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ORIGINAL ARTICLE

Improvement of saponification extraction method for fatty acids separation from geological samples

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Abstract The conventional saponification method could result in lower recoveries and artificial changes of long-chain fatty acids. The main reason is the error judgment of the intermediate layer suspended between the aqueous and organic layer during the liquid–liquid extraction process. This study shows that the intermediate layer consists of lots of medium- to long-chain carboxylic salts for their special physical and chemical properties. An improved saponification extraction method is also developed and the results show that the carboxylic salts distributed in the intermediate layer could be obtained completely, which greatly enhances the authenticity and accuracy of fatty acid analysis. Additionally, the possible reasons of formation of the intermediate layer are also discussed.

Keywords Fatty acid · Separation · Saponification extraction · Carboxylic salt · Geological sample

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1 Introduction

Fatty acids are key constituents of natural occurring lipids which are used as energy stores, cellular membrane components and external coatings by plants and animals. Meanwhile, as important precursors of hydrocarbon, they also attract many concerns from scientists in the field of geochemistry (Xu et al. 2001; Barakat et al. 2000; Wakeham 1999; Paul et al. 2002). The investigation of fatty acids in various depositional environments can provide a lot of information about the sources and diagenetic processes of organic matter (Russell et al. 1997; Gong and Hollander 1997; Sessions et al. 2002; Huang et al. 2002; Hu et al. 2003; Hughen et al. 2004). Therefore, it is particularly significant to isolate fatty acids from samples efficiently and completely.

Fatty acids can be separated by many methods, such as column chromatography, thin layer chromatography, high performance liquid chromatographic, solid layer extraction and so on. They share similar working principles, as they all utilize the different distribution capacity of materials between the solid phase and the fluent phase. But geological samples contain multiple compounds, and some complex structures will lead to greater steric hindrance to limit the elution efficiency (Zhang et al. 2004). Besides, researchers will spend lots of effort and time on each sample, or each fraction, in order to obtain optimized chromatographic conditions.

In contrast to the above methods, the saponification extraction method shows great advantages in terms of the quantity and purity of fatty acids, which is widely used in many research fields. The conventional steps are listed as follows (Tommaso et al. 2014; Sitindra and Mark 2013; Pisani et al. 2013; Guan et al. 2013; Leefmann et al. 2013): ① extraction of total lipids from samples; ② saponification

of free fatty acids; ③ separation of neutral and acidic fractions; ④ acidification of the aqueous layer (acidic fractions); ⑤ extraction of free fatty acids.

Its theoretical basis is that fatty acids can react with alkali and turn into water-soluble carboxylic salts. After extraction with organic solvents (step ③), all of the carboxylic salts could be isolated from other organic matter (unsaponifiable matter). Then the pure fatty acid fraction will be obtained by subsequent treatments (step ④ and ⑤).

However, serious problems have been found by our many experiments, especially in step ③. Actually, the carboxylic salts are amphiphilic molecules and have special physical and chemical properties, which is closer to anionic surfactants. During the liquid–liquid extraction process (step ③), complex micro interfacial phenomena will occur and, in most cases, an intermediate layer between the organic and the aqueous layers will appear. On the one hand, the intermediate layer will hinder the exchange of matter between the organic and the aqueous layers, resulting in lower extraction efficiency and operational difficulties (Logsdail and Slater 1993; Liu et al. 2002). On the other hand, lots of medium- to long-chain carboxylic salts can be found in this layer with further study. Unfortunately, the intermediate layer is often regarded as unsaponifiable matter, or even an impurity, and is always discarded, leading to great loss and artificial changes of medium- to long-chain fatty acids, which seriously affects the authenticity and accuracy in the laboratory analysis of fatty acids.

In fact, early researchers had already proven that saponification extraction would result in loss of fatty acids (Van der Veen et al. 1968). But they only chose several standard materials of fatty acids and did not give the theoretical analysis that accounted for such loss. So the problem did not attract enough attention and many researchers still used this conventional method for fatty acid separation (Shinya et al. 2013; Tommaso et al. 2014; Robert et al. 2014; Jorge et al. 2014; Shailesh et al. 2014).

