

A high-throughput method for profiling fatty acids in plant seeds based on one-step acid-catalyzed methylation followed by gas chromatography-mass spectrometry

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ABSTRACT

Fatty acids (FAs) in oilseeds are important for human health. Gas chromatography-mass spectrometry (GC-MS) is a popular analytical technology for FAs measurement. FAs need to be derivatized into fatty acid methyl esters (FAMES), which is essential for GC-MS analysis. The previous methods for methylation of FAs usually include reaction at high temperature and need specific vessels, for which samples with a large scale cannot be analyzed in a short time, leading to low efficiency. Here we report a high-throughput method for profiling fatty acids in plant seeds based on one-step acid-catalyzed methylation followed by GC-MS. This method for FA methylation *via* derivatization by sulfuric acid with methanol (5%, v/v) and incubation at 64°C for 4 h in a more convenient 2 mL tube, is efficient. Satisfactory methodological performance was achieved by optimization of the parameters. Only 5 mg seeds were needed as a microscale sample usage by this method. The method is a successful approach to increase the efficiency of sample preparation and analysis for large-scale experiments.

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Introduction

Edible vegetable oil is an essential part of human daily diet by providing necessary energy and nutritional components (e.g. fatty acid and vitamins). Oilseed crops are important sources of vegetable oils for food and industry. Soybean (*Glycine max*), rapeseed (*Brassica napus*), peanut (*Arachis hypogaea*) and sunflower (*Helianthus annuus*) are the top four oil crops in the world. The main storage form of vegetable oil is the triglycerides (about 95–98%), which are composed of fatty acids (FAs) and glycerol [1]. FAs from plants include saturated FAs (C16:0, C18:0, C22:0, etc.), mono-unsaturated FAs (C18:1, C22:1, etc.) and polyunsaturated FAs (C18:2, C18:3, C20:4, etc.) [2], in which polyunsaturated FAs is essential for human life. Some FAs are beneficial to human health, such as C18:1 and C18:2, which can effectively prevent coronary heart disease. On the contrary, long-chain saturated fatty acids (LCFA) containing 12:0–16:0 chain length would increase serum total cholesterol. High intake of these FAs will increase the level of cholesterol in the blood, and excessive intake of saturated fats is thought to

contribute to the development of heart disease, weight gain and obesity [3]. The oil quality and functionality mostly depend on the FAs composition. Therefore, a reliable quantification method to determine FAs in oil crops is extremely demanded.

Total FAs in edible oil could be traditionally detected by titrimetry as recommended by the American Oil Chemists' Society (AOCS) [4]. Liquid chromatography-mass spectrometry (LC-MS) has been also used for lipids analysis, but intensive sample purification for the enrichment of FAs is usually required before analysis in the complex fatty matrices. Gas chromatography (GC) [5], gas chromatography-flame ionization detection (GC-FID) [6, 7] and gas chromatography-mass spectrometry (GC-MS) [8–10] have been previously applied for FAs determination. In GC and GC-FID analysis, FAs composition is identified only by retention time without any information on molecular mass or other structural characteristics that can be used to discriminate between various FAs, which lead to some misinformation. The combination of mass spectrometry (MS) with GC could produce precise identification results for FAs composition by use of electron ionization. So GC-MS is frequently used for the

