Supporting Information

A feasible protocol to profile bile acids in rat dried blood spot using UHPLC-MS/MS method combining surrogate matrix

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List of Supporting Information

Experimental Details

Supporting Figures

All supporting figures can be found in this document (Word file).

S6 **Figure S1.** Morphology size of DBS corresponding to different blood sample volumes.
S6 **Figure S2.** Evaluation of water volume for DBS sufficient swelling.
S7 **Figure S3.** Optimization of gradient elution condition.
S8 **Figure S4.** Isolation efficiency of 9 pairs of isomers at different elution condition.
S9 **Figure S5.** Optimization of collision energy (CE) voltage of free BAs.
S10 **Figure S6.** Optimization of collision energy (CE) voltage of conjugated BAs.
S10 **Figure S7.** The H&E staining and Oil Red O staining of liver tissues in both Con group and ANIT group.

Supporting Tables

All supporting tables can be found in the additional sheets (Excel files).

Table S1. Concentration of series working solutions and LQC, MQC, HQC for different BAs.

Table S2. Structures and fragmentation patterns of all analytes along with deuterated internal standards.

Table S3. Descriptives and Tukey analysis data of all analytes.

Table S4. Summary of method validation results.

Table S5. Summary of stability results.

Table S6. Summary of BAs quantitation results.

Experimental Details

1. Preparation of stock solution and calibration solution

The range of concentration of each BA was shown in Table S4. The stocking solution of 21 analytes including UDCA, CDCA, ω-MA, α-MA, β-MA, γ-MA, CA, GLCA, GUDCA, GCDCA, GDCA, Gβ-MA, GCA, Glyco-3,7,12-Trione, TLCA, TUDCA, TCDCA, TDCA, Tβ-MA, THCA, and TCA were diluted with methanol to 2, 5, 10, 20, 50, 100, 200, 500, 1000, 1500, 2000 nmol/L as series working solutions for calibration curves. 1500 nmol/L was used as high concentration quality control (HQC), 200 nmol/L was used as medium concentration quality control (MQC), and 5 nmol/L was used as low concentration quality control (LQC). 7,12 dk-LCA was diluted with methanol to 20, 50, 100, 200, 500, 1000, 1500, 2000 nmol/L as working solutions. 1500 nmol/L was used as HQC, 200 nmol/L was used as MQC, and 50 nmol/L was used as LQC. The stocking solution of 12 analytes including ALCA, LCA, NDCA, ACA, IDCA, HDCA, DLCA, β-DCDCA, DCDCA, AlloCA, 12k-LCA, and 3,7,12-Trione were diluted with methanol to 50, 100, 200, 500, 1000, 1500, 2000 nmol/L as series working solutions for calibration curves.1500 nmol/L was used as HQC, 500 nmol/L was used as MQC, and 100 nmol/L was used as LQC. Methyl Ester was diluted with methanol to 100, 200, 500, 1000, 1500, 2000 nmol/L as series working solutions for calibration curves. 1500 nmol/L was used as HQC, 500 nmol/L was used as MQC, and 200 nmol/L was used as LQC.

2. Preparation of internal standard solutions

DBS samples and plasma samples differ in the manner of addition of IS solutions, so IS solutions were added in two different concentrations. The mixed solution of 100 nmol/L CA-d4, 100 μmol/L GCA-d4, and 100 μmol/L TCA-d4 was used for whole blood and plasma samples analysis. The mixed solution of 40 nmol/L CA-d4, 40 μmol/L GCA-d4 and 40 μmol/L TCA-d4 were used as extraction solvent for DBS analysis. All solutions were diluted with methanol (containing 0.05% BHT) from stocking solution.