In this study, an improved method of saponification extraction is proposed. Some standard substances of fatty acids are chosen to prove the repeatability of the improved method, and the applicability is proven by three kinds of geological samples. By comparing the obtained fatty acids with the two methods, the advantages of the improved method is highlighted. The reasons for the formation of the intermediate layer are also explained theoretically.

2 Materials and methods

2.1 Sample and reagents

The geological samples: Huangxian lignite was collected from the Beizao mine of the Huangxian coalfield in the Shandong Province; marsh sediment was obtained from a 4 m core located in the west of the Ruoergai marsh; peat sample was from a 4.5 m core collected at a location situated 2 km southeast of the city of Hongyuan.

Standard substances: palmitic acid, eicosanoic acid, tetracosanoic acid and octacosanoic acid were purchased from the Sinopharm Group Corp. (Shanghai, China); sodium palmitate, sodium arachidate and sodium nervonate were obtained from NU-CHEK-PREP, INC (USA).

The reagents used in the experiments: chloroform, dichloromethane, methanol, hydrochloric acid and sodium hydroxide were all analytical pure. They were obtained from the Sinopharm Group Corp. (Shanghai, China), and underwent second distillation before use. The boron trifluoride/methanol solution and erucic acid were bought from Fisher Corp. (Pittsburg, Pennsylvania, USA). The sodium palmitate, sodium arachidate and sodium nervonate were chromatographically pure (>99 %) and purchased from UN-CHEK-PREP, INC (Elysian, U.S.A). The vacuum rotary evaporator was bought from Labtechgroup Corp. (Beijing, China). Highly pure water was acquired from the Milli-Q water system (Millipore Corp., Billerica, MA, USA).

2.2 Analytical methods

2.2.1 Conventional saponification extraction method

All conventional method procedures are strictly in accordance with the reported literature (Tommaso et al. 2014; Sitindra and Mark 2013; Pisani et al. 2013; Guan et al. 2013; Leefmann et al. 2013).

2.2.1.1 Standard substances of fatty acid The quantitative palmitic acid, eicosanoic acid, tetracosanoic acid and octacosanoic acid were saponified with 6 % sodium hydroxide in methanol (w/v) for 2 h,. Then these saponified substances were extracted three times with dichloromethane three times. The aqueous layers were acidified to pH 1–2 with aqueous HCl 15 % (v/v) and extracted three times with dichloromethane. After evaporating and weighing the solvent, we obtained the quantity of obtained fatty acids.

2.2.1.2 Geological samples The fresh geological samples were dried at room temperature and ground to 100 mesh. Powdered samples were extracted in a Soxhlet apparatus with chloroform for 72 h. The extracts were saponified overnight with 6 % sodium hydroxide in methanol (w/v). Both the neutral and acidic fractions were successively recovered with dichloromethane three times, respectively. During every extraction process, three layers (aqueous layer, organic layer and intermediate layer) appeared. Only

Table 1 The recoveries of standard substances of fatty acid by the conventional and improved methods

Fatty acids	Times of experiments	Total weight (mg)	Conventional method (mg)	Recovery (%)	Improved method (mg)	Recovery (%)
Palmitic acid	1	5.6	1.0	17.86	3.9	69.64
	2	5.1	0.9	17.65	3.7	72.55
	3	4.9	1.0	20.41	3.4	69.39
Eicosanoic acid	1	6.7	0.5	7.46	5.5	82.09
	2	6.1	0.5	8.20	5.2	85.25
	3	5.9	0.4	6.78	4.7	79.66
Tetracosanoic acid	1	5.4	0.2	3.70	4.5	83.33
	2	4.7	0.2	4.26	3.8	80.85
	3	5.2	0.3	5.77	4.5	86.54
Octacosanoic acid	1	6.1	0.2	3.28	4.9	80.33
	2	6.6	0.3	4.55	5.4	81.82
	3	6.9	0.4	5.80	5.7	82.61

