**Supplementary Materials**

**1. Variations of steroidal saponins under different storage time conditions**

**1.1 Preparation of sample solutions**

CFT and SFT (120 g) were stored at ro om temperature, and each sample (20 g) was collected in 0, 3 rd, 6 th, 9 th and 12 th month. The extraction method was based on a previous study. 0.1 g of FT powder was placed into stoppered conical flask, and ultrasonically extracted twice with 20 ml 70% ethanol (v/v), each extraction lasting for 30 min. The extracts were filtered, then the filtrates were combined and the volume was made up to exactly 50 ml using 70% ethanol. The sample solution was filtered through 0.22 μm microporous membrane for UHPLC-MS/MS analysis.

**1.2 Preparation of standard solutions**

Stock solutions of reference standards were prepared individually using 70% acetonitrile (v/v). The standard stock solutions of each of the analytes included 300 μg/ml of FOT, 103 μg/ml of terrestrosin D, 230 μg/ml of tribuluside A, 320 μg/ml of 25*R*-tribulosin, 200 μg/ml of terrestrinin D, 40 μg/ml of terrestroside B, and 71 μg/ml of terrestrosin K. They were measured and transferred using a micropipette and then mixedand diluted to prepare the final mixed reference substance stock solution. All solutions were stored at 4 ℃.

**2. Determination of *β*-glucosidase activity in CFT and SFT**

**2.1 Preparation of sample solutions**

To determine the wavelength of the maximum absorption peak of p-nitrophenol. Six concentrations of p-nitrophenol (2.5, 5, 10, 20, 40, and 80 μmol/L) were prepared to build the calibration curve.

CFT and SFT were added with pre-cooled buffer solution, and ground into homogenates, respectively. The homogenates were centrifuged, the volume of supernatant was made up to exactly 10 mL using buffer solution. The control group test tube was added with 0.8 mL enzyme extract and 0.2 mL buffer solution. The same volume enzyme extract and pNPG solution were added to the sample group test tube. Both were heated in a water bath at 45 ℃ for 45 min. The sodium carbonate solution was added to terminate the reaction. Then, the reaction solution was diluted twice by adding 5 mL sodium carbonate solution. The absorbance at 401 nm was read using UV spectrophotometer. And the enzyme activity unit was calculated by enzyme activity formula.

Enzyme activity unit (U) = (YV2VN)/(KV1Mt)

Y: enzymatic reaction absorbance, V2: total reaction volume, V: enzymatic extract volume, N: dilution factor, K: p-nitrophenol standard curve slope, V1: enzymatic solution volume in the reaction system, M: sample mass, t: reaction time.

Enzyme activity unit (U) was defined as the amount of enzyme required to hydrolyze 1 μmol/L pNPG of per 1 mL enzyme solution within 1 min.

**3. Conversion of furostanol saponins by enzymatic hydrolysis**

**3.1 Preparation of *β*-D-glucosidase solution**

The *β*-D-glucosidase was accurately weighed, and dissolved in water.

**3.1 Preparation of standard solutions**

Stock solutions of reference standards were prepared individually using 70% acetonitrile (v/v). The mixture of standard stock solutions 1 included 210 μg/ml of FOT, and 40 μg/ml of terrestrosin D. They were measured and transferred using a micropipette and then mixedand diluted to prepare the final mixed reference substance stock solution. The mixture of standard stock solutions 2 included 300 μg/ml of tribuluside A, and 300 μg/ml of 25*R*-tribulosin. All solutions were stored at 4 ℃.

**3.2 Preparation of sample solutions**

The reference solutions of FOT and tribuluside A were accurately measured and transferred using a micropipette, thermostatically heated with *β*-D-glucosidase solution for 1.5 min at 40 ℃. The enzymatic hydrolysates of the two reference substances were dissolved in 1 mL of 70% acetonitrile (v/v).

**4. Method validation**

**4.1 Linearity and limits of detection and quantification**

The standard solutions were diluted with 70% acetonitrile (v/v) to provide a series of standard solutions with the appropriate concentrations. y = ax + b was used to express the standard curve for each compound, where x is the concentration of each compound and y is the peak area of each compound. The limits of detection and quantification (LOD and LOQ) were determined on the basis of response at a signal-to-noise ratio of 3 and 10, respectively.

