

## Determination of 13 Free Fatty Acids in *Pheretima* Using Ultra-Performance LC-ESI-MS

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### **Abstract:**

A simple and rapid ultra-performance liquid chromatography-electrospray ionization mass spectrometry method for the simultaneous determination of thirteen free fatty acids (FFAs) in *Pheretima* has been developed and validated. Measurements for each FFA were linear over a wide range (0.05–3.95  $\mu\text{g mL}^{-1}$ ) with good correlation coefficients (>0.99). The limit of detection and limit of quantification for all the fatty acids were below 26 and 78  $\text{ng mL}^{-1}$ , respectively. The intra- and inter-assay precision and accuracy for the thirteen FFAs fell well within the predefined limits of acceptability. Satisfactory recoveries were in the range of 96–103%.

### **Article:**

#### INTROCUPTION

*Pheretima* has been well known for its wide therapeutic properties such as anti-inflammatory, anti-oxidative [1], anti-asthmatic, thrombolytic, reducing symptoms of the central nervous system decline including memory loss in traditional Chinese medicine (TCM) for over 2,000 years [2]. Four kinds of earthworms, including *Pheretima aspergillum* (E. Perrier), *Pheretima guillelmi* (Michaelson), *Pheretima vulgaris* Chen, *Pheretima pectinifera* Michaelson are included in the Pharmacopoeia of the People's Republic of China (2005). Since patients with asthma and other forms of allergy have been repeatedly reported to present an abnormal plasma fatty acid pattern [3], fatty acids extensively occurring in animal origin TCM seem to play an important role and act as the bioactive components. In our previous work, the acidic fraction isolated from *Pheretima* containing free fatty acids (FFAs) showed obvious anti-asthmatic activities [4], and the FFAs in the active fraction probably contributed to these pharmacological effects. In order to facilitate the future researches about these activities, it is paramount to establish a qualitative and quantitative assay method for the determination of FFAs in *Pheretima*.

Although fatty acids have measurable absorbance in the range of 190–215 nm, the interference of most solvents is a limiting factor for sensitive detection when analyzed directly by liquid

chromatography-ultraviolet spectrometry (LC-UV). To increase sensitivity, gas chromatography (GC) has been widely applied in FFA analysis after the absolute necessary derivatization of FFAs by esterification to form relative low polar analytes.

Due to the high selectivity provided by mass spectrometric (MS) detection [2], and the capability of analyzing non-volatile compounds provided by liquid chromatography (LC), in most cases, derivatization to form UV-absorptive and volatile compounds is dispensable using LC combined with MS. Thus, LC-MS provides a relatively rapid, reproducible method which is suitable for the determination of thermolabile, non-volatile multi-components without obvious UV absorption. As the primary evolution of LC, ultra-performance liquid chromatography (UPLC) employs particles smaller than 2  $\mu\text{m}$  in diameter to achieve superior resolution, rapid speed and higher sensitivity compared to LC. Therefore, UPLC coupled with MS is suggested as a better alternative method for FFA assay in *Pheretima*.

Zehethofer et al. [5] had developed a method for profiling of 29 FFAs in plasma using UPLC-ESI-MS-MS with the positive ion mode by cationization of the FFAs via addition of barium ions for sensitive multiple reaction monitoring (MRM). However, to our knowledge the application of UPLC-MS to the analysis of FFAs in *Pheretima* raw materials has not been reported to date. In the present work, we aim to develop a simple UPLC electrospray ionization MS (UPLC-ESI-MS) method in the negative ion mode by selected ion monitoring (SIM) without adding any additives for the determination of FFAs in *Pheretima* raw materials and the isolated bioactive fraction to elucidate the composition variation between the two samples for further bioactive evaluation.

## EXPERIMENTAL

### *Material and Reagents*

The animal materials were purchased from Huayu Pharmaceutical Company in Shanghai. The authenticated samples were identified as *Pheretima vulgaris* Chen by Dr. Zhaohui Xu, and the voucher specimen (SJTU 05-12-01) were deposited in the Herbarium, School of Pharmacy, Shanghai Jiao Tong University. The earthworms were caught, killed and then eviscerated, with the viscera and organic components washed away, then dried and reduced to coarse powder.

Thirteen FFA standards including dodecanoic acid ( $\text{C}_{12-0}$ ), tri-decanoic acid ( $\text{C}_{13-0}$ ), 12-methyltridecanoic acid (Iso- $\text{C}_{14-0}$ ), tetra-decanoic acid ( $\text{C}_{14-0}$ ), 12-methyltetradecanoic acid (Iso- $\text{C}_{15-0}$ ), pentadecanoic acid ( $\text{C}_{15-0}$ ), hexadecanoic acid ( $\text{C}_{16-0}$ ), 14-methyl hexadecanoic acid (Iso- $\text{C}_{17-0}$ ), heptadecanoic acid ( $\text{C}_{17-0}$ ), octadecanoic acid ( $\text{C}_{18-0}$ ), *cis*-9-octadecenoic acid ( $\text{C}_{18-1}$ ), 9,12-octadecadienoic acid ( $\text{C}_{18-2}$ ), arachidic acid ( $\text{C}_{20-0}$ ) were obtained from Sigma (St. Louis, MO, USA).