Table 2 Distribution of carboxylic salts in the aqueous and the intermediate layers

Fatty acid	Times of experiments	Total weight (mg)	Aqueous layer (mg)	wt%	Intermediate layer (mg)	wt%
Sodium palmitate	1	3.8	0.7	19.45	2.7	70.58
	2	3.5	0.5	15.22	2.3	65.37
	3	4.1	0.8	18.89	3.0	72.07
Sodium arachidate	1	4.2	0.2	5.21	3.4	80.36
	2	4.5	0.4	8.49	3.6	79.84
	3	3.9	0.2	4.62	3.3	85.33
Sodium nervonate	1	3.6	0.1	4.08	3.2	88.16
	2	4.2	0.1	1.67	3.4	80.68
	3	4.0	0.1	3.47	3.3	82.19

the aqueous layer was acidified to pH <2 with hydrochloric acid (15 %, v/v), then dichloromethane was used to extract the fatty acid fraction. The separation procedures were repeated three times and the combined extracts were dried under a gentle stream of N2. A certain amount of erucic acid was added as an internal standard (IS) (no erucic acid component in these samples by our analysis). Then the fatty acids were esterfied overnight with 14 % a boron trifluoride/methanol solution prior to the GC/MS analysis and identification.

2.2.2 Improved saponification extraction method

The procedures of saponification and extraction of these fatty acids (standard substances and geological samples) were the same as Sects. 2.2.1.1 and 2.2.1.2. But during the separation process, the aqueous and the intermediate layers were combined and all were acidified to pH 1–2 with HCl 15 % (v/v) and extracted three times with dichloromethane. After evaporating and weighing the solvent, the quantity of obtained fatty acids was obtained.

2.2.3 Extraction experiments of standard substances of carboxylic salts

The quantitative sodium palmitate, sodium arachidate and sodium nervonate were dissolved in 6 % sodium hydroxide in methanol (w/v). Dichloromethane was added in and extracted three times. The aqueous and intermediate layers were isolated each time. After evaporating and weighing the solvent, the quantity of the carboxylic salts distributed in the two layers were obtained.

2.2.4 Gas chromatography/mass spectrometry (GC/MS)

All fatty acids were analyzed by gas chromatography-mass spectrometry (GC/MS), using a HP5890 N GC interfaced to a HP5973 N MS. An HP-5 capillary column was used (length 30 m; inner diameter 0.25 mm; film thickness 0.25 μm). Temperature programming: the temperature increases from 80 to 295 °C at a rate of 4 degrees per minute, with the initial and final hold times at 1 and 30 min, respectively. Highly pure helium was used as the

carrier gas and fed at a linear velocity of 37 cm/sec. The injector operated at a constant flow rate of 1.0 mL/min. Operating parameters of the mass-selective detector: ionization energy 70 eV, ion source temperature 230 °C, electron multiplier voltage 1800 V, and mass range 35–600 daltons. Identifications of individual compounds were based on the retention times of authentic standards and the comparison of their mass spectra with published MS data (Matsuda and Koyama 1977; Volkman et al. 1990).

All experiments were performed in triplicate, and Microsoft Excel software was used for data treatment.

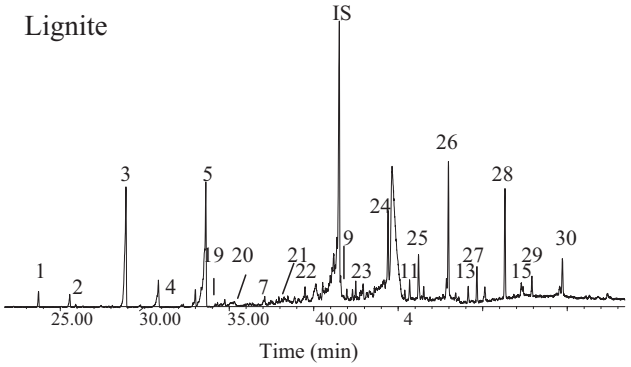


Fig. 1 Total ion current chromatogram of fatty acids obtained by the conventional method. Peak assignments are listed in Table 3