Experimental results

1. Optimization of gradient elution conditions

3 different gradient elution conditions of 15 min were tested to choose an optimal condition with good separation efficiency of nine pairs of isomers in the shortest possible time: Condition (A): 0-2 min 30%B-80%B, 2-13 min 80%B-98%B, 13-13.5 min 98%B-30%B, 13.5-15 min 30%B; Condition (B): 0-2 min 30%B-60%B, 2-13 min 60%B-98%B, 13-13.5 min 98%B-30%B, 13.5-15 min 30%B; Condition (C): 0-2 min 30%B-50%B, 2-13 min 50%B-98%B, 13-13.5 min 98%B-30%B, 13.5-15 min 30%B. Isomer m/z 407 has 5 compounds, which has high requirements of elution condition, inappropriate elution condition can't separate target compounds. Therefore, the isolation efficiency of isomer m/z 407 was used as a standard to choose an optimal condition. Condition (A) and (B) had a poor separation efficiency of isomer m/z 407, but condition (C) generally performed well so we selected this it (Figure S1A-C). See Figure S1D for good isolation efficiency of 9 pairs of isomers with the selected elution condition C.

Based on the chosen 15 min elution condition, we next reduced analysis time to 12 min, 10 min, 8 min and 6 min: (A) 0-2 min 30%B-50%B, 2-13 min 50%B-98%B, 13-13.5 min 98%B-30%B, 13.5-15 min 30%B. (B) 0-2 min 30%B-50%B, 2-9 min 50%B-98%B, 9-10.5 min 98%B-30%B, 10.5-12 min 30%B. (C) 0-2 min 30%B-50%B, 2-8 min 50%B-98%B, 8-8.5 min 98%B-30%B, 8.5-10 min 30%B. (D) 0-2 min 30%B-80%B, 2-6 min 80%B-98%B, 6-6.5 min 98%B-30%B, 6.5-8 min 30%B. (E) 0-2 min 30%B-50%B,2-5 min 50%B-98%B, 5-5.5 min 98%B-30%B, 5.5-6 min 30%B. Isolation efficiency 9 pairs of isomers were shown in Figure S2A-E. As 6 min elution condition had a poor separation efficiency of

compound 10 and 11 (Figure S2E), we applied 8 min as the shortest possible time (gradient elution condition: 0-2 min 30%B-80%B, 2-6 min 80%B-98%B, 6-6.5 min 98%B-30%B, 6.5-8 min 30%B.).

2. Optimization of CE voltage

Figure S3-4 showed that the best CE voltage was 10 eV for free BAs, 45 eV for glycine-conjugated BAs and 60 eV for taurine-conjugated BAs.

3. Determination of aqueous phase volume required for expansion

20 μL, 50 μL, 100 μL, and 200 μL of water were utilize to determine volume of water required for DBS expansion. The results demonstrated that, for the majority of bile acid compounds within DBSs, the optimal extraction efficiency and minimal standard deviation (n=3) were achieved using 100 μL of water, preventing incomplete wetting of the DBS or excessive residue.

4. The H&E staining and Oil Red O staining results

An established model for inducing intrahepatic cholestasis in rodents is ANIT treatment; it leads to acute cholestasis, obstructive cholestasis, and significant bile duct epithelial cell damage. In this study, a rat model of intrahepatic cholestasis was established after an oral dose of ANIT. We observed pathological changes in the liver by Hematoxylin and Eosin (HE) staining (Figure S7, scale bars: 100 μm); results presented the infiltration of inflammatory cells, suggesting the presence of liver cell damage and impaired liver function. Moreover, we found Oil Red O staining revealing red-stained lipid droplets (Figure S7, scale bars: 100 μm), indicating the establishment of hepatic steatosis. Results above suggested that intrahepatic cholestasis modeling was successful in all 6 rats.

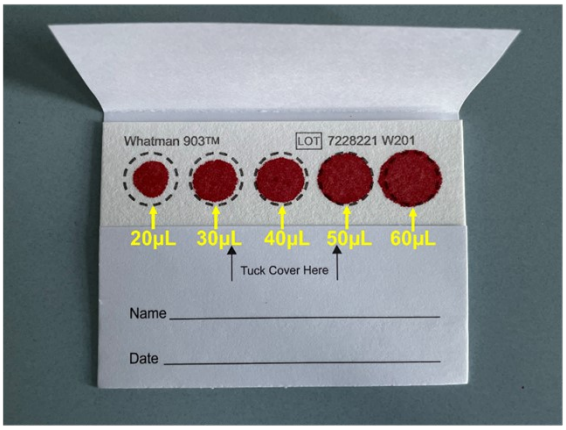

Figure S1. Morphology size of DBS corresponding to different blood sample volumes.

