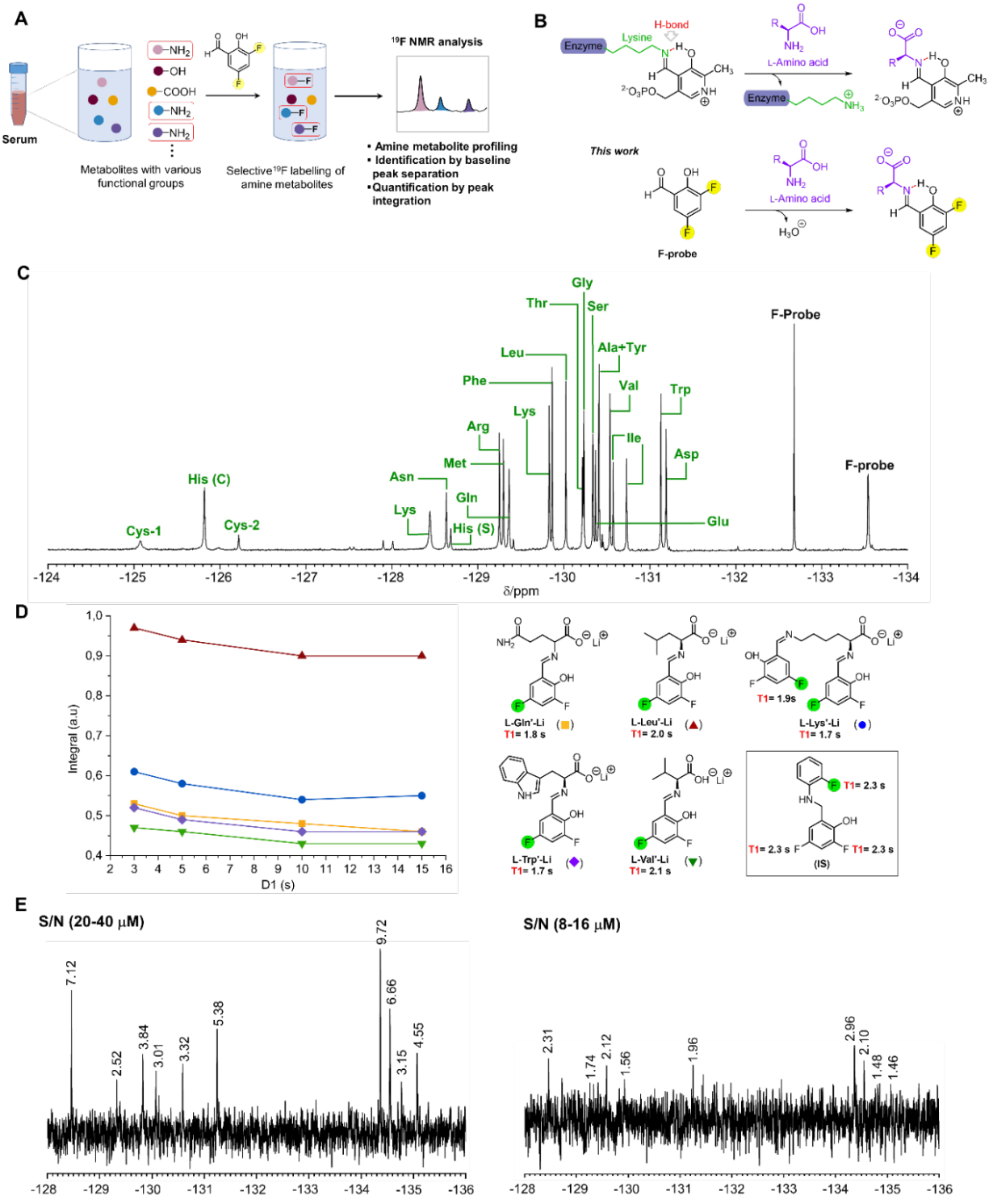

Figure 1. ^{19}F NMR-based detection of fluorine labeled amino acids. (A) F-Labeling Strategy for Specific Analysis of Primary Amine Metabolites. (B) Pyridoxal phosphate-dependent transamination and biomimetic imine formation. (C) Simultaneous detection of 19 amino acids. (D) Correlation analysis of D1 and integration for imines and internal standard. (E) Limit of detection determination through 376 MHz ^{19}F{^{1}H} NMR spectroscopy.