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identification and quantification of FAs in plants and animals in recent years, owing to its effectiveness and high sensitivity [11, 12]. The main challenges for FAs assay include the unstable retention time and difficult separation, since these long-chain FAs with high polarity [13] and high boiling points (BP) are not directly volatilizable under general conditions. Generally, derivatization steps are required in GC-MS analysis. FAs needed to be methylated and converted into fatty-acid methyl esters (FAMES), being turned into volatiles at low BP, thus providing higher resolution for fatty-acid peaks under reduced vaporization temperature [11, 14]. As a result, the methylation derivatization of FAs plays a vital role for FAs analysis. The methylation methods for FAs include acid catalysis (i.e. the method of converting FAs into FAMES by use of reaction mixture buffer of sulfuric acid or hydrochloric acid and methanol) [5, 6], base catalysis [7] and other methods. The advantages and disadvantages of FA methylation methods are summarized in Table 1. Currently, the acid-catalyzed methylation method has been widely used to analyze FAs in plant samples, among which the methylation using sulfuric acid with methanol (5%, v/v) has been widely used for *Arabidopsis thaliana* seeds and oilseeds. The acid catalysis operations reported in previous literature sources were complicated with high reaction temperature and complex instruments, by which the experimental efficiency was limited. The temperature and time of the methylation was 90-95 °C and 1.5 h, respectively [8, 20, 21]. In order to improve the efficiency of acid-catalysis, the reaction temperature was usually set to 70-100 °C [8-10, 22-24]. It is noteworthy that the conjugated linoleic acids profile would alter *via* geometric isomerization at high temperature by acid-catalyzed methylation, which led to a decrease in *cis/trans* C18:2c9t11 and an increase in *trans/trans* C18:2t9t11 [15, 25]. Concurrently, a tightly sealed reaction container is required (e.g. Teflon-lined screw cap glass tube and vacuum rotary evaporator) in the process of methylation at high temperature [8-10, 20, 21, 23, 24], which leads to low efficiency for experiment operation, and it is impossible to

carry out mass sample methylation at the same time. Therefore, a simple, efficient and gentle FAs methylation method needs to be explored.

The objective of the present work was to develop a one-step acid-catalyzed methylation method based on sulfuric acid catalysis [20] for the determination of oil content and FAs composition in *Arabidopsis thaliana* seeds. With less seed sample in a small sized 2-mL Eppendorf (EP) tube as an alternative reaction container, we demonstrated that FAs can be well methylated at a lower temperature (64 °C). The present method was validated by using different oilseeds (e.g. *Brassica juncea* seeds, *Arachis hypogaea*, *Sesamum indicum*, *Helianthus annuus*, and *Glycine max*).

Materials and methods

Reagents

Analytical grade sulfuric acid was purchased from Huafu Chemical (Jiangsu, China), Chromatographic grade methanol and *n*-hexane were purchased from Merck (USA) and TEDIA (USA), respectively. The carrier gas was high purity helium (99.9999%, Saizhong Ltd. Changsha, China) for GC-MS. The volatile fatty acid standards of palmitic acid ($\geq 99.0\%$), hexadecenoic acid (*cis*-9) ($\geq 99.0\%$), stearic acid ($\geq 99.5\%$), octadecenoic acid (*cis*-9) ($\geq 99.0\%$), oleic acid ($\geq 99.0\%$), linolenic acid ($\geq 99.0\%$), arachidic acid ($\geq 99.0\%$) and erucic acid ($\geq 99.0\%$) were purchased from Aladdin (Shanghai, China). The mixed FAs standard solution (6.7 $\mu\text{g}/\mu\text{L}$ C16:0, 0.7 $\mu\text{g}/\mu\text{L}$ C16:1, 2.7 $\mu\text{g}/\mu\text{L}$ C18:0, 10.9 $\mu\text{g}/\mu\text{L}$ C18:1n9c, 24.7 $\mu\text{g}/\mu\text{L}$ C18:2n6c, 1.35 $\mu\text{g}/\mu\text{L}$ C20:0, 16.75 $\mu\text{g}/\mu\text{L}$ C18:3n6, 1.0 $\mu\text{g}/\mu\text{L}$ C22:1n9) was prepared in *n*-hexane and kept at -20°C in the dark.

Sampling

Seeds of *Arabidopsis thaliana* ecotype Columbia (Col-0) were surface-sterilized in 70% alcohol for 15 min, followed by washing in sterile distilled water 3-5 times. Sterilized seeds were dispersed in 0.1% agar and

Table 1. Comparison of different methods of fatty acid methylation.