**4.2 Precision**

The intra- and inter-day variabilities were measured to determine the precision of our methods. The sample solution was analyzed six times on the same day and on three consecutive days to obtain intra- and inter-day variabilities. The relative standard deviation (RSD) was calculated to evaluate precision.

**4.3 Stability and repeatability**

Replicate injections of the solution were performed at room temperature at 0, 2, 4, 6, 8 and 12 months to investigate the stability of the solution. The RSD was calculated as a measure of stability. To assess the repeatability of the method, six samples from the same raw FT were analyzed with the proposed method, and the RSD was calculated.

**4.4 Recoveries**

Certain amounts of reference substances (low, medium, and high concentrations) were added to a certain amount of raw FT sample. The resultant samples were extracted and analyzed with the proposed method and three replicates were performed at each level. The percentage recoveries were calculated according to the following equation: (detected amount – original amount)/spiked amount × 100%.

**5. Method validation**

**5.1 Linearity and limits of detection and quantification**

The calibration curves were determined by the ratio of the peak area of the measured component to the component concentration using the method described previously. The LODs and LOQs under the present chromatographic conditions were determined at signal-to-noise ratios of 3 and 10, respectively. The calibration curve, correlation coefficient, linear range, LOD and LOQ for each analyte are presented in Supplementary Table S1.

**5.2 Precision**

In order to investigate the precision of the samples, the samples were analyzed six times on the same day and on three consecutive days to examine the changes in intra-day and inter-day precision. The RSDs were 2.19%-3.12% and 2.39%-3.08%. All the data showed that the instrument has good precision.

**5.3 Stability and repeatability**

The sample solutions were injected and analyzed at 0, 2, 4, 6, 8, and 12 months, respectively. The RSDs of the four analytes were 3.01%-3.51%. All data showed that the sample solution was stable within 12 months. Six sample solutions were analyzed as previously described. The RSDs are presented in Supplementary Table S1, indicating that the method has good repeatability.

**5.4 Recoveries**

The different concentrations of spiked sample solutions were extracted and analyzed with the proposed method and three replicates were performed at each level. The recovery of the four analytes was in the range of 99.00–100.76%, and the RSD was ≤ 2.56%. It shows that the method has good accuracy and is suitable for quantitative determination of four analytes.

**Table S1**

Calibration curves, correlation coefficients, linearity range, limits of detection (LOD), limits of quantitation (LOQ), intra- and inter-day precision, stability, repeatability and recovery of the analyte

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Analytes | Calibration curve | *R*2 | Linear range（ng/mL） | LOD（ng/mL） | LOQ（ng/mL） | Precision (%, RSD) | | Stability  (%, RSD) | Repeatability  (%, RSD） | Recovery | |
| Intra-day  (*n* = 6) | Inter-day  (*n* = 3) | Mean recovery  (%) | RSD (%) |
| FOT | y = 55.606x + 69.374 | 0.9997 | 594.51-608778 | 178.49 | 309.38 | 2.24 | 2.78 | 3.51 | 3.45 | 98.49 | 1.72 |
| terrestrosin D | y = 750.51x - 31.5 | 0.9999 | 78.51-20098 | 5.58 | 17.15 | 2.19 | 2.39 | 3.27 | 2.77 | 100.04 | 2.46 |
| tribuluside A | y = 88.146x + 0.0789 | 0.9998 | 140.25-35904 | 3.55 | 12.07 | 2.52 | 2.69 | 3.30 | 2.95 | 98.38 | 2.34 |
| 25*R*-tribulosin | y = 666.83x - 8.0702 | 0.9999 | 29.27-7493 | 10.92 | 27.38 | 3.12 | 3.8 | 3.01 | 3.25 | 100.26 | 2.56 |