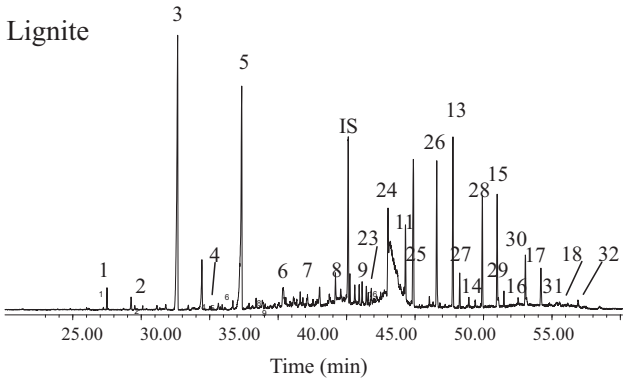


Fig. 2 Total ion current chromatogram of fatty acids obtained by the improved method. Peak assignments are listed in Table 3

3 Results and discussion

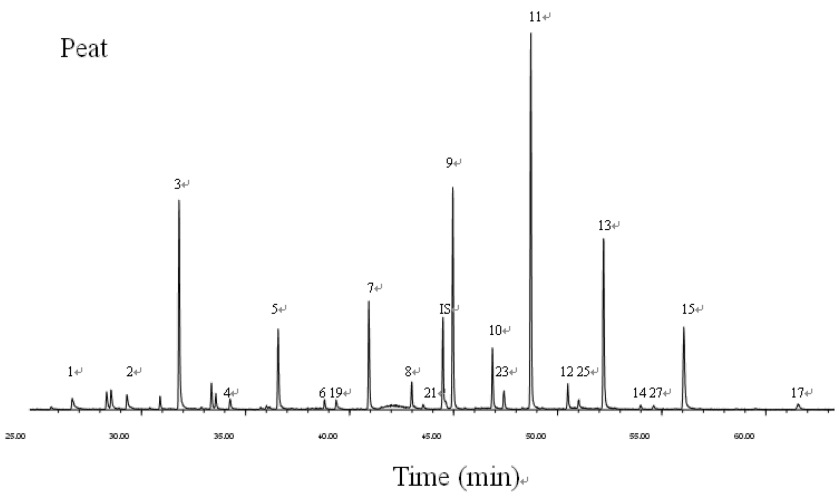
3.1 Standard substances of fatty acid

The recoveries of standard substances of fatty acids by conventional and improved methods are shown in Table 1. The recoveries of different carbon-chain length fatty acids were greatly improved with the new method, from 3.28 %–20.41 % to 69.39 %–86.54 %. The longer carbon-chain fatty acid is, the higher recovery gets.

In order to further prove the above point, several standard materials of carboxylic salts (sodium palmitate, sodium arachidate and sodium nervonate) were chosen to be extracted in the method mentioned in Sect. 2.2.3 and the distribution results in the aqueous and the intermediate layer are shown in Table 2. It is obvious that most of the short carbon-chain carboxylic salts are distributed in the aqueous layer, and most of the long carbon-chain carboxylic salts are distributed in the intermediate layer. And the longer the carbon-chain carboxylic salt is, the more it distributes in the intermediate layer.

Here are some further explanations on the reasons for the distribution of carboxylic salts in the intermediate layer. Liquid–liquid extraction of carboxylic salts relates to many factors, such as the chemical properties of solvents, extracts, organic layer, aqueous layer and so on (Szymaoowski and Cierpiszewski 1992; Szymanowski and Tondre 1994; Menon and Wasan 1988). Firstly, as a

Fig. 3 Total ion current chromatogram of fatty acids obtained by the conventional method. Peak assignments are listed in Table 3