Methods of fatty acid methylation	Advantages	Disadvantages	Ref.
Acid catalyzed methylation	Able to methylate both free FA and bounded FA (in the form of triglycerides, phospholipids, etc.)	Time consuming and higher temperatures (80-100 °C) are generally required, can modify conjugated linoleic acids profile	[15]
Alkaline- catalyzed methylation	Time is very short, FAs are relatively stable and do not form isomers	Free FA and sphingomyelins cannot be methylated	[15, 16]
BF ₃	A fast and effective methylation	The reagent is expensive and does not have a long shelf-life	[17, 18]
Diazomethane method	A good derivatization reagent for free FA, minimum change to original compounds, mild conditions and fast	Toxicity, higher acids are not completely esterified	[18, 19]

plated on 1/2MS medium solidified by 0.8% agar. Seeds were placed at 4°C for 3 days of vernalization, and then plants were grown at 22°C under 16 h light/8 h dark [26, 27]. Then, 7-day old seedlings were transplanted into soil and kept under the same conditions. Eight weeks later, mature seeds were harvested, cleaned and dried. *Brassica juncea*, Sesame (*Sesamum indicum*), peanut (*Arachis hypogaea*), edamame (*Glycine max*) and sunflower (*Helianthus annuus*) seeds were purchased from the local market. The seeds were well ground in liquid nitrogen because of their thick testa. Five milligrams of homogenized seeds were weighed and transferred to a 2 -mL EP tube (no mixed standards added) for further operations.

Preparation of calibration curve

Ten FAs (C16:0, C16:1, C18:0, C18:1, C18:2, C18:3, C20:2, C20:1, C20:2 and C22:1) were quantitatively analyzed using mixed FAs standard solution containing 37 methylated FAs (Sigma-Aldrich, 18919-1AMP). A calibration curve was constructed using a standard solution of 37 mixed methylated FAs [28, 29] at 7 different concentrations ranging from 0.8 to 25 µg/µL (0.8, 1, 2, 4, 8, 10, and 25 µg/µL).

Preparation of FAME

High temperature acid-catalyzed method: 10 mg intact *A. thaliana* seeds were weighed on an analytical balance and were placed into a 10 mL round bottom capped glass tube, then 1 mL sulfuric acid with methanol (5%, v/v) was added, and 20 µL of mixed FAs standards for quantification was also added into the mixture. Afterwards, the mixture was subjected to vortexing. The methylation reaction was performed at 85°C for 2 h [5, 6]. After cooling to room temperature, FAMES were extracted with 800 µL of hexane. The mixture was vortexed for 30 s and then left to stand for 2 min. Finally, the supernatant

of organic phase was collected and filtered for GC-MS analysis.

Lower temperature acid-catalyzed method: 10 mg of *A. thaliana* seeds or indicated weight (0.5, 1, 2, 5, and 10 mg) were transferred to a 2-mL EP tube, the seeds were mixed with 1 mL of sulfuric acid with methanol (5%, v/v) and 20 µL of mixed standards. Then the tube was vortexed for 5 s and heated at the indicated temperature (25°C, 37°C, 42°C and 64°C) or 64°C for the indicated time (2, 4, 8, 12 and 16 h). After cooling to room temperature, FAMES were extracted with 800 µL of hexane. The extraction mixture was again vortexed for 30 s and then was centrifuged at 12,000 *g* for 2 min. The upper organic layer containing the FAME was transferred to a vial and stored at -20°C for the subsequent GC-MS analysis.

GC-MS analysis

The concentration of FAMES was determined by GC-MS. GC-MS analyses were carried out with a GC MS-TQ8050 (Shimadzu Corporation, Japan) equipped with a SH-Rt-2560 column sized as 100 m × 0.25 mm × 0.25 µm (Shimadzu Corporation, Japan). GC inlet temperature was 240°C. The total flow rate, column flow rate, linear velocity and purge flow rate were 100.0 mL/min, 1.50 mL/min, 25.2 cm/s, and 3.0 mL/min, respectively. The oven temperature program employed for separation of FAMES was as follows: the initial temperature of 125°C was increased to 180°C at an increment of 10°C/min, held for 2 min, and then programmed at 200°C at a rate of 3°C/min, held for 20 min and finally increased to 230°C at 5°C/min for 10 min. The injection volume was 1.0 µL and splitless. The ion source (electron ionization, EI) detector temperature was maintained at 240°C, and the solvent delay was set at 12 min. The MS system was run in a scan mode of acquisition for selection of appropriate EI mass fragments for each analysis [11, 23].

