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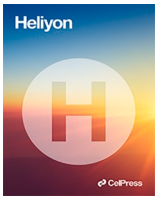

Research article

# A UPLC-MS/MS method for simultaneous determination of arachidonic acid, stearic acid, and related endocannabinoids in human plasma

Xiaojing Qian [a,b,1], Wangzhenzu Liu [a,1], Ying Chen [c], Jiaqi Zhang [b], Yuanye Jiang [d], Lingyun Pan [a,*], Cheng Hu [a,**]

[a] *Shanghai University of Traditional Chinese Medicine, Shanghai, 201203, China*
[b] *Department of Pharmacy, Shanghai Municipal Hospital of Traditional Chinese Medicine, Shanghai University of Traditional Chinese Medicine, Shanghai, 200071, China*
[c] *Shanghai TCM-Integrated Hospital Affiliated to Shanghai University of Traditional Chinese Medicine, Shanghai University of Traditional Chinese Medicine, Shanghai, 200082, China*
[d] *Department of Gastroenterology, Putuo Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai, 200062, China*

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ABSTRACT

Endocannabinoids (eCBs) exert considerable influence over energy metabolism, lipid metabolism, and glucose metabolism within the human body. Among the most biologically active cannabinoids identified thus far are 2-arachidonoylglycerol (2-AG), arachidonoyl ethanolamide (AEA), 1-stearoylglycerol (1-SRG), and stearoyl ethanolamide (SEA), which are derived from arachidonic acid (AA) and stearic acid (SA). However, despite the unique in bioactivities exhibited by eCBs, their determination in plasma has been hindered by the lack of sensitive analytical methods. The aim of this study was to develop and validate a highly sensitive and rapid method using ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) for accurate measurement of AEA, SEA, 2-AG, 1-SRG, AA, and SA levels in human plasma samples. Sample preparation involved a protein precipitation method and a methyl *tert*-butyl ether liquid-liquid extraction method. Chromatographic separation was accomplished by utilizing an ACQUITY UPLC BEH $C_8$ column with a mobile phase of acetonitrile containing 0.1% formic acid and water containing 0.1% formic acid, flowing at a rate of 0.35 mL/min. AA-$d_8$, 2-AG-$d_5$, and AEA-$d_8$ were selected as deuterated internal standards. The analytes were determined with MRM in both positive and negative ion mode. The lower limit of quantification ranged from 0.1 to 400 ng/mL, and the correlation coefficient ($R^2$) was >0.99. Inter-day and intra-day precision exhibited values of 0.55–13.29% and 0.62%–13.90%, respectively. Recovery and matrix effect were within the range of 77.7%–109.7%, and 90.0%–113.5%, respectively. Stability tests confirmed the acceptability of all analytes. To demonstrate the effectiveness of the approach, it was implemented to assess and compare plasma samples from healthy volunteers (n = 49) and individuals with non-

* Corresponding author. Experiment Center for Science and Technology, Shanghai University of Traditional Chinese Medicine, Shanghai, 201203, China.
** Corresponding author. Experiment Center for Science and Technology, Shanghai University of Traditional Chinese Medicine, Shanghai, 201203, China.
*E-mail addresses:* kelley007@163.com (L. Pan), hucheng10200@163.com (C. Hu).
[1] These authors contribute equally to this work.

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alcoholic fatty liver disease (NAFLD) (n = 62). The study revealed significant differences in AEA, SEA, AA, and SA levels between the two groups.

**Abbreviation**

| | |
|---|---|
| 2-AG | 2-arachidonoylglycerol |
| AEA | Arachidonoyl ethanolamide |
| 1-SRG | 1-stearoylglycerol |
| SEA | Stearoyl ethanolamide |
| AA | Arachidonic acid |
| SA | Stearic acid |
| ECs | Endocannabinoid system |
| CBRs | Cannabinoid receptors |
| eCBs | Endocannabinoids |
| FAAH | Fatty acid amide hydrolase |
| MAGL | Monoacylglycerol lipase |
| WBC | White blood cells |
| RBC | Red blood cells |
| HGB | Haemoglobin |
| HCT | Hematocrit |
| MCV | Mean corpuscular volume |
| MCH | Mean corpuscular hemoglobin |
| MCHC | Mean corpuscular hemoglobin concentration |
| PLT | Platelet |
| RDW | Red blood cell distribution width |
| MPV | Mean platelet volume |
| NE | Neutrophilicgranulocyte |
| LY | Lymphocyte |
| MO | Monocytes |
| EO | Eosinophil |
| BASO | Basophilicgranulocyte |
| GFR | Glomerular filtration rate |
| TB | Total bilirubin |
| DB | Direct bilirubin |
| AKP | Alkaline phosphatase |
| TP | Total protein |
| ALB | Albumin |
| γ-GT | γ-gamma-glutamyltransferase |
| CHE | Cholinesterase |
| ALT | Alanine aminotransferase |
| AST | Aspartate aminotransferase |
| TBA | Total bile acid |
| Glu | Glucose |
| HDL-C | High density lipoprotein |
| LDL-C | Low density lipoprotein |
| TC | Total cholesterol |
| TG | Triglyceride |
| APOA1 | Apolipoprotein A-1 |
| APOB | Apolipoprotein B |

## 1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a chronic condition marked by lipid accumulation in the liver. Since the 21st century, NAFLD has emerged as the most common liver disease, affecting individuals of all races and age groups without limitation [1]. Moreover, it is considered the rapidly escalating cause of hepatocellular carcinoma (HCC) among candidates for liver transplantation [2].

Numerous studies have revealed the significant role of the endocannabinoid system (ECs) in various diseases, including NAFLD, nonalcoholic steatohepatitis, and liver cirrhosis [3–5]. The ECs primarily consists of cannabinoid receptors (CBRs), endocannabinoids (eCBs), and associated enzymes [6]. This system is involved in a wide range of pathophysiological activities within the body, including energy metabolism, modulation of food intake, adipose tissue regulation, digestion, glucose utilization, and liver lipid metabolism [7–10]. Furthermore, eCBs show great promise as potential targets for pharmacological intervention in obesity therapy [11,12]. The most biologically active cannabinoids identified so far include 2-arachidonoylglycerol (2-AG), arachidonoyl ethanolamide (AEA), 1-stearoylglycerol (1-SRG), and stearoyl ethanolamide (SEA). These eCBs are derived from arachidonic acid (AA) and stearic acid (SA) and exert their effects by binding to CBRs receptors [13,14]. AA, a member of the n-6 polyunsaturated fatty acids (PUFAs) family, plays a crucial role as a fundamental building block for the synthesis of both pro-inflammatory and anti-inflammatory eicosanoids [15,16]. On the other hand, SA possesses the capability to alleviate oxidative stress and inflammation [17]. A study has shown that SA-treated hepatocytes showing elevated production of the anti-inflammatory cytokine interleukin-10 (IL-10) [18]. Notably, fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) have been identified as playing a rapid metabolizing role in intracellular endocannabinoids [19]. Understanding the complex interactions of the ECs and its components offers promising avenues for potential therapeutic interventions in various diseases.