**Table S2** Optimized multiple reaction monitoring parameters of the four analytes

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Analytes | Precursor ion  (*m/z*) | Fragmentor voltage  (V) | Product ion  (*m/z*) | Collision  energy  (V) | Detected  ion |
| FOT | 1211.5 | 75 | 1049.5 | 20 | [M+H-H2O]+ |
| terrestrosin D | 1071.5 | 255 | 939.6 | 80 | [M+Na]+ |
| tribuluside A | 1313.6 | 320 | 887.4 | 30 | [M+H-H2O]+ |
| 25*R*-tribulosin | 1173.6 | 310 | 1027.6 | 85 | [M+Na]+ |

**Table S3** Four compounds identified from enzymatic hydrolysates

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Peak | *t*r (min) | Formula | Precursor  Ion | Selective Ion  MS2 | Fragmentation | Identification |
| 1 | 7.17 | C56H92O29 | 1227.5635 | [M-H]- | 1095.5205, 933.4679, 771.4141, 609.3619 | 26-*O*-*β*-D-glucopyranosyl-3*β*,22*α*,26-triol-(25*R*)-5*α*-furostan-12-one-3-*O*-*β*-D-galactopyranosyl-(1→2)-  [*β*-D-xylopyranosyl-(1→3)]-*β*-D-glucopyranosyl-(1→4)-*β*-D-galactopyranoside |
| 2 | 26.01 | C50H80O23 | 1047.4956 | [M-H]- | 1093.5124, 1047.4957, 915.4617, 753.4058, 591.3602 | Terrestrosin D |
| 3 | 18.89 | C61H102O31 | 1329.6353 | [M-H]- | 1197.5852, 1065.5418, 1051.5361, 919.4877, 757.4336 | Tribuluside A |
| 4 | 29.74 | C55H90O25 | 1149.5686 | [M-H]- | 1017.5231, 885.4907, 739.4298 | 25*R*-tribulosin |