Our development of an F-labeling reagent was inspired by the biological cofactor, pyridoxal phosphate (PLP), which is involved in amino acid metabolism, particularly in the transamination, decarboxylation, and deamination of amino acids (Figure 1B).[19] The reagent uses an aldehyde functionality to react with amino acids to form Schiff bases, which are further stabilized by the 2-hydroxyl group with intramolecular hydrogen bonds. Taking inspiration from this natural Schiff base formation, we utilized fluoro-salicylaldehyde as the fluorine-labeling group for amine metabolites. Due to the stabilization by the intramolecular hydrogen bonding, the imine formation was complete immediately after mixing reagents and the Schiff base products were stable in solutions. Out of the 20 amino acids, all except proline have the ability to react with fluoro-salicylaldehyde, leading to the formation of Schiff bases. The formation of Schiff bases was successfully confirmed, and they were isolated and identified without ambiguity (Supplementary Scheme 1). Notably, Cys and His undergo the formation of cyclic products through reactions involving the imine bonds and their respective side chain thiol and imidazole functionalities. The NMR spectra of these cyclic products display two distinct sets of ^{19}F signals, suggesting the existence of an equilibrium between the Schiff bases and the cyclic forms.

We investigated to assess the peak resolution of Schiff base mixtures derived from 19 amino acids using $^{19}F\{^{1}H\}$ NMR spectra. Our findings indicate that the fluorine atom positioned at the 5-position of the salicylaldehyde is more effective in spreading signals compared to the 3-position. Significantly, the combination of two fluorine groups in 3,5-difluoro salicylaldehyde led to a remarkable improvement in signal resolution, particularly for the fluorine signals located at the 5-position (Supplementary Figure 1). In contrast to the typical F-F coupling observed in difluoro benzenes, the existence of an electronegative oxygen substituent in 3,5-difluoro salicylaldehyde yields singlet fluorine signals. This characteristic enables easy and accurate detection of individual metabolites. In this study, we selected 3,5-difluoro salicylaldehyde as the F-probe, primarily utilizing the F signals at the 5-position to identify and quantify the Schiff bases. The utilization of the F-probe successfully achieved a full separation of baseline peaks for 19 amino acids in the $^{19}F\{^{1}H\}$ NMR spectra (Figure 1C). The majority of amino acid signals were detected within a range of 4 ppm (-131.5 to -128.5 ppm), whereas cysteine and histidine displayed signals at -126 ppm. These results clearly demonstrate that the F-probe provides a satisfactory level of peak resolution for identifying and quantifying the 19 amino acids, as evident from the $^{19}F\{^{1}H\}$ NMR spectra of the Schiff bases (Supplementary Figure 2).

Accurate quantification of metabolites is crucial for metabolite research. However, the application of ^{19}F NMR is hindered by the wide bandwidth (± 300 ppm), which leads to significant resonance offset effects.[20] These effects distort both signal intensities and signal phases, ultimately compromising the accuracy of signal integration. To minimize the resonance offset effect, it is essential to use analytes and reference materials with similar spin-lattice relaxation time (T1).[21] Fortunately, the T1 values of five Schiff bases, measured to be 1.7 - 2.1 s, indicate that F-labelled Schiff bases possess similar T1 values (Figure 1D). This similarity ensures precise and reliable integration. Furthermore, for absolute concentration determination, a suitable internal standard is necessary. To address this requirement, we prepared a well-suited internal standard (IS) containing three F groups, exhibiting T1 values of 2.3 s (Supplementary Scheme 2). Through