In a normal liver, CBRs are either not expressed or expressed in small amounts. However, a high-fat diet, alcohol, and endotoxin can all stimulate the production of 2-AG, AEA, 1-SRG, and SEA, while inhibiting the activity of FAAH, thereby reducing the degradation of AEA and SEA. The levels of SRG and SEA are significantly increased, which leads to the decline of energy metabolism and lipid metabolism through the activation of CBRs, and finally causes fatty liver [20].

Due to their participation in numerous biochemical pathways, various analytical methods have been developed over the years to quantify classic and endocannabinoid-like compounds in different biological matrices. LC-MS/MS and GC-MS/MS methods are frequently used techniques for quantitatively determining 2-AG and AEA in biological samples [21–24]. Representative analysis methods are listed in Table 1. Detection methods using GC-MS necessitate derivatization [21,22], while LC-MS/MS detection methods can achieve satisfactory limits of quantification for samples subjected to solid-phase extraction [23,24]. In terms of methodological validation, calibration curves are typically constructed using pure solvents or surrogate or naive matrices. However, to date, no comprehensive assay method is available for the simultaneous determination of these two closely related sets of components in biological samples: 2-AG, AEA, AA, and 1-SRG, SEA, SA.

Therefore, in this study, a highly sensitive method utilizing UPLC-MS/MS was developed for quantifying AEA, SEA, 2-AG, 1-SRG, AA, and SA in human plasma according to acceptance criteria of FDA recommendations, including linearity, LLOQ, precision and accuracy, extraction recovery, matrix effects, and stability [25–27]. The chemical structures of these compounds are shown in Fig. 1. This newly developed method offers fast and efficient analysis, making it suitable for high-throughput applications.

## 2. Methods and materials

### 2.1. Chemicals and reagents

Arachidonic acid (AA, 0525560–65), 2-arachidonoylglycerol (2-AG, 0613326–2), arachidonoyl ethanolamide (AEA, 0628207–1), stearic acid (SA, 1001002752), 1-stearoylglycerol (1-SRG, 1001036188), stearoyl ethanolamide (SEA, SLCF4292), arachidonic acid-$d_8$ (AA-$d_8$, 0524707–106), 2-arachidonoylglycerol-$d_5$ (2-AG-$d_5$, 18469), and arachidonoyl ethanolamide-$d_8$ (AEA-$d_8$, 0489823–42) were purchased from Cayman Chemical. Methanol, acetonitrile, and ammonium acetate (HPLC grade) were purchased from Merck (Darmstadt, Germany). Ultrapure water was purified using a Milli-Q Reagent system (Millipore, Billerica, USA). All additional reagents utilized were of analytical grade.

### 2.2. Participants, inclusion and exclusion criteria

Plasma samples were collected from 62 patients with NAFLD and 49 healthy controls for the purpose of method development and determination. The inclusion and exclusion criteria was shown in our previous study [28], and the study protocol received approval from the Medical Ethics Committee of the Putuo Hospital Affiliated to Shanghai University of Traditional Chinese Medicine, with an assigned ethics approval number of PTEC-A-2018-49-1. Before sample collection, all participants fasted overnight for 12 h, and blood samples were collected in the early morning from their veins. An automated chemistry analyzer (Hitachi 7600d-210, Japan) was employed to conduct measurements of blood glucose, lipids, liver function, and other plasma indicators.

**Table 1**
Comparative analysis of analytical methods for determination of AA, SA, and related endocannabinoids.

| No. | Anayltes | Extraction method | Analytical technique | Sample type | Ref. |
|---|---|---|---|---|---|
| 1 | Medium and long-chain fatty acid (eg. AA, SA) | Protein precipitation and derivatization | GC- MS | Mouse plasma | [21] |
| 2 | AEA, 2-AG | Liquid-liquid extraction and derivatization | GC- MS | Human plasma | [22] |
| 3 | AEA, 2-AG , *N*-arachidonoyl amino acids | Micro solid phase extraction | UHPLC-MS/MS | Mouse brains | [23] |
| 4 | AEA, AA | Solid phase extraction | LC-MS/MS | Human plasma | [24] |

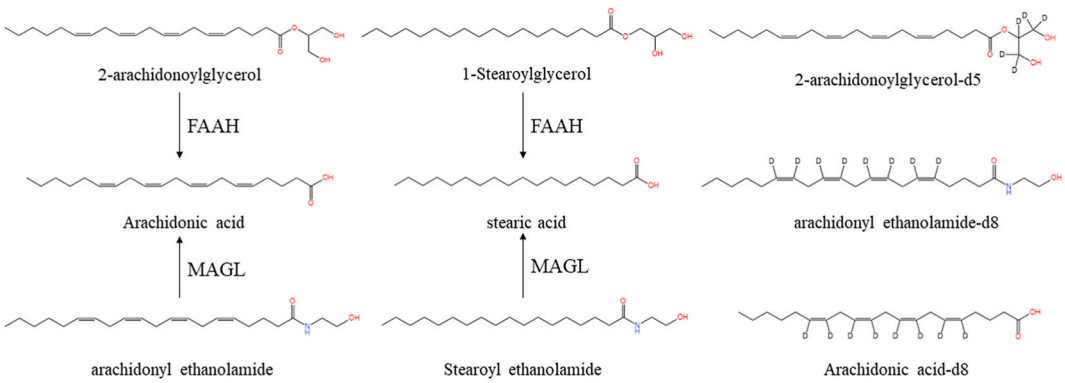


**Fig. 1.** Chemical structures of six analytes and three internal standards.

### 2.3. Standard solutions and quality control solutions

Accurate amounts of AA, SA, SEA, AEA, 2-AG, 1-SRG, and internal standards (IS) were weighed and dissolved in methanol to prepare stock solutions. The concentrations of the stock solutions were set at 1.0 mg/mL for the six target compounds and 100 μg/mL for the IS. A portion of the standard solution was diluted continuously with methanol to prepare mixed standard solutions with varying concentrations for later use.

To validate the analysis, baseline plasma samples were obtained from volunteers and combined to create a 10 mL pooled human plasma sample. For the subsequent step of quality control (QC) sample preparation, 60 μL of the pooled plasma sample was extracted, and appropriate amounts of AA, SA, SEA, AEA, 2-AG, 1-SRG standards were added, along with 14 μL of the IS. The concentrations of the analytes in the QC solution were as follows: AEA at 0.2, 5, and 40 ng/mL; SEA at 20, 200, and 500 ng/mL; 2-AG at 2, 20, and 50 ng/mL; 1-SRG at 200, 2,000, and 5000 ng/mL; AA at 50, 500, and 1000 ng/mL; and SA at 0.5, 10, and 20 μg/mL.