**Table S4** Steroidal saponins identified from ethanolic extracts

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Peak | *t*r  (min) | Formula | Precursor  Ion | Selective Ion  MS2 | Fragmentation | Identification |
| 1 | 4.33 | C45H74O21 | 949.4602 | [M-H]- | 787.4095, 625.3556 | Tribufuroside E [3] |
| 2 | 5.98 | C51H84O25 | 1095.5175 | [M-H]- | 933.4668, 771.4203, 609.3651 | Terrestrosin I [1] |
| 3 | 6.88 | C51H84O24 | 1079.5214 | [M-H]- | 933.4633, 917.4761, 771.4146 | 26-*O*-*β*-D-glucopyranosyl-3*β*,22*α*,26-triol-(25*S*)-5*α*-furostan-12-one-3-*O*-*β*-D-glucopyranosyl-(1→4)-  [*α*-L-rhamnopyranosyl-(1→2)]-*β*-D-galactopyranoside [4] |
| 4 | 7.17 | C56H92O29 | 1227.5621 | [M-H]- | 1095.5204, 933.4679, 771.4142, 609.3618 | 26-*O*-*β*-D-glucopyranosyl-3*β*,22*α*,26-triol-(25*R*)-5*α*-furostan-12-one-3-*O*-*β*-D-galactopyranosyl-(1→2)-  [*β*-D-xylopyranosyl-(1→3)]-*β*-D-glucopyranosyl-(1→4)-*β*-D-galactopyranoside a |
| 5 | 7.21 | C45H74O20 | 933.4636 | [M-H]- | 771.4140, 609.3648, 447.3101 | 26-*O*-*β*-D-glucopyranosyl3*β*,22*α*,26-triol-(25*R*)-5*α*-furostan-12-one-  -3-*O*-*β*-D-  glucopyranosyl-(1→4)-β-  D-galactopyranoside [5] |
| 6 | 8.89 | C51H86O25 | 1097.5401 | [M-H]- | 935.4867, 773.4305, 611.3793 | Terrestrosin G [1] |
| 7 | 8.99 | C33H54O10 | 609.3644 | [M-H]- | 447.3121 | Terrestrinin F [6] |
| 8 | 10.03 | C45H76O20 | 981.4860 | [M+HCOO]- | 935.4806, 773.4316, 611.3786 | Terrestrosin F [1] |
| 9 | 12.52 | C45H72O20 | 931.4532 | [M-H]- | 769.4015, 607.3471, 445.2950 | Tribufuroside J [7] |
| 10 | 15.14 | C51H82O24 | 1077.5129 | [M-H]- | 915.4628, 753.4051, 591.3566 | Terrestrosin K a |
| 11 | 15.89 | C33H50O10 | 651.3395 | [M+HCOO]- | 605.3319, 443.2803 | Terrestrinin D a |
| 12 | 16.02 | C56H94O28 | 1213.5832 | [M-H]- | 1081.5396, 1051.5282, 919.4908 | Uttroside B [2] |
| 13 | 16.07 | C51H84O23 | 1063.5290 | [M-H]- | 917.4765, 901.4837, 755.4201, 593.3695 | Protogracillin [2] |
| 14 | 16.14 | C51H82O23 | 1061.5130 | [M-H]- | 915.4571, 899.4602, 753.4071, 591.3530, 429.3208 | 26-*O*-*β*-D-glucopyranosyl-3*β*,26-diol-  (25*S*)-5*α*-furostan-12-one-  20(22)-en-3-*O*-  *β*-D-glucopyranosyl-(1→4)-  [*α*-L-rhamnopyranosyl-(1→2)]-*β*-D-galactopyranoside [8] |
| 15 | 16.41 | C45H72O19 | 915.4594 | [M-H]- | 753.4022, 591.3543 | 26-*O*-*β*-D-glucopyranosyl-3*β*,26-diol-  (25*R*)-5*α*-furostan-12-one-  20(22)-en-3-*O*-*β*  -D-glucopyranosyl-(1→4)-  *β*-D-galactopyranoside [9] |
| 16 | 16.77 | C51H86O22 | 1049.5568 | [M-H]- | 903.4903, 757.4332 | Neoprotodioscin [10] |
| 17 | 18.07 | C51H84O24 | 1079.5258 | [M-H]- | 917.4718, 755.4264, 593.3685 | 26-*O*-*β*-D-glucopyranosyl-2*α*,3*β*,26-triol-(25*S*)-5*α*-furostan-20(22)-  en-3-*O*-*β*-D-  galactopyranosyl-(1→2)-*β*-  D-glucopyranosyl-(1→2)-*β*-  D-galactopyranoside [11] |
| 18 | 18.26 | C61H102O31 | 1329.6302 | [M-H]- | 1197.5853, 1065.5417, 1051.5361, 919.4877, 757.4337 | Tribuluside A a |
| 19 | 18.61 | C45H74O19 | 917.4726 | [M-H]- | 755.4192, 593.3650 | 26-*O*-*β*-D-glucopyranosyl-2*α*,3*β*,26-triol-(25*R*)-5*α*-furostan-20(22)-  en-3-*O*-*β*-D-  glucopyranosyl-(1→4)-*β*-  D-galactopyranoside [12] |
| 20 | 20.99 | C33H52O9 | 591.3545 | [M-H]- | 429.2998 | 26-*O*-*β*-D-glucopyranosyl-3*β*,26-diol-  (25*S*)-5*α*-furostan-12-one-  20(22)-en [11] |
| 21 | 21.04 | C56H92O27 | 1195.5690 | [M-H]- | 1063.5263, 1033.5172, 901.4755, 739.4245 | Terrestrinin T [9] |
| 22 | 21.85 | C51H82O22 | 1045.5188 | [M-H]- | 899.4602, 737.4093, 575.3621 | Pseudoprotogracillin [9] |
| 23 | 22.11 | C33H48O9 | 633.3266 | [M+HCOO]- | 587.3244, 425.2681 | Terrestrinin U [9] |
| 24 | 22.54 | C61H100O30 | 1311.6240 | [M-H]- | 1165.5575 ,1033.5267, 901.4854 | Terrestroside B a |
| 25 | 25.42 | C50H80O23 | 1047.4956 | [M-H]- | 1093.5123, 1047.4957, 915.4618, 753.4058, 591.3602 | Terrestrosin D |
| 26 | 25.59 | C45H72O19 | 961.4622 | [M+HCOO]- | 915.4606, 753.4101, 591.3563 | Terreside A [9] |
| 27 | 26.87 | C50H82O23 | 1049.5207 | [M-H]- | 917.4698, 755.4245 | Desglucolanatigonin II [9] |
| 28 | 28.77 | C50H82O22 | 1079.5228 | [M+HCOO]- | 1033.5178, 901.4748, 739.4299, 577.3754 | Gitonin [9] |
| 29 | 28.78 | C55H90O25 | 1149.5682 | [M-H]- | 1017.5212, 885.4863, 739.4255, 577.3772 | Tribulosin [9] |
| 30 | 29.04 | C55H90O25 | 1149.5641 | [M-H]- | 1017.5231, 885.4907, 739.4298 | 25*R*-tribulosin a |
| 31 | 33.69 | C39H62O12 | 767.4235 | [M+HCOO]- | 721.4148, 575.3598 | Tribestin [9] |