Methanol and acetonitrile were purchased from Merck (New Jersey, USA). Ultra-pure water from a Millipore system (Millipore, Billerica, USA) was used throughout the work.

### *Standard Solutions Preparation*

Stock solutions containing 0.1–1  $\text{mg mL}^{-1}$  of various FFAs were prepared in LC-grade methanol and stored in the dark at 4  $^{\circ}\text{C}$  for a month. Working standard solutions were prepared from these stock solutions and diluted with methanol prior to analysis.

### *Sample Solutions Preparation*

In order to obtain homogenous raw material used for sample preparation, the crude powder of *Pheretima* at 20–40 mesh was obtained after comminuting and sieving. In the present work, the extraction efficiency of different ratios of methanol/water (*v/v*) were compared, especially, 10 mg raw material was ultrasonically treated three times by 10 mL 50, 80 and 100% methanol at 40 °C respectively for 1 h each time. The individual three suspensions were pooled and then centrifuged (14,000 rpm for 5 min at 4 °C). After partial solvent of the supernatant removed by rotatory evaporation at 40 °C, the final volume of the total extract was quantified to 10 mL with methanol. The bioactive fraction of *Pheretima* was prepared according to the process reported previously [4], thus 15.5 µg mL<sup>-1</sup> solution of the fraction was obtained by dissolving in 80% methanol. Both the total extract and the bioactive fraction solution were subsequently passed through a syringe filter of 0.22 µm, and then the filtrate was injected into the UPLC-MS for analysis.

### *Apparatus and Chromatographic Conditions*

The UPLC-ESI-MS system was performed on an ACQUITY UPLC system (Waters, MA, USA), equipped with a binary solvent delivery system and an autosampler. Chromatographic separation was carried out on an ACQUITY UPLC BEH C<sub>18</sub> column (2.1 mm × 50 mm, 1.7 µm particle size) (Waters, MA, USA). The column was maintained at 40 °C. The mobile phase consisted of water as solvent A and acetonitrile as solvent B. Separation was performed by gradient elution: The ratio of solvent A to solvent B decreased linearly from 70:30 (solvent A: solvent B, *v/v*) to 25:75 (*v/v*) over the first 10 min, then increased to 0:100 (*v/v*) over next 10 min. The flow rate was 0.2 mL min<sup>-1</sup>. The injection volume was 5 µL. Each washing cycle consisted of 200 µL of strong washing solvent (acetonitrile:water, 90:10) and 600 µL of weak washing solvent (acetonitrile:water, 10:90).

Mass spectrum was carried out on a Micromass-ZQ mass spectrometer (Waters, MA, USA). ESI mass spectra were acquired in the negative ion mode by SIM mode. The [M-H]<sup>-</sup> ion was used to monitor all FFA responses. The parameters used for the mass spectrometer were: capillary voltage 3.0 kV, cone voltage -35 V, extractor -5 V, Rf lens -0.1 V, source temperature 100 °C, desolvation temperature 200 °C, desolvation gas flow rate 600 L h<sup>-1</sup>, cone gas flow rate 50 L h<sup>-1</sup>, low and high mass resolution 15.0, ion energy 0.5 V, and electron multiplier voltage 650 V. The dwell time for each channel was 0.08 s.

### *Method Validation*

To assess linearity, a series of standard solutions of FFAs were detected and the calibration curves were obtained by plotting the nominal standard concentration (*x*) versus the peak area (*y*) of the analytes. The limit of detection (LOD) was calculated by measuring the standards of decreasing concentrations to establish the lowest concentration that the method can detect with a suitable response in the UPLC-ESI-MS (the ratio of the testing peak signal-to-noise, S/N = 3). The limit of quantification (LOQ) was defined as the lowest concentration that the method can quantify at acceptable UPLC-ESI-MS criteria with an S/N of 10. The intra-day and inter-day assay precision and accuracy of the method were assessed at three concentration levels, each concentration level with six replicates in a single day and on six consecutive days, respectively. The relative standard deviation (RSD) was calculated from the observed mass concentrations as

follows:  $RSD (\%) = [\text{standard deviation (SD)}/C_{\text{obs}}] \times 100$ . The accuracy (% bias) was calculated from the nominal mass concentration ( $C_{\text{nom}}$ ) and the mean value of the observed concentration ( $C_{\text{obs}}$ ) as follows:  $\text{bias} (\%) = [(C_{\text{obs}} - C_{\text{nom}})/(C_{\text{nom}})] \times 100$ . The recovery was performed by adding a certain amount of individual standard into a certain amount namely 10 mg of raw *Pheretima* powder. The mixture was extracted and analyzed using the method mentioned above. Three replicates were performed for the tests.