Table 2. Performance of the proposed method for ten FAs.

Analytes	RT (min)	Linear range (µg/µL)	Calibration curve	R ²	m/z (relative intensity)
C16:0	15.935	0.048-1.5	$y = 1.128605 \times 10^3 x$	0.9985	74.05 (100)
C16:1	17.05	0.016-0.5	$y = 1.637546 \times 10^3 x$	0.9998	55.05 (100)
C18:0	19.105	0.032-1.0	$y = 1.606998 \times 10^3 x$	0.9997	74.05 (100)
C18:1	20.42	0.032-1.0	$y = 1.65982 \times 10^3 x$	0.9996	55.05 (100)
C18:2	22.59	0.016-0.5	$y = 1.772876 \times 10^3 x$	0.9999	81.10 (100)
C20:0	23.475	0.032-1.0	$y = 1.967572 \times 10^3 x$	0.9996	74.05 (100)
C20:1	25.255	0.016-0.5	$y = 2.031918 \times 10^3 x$	0.9993	55.05 (100)
C18:3	25.595	0.016-0.5	$y = 1.690173 \times 10^3 x$	0.9996	55.05 (100)
C20:2	28.37	0.016-0.5	$y = 1.970988 \times 10^3 x$	0.9990	81.10 (100)
C22:1	32.395	0.016-0.5	$y = 2.200888 \times 10^3 x$	0.9999	55.05 (100)