the implementation of relaxation delays (D1) ranging from 1 to 15 s, consistent and stable integration results were observed when using this IS (Figure 1D). In contrast, the utilization of fluorobenzene, 4-fluorobenzophenone, and 2,4-difluorophenol, with T1 values of 4.0 s, 4.1 s, and 4.5 s respectively, exhibited inconsistent responses at different D1 values (Supplementary Figure 4). It is widely acknowledged that quantitative NMR analysis necessitates multiplying D1 by a factor of at least five times the longest resulting T1.[21] Our measurements revealed that a D1 value of 10 s ensures reliable quantitative analysis.

The investigation also involved assessing the limit of detection (LOD). Signal-to-noise (S/N) values were determined using 376 MHz $^{19}F\{^{1}H\}$ NMR spectroscopy. S/N values ranging from 2.5 to 9.7 were observed for samples in the 20-40 μM range. For samples in the 8-16 μM range, marginal S/N values of 1.5 to 3.0 were obtained, suggesting a lower detection limit of approximately 15 μM. The study aimed to showcase the profiling of amino acids using easily accessible NMR instruments. However, to improve the detection limit, it is advisable to increase the magnetic field strength and utilize a cryogenic probe.[13]

The ^{19}F NMR analysis with the F-probe was performed ^{19}F NMR analysis of human serum. Following a previously established protocol for metabolite extraction, 1 mL of serum was treated with 2 mL of MeOH. Protein precipitates were formed by placing the mixture at -20°C for 20 minutes, and the resulting aqueous methanol solution was filtered to obtain the metabolite mixtures for analysis.[22] The volatiles were then evaporated under reduced pressure. Subsequently, a 0.01 M F-probe solution in MeOH (4 mL) was added, and the solution was sonicated for 15 to 20 minutes to ensure the complete formation of Schiff bases. To prevent inconsistencies in peak positions and resolution, the methanolic solution was concentrated and completely dried to remove any residual water. For the NMR measurement, the dried residue was dissolved in 0.75 mL of CD_3OD, and an internal standard (IS, 20 μL) was added. The ^{19}F NMR measurements were performed on 400 MHz NMR instruments (376 MHz for ^{19}F) using a pulse angle of 90°, 200 scans, a relaxation delay (D1) of 10 s, and an acquisition time of 1.4 s. The entire measurement process took approximately 40 minutes.