## RESULTS AND DISCUSSION

### *Chromatographic and Mass Spectrometric Behavior of Thirteen Free Fatty Acids*

The mixed standard solution with about  $2 \mu\text{g mL}^{-1}$  individual compound was subjected to the UPLC-ESI-MS analysis described above, each compound provided the deprotonated molecule  $[M-H]^-$  as the base ion peaks. The typical chromatogram on different channels of the mixed standard solution demonstrated the deprotonated molecules and corresponding retention time (Fig. 1), where satisfactory separation of the thirteen FFAs in the mixed standard solution was achieved within 19 min (eleven FFAs including two pairs of isomers (Iso- $C_{15-0}$  and  $C_{15-0}$ , Iso- $C_{17-0}$  and  $C_{17-0}$ ) could be baseline separated except that the resolution of isomer of Iso- $C_{14-0}$  and  $C_{14-0}$  was more than 1.2). But the assay of long chain FFAs by LC is a very time-consuming process because of their high hydrophobic retention. It is reported that the assay of FFAs using  $C_{18}$  column needs a fairly long analytical time up to 40–70 min [6, 7]. Due to UPLC involved, the present analytical time was greatly shortened without compromising the high resolution.

The chromatograms of the mixed standard solution (Fig. 1) clearly demonstrated that the retention time was directly proportional to chain length and inversely proportional to the degrees of unsaturation of the FFAs, which was consistent with the previous observations by some researchers with LC [8].

### *Optimization of the Extraction Method*

An amount of 50, 80 and 100% methanol extraction solution of *Pheretima* with an identical concentration were analyzed using the UPLC-MS conditions described above except the initial mobile phase concentration in the chromatographic separation was 50% acetonitrile other than the 30% one. The signal response comparison demonstrated that 50% methanol extract displayed high intensity of ions with  $C_{12-15}$  chain, but consisted of more solutes corresponding to nonvolatile or less volatile solutes. These solutes not only affected the separation of interested compounds, but also interfered with identifying interested ions in mass detection, because they can influence the spray droplet solution properties and then cause ion suppression affecting the quantitative performance of the mass detector. About 80 and 100% methanol extracts displayed higher ions response intensity as a whole besides the  $C_{16-20}$  chains, and contained fewer polar solutes which were liable to cause ion suppression. Furthermore, 80% methanol extract displayed the higher whole intensity response than those of 100% extracts (Fig. 2) probably due to its higher extraction efficiency. As a result, 80% methanol was chosen to be the suitable extraction solvent. This extraction method is simpler compared to previously reported methods using methanol, chloroform and deionized water as extraction solvents [9].