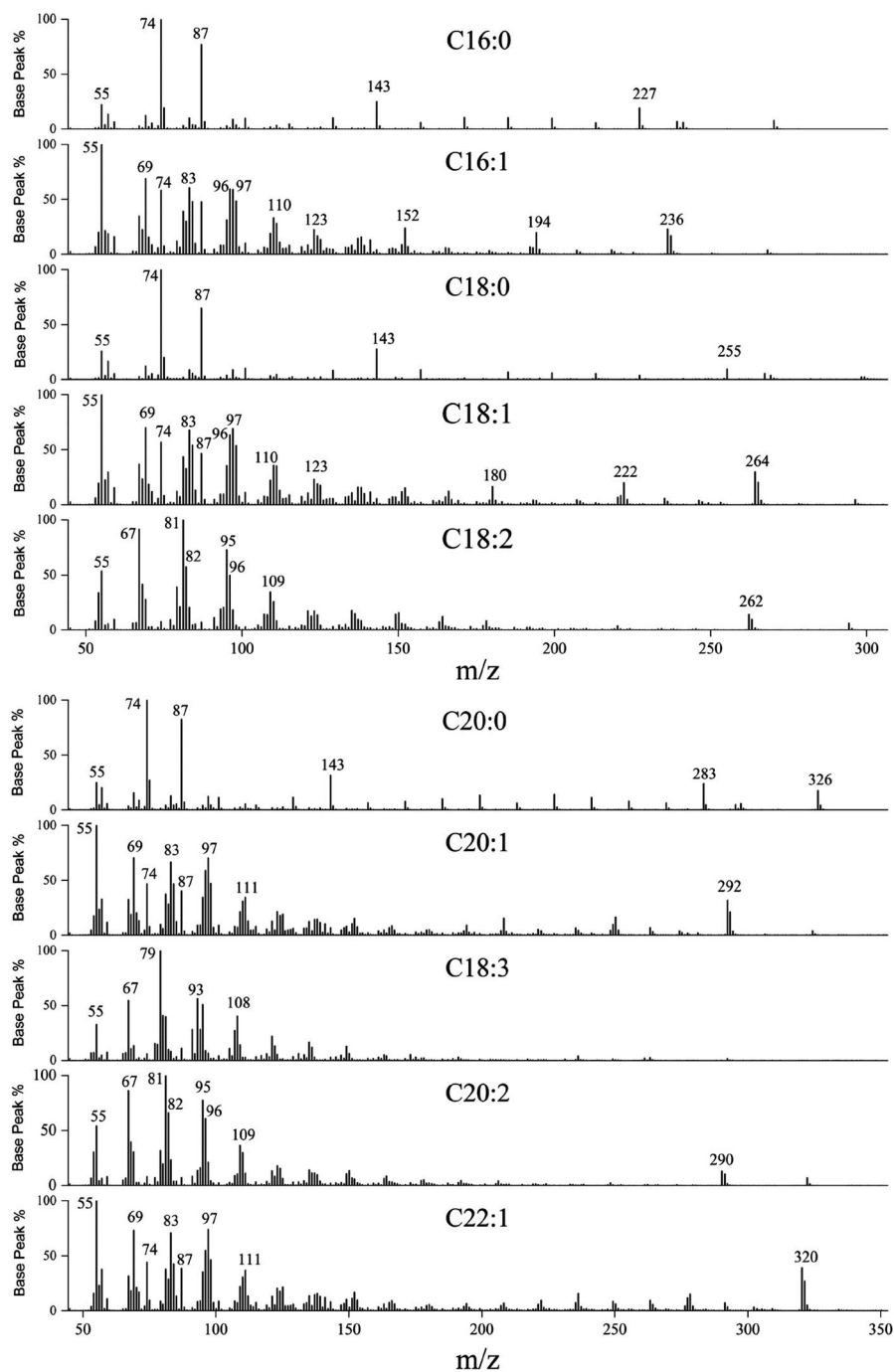


Figure 1. GC/MS chromatogram of derivatized methanol for the ten FAs. C16:0, C16:1, C18:0, C18:1, C18:2, C20:0, C20:1, C18:3, C20:2, C22:1.

Results

Establishment of calibration curve

In order to study the efficiency of our pretreatment method, firstly the calibration curve was established and the GC-MS analysis conditions of each component were determined. Ten kinds of common FAs in vegetable oil (C16:0, C16:1, C18:0, C18:1, C18:2, C18:3, C20:2, C20:1, C20:2 and C22:1) were analyzed. As depicted in Table 2, the calibration curves of all the

analytes exhibited good linear determination coefficients R^2 (0.9985–0.9999), which demonstrated good linear relationship between the concentration of FA (y) and peak area (x) in GC-MS detection among linear ranges. In this work, the linear range for C16:0 was 0.048–1.5 $\mu\text{g}/\mu\text{L}$, for C18:0, C18:1 and C20:1 it was 0.032–1 $\mu\text{g}/\mu\text{L}$, and for C16:1, C18:2, C18:3, C20:0, C20:2 and C22:1 it was 0.016–0.5 $\mu\text{g}/\mu\text{L}$, respectively). The electron ionization mass spectrum of the 10 FAs is shown in Figure 1.

Optimization of derivatization conditions

Comparison of the recoveries between the two methods

In order to establish an efficient method for FAs methylation, *A. thaliana* seeds were used as the experimental material to optimize the experimental conditions including reaction temperature, time and reaction container. Considering the influence of methanol volatilization on methylation efficiency, we firstly chose 64°C, which is below the methanol volatilization point (64.7°C), as the test temperature for our experiment. The recovery rates of FAs were compared in the case of 85°C and 64°C methylation temperatures and the reaction took 2 h (85°C) in a 10-mL round bottom capped glass tube and 4 h (64°C) in a 2-mL EP tube, respectively, as described in the Materials and Methods section (Figure 2) [30]. In previous studies, the temperature of FAs methylation ranged from 70°C to 95°C, at which the experimental vessels with good sealing (e.g. Teflon-lined screw cap glass tube, vacuum rotary evaporator) were required (Figure 2A). Therefore, a large number of samples cannot be placed at once. As shown in Table 3, the recoveries of most FA components (C16:1, C18:1, C18:2, C18:3 and C22:1) at 64°C were a little higher than or close to the recoveries at 85°C. Although the recoveries of some FA components (C16:0, C18:0 and C20:0) at 64°C were a little lower than those at 85°C, the recoveries of the three FAs were in accordance with the standards of 80–120%, so it is feasible to choose 64°C as the methylation temperature. Moreover, 2-mL EP tube can be applied as the optimum selection for a reaction container with low cost and high experimental efficiency in the laboratory.