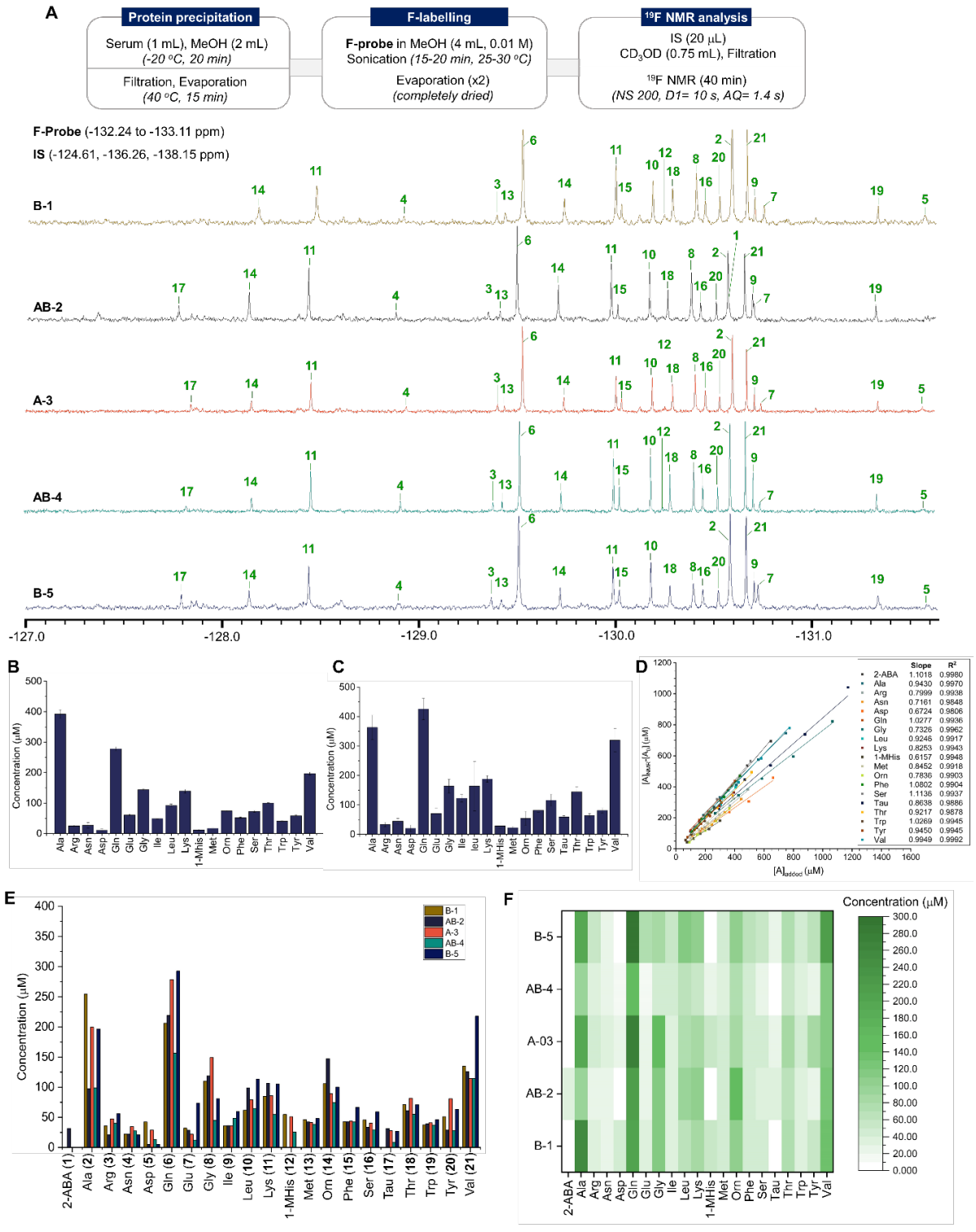

Figure 2. The detection and quantification of amine metabolites in human serum. (A) $^{19}F\{^{1}H\}$ NMR spectra of human serums with F-labelling. (B) Amine metabolites obtained from five repeated measurements of filtered serum B-1. (C) Amine metabolites obtained from 5 replicates of 1 mL serum AB-4. (D) Calibration curves for amino acids and amines (E) Absolute concentrations of amino acids and amines calibrated in five human serum samples. (F) Heatmap representation of detected metabolites in

five human serum samples. The color scale ranges from highest abundance (green) to lowest abundance (white) to indicate the relative levels of metabolites.

The $^{19}F\{^{1}H\}$ NMR spectra for five serum samples (B-1, AB-2, A-3, AB-4 and B-5) were obtained from the above procedure, showing amino acids and amines were clearly analyzed (Figure 2A). In the region of -131.5 to -127.5 ppm, seventeen amino acids, three amino acid derivatives such as L-Ornithine, 1-Methyl-Histidine, and 2-Aminobutyric acid, and one biogenic amine, Taurine, were clearly detected due to the baseline peak separation of all metabolites. However, His and Cys, which exists as an equilibrium mixture of Schiff base and the cyclic form, showed weak, broad, and variable signals that are quite difficult to correctly assign their concentration. Accordingly, the quantification of His and Cis is not applied to human serum. In addition, the spiking experiments indicated that no traces of α(2)-Aminobutyric acid, β(3)-Aminobutyric acid, ɣ(4)-Aminobutyric acid, N_{α}-Acetyl-D-Lysine, β-Homoleucine, L-2-Aminoadipic acid, Tryptamine, Putrescine, Methylamine, Tyramine, and DL-Norepinephrine were found in the examined human serums.