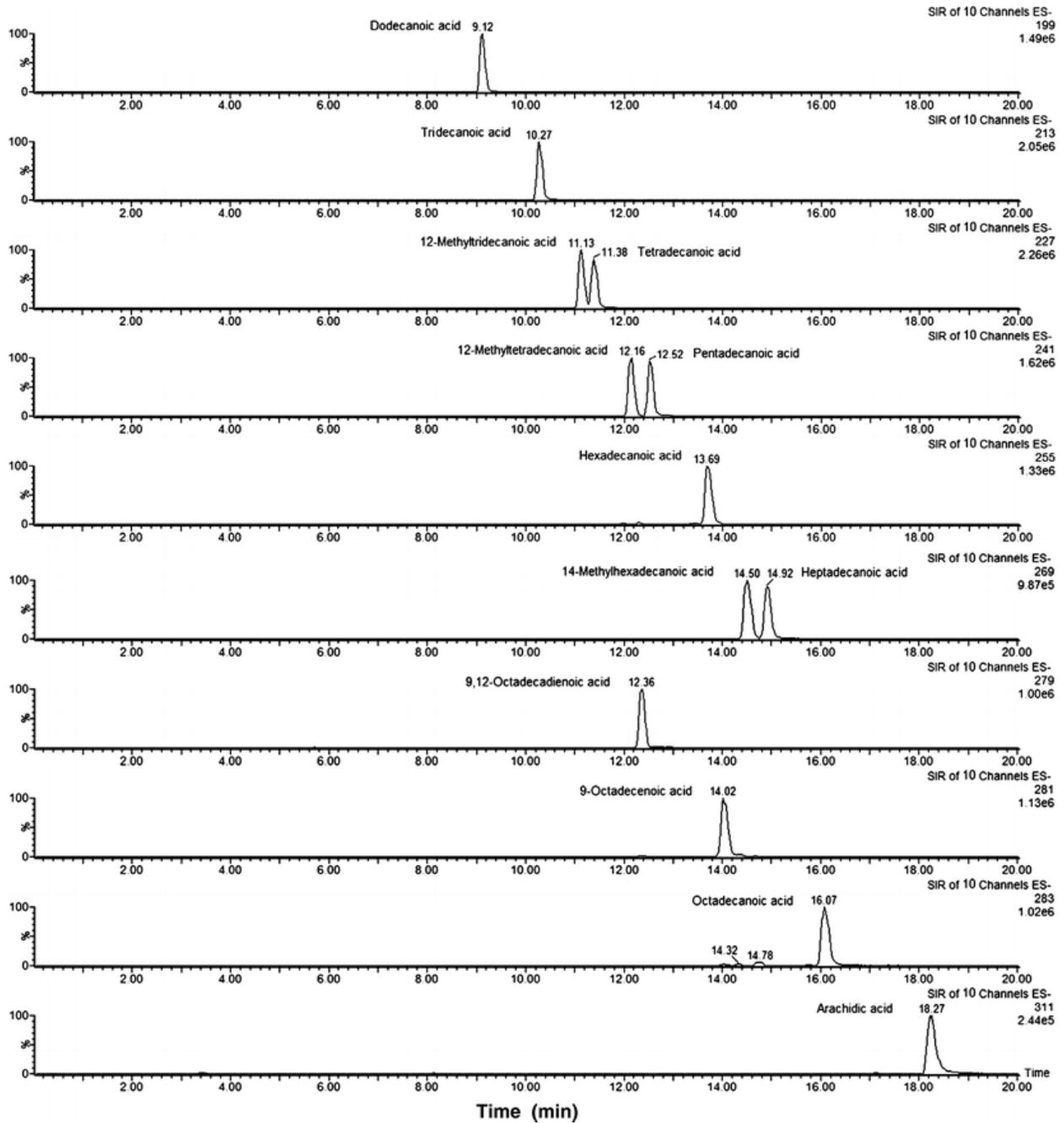
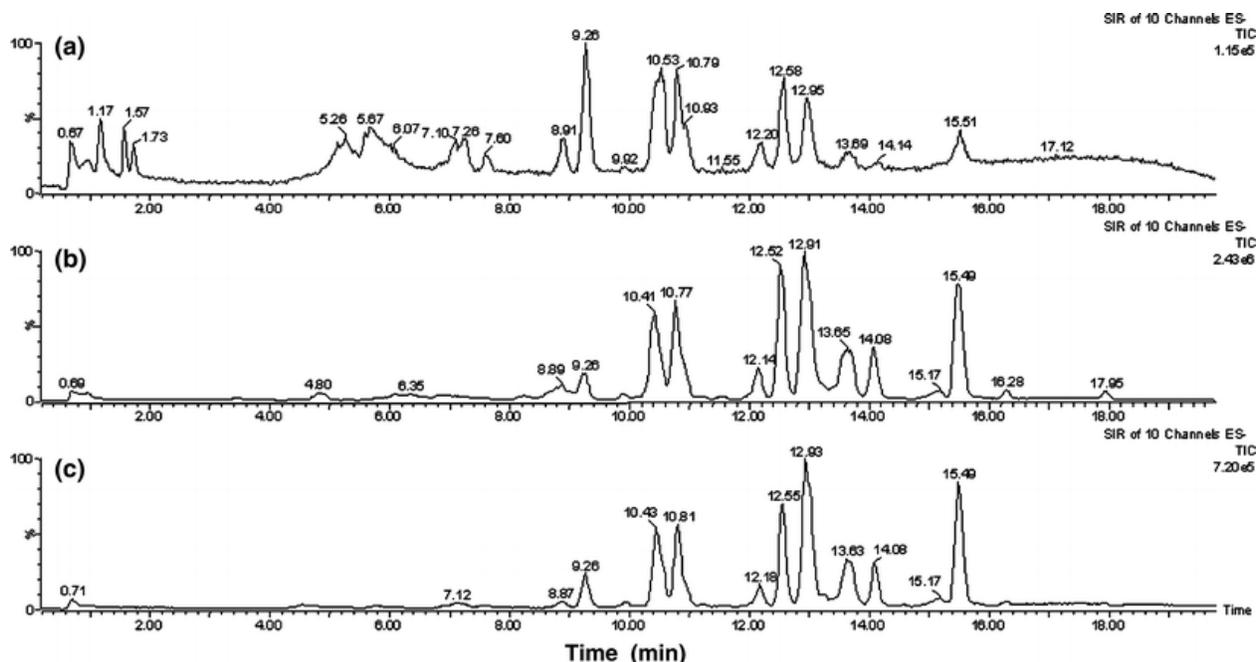