Optimization of methylation temperature

The recoveries at 64°C and 85°C were compared in the previous part. The results showed that the better recoveries can be obtained by lowering the temperature to 64°C. We compared the FAs methylation results at 25°C (close to room temperature), 37°C (the general optimal temperature for enzymes), 42°C (often used in molecular experiments) and 64°C. As shown in Figure 3, two to four of the ten target FAs could not be detected when the methylation temperature was 25°C, 37°C or 42°C. However, the ten target FAs could be detected at 64°C, and the relative contents of FAs were similar with earlier reports [5, 20]. Therefore, the subsequent optimization experiment is based on the methylation temperature of 64°C.

Optimization of methylation time

At the optimized methylation temperature of 64°C, we further explored the effect of methylation time (2 h, 4 h, 8 h, 12 h and 16 h) on FAs analysis. After methylation for 2 h, FAs with lower abundance such as C20:2 and C22:1 could not be detected. However, 10 FAs were detected after methylation for 4 h, 8 h, 12 h, 16 h, and the relative contents of FAs (Figure 4) were similar to previous reports [20, 31]. Considering the reliability and experimental efficiency, 4 h was chosen to complete the FAs methylation.

Determination of sample usage on the quantification of FAs in Arabidopsis thaliana seeds. The relative contents of FAs composition respectively in 0.5, 1, 2, 5 and 10 mg of *A. thaliana* seeds were analyzed. The result in Figure 5 demonstrated that C16:1, C20:2 and C22:2 could not be detected in 0.5 mg and 1 mg seeds, neither C22:1 in 2 mg seeds. However, all 10 target FAs could be detected in 5 mg and 10 mg seeds (Figure 5), and the relative contents of FAs were similar with earlier reports [5, 20, 31]. In conclusion, both 5 mg and 10 mg seeds were used as sample amount for GC-MS analysis, and 5 mg could be suitable for FAs analysis of precious seeds.

Application of the method in different seeds

Now that we had constructed a new method for FAs methylation, we also studied whether it can be applied in other oil seeds. Soybean (*Glycine max*), rapeseed (*Brassica napus*), peanut (*Arachis hypogaea*) and sunflower (*Helianthus annuus*) are the top 4 oil crops in the world, and sesame (*Sesamum indicum*) is also rich in oil, so the above 5 kinds of seeds were chosen as material for the method application experiment. Because these seeds are larger than *A. thaliana* seeds, and the seed coat may have an inhibitory effect on FAs methylation, these oilseeds were first ground with liquid nitrogen and then 5 mg were weighed out for the methylation reaction. The differences in the oil content and FAs types in these oilseeds are respectively presented in Table 4 and Figure 6. Seven FAs were detected in *Glycine max*, 10 target FAs in *Brassica juncea* seeds, and 6 FAs in *Arachis hypogaea*, *Helianthus annuus* and *Sesamum indicum* seeds, respectively, and there were obvious differences in their relative contents. The FAs content of *Sesamum indicum* was the highest, followed by *Arachis hypogaea*, *Helianthus annuus*, *Brassica juncea* and *Glycine max*. The oil contents of these oilseeds were consistent with previous reports [7, 13], and the majority of the 10 target FAs in the oilseeds could be detected [7], such as C16:0, C18:0,