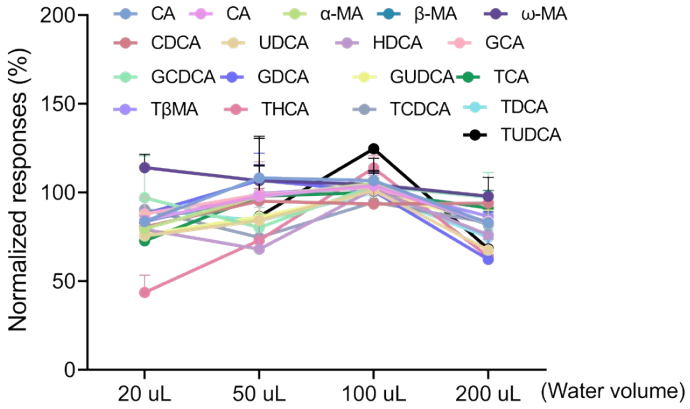

Figure S2. Evaluation of water volume for DBS sufficient swelling.

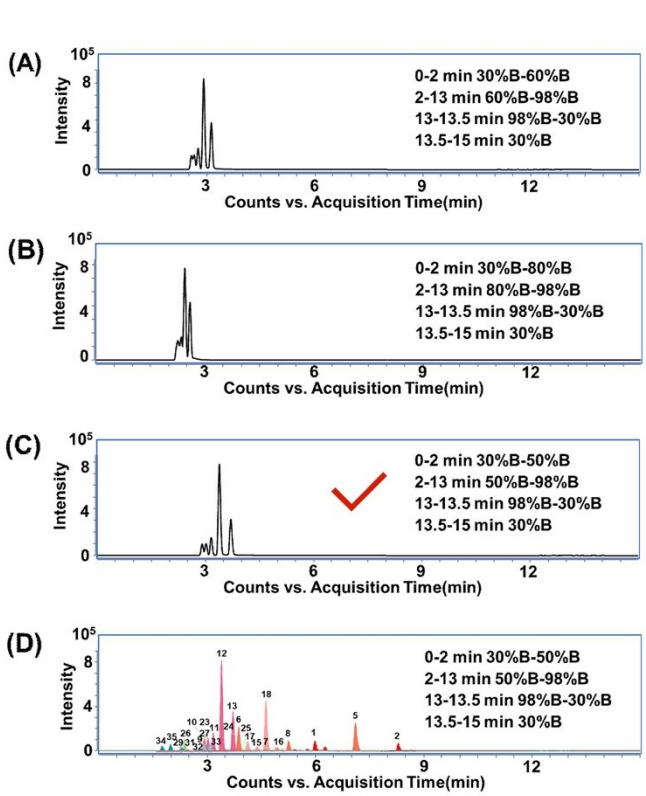

Figure S3. Optimization of gradient elution condition. (A-C) Optimization of 3 different gradient elution conditions: (A) 15 min: 0-2 min 30%B-80%B, 2-13 min 80%B-98%B, 13-13.5 min 98%B-30%B, 13.5-15 min 30%B; (B) 0-2 min 30%B-60%B, 2-13 min 60%B-98%B, 13-13.5 min 98%B-30%B, 13.5-15 min 30%B; (C) 0-2 min 30%B-50%B, 2-13 min 50%B-98%B, 13-13.5 min 98%B-30%B, 13.5-15 min 30%B. (D) isolation efficiency 9 pairs of isomers with the selected elution condition.