### 2.4. Sample preparation

After thawing at room temperature, the plasma samples underwent a division into two distinct groups to facilitate subsequent separate pre-processing and injection. Group 1 (AEA): 60 μL of plasma sample was mixed with 14 μL of AEA-$d_8$ solution (1.0 μg/mL) and 20 μL of methanol (QC or standard solution). Subsequently, 1000 μL of methyl *tert*-butyl ether (MTBE), along with 300 μL of methanol and 290 μL of pure water (in a ratio of MTBE: methanol: water, 5:1.5:1.45), were added to the mixture. The mixture was shaken for 2 min, followed by centrifugation for 5 min at 5000 rpm. The upper layer solution was dried, and the residue was reconstituted by adding 100 μL of an acetonitrile-0.1% formic acid water solution (70/30, v/v). The reconstituted mixture was sonicated for 2 min, followed by centrifugation at 10,000 rpm for 10 min at 4 °C. Then, 80 μL of the supernatant was transferred to a vial for injection, with an injection volume of 10 μL.

Group 2 (SEA, 2-AG, 1-SRG, AA, SA): 10 μL of plasma sample was combined with 100 μL of methanol (QC or standard solution). To this mixture, 14 μL of an IS solution containing 1 μg/mL of AA-$d_8$ and 1 μg/mL of 2-AG-$d_5$ was added. Following the addition of 90 μL of acetonitrile and thorough shaking for 2 min, the mixture underwent centrifugation at 3000 rpm for 10 min. The clear supernatant was carefully transferred to a fresh tube and subjected to a drying process. Subsequently, the treated dried residue underwent the identical procedural steps as outlined for Group 1.

### 2.5. Analytical instruments and conditions

The content of AA, SA, and related eCBs in human plasma was detected on an Ultra-high performance liquid chromatography

**Table 2**
The detection settings for mass spectrometry conditions.

| Parameter name | Parameter value |
|---|---|
| Ion spray voltage (positive mode) | +5500 V |
| Ion spray voltage (negative mode) | −4500 V |
| Source temperature | 550 °C |
| Entrance potential | 10 V |
| Collision gas | Nitrogen |
| Curtain gas | 241.3 kPa |
| Nebulizer gas | 413.7 kPa |
| Heater gas | 413.7 kPa |
| Dwell time | 60 ms |

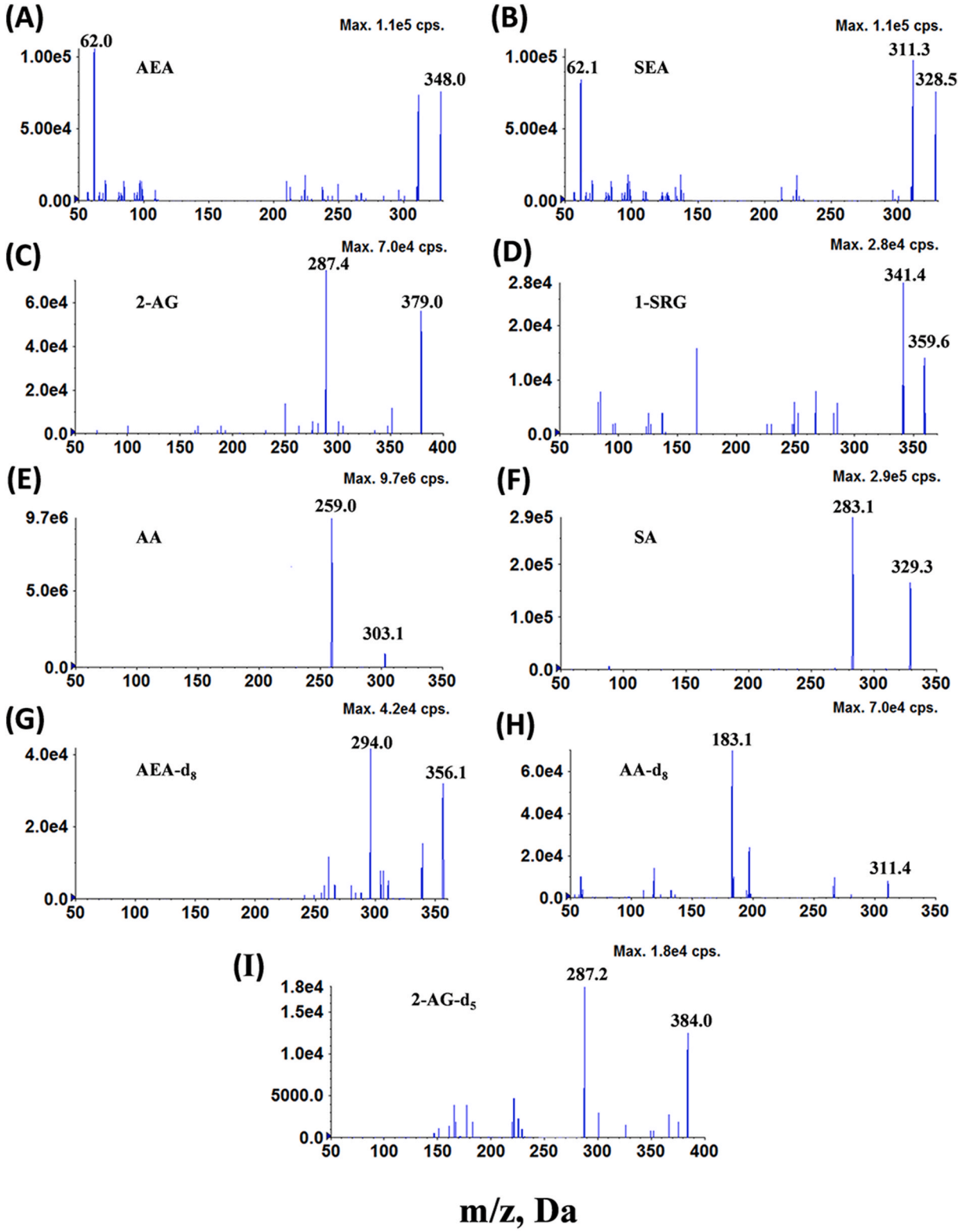


**Fig. 2.** Parent ion and product ion scan spectra of six analytes and three internal standards: (A) AEA; (B) SEA; (C) 2-AG; (D) 1-SRG; (E) AA; (F) SA; (G) AEA-$d_8$; (H) AA-$d_8$; (I) 2-AG-$d_5$.

(Waters H-Class Acquity) tandem mass spectrometry system (AB SCIEX 6500 triple quadrupole). During the separation process, processed samples underwent separation utilizing an Acquity UPLC BEH $C_8$ column (2.1 mm × 100 mm, 1.7 μm) by maintaining the column oven temperature at 40 °C and a flow rate of 0.35 mL/min. The chromatographic separation utilized a gradient of acetonitrile with 0.1% formic acid (mobile phase B) and water with 0.1% formic acid (mobile phase A). The elution profile involved a sequence of changes: 0–1 min, 50%A; 1–5 min, 50-20%A; 5–6.5 min, 20-5%A; 6.5–9min, 5%A; 9–10 min, 50%A. Mass spectrometry conditions are provided in Table 2, and the precursor-product ion transitions of AEA, SEA, 2-AG, 1-SRG, AA, SA, AEA-$d_8$, AA-$d_8$, and 2-AG-$d_5$ are shown in Fig. 2(A)-2(I).