Figure 1: Typical chromatograms of mixed standard solution on different channels



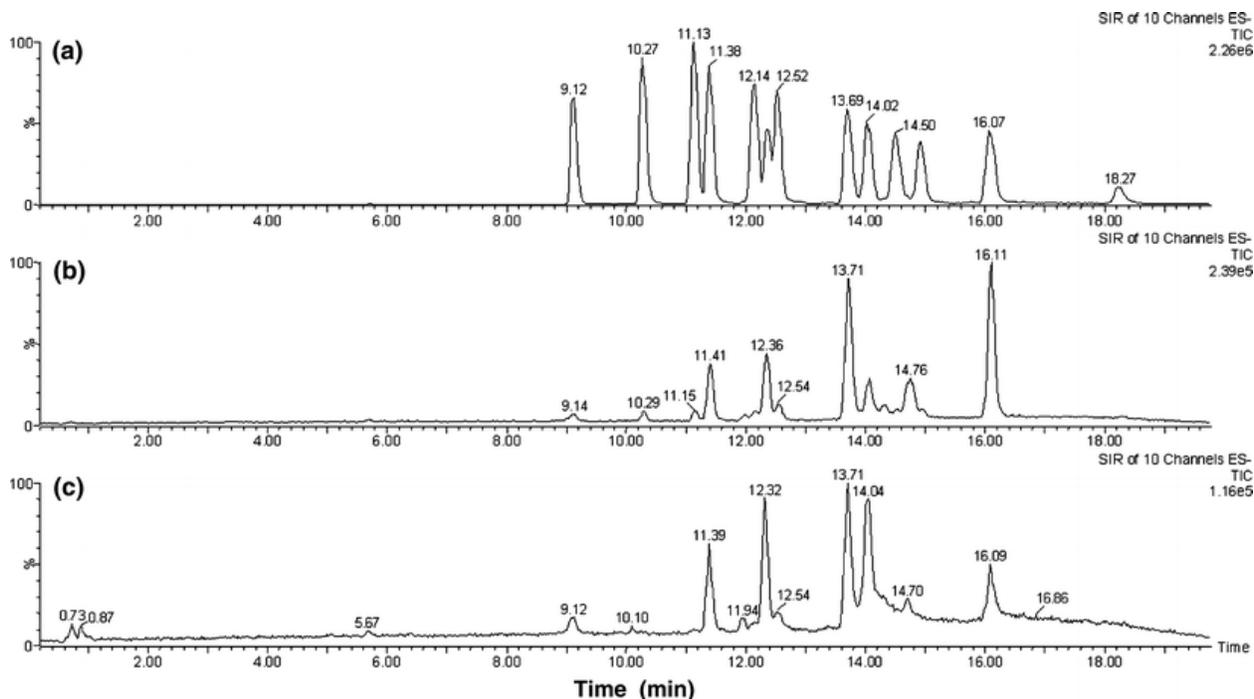
**Figure 2:** Extraction efficiency of three different solvents. **a** 50% methanol, **b** 80% methanol, **c** 100% methanol

### *Optimization of the Chromatography Separation*

Mass spectra for each FFA were acquired for further studies using direct sample infusion by continuous infusion  $10 \mu\text{L min}^{-1}$ . ESI was used as ionization sources both in negative and positive modes, changing the cone voltage between 25 and 70 V, extractor voltage between 3 and 10 V, in a range of  $m/z$  between 70 and 1,000 but only effective results were obtained using in negative mode. We selected the adductive ion of  $[\text{M}-\text{H}]^-$  as the target  $m/z$  as it showed the best sensitivity and stability. Since the extraction solution is a complicated mixture of compounds with diverse chemical structures, the SIM mode was particularly employed. The MS parameters were optimized as described before.

For the optimization of the separation and ionization, the identification of individual FFA in the *Pheretima* extract was performed by comparing the mass spectra and retention time to those of standards available, then several elution systems were tested, including acetate-ammonium or 0.01% acetic acid added in mobile phase. The results showed that the mobile phase consisting of pure water and acetonitrile could provide better ionization efficiency than that of the aforementioned additives. These additives presumably resulted in ion suppression in the extremely complicated mixture including large amounts of acidic and alkali compounds themselves. In addition, the mobile phase composition without additives is preferable in that additives may shorten column lifespan [7]. Therefore the mixture of acetonitrile and water was finally used as the mobile phase for separation of FFAs. Better separation for all FFAs was achieved by linear gradient elution other than isocratic elution. The different gradient elution condition and flow rates were also optimized. The ultimate optimized UPLC conditions were presented above. To provide a good separation of all FFAs in the complicated samples with highly similar structures including three pairs of isomers, we found that the initial 9 min elution were critical to eliminate the interference of the matrix before the first analyte of interest was

eluted (Fig. 3, 4) using the optimized condition, otherwise, the isomer of iso-C<sub>14:0</sub> and C<sub>14:0</sub> could hardly be separated.