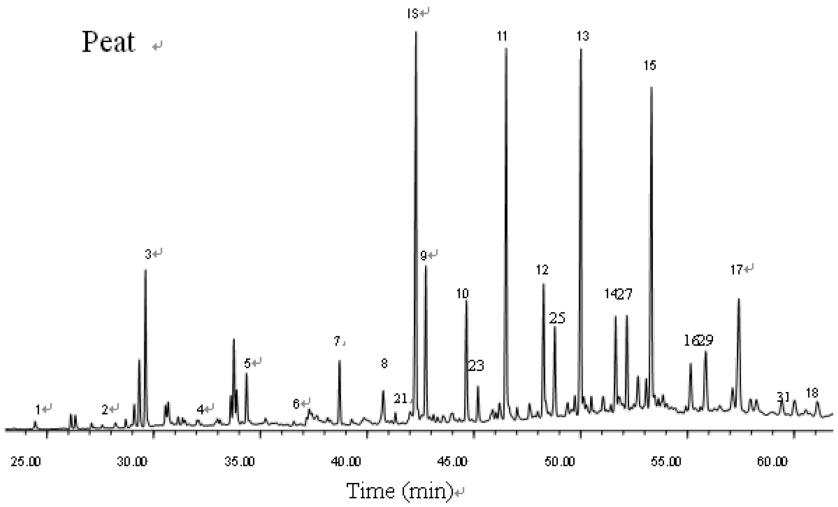


Fig. 4 Total ion current chromatogram of fatty acids obtained by the improved method. Peak assignments are listed in Table 3

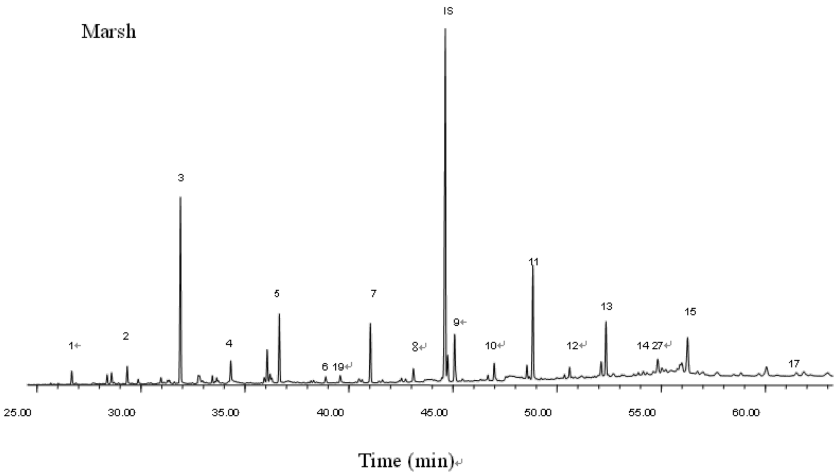


Fig. 5 Total ion current chromatogram of fatty acids obtained by the conventional method. Peak assignments are listed in Table 3

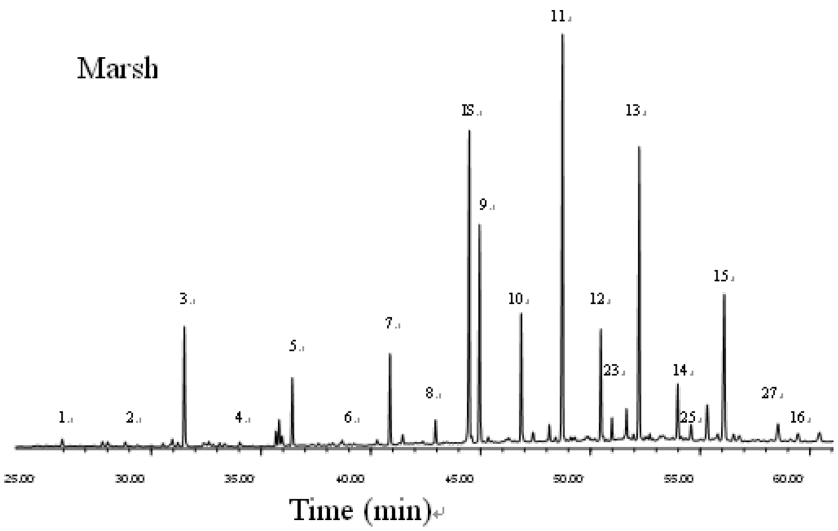


Fig. 6 Total ion current chromatogram of fatty acids obtained by the improved method. Peak assignments are listed in Table 3