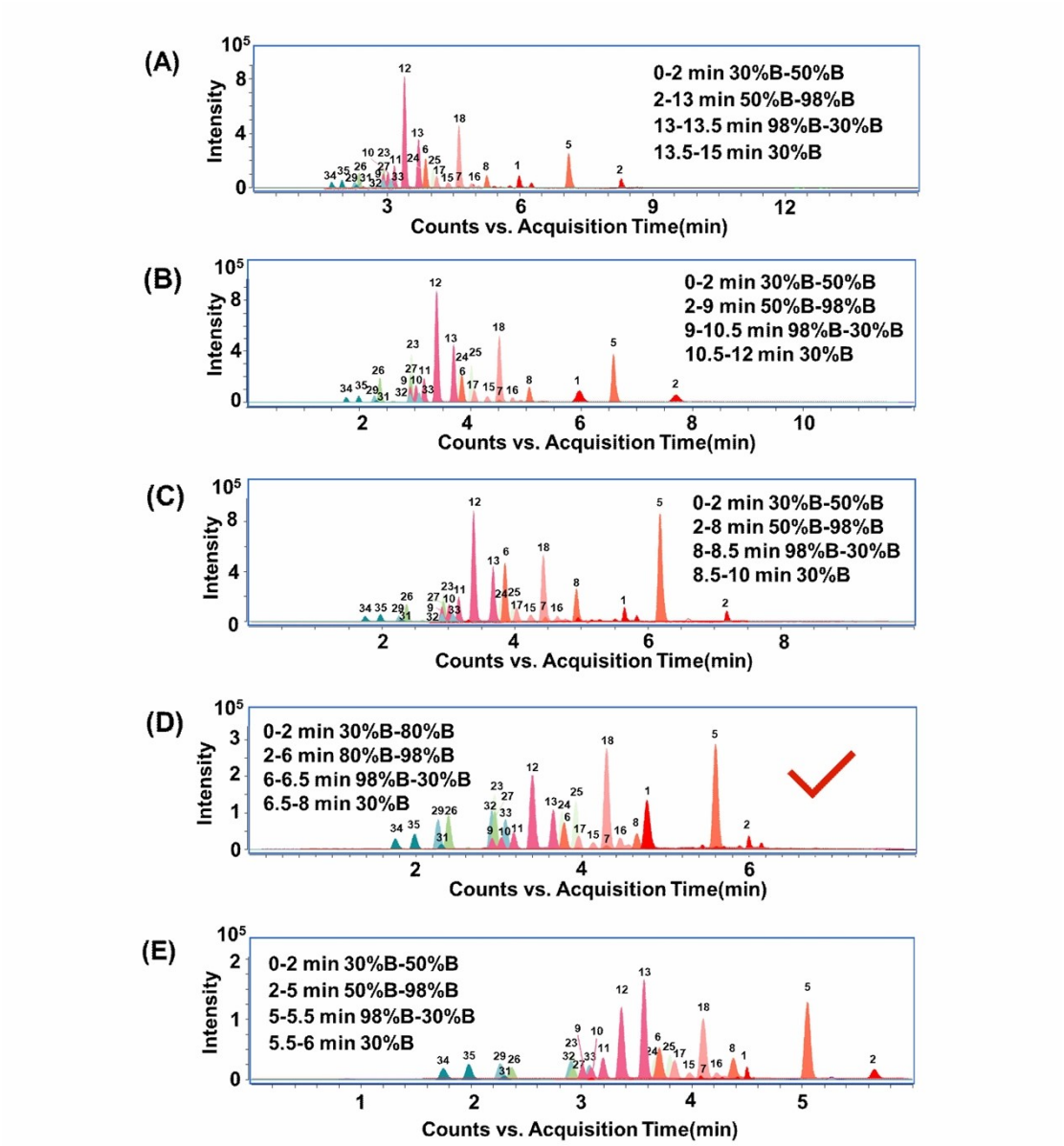

Figure S4. Isolation efficiency of 9 pairs of isomers at different elution condition: (A) 0-2 min 30%B-50%B, 2-13 min 50%B-98%B, 13-13.5 min 98%B-30%B, 13.5-15 min 30%B. (B) 0-2 min 30%B-50%B, 2-9 min 50%B-98%B, 9-10.5 min 98%B-30%B, 10.5-12 min 30%B. (C) 0-2 min 30%B-50%B, 2-8 min 50%B-98%B, 8-8.5 min 98%B-30%B, 8.5-10 min 30%B. (D) 0-2 min 30%B-80%B, 2-6 min 80%B-98%B, 6-6.5 min 98%B-30%B, 6.5-8 min 30%B. (E) 0-2 min 30%B-50%B,2-5 min 50%B-98%B, 5-5.5 min 98%B-30%B, 5.5-6 min 30%B.