**Figure 3:** Comparison of two applied samples to mixed standard solution. **a** Typical total ion current chromatogram of the mixed standard solution, **b** Typical total ion current chromatogram of the total extraction solution of *Pheretima*, **c** Typical total ion current chromatogram of the bioactive fraction

### Method Validation

The SIM chromatograms for FFAs in the total extraction solution of *Pheretima* indicated no interfering peaks in the range of the targeted FFAs retention times and mass-to-charge ratios (Fig. 4). Though the isomers of iso-C<sub>14:0</sub> and C<sub>14:0</sub>, iso-C<sub>15:0</sub> and C<sub>15:0</sub>, iso-C<sub>17:0</sub> and C<sub>17:0</sub> were closely eluted, all of the three groups of isomers were successfully resolved with good symmetry due to the high specificity of the SIM. The peak purities of the samples were confirmed with mobile phase of 10% acetonitrile increased to 100% over 10, 20 and 30 min respectively at 0.2 mL min<sup>-1</sup>, resulting in a corresponding different retention time of each analyte for consistent identification with that of each standard, which indicated that the developed method has good assay specificity. The good specificity is acquired without addressing any lengthy and cumbersome partition pretreatments, such as liquid-liquid extraction or column extraction by using the stationary phase of sephadex, cellite or cellulose, which is usually needed for the analysis of FFAs in the extremely complicated natural sample such as the *Pheretima* extract [8]. Measurements for each FFA were linear over a wide range (0.05–3.95 µg mL<sup>-1</sup>) with good correlation coefficients (>0.99) as shown in Table 1. The linearity range was considered adequate for the purpose of the sample analysis. From Table 1, LOD and LOQ for all the fatty acids were below 26 and 78 ng mL<sup>-1</sup> respectively. The intra- and inter-assay precision and accuracy for the thirteen FFAs were below 5.2% (R.S.D.) and 6.3% (bias) respectively (Table 2).

Satisfactory recoveries, ranging from 96 to 103%, were acquired (Table 3). The good precision and accuracy were adequate for the FFAs quantification in the complicated sample.

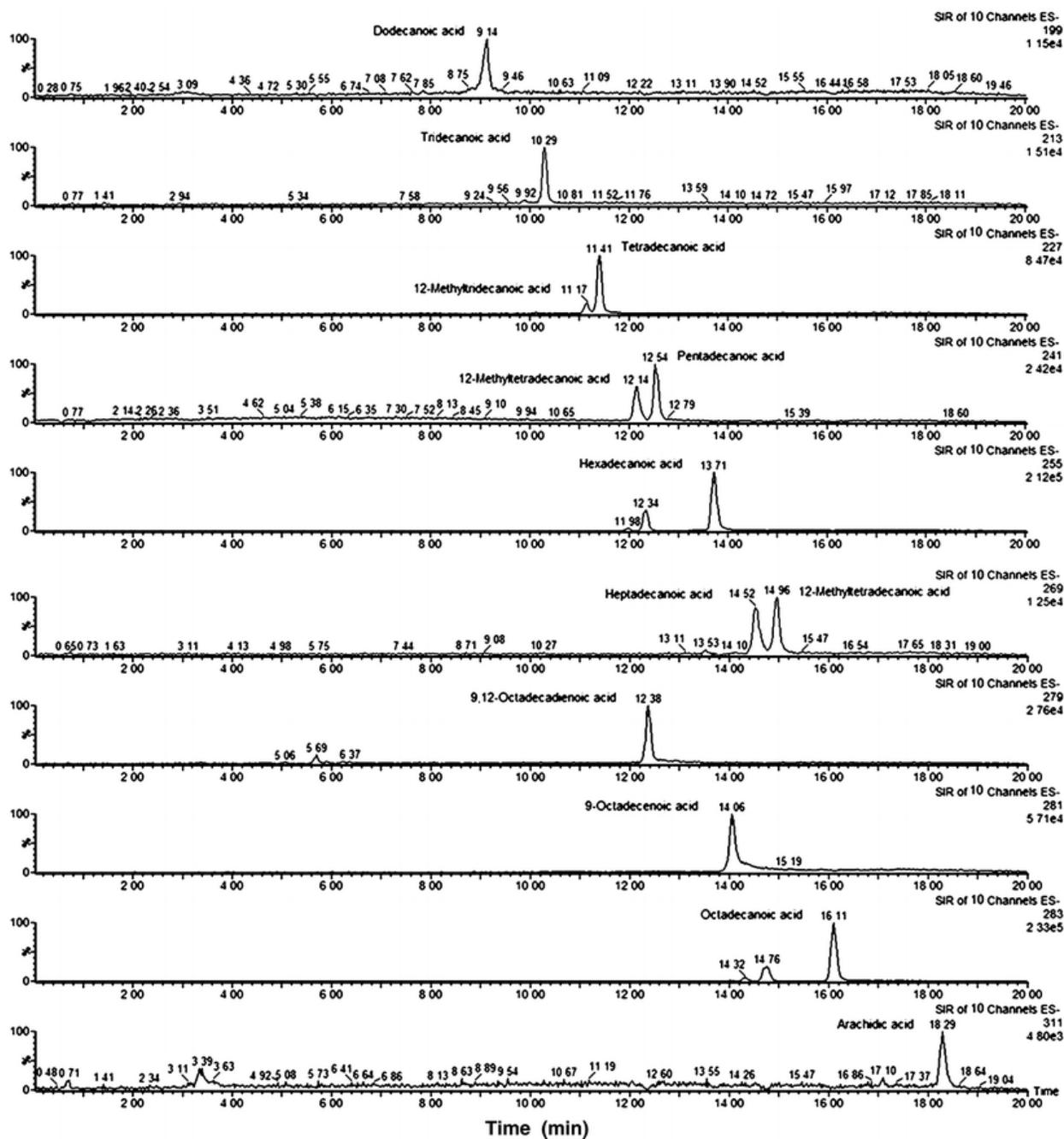


Figure 4: Typical chromatograms of the total extraction solution of *Pheretima* on different channels