### 2.6. Linearity and LLOQ

To assess the linearity, the calibration curve was created by plotting the ratio of peak areas (analyte/IS) after subtracting the blank, against the known concentrations of the analytes using a $1/X^2$ weighted least-squares linear regression model. Since stable isotope-labeled internal standards for SEA,1-SRG, and SA were not commercially available during the method development, AEA-$d_8$ was used as the internal standard for AEA and SEA, 2-AG-$d_5$ was used for 2-AG and 1-SRG, and AA-$d_8$ was used for AA and SA. The calibration curve necessitated an R-squared coefficient exceeding 0.99. To establish the LLOQ, plasma samples were spiked with analytes present endogenously, ensuring a signal-to-noise ratio of 10, and the determination involved subtracting the blank, yielding a positive value.

### 2.7. Precision and accuracy

The precision and accuracy of QC were assessed through both intra- and inter-assay evaluations. Six replicates were analyzed on a single day, and this process was repeated over three consecutive days. Accuracy was determined by calculating the percentage error (RE%) after subtracting the blank, while precision was quantified as the relative standard deviation (RSD%) of measured

**Table 3**
Baseline characteristics and plasma biochemical indexes of subjects.

| Clinical Indicators | Normal control (n = 49) | NAFLD (n = 62) | Statistical value | P value |
|---|---|---|---|---|
| Gender (male/female) | 12/37 | 33/29 | | |
| Height (cm) | 164.6 ± 7.6 | 168.6 ± 8.3 | t = 2.626 | 0.010 |
| Weight (kg) | 57.0 ± 6.6 | 75.6 ± 10.6 | t = 10.692 | <0.001 |
| BMI | 20.8 ± 6.6 | 26.5 ± 2.4 | t = 14.214 | <0.001 |
| WBC( × $10^9$/L) | 5.4 ± 1.5 | 6.3 ± 1.6 | t = 3.008 | 0.003 |
| RBC(%) | 4.4 ± 0.7 | 4.8 ± 0.4 | t = 4.453 | <0.001 |
| HGB (g/L) | 129.1 ± 22.1 | 147.4 ± 15.7 | t = 5.089 | <0.001 |
| HCT (%) | 38.80 (36.30,41.40) | 44.10 (41.50,46.35) | Z = −5.262 | <0.001 |
| MCV(fl) | 91.00 (89.05,93.25) | 90.90 (87.85,93.38) | Z = 0.404 | 0.686 |
| MCH(pg) | 30.90 (29.75,31.45) | 30.95 (29.80,31.83) | Z = 0.377 | 0.706 |
| MCHC(g/L) | 338.00 (331.75,345.25) | 334.00 (330.00,341.50) | Z = −1.771 | 0.077 |
| PLT ( × $10^9$/L) | 232.1 ± 77.7 | 234.5 ± 61.7 | t = 0.188 | 0.851 |
| RDW ( × $10^{12}$/L) | 12.40 (11.90,12.80) | 12.50 (12.10,13.20) | Z = −1.363 | 0.173 |
| MPV(fl) | 10.40 (10.00,11.20) | 10.50 (9.78,11.33) | Z = −0.095 | 0.924 |
| NE (%) | 55.0 ± 7.9 | 57.0 ± 8.7 | t = 1.230 | 0.221 |
| LY (%) | 34.3 ± 8.4 | 32.5 ± 8.4 | t = −1.145 | 0.109 |
| MO(%) | 6.8 ± 1.7 | 7.3 ± 1.8 | t = 1.460 | 0.109 |
| EO (%) | 1.70 (1.05,2.90) | 2.10 (1.40,3.25) | Z = −1.385 | 0.166 |
| BASO(%) | 0.60 (0.35,0.75) | 0.50 (0.40,0.70) | Z = −0.179 | 0.858 |
| Urea (mmol/L) | 2.0 ± 1.1 | 5.5 ± 1.2 | t = 2.018 | 0.107 |
| Creatinine (μmol/L) | 59.50 (54.00,71.25) | 74.00 (68.00,83.25) | Z = −4.302 | <0.001 |
| Uric acid (μmol/L) | 298.00 (241.50,348.75) | 401.00 (336.25,483.00) | Z = −4.902 | <0.001 |
| GFR (ml/min) | 107.7 ± 16.9 | 93.9 ± 17.9 | t = −3.050 | 0.003 |
| TB (μmol/L) | 14.00 (10.00,16.00) | 14.50 (11.00,19.20) | Z = −1.319 | 0.187 |
| DB (μmol/L) | 2.6 ± 0.9 | 3.0 ± 1.5 | t = 1.584 | 0.107 |
| AKP(U/L) | 70.8 ± 23.4 | 81.4 ± 19.1 | t = 2.589 | 0.107 |
| TP (g/L) | 73.00 (70.00,75.00) | 74.50 (72.00,77.00) | Z = −1.750 | 0.080 |
| ALB (g/L) | 44.00 (43.00,46.00) | 44.00 (42.00,46.00) | Z = −0.003 | 0.998 |
| γ-GT (U/L) | 17.00 (15.00,31.00) | 32.50 (23.00,42.25) | Z = −4.213 | <0.001 |
| CHE(U/L) | 7757.4 ± 1295.9 | 9229.4 ± 1647.4 | t = 4.978 | 0.105 |
| ALT (U/L) | 10.00 (7.50,16.50) | 17.00 (13.00,27.00) | Z = −4.052 | <0.001 |
| AST (U/L) | 21.00 (17.00,27.00) | 25.50 (21.00,31.00) | Z = −2.929 | 0.003 |
| TBA (μmol/L) | 3.00 (2.00,5.00) | 3.00 (2.00,5.00) | Z = −0.840 | 0.401 |
| Glu (mmol/L) | 4.85 (4.50,5.20) | 5.40 (4.90,6.13) | Z = −4.777 | <0.001 |
| HDL-C (μmol/L) | 1.5 ± 0.3 | 1.1 ± 0.2 | t = −7.634 | 0.107 |
| LDL-C (μmol/L) | 3.3 ± 0.8 | 3.6 ± 0.7 | t = 2.240 | 0.107 |
| TC (μmol/L) | 5.2 ± 1.1 | 5.4 ± 1.0 | t = 0.956 | 0.109 |
| TG (μmol/L) | 1.06 (0.70,1.39) | 1.78 (1.28,2.58) | Z = −5.559 | <0.001 |
| APOA1 (g/L) | 1.6 ± 0.3 | 1.3 ± 0.2 | t = −4.475 | 0.105 |
| APOB(g/L) | 0.9 ± 0.2 | 1.1 ± 0.2 | t = 4.616 | 0.103 |

concentrations. For both intra- and inter-assay precision and accuracy, the acceptance criteria were established at 15% for the concentrations of low QC, medium QC, and high QC.