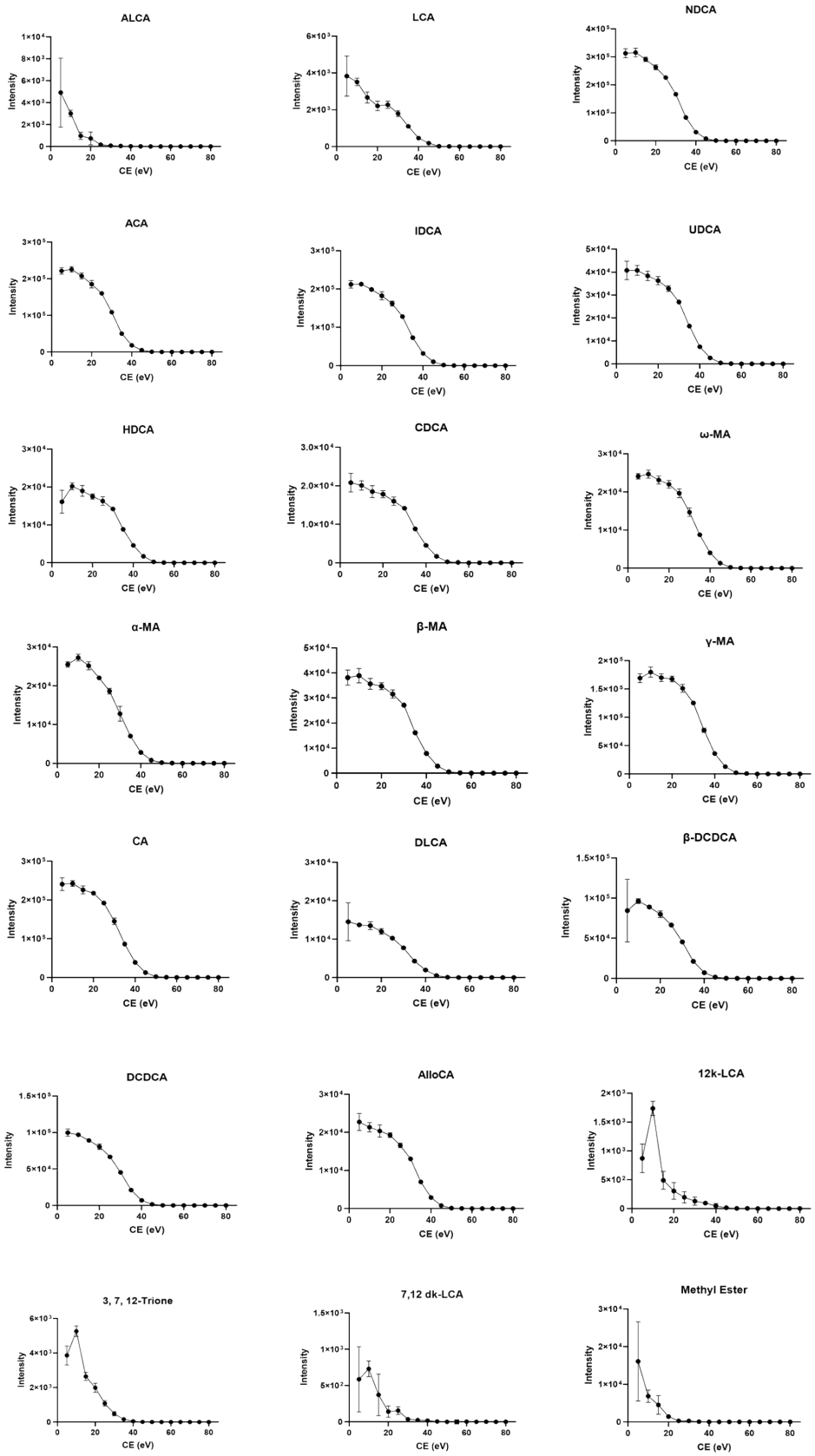

Figure S5. Optimization of collision energy (CE) voltage of free BAs.