**Table 1:** Linearity and sensitivity

Compound	Calibration curve	$R^2$	Linear range ( $\mu\text{g mL}^{-1}$ )	LOD ( $\text{ng mL}^{-1}$ )	LOQ ( $\text{ng mL}^{-1}$ )
C <sub>12-0</sub>	$y = 138,296x + 9724.3$	0.9988	0.09–3.39	7.16	21.69
C <sub>13-0</sub>	$y = 123,699x + 9350.1$	0.9991	0.09–3.36	8.00	24.25
Iso-C <sub>14-0</sub>	$y = 182,157x + 21861$	0.9983	0.11–3.95	10.87	32.94
C <sub>14-0</sub>	$y = 77,836x + 8488.8$	0.9973	0.05–1.67	6.78	20.55
Iso-C <sub>15-0</sub>	$y = 204,477x + 25,071$	0.9978	0.07–2.44	9.68	29.34
C <sub>15-0</sub>	$y = 50,983x + 15,669$	0.9963	0.09–3.33	19.42	58.84
C <sub>16-0</sub>	$y = 116,654x + 54,443$	0.9921	0.06–2.25	25.46	77.14
Iso-C <sub>17-0</sub>	$y = 119,365x + 12,667$	0.9965	0.09–3.41	8.29	25.13
C <sub>17-0</sub>	$y = 98,789x + 9168.9$	0.9971	0.07–2.67	8.35	25.30
C <sub>18-2</sub>	$y = 88,232x + 5,964$	0.9996	0.10–3.50	9.35	28.33
C <sub>18-1</sub>	$y = 111,686x + 13,272$	0.9987	0.10–3.74	8.86	26.86
C <sub>18-0</sub>	$y = 154,923x + 63,981$	0.9956	0.07–2.49	19.17	58.09
C <sub>20-0</sub>	$y = 25,047x + 6,476$	0.9921	0.08–2.96	7.02	21.28

**Table 2:** Intra-day and inter-day precision and accuracy for FFAs ( $n = 6$ )

Compound	Concentration ( $\text{ng mL}^{-1}$ )	Intra-day precision (CV) (%)	Intra-day accuracy (%)	Inter-day precision (CV) (%)	Inter-day accuracy (%)
C <sub>12-0</sub>	100	2.1	103.2	3.2	103.5
	500	1.4	98.8	2.7	97.9
	2,500	1.8	97.9	1.8	96.8
C <sub>13-0</sub>	100	1.9	102.3	3.8	101.6
	500	1.3	98.6	3.1	102.1
	2,500	1.1	98.1	1.5	98.3
Iso-C <sub>14-0</sub>	120	3.3	102.1	2.4	102.4
	600	0.9	97.9	3.1	97.3
	3,000	1.8	97.5	1.9	98.4
C <sub>14-0</sub>	50	3.9	104.7	5.3	106.3
	250	2	97.4	3.5	102.5
	1,250	2.8	102.1	2.6	102.7
Iso-C <sub>15-0</sub>	70	3.1	102.8	4.3	102.8
	350	1.1	103.1	1.7	96.7
	1,750	3.2	97.9	2.5	98.1
C <sub>15-0</sub>	90	4.8	105.2	4.7	105.4
	450	2.6	96.9	2.7	94.8
	2,250	1.3	97.1	4	96.9
C <sub>16-0</sub>	60	3.5	102.4	3.6	103.1
	300	2.3	97.5	2.2	97.7
	1,500	2.8	101.1	2.7	102.8
Iso-C <sub>17-0</sub>	90	2.7	102.5	3.3	102.4
	450	1.2	105.2	2.6	102.3
	2,250	1.4	97.9	2.4	97.8
C <sub>17-0</sub>	80	3.7	100.6	1.8	103
	400	1.2	103.9	2.2	105.8
	2,000	1.7	98.8	2.3	98.7
C <sub>18-2</sub>	100	1.2	102.8	2.7	102.3
	500	2.7	101.5	3.1	95.1
	2,500	1.3	97	3.6	98.2
C <sub>18-1</sub>	100	3.2	101.1	2.5	102.7
	500	1.5	99.1	2.8	96.9
	2,500	1.1	98.1	3.6	97.5
C <sub>18-0</sub>	80	1.3	101.1	1.5	102.9
	400	0.9	98.1	1.8	98.4
	2,000	2.3	102.6	3.1	96.8
C <sub>20-0</sub>	90	1.5	101.9	3.7	102.4
	450	1.2	101.5	2.8	97.4
	2,250	1.8	97.7	3.4	97.9