To determine the absolute concentration of serum amine metabolites, we conducted validation and verification experiments to confirm the quantification method. Initially, we prepared analytical mixtures by removing proteins from 5 mL of serum through filtration. Following the addition of an internal standard (IS), the filtrate was divided into five vials and individually analyzed using the F-probe. The concentrations of amino acids exceeding 40 μM exhibited consistent results, with a relative standard deviation (RSD) ranging from 1.9% to 5.3%. However, amino acids with concentrations ranging from 11-25 μM displayed considerable variability, with RSD values ranging from 13.6% to 42.6%. While experimental errors are expected for analytes with low concentrations, the concentration of F-labelled amino acid metabolites was accurately determined.

Moreover, we obtained five separate 1 mL serum samples and analyzed them independently. These results confirm the reliability of the F-labelling protocol and the metabolite extraction method using MeOH. Specifically, the RSD values for amino acid concentrations exceeding 40 μM ranged from 5.8% to 21.6%. However, the errors observed for low-concentration metabolites became more pronounced, reaching up to 54%. These replicate experiments underscore the reliability of ^{19}F NMR analysis for F-labelled amino acids and highlight the need for further optimization of the serum extraction method to ensure consistent and comparable results.

Calibration is then conducted to determine the actual concentration. Analysis of amino acid mixtures at various concentrations consistently reveals strong linear relationships, with all plots exhibiting average R^2 values of 0.9941, indicating excellent correlation. Moreover, reference standards with known concentrations are added to the serum extracts, resulting in linear plots depicting increased NMR intensities corresponding to the added concentrations. These plots also demonstrate reliable calibration, with average R^2 values of 0.99. The slopes obtained, ranging from 0.616 to 1.11, serve as conversion factors for determining the actual concentrations. Applying these conversion factors, we successfully determine the actual concentrations of five serum samples, B-1, AB-2, A-3, AB-4, and B-5 and their relative concentrations are

illustrated in Figure 2E. Additionally, a contour plot is provided to visually depict the relative concentrations of each amino acid metabolite.

In conclusion, the development of the F-labeling reagent inspired by pyridoxal phosphate (PLP) has proven to be a successful approach for amino acid profiling using ^{19}F NMR spectroscopy. The utilization of fluoro-salicylaldehyde as the fluorine-labeling group enabled the formation of Schiff bases, which were stable and easily detectable in solutions. The F-probe, specifically 3,5-difluoro salicylaldehyde, demonstrated excellent peak resolution for the 19 amino acids, allowing for their identification and quantification in ^{19}F NMR spectra. The use of suitable internal standards and optimization of relaxation delays ensured accurate and reliable quantification of metabolites. The ^{19}F NMR analysis of human serum samples validated the applicability of the developed method, enabling the clear detection and quantification of amino acids and amines. However, further optimization of the serum extraction method is required to achieve consistent results for low-concentration metabolites. Overall, this study provides valuable insights into amino acid profiling using F-labeling and ^{19}F NMR spectroscopy, laying the foundation for future research in metabolomics and advancing our understanding of physiological processes and disease mechanisms.

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Methods

Preparation of Schiff bases from amino acids

To the solution of amino acid (1 mmol) in MeOH (Samchun) (3 mL) was sequentially added LiOH·H_2O or tetramethylammonium hydroxide (TMAOH) (Sigma Aldrich) (199 mg, 1 mmol) and 3,5-difluorosalicylaldehyde (Manchester Organics) (140 mg, 1 mmol). The reaction was stirred for 1 h at room temperature. After all volatile residues were removed under reduced pressure, the resulting mixture was washed with CH_2Cl_2 : *n*-hexane (1:1) to afford L-AA'-Li or L-AA'-TMA as a yellow solid.