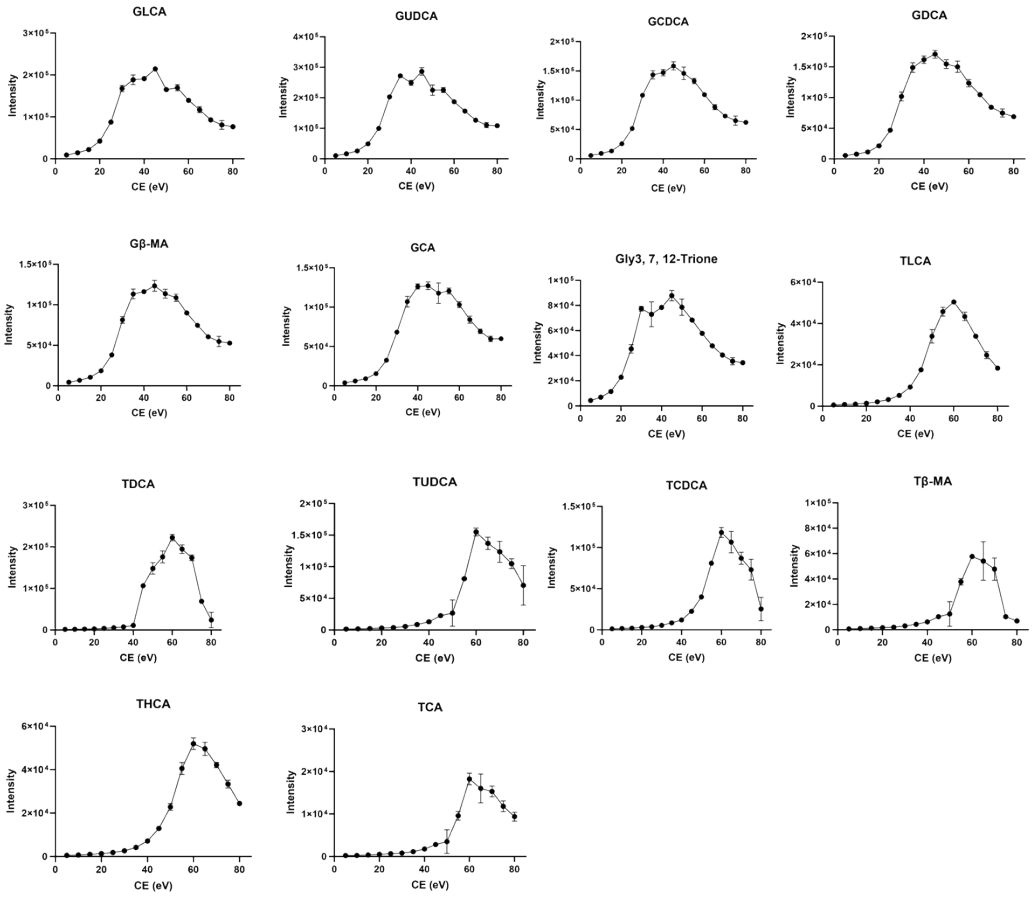

Figure S6. Optimization of collision energy (CE) voltage of conjugated BAs.

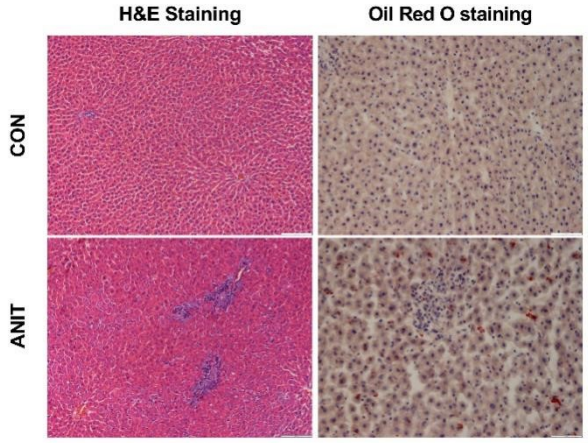

Figure S7. The H&E staining (scale bars: 100 μm) and Oil Red O staining (scale bars: 100 μm) of liver tissues in both Con group and ANIT group.