**Table 3:** Recovery for the method validation of 13 FFAs ( $n = 3$ )

Compound	Normal mass concentration (ng mL <sup>-1</sup> )	Observed mass concentration (ng mL <sup>-1</sup> )	Recovery (%)
C <sub>12-0</sub>	2,037	1,988 ± 48	97.6
C <sub>13-0</sub>	2,015	1,951 ± 50	96.8
Iso-C <sub>14-0</sub>	2,367	2,400 ± 66	101.4
C <sub>14-0</sub>	1,002	972 ± 35	97.0
Iso-C <sub>15-0</sub>	1,467	1,448 ± 54	98.7
C <sub>15-0</sub>	1,996	2,018 ± 50	101.1
C <sub>16-0</sub>	1,350	1,314 ± 39	97.3
Iso-C <sub>17-0</sub>	2,043	2,004 ± 47	98.1
C <sub>17-0</sub>	1,605	1,581 ± 27	98.5
C <sub>18-2</sub>	2,097	2,053 ± 38	97.9
C <sub>18-1</sub>	2,244	2,291 ± 47	102.1
C <sub>18-0</sub>	1,497	1,534 ± 37	102.5
C <sub>20-0</sub>	1,773	1,739 ± 69	98.1

### Application

The developed method was applied to determine the FFAs in the total extraction solution collected according to the procedure described in sample solutions preparation and the bioactive fraction of *Pheretima*. The bioactive fraction was isolated from *Pheretima* water extract through anion exchange resin and its biological activity had been evaluated through a series of pharmacological evaluation in vitro and in vivo [4]. As shown in Table 4, the results of the FFA contents expressed in mg for each FFA per 10 g the dry *Pheretima* powder or the active fraction, respectively, and the most abundant compounds in the dry *Pheretima* powder were C<sub>14-0</sub>, C<sub>16-0</sub>, C<sub>18-2</sub>, C<sub>18-1</sub>, C<sub>18-0</sub>, in contrast, the contents of C<sub>14-0</sub>, C<sub>16-0</sub>, C<sub>18-1</sub>, C<sub>18-0</sub> in the active fraction were increased, the contents of C<sub>18-2</sub> was decreased, C<sub>20-0</sub> was not detected, probably due to the loss in the isolation procedure.

**Table 4:** Quantification results of the samples

Compound	Phereima crude material (mg/10 g)	Bioactive fraction (mg/10 g)
C <sub>12-0</sub>	0.72 ± 0.02	4.22 ± 0.17
C <sub>13-0</sub>	2.01 ± 0.08	1.26 ± 0.05
Iso-C <sub>14-0</sub>	1.41 ± 0.06	8.61 ± 0.31
C <sub>14-0</sub>	9.02 ± 0.29	44.09 ± 1.68
Iso-C <sub>15-0</sub>	1.65 ± 0.05	2.22 ± 0.06
C <sub>15-0</sub>	1.34 ± 0.03	4.59 ± 0.1
C <sub>16-0</sub>	17.36 ± 0.66	56.34 ± 2.59
Iso-C <sub>17-0</sub>	3.55 ± 0.15	0.96 ± 0.02
C <sub>17-0</sub>	5.63 ± 0.22	2.05 ± 0.08
C <sub>18-2</sub>	20.53 ± 0.94	3.89 ± 0.15
C <sub>18-1</sub>	58.17 ± 2.15	84.5 ± 2.62
C <sub>18-0</sub>	15.05 ± 0.59	72.97 ± 1.97
C <sub>20-0</sub>	nq <sup>a</sup>	nd <sup>b</sup>
Sum	136.44 ± 4.79	285.68 ± 9.93

<sup>a</sup>Not quantified

<sup>b</sup>Not detected

### CONCLUSION AND DISCUSSION

The UPLC-ESI-MS method for determination of FFAs in the crude extraction solution and the isolated active fraction of *Pheretima* had been successfully established. The method demonstrated good precision and accuracy with high sensitivity regardless of the extremely complicated compounds in the extraction solution of *Pheretima* including three pairs of isomers.

The ionization suppression effects were minimized both by optimization of the extraction procedure and by chromatographic condition, and the possible ionization suppression effects had been reduced to a degree so low that satisfactory recoveries in the range of 96–103% could be acquired. Furthermore, the overall analytical time was greatly shortened compared to other analytical methods like LC-MS for FFAs [6, 7] due to the simple procedures without any pretreatment procedures such as partition and derivatization, combined with the advantage of rapidness provided by UPLC.

In summary, this method provides a simple and rapid quantification tool for the determination of FFAs in *Pheretima* as well as possibly other natural products widely containing diversified FFAs, helping to understanding the FFA variation and further evaluation of their bioactivities.

#### ACKNOWLEDGMENTS

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