Table 3 The results of fatty acids obtained from geological samples by the conventional and improved methods

Fatty acid type	Peak	Carbon number	Huangxian lignite			Peat sediment			Marsh sediment		
			Conventional method (μg/g)	Improved method (μg/g)	Times (improved/conventional)	Conventional method (μg/g)	Improved method (μg/g)	Times (improved/conventional)	Conventional method (μg/g)	Improved method (μg/g)	Times (improved/conventional)
Monocarboxylic acid	1	C_{14}	2.0	5.3	2.7	3.1	0.9	1.3	1.5	2.3	2.6
	2	C_{15}	0.2	0.8	5.2	1.7	0.5	1.3	0.8	2.1	3.5
	3	C_{16}	15.2	149.0	9.8	19.6	18.9	2.0	10.1	24.9	3.5
	4	C_{17}	0.0	0.8	25.2	1.0	0.5	1.5	0.7	1.5	3.0
	5	C_{18}	16.8	117.2	7.0	9.1	10.8	2.2	5.8	19.4	4.3
	6	C_{19}	–	0.7	–	0.9	0.7	1.8	0.5	3.2	7.3
	7	C_{20}	1.3	17.6	13.1	8.6	14.6	2.7	4.6	37.9	9.2
	8	C_{21}	0.1	7.2	139.6	1.6	4.0	3.5	1.7	20.9	13.6
	9	C_{22}	1.6	21.3	13.7	23.0	34.9	2.5	4.5	196.2	20.9
	10	C_{23}	0.1	13.9	140.1	10.6	20.6	2.9	2.3	66.9	30.4
	11	C_{24}	1.9	96.0	51.7	5.0	65.3	14.0	5.6	320.2	39.2
	12	C_{25}	0.1	11.7	117	14.5	18.2	2.3	1.0	43.0	45.7
	13	C_{26}	1.5	177.3	121.5	4.1	57.5	12.6	3.6	202.3	57.7
	14	C_{27}	0.1	11.6	116	8.4	9.4	2.1	–	12.4	–
	15	C_{28}	1.3	119.3	94.4	0.9	53.6	26.6	1.2	75.5	61.7
	16	C_{29}	–	14.0	–	4.3	3.2	1.7	–	2.9	–
	17	C_{30}	–	54.2	–	1.0	6.0	7.2	–	10.5	–
	18	C_{31}	–	4.0	–	1.2	–	–	–	–	–
Dicarboxylic acid	19	C_{15}	1.0	–	–	1.5	–	–	0.3	–	–
	20	C_{16}	0.9	0.9	1.1	–	–	–	–	–	–
	21	C_{17}	2.7	3.0	1.1	1.1	2.0	1.8	–	–	–
	22	C_{18}	1.7	1.8	1.1	–	–	–	–	–	–
	23	C_{19}	2.2	24.2	11.2	3.1	3.8	0.3	–	–	–
	24	C_{20}	8.9	85.9	9.7	–	–	–	–	–	–
	25	C_{21}	4.6	35.1	7.7	2.1	6.2	2.1	–	–	–
	26	C_{22}	15.9	159.8	10.1	–	–	–	–	–	–
	27	C_{23}	4.3	36.6	8.4	1.1	6.4	2.1	1.0	6.1	–
	28	C_{24}	15.1	143.3	9.5	–	–	–	–	–	–
	29	C_{25}	3.0	15.5	5.2	–	5.5	–	–	4.3	–
	30	C_{26}	5.5	52.3	9.5	–	–	–	–	–	–
	31	C_{27}	-.	4.3	–	–	2.1	–	–	3.9	–
	32	C_{28}	–	12.4	–	–	–	–	–	–	–
Total			107.5	1391.0	12.9	127.1	378.2	3.1	52.8	1086.5	20.6