a identified by comparing with reference standards

[1] Wang Y, Ohtani K, Kasai R, et al. Steroidal saponins from fruits of *Tribulus terrestris*[J]. Phytochemistry,1997,45(4):811-817.

[2] Zheng W, Wang FX, Zhao Y, et al. Rapid Characterization of Constituents in *Tribulus terrestris* from Different Habitats by UHPLC/Q-TOF MS[J]. J Am Soc Mass Spectrom,2017,28(11):2302-2318.

[3] Xu YJ, Xu TH, Liu Y, et al. Two new steroidal glucosides from *Tribulus terrestris* L.[J]. J Asian Nat Prod Res,2009,11(6):548-553.

[4] Cai LF, Wu YJ, Zhang JG, et al. Steroidal Saponins from *Tribulus terrestris*[J]. Planta Med,2001,67(2):196-198.

[5] Chen G, Su L, Feng SG, et al. Furostanol saponins from the fruits of *Tribulus terrestris*[J]. Nat Prod Res,2013,27(13):1186-1190.

[6] Kang LP, Wu KL, Yu HS, et al. Steroidal saponins from *Tribulus terrestris*[J]. Phytochemistry,2014,107:182-189.

[7] Xu TH, Xu YJ, Liu Y, et al. Two new furostanol saponins from *Tribulus terrestris* L[J]. Fitoterapia,2009,80(6):354-357.

[8] Xu YJ, Liu YH, Xu TH, et al. A New Furostanol Glycoside from *Tribulus terrestris*[J]. Molecules,2010,15(2):613-618.

[9] Zheng W, Wang FX, Zhao Y, et al. Rapid Characterization of Constituents in *Tribulus terrestris* from Different Habitats by UHPLC/Q-TOF MS[J]. J Am Soc Mass Spectrom,2017,28(11):2302-2318.

[10] De Combarieu E, Fuzzati N, Lovati M, et al. Furostanol saponins from *Tribulus terrestris*[J]. Fitoterapia,2003,74(6):583-591.

[11] Xu TH, Xu YJ, Xie SX, et al. Two new furostanol saponins from *Tribulus terrestris* L.[J]. J Asian Nat Prod Res,2008,10(5):419-423.

[12] Liu T, Chen G, Yi GQ, et al. New pregnane and steroidal glycosides from *Tribulus terrestris* L.[J]. J Asian Nat Prod Res,2010,12(3):209-214.

**Table S5** Cytotoxicities of steroidal saponins on LO2 and 293T cells (*n* = 6).

|  |  |  |  |
| --- | --- | --- | --- |
| Compound | Concentration  (μM) | Inhibition rate (%) | |
| LO2 | 293T |
| FOT | 41.3 | 28.42±2.73 | 17.27±0.56 |
| 20.6 | 22.01±1.40 | 14.66±1.33 |
| 10.3 | 18.27±2.05 | 10.69±1.89 |
| 5.2 | 12.72±0.38 | 8.16±0.56 |
| 2.6 | 7.16±0.77 | 6.16±0.53 |
| terrestrosin D | 41.3 | 74.68±1.33 | 59.12±2.43 |
| 20.6 | 62.65±1.91 | 50.67±1.69 |
| 10.3 | 40.11±1.60 | 48.31±1.67 |
| 5.2 | 7.44±1.78 | 6.21±1.43 |
| 2.6 | 2.55±0.19 | 2.69±0.39 |



**Fig. S1** Flowchart of the strategy of studies on “enzyme inactivation and toxicity reduction”