### 2.8. Matrix effect and extraction recovery

The assessment of extraction recovery for QC samples involved comparing peak area ratios by spiking analytes into pooled plasma before and after the extraction process at three different concentration levels. The extraction recovery (R) was determined through the formula: $R = 100 \times [(A2 - Ap)/(A1 - Ap)]$, where A2 represents the peak area of QC samples, A1 represents the peak area in post-extraction samples spiked with an equivalent concentration of QCs, and Ap (endogenous) refers to the peak area in pooled samples without spiking. Additionally, to investigate the matrix effect, peak area ratios of post-extraction analytes from six distinct samples (after subtracting the blank) were compared with the areas derived from their corresponding neat standard QC.

### 2.9. Stability and carryover

Validation requires a thorough assessment of analyte stability. To evaluate stability, biological samples were subjected to various storage conditions. Short-term stability was assessed by exposing the samples to room temperature for 4 h. Autosampler stability was achieved by storing the samples at 4 °C for 12 h. Freeze-thaw stability was assessed through three cycles of freezing and thawing, and long-term stability involved keeping samples at −80 °C for 20 days. Each stability condition was tested in replicates, with five tests conducted for each condition ($n = 5$). The acceptance criteria were set at ±15% relative to the nominal values. Moreover, to assess carryover effects, blank samples (methanol) injected immediately after the highest point on the calibration curve. The blank samples should exhibit peak areas less than 20% compared to the respective analytes' peak areas at LLOQ.

### 2.10. ECBs determination in human plasma

To validate the developed method, plasma samples from two groups were analyzed: a group comprising 49 healthy individuals and another consisting of 62 patients diagnosed with non-alcoholic fatty liver disease. Approximately 3–5 mL of blood samples were collected and promptly stored at −80 °C. The levels of AEA, SEA, 2-AG, 1-SRG, AA, and SA in the plasma were measured for each participant.

## 3. Results

### 3.1. Demographic characteristics and plasma index

We found significant differences between healthy volunteers and NAFLD patients in demographic characteristics, as shown in Table 3, which includes height, body weight, and BMI ($p < 0.001$). Furthermore, there were notable distinctions in various biochemical indexes, including creatinine, uric acid, GFR, γ-GT, ALT, AST, Glu, and TG, between the two groups ($p < 0.01$). No significant differences ($p > 0.05$) were found in the following parameters: MCV, MCH, MCHC, PLT, RDW, MPV, NE, LY, MO, EO, BASO, Urea, TB, TP, ALB, CHE, HDL-C, LDL-C, TC, APOA1, APOB.

### 3.2. Selectivity

Table 4 presents the MRM acquisition parameters, including DP, CE, CXP, MRM channel, and retention time for the target compounds. The MRM chromatograms of the mixed standard solvent, along with the three internal standards, are shown in Fig. 3(A), while the MRM chromatograms of the human plasma sample (NAFLD patient) with internal standard are shown in Fig. 3(B). As depicted in Fig. 3(A) and B, the detection of the target compounds was not affected by any other endogenous components present in human plasma.

**Table 4**
MRM acquisition settings and retention time of the target compounds.

| Analytes | $t_R$/min | DP (V) | CE (V) | CXP(V) | MRM channel (Q1/Q3) |
|---|---|---|---|---|---|
| AEA | 5.56 | 56.0 | 42.0 | 10.0 | 348.0/62.0 |
| SEA | 6.94 | 160.0 | 20.0 | 16.0 | 328.5/311.3 |
| 2-AG | 6.28 | 163.9 | 20.1 | 13.1 | 379.0/287.4 |
| 1-SRG | 7.67 | 160.0 | 12.5 | 21.0 | 359.6/341.4 |
| AA | 2.57 | −73.9 | −16.1 | −13.0 | 303.1/259.0 |
| SA | 4.92 | −24.0 | −14.8 | −30.9 | 329.3/283.1 |
| AEA-$d_8$ | 5.54 | 132.7 | 21.6 | 12.6 | 356.1/294.0 |
| AA-$d_8$ | 2.53 | −104.5 | −43.1 | −26.0 | 311.4/183.1 |
| 2-AG-$d_5$ | 6.26 | 73.3 | 17.5 | 15.2 | 384.0/287.2 |