–, below detection limit

kind of salt molecule, short-chain carboxylic salts are easily ionized and dissolved in the aqueous layer completely. But long-chain carboxylic salts are weak electrolytes in their larger hydrocarbon chains, showing weak polarities and difficult ionization. Secondly, the physical and chemical properties of long-chain carboxylic salts are similar to the anionic surfactants, which are amphiphilic molecules with lipophilic tails (carbon chain) and hydrophilic heads (carboxylate ion) (Rosen 1978). During the liquid–liquid extraction process, these medium- to long-chain carboxylic salts will form the intermediate layer with the hydrophilic heads facing the aqueous layer and the hydrophobic tails inserting the organic layer. Thus the intermediate layer is actually composed of long-chain carboxylic salts.

The repeatability of the improved method has been proven in Table 1. Then three kinds of geological sample were chosen to prove the applicability of this method.

3.2 Geological samples

From a qualitative point of view, in the Huangxian lignite (Figs. 1, 2), C14–C28 monocarboxylic acids and C15–C26 dicarboxylic acids were obtained and exhibited bimodal distribution with the main peaks at C16 (monocarboxylic acid) and C22 (dicarboxylic acid) with the conventional saponification extraction method. Whe using the improved method, C14–C31 monocarboxylic acids and C15–C28 dicarboxylic acids were isolated. Monocarboxylic acids exhibit bimodal distribution with the main peaks at C16 and C26, dicarboxylic acids exhibit unimodal distribution with the main peaks at C22. It is obvious that the carbon number range expanded and the peak distribution changed fundamentally, especially in the monocarboxylic acids. The distribution of monocarboxylic acids varies from unimodal to bimodal peak and the main peak is from C16 to C16 and C26.

With the conventional method, in the peat sediment (Figs. 3, 4), the C14–C30 monocarboxylic acids and C15–C23 dicarboxylic acids were obtained, and exhibited bimodal distribution, with the main peaks at C24 (monocarboxylic acid). By using the improved method, the C14–C31 monocarboxylic acids and C17–C25 dicarboxylic acids are isolated. Monocarboxylic acids exhibit bimodal distribution with the main peaks at C16 and C24, dicarboxylic acids exhibit unimodal distribution with the main peaks at C21. The carbon number range is expanded and the peak distribution is changed fundamentally.

The distribution of monocarboxylic acids is from unimodal to bimodal peaks and the main peak is from C16 to C16 and C24.

In the marsh sediment (Figs. 5, 6), the main difference is the peak distribution. The distribution of monocarboxylic acids is from unimodal to bimodal peaks and the main peak is from C16 to C24.

The comparison of quantitative results is shown in Table 3. The fatty acids obtained from these samples with the traditional method are all lower than those with the improved method. Furthermore, the individual fatty acid, especially the medium- to long-chain fatty acids, shows dozens or even hundreds of times the increase compared to those by the conventional method, enhancing the authenticity and accuracy of the fatty acid analysis. As a result, it is inaccurate to regard the intermediate layer as unsaponifiable matter or impurity and throw it away.

4 Conclusions

The conventional saponification extraction of fatty acids only focuses on the aqueous layer, leading to great loss and artificial changes of the series of fatty acids. The main reason is the error judgment for the intermediate layer during the liquid–liquid extraction process. In fact, the intermediate layer is gathered by plenty of medium- to long-chain carboxylic salts. In this study, an improved saponification extraction method is proposed and the results show that the fatty acids in the intermediate layer could be obtained completely, which greatly reduces the loss of fatty acids, especially in medium-to long-chain fatty acids. The improved method enhances the accuracy and authenticity of fatty acids separation in laboratory analysis, and also provides new ideas or approaches to reduce the formation of the intermediate layer in the liquid–liquid extraction procedure.

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