^{19}F{^{1}H} NMR data acquisition and processing. All ^{19}F NMR experiments were performed at 298 K on a Bruker AVANCE III HD (9.4 T) 400 MHz spectrometer equipped with 60 slot automatic sample handler and BBFO 400Mhz S1 5mm with z-gradient or Bruker AVANCE NEO Nanobay (9.4 T) 400 MHz with iProbe HR Liquids. ^{19}F{^{1}H} NMR (Inverse-gated decoupling) spectra were recorded at 376 MHz using the Brusker 'zgfhigqn' pulse sequence with the optimized parameters (200 scans for human serum samples and other validation experiments) an acquisition time of 1.4 s, pulse angle of 90°). Topspin 3.6.5 or 4.1.4 on CentOS software packages were used for NMR data acquisition. Total experiment time with the optimized parameters for the detection and quantification of amine metabolites was around 40 min.

^{19}F{^{1}H} NMR spectra were processed and analyzed using MNOVA Software (Mestrelab, version 12.0.3). Spectra were phased and corrected using manual correction or automatic adjustment (Splines and Whittaker Smoother function) to achieve a flat baseline. The signal of noise (S/N) was increased by setting of Apodization (Exponential at 1 Hz). The chemical shifts were reported in parts per million (ppm) and

referenced to ^{19}F signals of IS whose NMR signal was set at -138.15 ppm. Peak picking and integration were performed manually.

Identification of F-labelled amino acid mixtures in $^{19}F\{^{1}H\}$ NMR spectra

A 1 M stock solution of individual amino acids (TCI or Sigma) (1 mmol) and TMAOH (Sigma) (181 mg, 1 mmol) was prepared in a volumetric flask by dissolving them in 1 mL of CD_3OD (Deutero). In a vial, a mixture of amino acids was created by combining 10 µL of each individual amino acid stock solution (1 M) with 0.47 mL of CD_3OD (Deutero). To this solution, 3,5-difluorosalicylaldehyde (Manchester Organics) (4.2 mg, 0.03 mmol) was added and stirred for 10-15 minutes. Firstly, mixtures of three amino acids were prepared and identified. Secondly, two mixtures containing nine amino acids each were prepared and identified by comparing the $^{19}F\{^{1}H\}$ NMR spectra of the three mixtures. Finally, a mixture of 19 amino acids was created and simultaneous detection was performed (Figure 1C and Supplementary Figure 2).

Calibration curve (L-AA'-TMA). Linearity was investigated at five concentration points. Stock solutions of five or six amino acids (TCI or Sigma Aldrich) with varying concentrations (2-35 mM) were prepared in 4 mL of MeOH (Samchun) in the presence of TMAOH (an equivalent amount of TMAOH was added until the solid completely dissolved in MeOH). Study samples were prepared by diluting the stock solution to 0.5 mL with MeOH (Samchun) to achieve concentrations within the desired range (dilution factors of 10, 13, 17, 20, and 25). After adding 3,5-difluorosalicylaldehyde (Manchester Organics) (2-3 mg) and stirring for 10 min at 25 - 30 °C, the resulting mixture was evaporated and dissolved in 0.5 mL of CD_3OD (Deutero) in an NMR tube (Deutero, No. Boroeco-5-7). $^{19}F\{^{1}H\}$ NMR spectra were recorded, and plots were generated based on the peak area of each component at each concentration level. The slope and correlation coefficient (R2) were determined using regression analysis (Supplementary Figure 5).