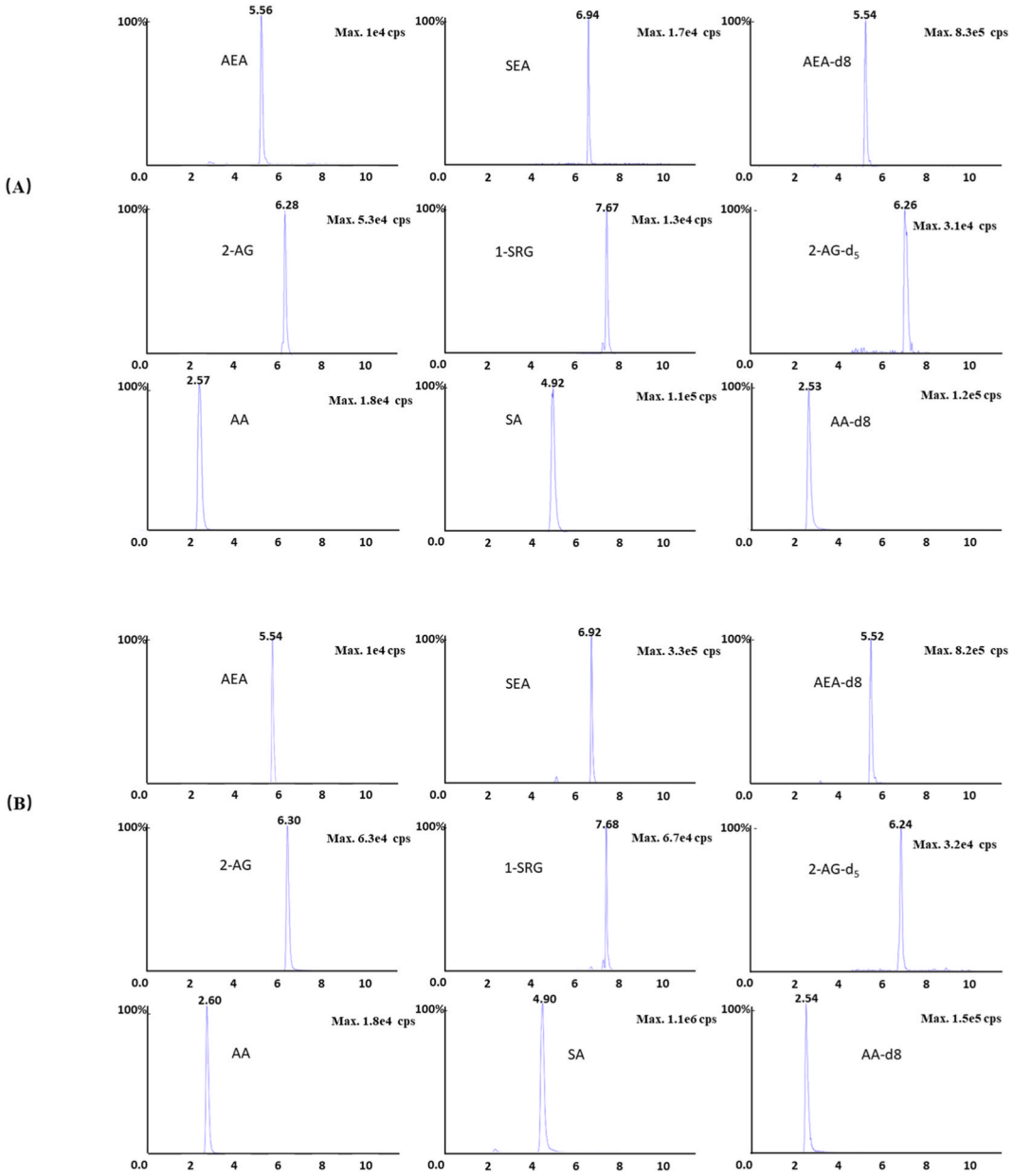


**Fig. 3.** Typical MRM chromatograms of all analytes and internal standards: (A) Mixed standard solvent with internal standard (LLOQ); (B) human plasma sample (NAFLD patient) with internal standard.

**Table 5**
Regression equations, linear ranges, and correlation coefficients of the target analytes.

| Analyte | Regression equation | Linear range ( ng/mL ) | $R^2$ | LLOQ ( ng/mL ) |
|---|---|---|---|---|
| AEA | $Y = 2.82\times10^{-2}\,X + 4.06\times10^{-2}$ | 0.1–50 | 0.9993 | 0.1 |
| SEA | $Y = 6.02\times10^{-1}\,X$-1.04 | 10–625 | 0.9975 | 10 |
| 2-AG | $Y = 2.04\times10^{-1}\,X$-$1.76\times10^{-1}$ | 1–62.5 | 0.9997 | 1 |
| 1-SRG | $Y = 3.80\times10^{-2}\,X + 3.0\times10^{-1}$ | 100-6250 | 0.9902 | 100 |
| AA | $Y = 3.00\times10^{-1}\,X + 2.68$ | 40-1250 | 0.9978 | 40 |
| SA | $Y = 2.01\times10^{-1}\,X$+1.53 | 400-25,000 | 0.9951 | 400 |

### 3.3. Linearity and LLOQ

The representative calibration curves, linear ranges, correlation coefficients ($R^2$), and LLOQs of the six analytes are shown in Table 5. The standard curves of all analytes have good linearity, and the regression coefficient was over 0.99, which is suitable for the detection of human plasma samples. The LLOQs of AEA, SEA, 2-AG, 1-SRG, AA, and SA were determined as 0.1, 10, 1, 100, 40 and 400 ng/mL, respectively.

### 3.4. Intra- and inter-day precision and accuracy

Table 6 presents the results of the intra- and inter-day accuracy and precision. The RSD for all QC samples was found to be below 13.90%, signifying good precision. Furthermore, the accuracy values ranged from 85.2% to 113.4%, demonstrating that the analytical method was reliable and reproducible, as it met the acceptance criterion.

### 3.5. Extraction recovery and matrix effect

The mean extraction recovery and matrix effect for the six compounds are listed in Table 7. The extraction recovery of all analytes at three levels was in the range of 77.7–109.7%, with RSD <10.2%, whereas the matrix effect was in the range of 90.0–113.5%, with RSD <13.7%. These results showed that matrix effects and recoveries in human plasma are acceptable.

### 3.6. Stability

The results of stability study are shown in Table 8. The RE ranged from −6.25 to 11.95%, and the RSD was less than 10.19%. Therefore, all analytes were stable under the storage conditions used in the study, with little impact on the determination in human plasma samples. Additionally, the peak areas of the blank samples, measured after the injection of ULOQ, were found to be less than 10 % of LLOQ. This result indicates the absence of a carry-over effect.

### 3.7. Application in human plasma

The plasma levels of various analytes were analyzed in both NAFLD patients (n = 49) and normal individuals (n = 62). The results, presented in Table 9, demonstrate significant differences between the two groups. Specifically, NAFLD patients exhibited significantly lower levels of AA and SA compared to the normal group ($p < 0.01$). On the other hand, the contents of 1-SRG and AEA showed a significant increase in NAFLD patients ($p < 0.01$). However, the levels of SEA and 2-AG did not demonstrate significant changes.

## 4. Discussion

The aim of this study was to establish and validate a UPLC-MS/MS method capable of simultaneously and accurately measuring AEA, SEA, 2-AG, 1-SRG, AA, and SA levels in human plasma. During the method development phase, various chromatographic conditions, such as the choice of column and mobile phase composition, were optimized to separate the target analyte in a shorter time.

**Table 6**
Accuracy and precision of analytes in human plamsa (n = 6).

| Analyte | Concentration ( ng/mL ) | Intra-day | | | Inter-day | | |
|---|---|---|---|---|---|---|---|
| | | Measured concentration ( ng/mL ) | Accuracy ( % ) | Precision ( RSD,% ) | Measured concentration ( ng/mL ) | Accuracy ( % ) | Precision ( RSD,% ) |
| AEA | 0.2 | 0.18 ± 0.01 | 90.1 | 9.82 | 0.18 ± 0.00 | 88.4 | 1.93 |
| | 5 | 4.41 ± 0.28 | 88.3 | 6.38 | 4.33 ± 0.03 | 86.5 | 0.62 |
| | 40 | 34.66 ± 4.60 | 86.6 | 13.26 | 34.09 ± 0.51 | 85.2 | 1.49 |
| SEA | 20 | 22.42 ± 0.23 | 112.1 | 1.03 | 22.32 ± 0.29 | 111.6 | 1.29 |
| | 200 | 179.26 ± 8.37 | 89.6 | 4.67 | 181.28 ± 6.55 | 90.6 | 3.61 |
| | 500 | 466.06 ± 11.31 | 93.2 | 2.53 | 440.17 ± 10.51 | 88.0 | 2.39 |
| 2-AG | 2 | 2.04 ± 0.01 | 102.3 | 0.55 | 2.13 ± 0.04 | 106.6 | 1.65 |
| | 20 | 21.26 ± 0.37 | 106.3 | 1.74 | 20.79 ± 0.41 | 104.0 | 1.98 |
| | 50 | 49.41 ± 1.08 | 98.8 | 2.18 | 48.42 ± 0.85 | 96.8 | 1.75 |
| 1-SRG | 200 | 222.64 ± 24.60 | 111.3 | 11.05 | 224.08 ± 21.57 | 112.0 | 9.62 |
| | 2000 | 2154.54 ± 220.90 | 107.7 | 10.25 | 2267.57 ± 177.75 | 113.4 | 7.83 |
| | 5000 | 5471.34 ± 225.32 | 109.4 | 4.12 | 5625.64 ± 250.96 | 112.5 | 4.46 |
| AA | 50 | 50.75 ± 0.93 | 101.5 | 1.82 | 48.11 ± 1.05 | 96.2 | 2.18 |
| | 500 | 537.40 ± 9.90 | 107.5 | 1.84 | 510.35 ± 6.33 | 102.1 | 1.24 |
| | 1000 | 1038.76 ± 12.72 | 103.9 | 1.22 | 992.62 ± 15.82 | 99.3 | 1.59 |
| SA | 500 | 482.87 ± 64.18 | 96.6 | 13.29 | 533.54 ± 74.16 | 106.7 | 13.90 |
| | 10,000 | 10,695.70 ± 790.49 | 107.0 | 7.39 | 11,034.19 ± 990.92 | 110.3 | 8.98 |
| | 20,000 | 19,464.64 ± 1898.27 | 97.3 | 9.75 | 22,092.53 ± 1615.05 | 110.5 | 7.31 |

**Table 7**
Extraction recovery and matrix effect of analytes in human plamsa (n = 6).

| Analyte | Spiked concentration (ng/mL) | Extraction recovery (%) | RSD (%) | Matrix effect (%) | RSD (%) |
|---|---|---|---|---|---|
| AEA | 0.2 | 77.7 ± 1.5 | 1.9 | 105.5 ± 14.4 | 13.7 |
| | 5 | 85.4 ± 1.2 | 2.1 | 111.3 ± 9.1 | 8.2 |
| | 40 | 87.8 ± 0.7 | 1.2 | 109.4 ± 9.7 | 8.9 |
| SEA | 20 | 93.5 ± 3.4 | 3.7 | 103.7 ± 1.6 | 1.5 |
| | 200 | 106.3 ± 3.6 | 3.4 | 112.6 ± 4.6 | 4.0 |
| | 500 | 106.1 ± 2.3 | 2.1 | 113.5 ± 5.5 | 4.8 |
| 2-AG | 2 | 92.8 ± 4.9 | 5.3 | 96.6 ± 11.4 | 11.9 |
| | 20 | 98.3 ± 3.3 | 3.4 | 105.6 ± 2.8 | 2.7 |
| | 50 | 87.8 ± 2.3 | 2.6 | 106.9 ± 2.0 | 1.9 |
| 1-SRG | 200 | 97.8 ± 4.1 | 4.2 | 91.4 ± 7.7 | 8.4 |
| | 2000 | 98.4 ± 6.3 | 6.4 | 111.8 ± 5.0 | 4.5 |
| | 5000 | 92.8 ± 9.5 | 10.2 | 107.8 ± 7.1 | 6.6 |
| AA | 50 | 98.4 ± 4.4 | 4.4 | 91.6 ± 8.2 | 9.0 |
| | 500 | 109.7 ± 2.5 | 2.2 | 90.0 ± 5.9 | 6.6 |
| | 1000 | 99.0 ± 4.1 | 4.1 | 107.5 ± 3.1 | 2.9 |
| SA | 500 | 102.2 ± 3.6 | 3.5 | 101.2 ± 3.8 | 3.8 |
| | 10,000 | 102.6 ± 6.7 | 6.5 | 108.8 ± 2.3 | 2.1 |
| | 20,000 | 100.1 ± 3.8 | 3.8 | 96.5 ± 1.3 | 1.3 |

**Table 8**
The stability of analytes in human plasma (n = 5).

| Analyte | Spiked concentration (ng/mL) | Room temperature for 4 h | | In autosampler vials for 12 h | | Three freeze-thaw cycles | | −80 °C for 20 days | |
|---|---|---|---|---|---|---|---|---|---|
| | | RSD (%) | RE (%) | RSD (%) | RE (%) | RSD (%) | RE (%) | RSD (%) | RE (%) |
| AEA | 0.2 | 1.36 | −0.73 | 1.16 | −1.89 | 0.11 | −2.28 | 2.81 | 3.86 |
| | 5 | 2.67 | 3.79 | 0.90 | 3.10 | 2.58 | −1.87 | 3.41 | 4.56 |
| | 40 | 3.42 | 7.34 | 2.65 | −2.02 | 3.96 | 4.22 | 4.23 | 7.81 |
| SEA | 20 | 4.38 | 3.81 | 4.53 | 4.10 | 4.16 | 3.33 | 3.69 | 4.28 |
| | 200 | 6.65 | 4.16 | 4.65 | 2.72 | 4.89 | 7.78 | 3.80 | 0.62 |
| | 500 | 4.68 | 5.17 | 4.05 | 1.02 | 5.05 | 4.54 | 2.03 | 1.90 |
| 2-AG | 2 | 3.87 | 5.44 | 3.55 | −1.31 | 3.36 | 3.88 | 4.89 | −5.54 |
| | 20 | 3.40 | 3.05 | 2.94 | 4.69 | 2.89 | 5.73 | 4.11 | 3.32 |
| | 50 | 7.33 | 4.92 | 6.91 | 4.06 | 9.55 | 5.97 | 2.10 | 9.64 |
| 1-SRG | 200 | 2.31 | 4.21 | 3.61 | 7.66 | 4.28 | −1.18 | 5.34 | −3.08 |
| | 2000 | 3.07 | −3.22 | 3.02 | 4.63 | 3.96 | −2.16 | 3.40 | 5.04 |
| | 5000 | 10.19 | 2.45 | 4.01 | 3.02 | 4.02 | 3.08 | 3.77 | 11.95 |
| AA | 50 | 3.25 | 5.10 | 3.89 | 9.10 | 3.78 | 3.11 | 2.04 | 4.15 |
| | 500 | 8.64 | 3.80 | 3.32 | 4.43 | 5.76 | 4.32 | 2.58 | 4.27 |
| | 1000 | 6.62 | 8.24 | 9.60 | 5.03 | 3.87 | 5.46 | 3.84 | −6.25 |
| SA | 500 | 5.25 | −1.28 | 4.68 | 2.97 | 5.49 | 10.54 | 4.26 | 4.03 |
| | 10,000 | 3.83 | 2.08 | 8.48 | 3.74 | 4.60 | 6.10 | 3.68 | 4.68 |
| | 20,000 | 4.71 | 6.41 | 2.37 | 4.30 | 3.81 | 7.37 | 3.93 | −1.89 |

**Table 9**
Application in NAFLD patients and normal individuals.

| Analyte | NAFLD ( ng/mL ) | Healthy control ( ng/mL ) |
|---|---|---|
| AEA | 0.37 ± 0.1** | 0.2 ± 0.1 |
| SEA | 24.7 ± 1.1 | 25.6 ± 1.2 |
| 2-AG | 9.3 ± 4.6 | 10.4 ± 6.1 |
| 1-SRG | 694.5 ± 161.6** | 634.2 ± 145.6 |
| AA | 66.3 ± 20.6** | 81.8 ± 26.9 |
| SA | 9568.8 ± 3230.7** | 13,875.5 ± 3603.6 |

**$p < 0.01$, as compared with the healthy group.

Among the columns tested (Acquity BEH $C_{18}$, Acquity BEH $C_8$, and HSS T3), the Acquity BEH $C_8$ column (2.1 mm × 100 mm, 1.7 μm) exhibited superior separation and peak shape. Several mobile phases, including acetonitrile and methanol, and buffer salt systems (0.1%–0.2% formic acid, and 5 mM ammonium acetate plus 0.1% formic acid), were compared to optimize chromatographic conditions. It was determined that a mobile phase consisting of acetonitrile containing 0.1% formic acid (mobile phase B) and water with 0.1% formic acid (mobile phase A) yielded the optimal balance for separation and peak shape, particularly for 2-AG and 1-SRG.