Calibration curve (Serum). To prepare the stock solution, 23 selected amino acids and amines (1.9-5.5 mg) were dissolved in a mixture of MeOH:H_2O (8:2, 10 mL). A total of 5 mL of human serum (AB-4) was stirred with 10 mL of MeOH (Samchun) for 30 seconds and then placed in the freezer at -20 °C for 20 min. The protein precipitate was filtered out, resulting in 15 mL of filtrate. For the initial sample, 2 mL of the filtrate was transferred to a vial. The remaining filtrate was mixed with an internal standard (1.4 mg) and divided into 5 vials (2 mL each). To each vial, 200 µL, 150 µL, 110 µL, 80 µL, and 50 µL of the amino acid and amine stock solution were added, respectively. Subsequently, 3,5-difluorosalicylaldehyde (Manchester Organics) (6.3 mg) was added to each vial along with 4 mL of MeOH (Samchun), and the solution was sonicated at 25 – 30 °C for 15 - 20 min. The resulting mixtures were evaporated to remove the solvent and water residue. NMR samples were prepared in CD_3OD (Deutero) (0.75 mL) in NMR tubes (Deutero, No. D400-5-8). $^{19}F\{^{1}H\}$ NMR spectra were recorded, and the concentration of each component was determined based on the relative integration and the concentration of the internal standard. Plots were generated based on the difference in the NMR response of each compound in the initial and spike-in samples at each

concentration level. The slope and correlation coefficient (R2) were determined using regression analysis in Origin Lab (Figure 2D and Supplementary Table 1).

Serum samples. All human serum samples (fresh frozen plasma (FFP)) were acquired from the Korean Red Cross Daejeon Sejong Chungnam Blood Center in Daejeon, with approval from the KAIST Institutional Review Board. The FFP samples were prepared from five healthy blood-donor volunteers with blood group RH+/AB (No. 09-2303-2 and 09-2303-4), RH+/B (No. 09-2303-1 and 09-2303-5), and RH+/A (No. 09-2303-03). The plasma was separated from the whole blood, and the FFP samples were immediately stored at -18 °C after collection. Each blood type's serum was named as AB-2, AB-4, B-1, B-5, and A-3 and was aliquoted into separate 1 mL vials, which were promptly stored at -20°C or below until analysis. Prior to use, the 1 mL aliquot of frozen serum sample was thawed at room temperature for 15 minutes.

Detection and quantification of amino acids and amines in serum. To precipitate proteins, 2 mL of MeOH (Samchun) was added to a 1 mL serum aliquot (1:2 v/v ratio).[22] The mixture was incubated at -20 °C for 20 min before being filtered through a syringe filter (Advantec, cat. no. AD.25HP020AN, 0.2 μm, 25 mm) for metabolite extraction. The resulting filtrate was concentrated to less than 0.1 mL using a rotary evaporator (Buchi) and was then ready for reaction with 3,5-difluorosalicylaldehyde (Manchester Organics) (6.3 mg, 0.01 M in MeOH). After adding the fluorine reagent, the sample was stirred at 25 – 30 °C for 15-20 min, followed by evaporation of the solvent. The dried sample was dissolved in 2 mL of MeOH (Samchun) and evaporated using a rotary evaporator (Buchi). This step was repeated three times to remove any residual water. An NMR sample was prepared in 0.75 mL of CD_3OD with the presence of an internal standard (IS) (9.1 mM, 10 - 20 μL) and transferred to an NMR tube (Deutero, No. D400-5-8). For the detection of amino acids, 10 μL of AA'-TMA and Amine-TMA stock solutions were added to the sample. $^{19}F\{^1H\}$ NMR spectra (376 MHz) of the solution were recorded, and the ^{19}F signal of the target compounds was identified based on the increase in peak intensity (Supplementary Figure 6). The absolute concentration of the detected amino acids and amines was calculated based on the concentration of the internal standard in the $^{19}F\{^1H\}$ NMR spectra (376 MHz) and corrected using the slope and intercept values from the calibration curve (Supplementary Table 4).

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