During the chromatographic separation process, it was observed that 2-AG and 1-SRG exhibited double peaks after optimizing the

separation conditions. This phenomenon can be attributed to acyl migration and the spontaneous generation of their own isomers [29,30]. Since these paired isomers can interconvert in vivo, their total peak area was considered for analysis, as previously reported [31,32]. Furthermore, a comparison of positive and negative ion modes revealed that AEA, SEA, 2-AG, and 1-SRG exhibited better responses in the positive mode (ESI+). Conversely, AA and SA exhibited higher sensitivity in the negative ion mode (ESI-).

Previously reported by Gachet et al. [24], the Folch extraction method, commonly used in MS analysis of fetal bovine serum, followed by SPE clean-up, did not effectively recover PEA and SEA in plasma samples from humans or rodents. Consequently, this study investigated various extraction methods to address this limitation. Three methods were compared for their extraction efficiency: the conventional approach using a combination of MTBE and methanol for lipid extraction, direct protein precipitation with methanol, and liquid-liquid extraction using ethyl acetate. Through careful comparison, it was determined that the liquid-liquid extraction method involving MTBE and methanol is suitable for extracting low-content components such as AEA. On the other hand, the methanol precipitation method is suitable for extracting high-content components like 2-AG, SEA, 1-SRG, AA, and SA. However, it is worth noting that Fanelli et al. reported that the recovery rate and stability of 1/2-AG were unsatisfactory using the methanol extraction method. The main reason for this was speculated to be degradation, with 1/2-AG potentially hydrolyzing into arachidonic acid and glycerol [32]. Therefore, both methanol and acetonitrile were used for protein precipitation, and acetonitrile was also used for reconstitution. Additionally, during the final reconstitution step, different proportions of the reconstitution solution were attempted: acetonitrile-0.1% formic acid water solution (50/50, 70/30, v/v) and 100% acetonitrile. It was found that the ratio of 70/30 can dissolve the residue effectively and reduce the matrix effect efficiently. Consequently, these two pretreatment methods were chosen as the extraction techniques for this study to attain a lower limit of quantitation, enhanced recovery, and obtain a comprehensive understanding of changes in endocannabinoids.

Blood endocannabinoids such as AEA, PEA, and OEA can be synthesized ex vivo during collection, and their concentrations tend to increase with time and temperature, especially after 4 h [33,34]. Therefore, it is necessary to pay attention to the storage time of blood samples at room temperature. In our study, we found that AEA remained stable for up to 4 h at room temperature after sample extraction. Additionally, it showed stability for as long as 12 h when kept in the autosampler.

Fatty acids are the basic components of fat-triglyceride and play a key role in body's metabolism. SA, for instance, can reduce low-density lipoprotein levels and increase high-density lipoprotein levels. Additionally, as an essential fatty acid on cell membrane, AA is involved in cell signal transmission of various inflammatory reactions in vivo [35]. An increase in AA content can cause oxidative stress in mitochondria of hepatocytes, which leads to the formation of lipid peroxide and finally accelerates the progress of NAFLD to NASH. When analyzing plasma from NAFLD patients and healthy volunteers, it was found that the levels of AA and SA decreased significantly ($p < 0.05$). This indicates that NAFLD patients in our study are in the stage of lipid accumulation. Previous reports suggest that low plasma AA content may be associated with a higher risk of metabolic diseases [36]. It is possible that fatty acid content may increase as the disease progresses. To verify our hypothesis, we will collect patients with NASH and fibrosis.

ECs is an endogenous lipid signaling system that plays a crucial role in the regulation of fat accumulation, glucose metabolism, and lipid metabolism [37]. Bioactive cannabinoids are involved in the modulation of various fundamental biological processes, including cell survival and death, immune response, and energy homeostasis. Changes in plasma levels of eCBs have been implicated in the development of obesity and related diseases [6]. In recent years, it has been found that intestinal flora disorder is an important cause of NAFLD and liver inflammation, and this process is regulated by intestinal ECs. Cannabinoid type 1 receptor (CB1) and cannabinoid type 2 receptor (CB2) belong to the G protein-coupled receptor family, which mediate the biological effects of plant and endogenous cannabinoids. Studies have shown that inhibiting CB1 can reduce intestinal permeability and activating CB2 can reduce intestinal inflammation [38]. Compared with normal mice, when the CB1 receptor is specifically knocked out and given a high-fat diet, the weight gain of mice is similar to that of the normal group, but there is less liver steatosis [39]. ECs ligands are mainly fatty acyl glycerides and fatty acyl ethanolamines, among which AEA and SEA can bind with CB1 receptor to destruct intestinal mucosal barrier function; while 2-AG, 1-SRG can bind with CB2 receptor to maintain function [40]. In this study, we found that the levels of CB2 ligands (2-AG and 1-SRG) in NAFLD patients remained unchanged, while the contents of CB1 ligands (AEA and SEA) increased significantly. Hence, simultaneous quantification of these important modulatory analytes can provide a deeper understanding of the changes in ECs associated with various diseases.

## 5. Conclusion

The robust and sensitive UPLC-MS/MS method was successfully validated and applied to simultaneously quantify four eCBs (AEA, SEA, 2-AG, and 1-SRG), as well as AA and SA in human plasma. This analytical approach was utilized to compare the analysis between healthy volunteers and individuals with NAFLD. The results of this study revealed significant differences in the levels of AEA, SEA, AA, and SA between the two groups. These findings can provide valuable information for further development of utilizing plasma eCBs composition as a potential clinical pharmacodynamic marker.

## Data availability statement

Data included in article/supplementary material/referenced in article.

## CRediT authorship contribution statement

**Xiaojing Qian:** Methodology. **Wangzhenzu Liu:** Methodology, Investigation. **Ying Chen:** Formal analysis. **Jiaqi Zhang:** Formal

analysis. **Yuanye Jiang:** Validation, Methodology. **Lingyun Pan:** Writing – original draft, Formal analysis. **Cheng Hu:** Writing – review & editing.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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