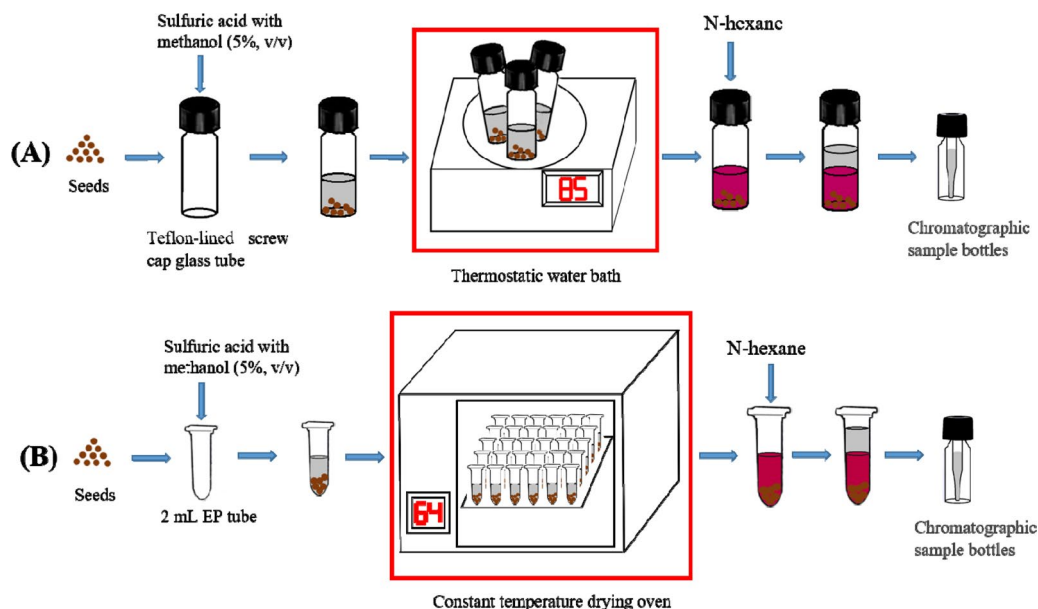


Figure 2. Scheme of the FAME extraction at different temperatures. Previous fatty acid methylation program (reaction at 85°C for 2 h in Teflon-lined screw cap glass tube by use of Thermostatic water bath) (A), Optimized fatty acid methylation program (reaction at 64°C for 4 h in a 2-mL EP tube by use of a constant temperature drying oven) (B). The red box illustrates the difference in sample throughput between the two methods.

C18:1, C18:2, C20:0 and C18:3. Moreover, *Brassica juncea* seeds are richer in erucic acid (C22:1) [32] than other oilseeds analyzed in this study (Figure 6). It was found that the present method is also suitable for the FAs analysis of different plant seeds (e.g. *Brassica juncea* seeds, *Arachis hypogaea*, *Sesamum indicum*, *Helianthus annuus* and *Glycine max*).

Discussion

In this study, we optimized and validated a one-step acid-catalyzed methylation method in treatment conditions including reaction container, temperature, time and sample usage. Firstly, *Arabidopsis thaliana* were selected as experimental materials for method optimization, which provides an ideal system for the study of lipid biosynthetic and metabolism pathways in oilseeds, due to its popularity as a model plant and close relationship with *Brassica napus*, a major oilseed crop [33, 34].

The fatty acid pretreatment methods in the previous literature require the use of well-sealed containers at high temperatures, such as rotary evaporators, Teflon-lined screw cap glass tubes, etc., which is the key point to limit the efficiency of the previous methylation methods. This prompted us to explore whether the efficiency could be improved by reducing the reaction temperature and prolonging the reaction time. Considering that while reducing the reaction

Table 3. Recoveries of FAs composition at different methylation temperatures.

Fatty acids	Recoveries (% , n=3)	
	Methylation temperature (64 °C)	Methylation temperature (85 °C)
C16:0	90.72 ± 3.45	103.48 ± 4.10
C16:1	94.34 ± 5.75	81.52 ± 3.86
C18:0	80.62 ± 0.48	108.13 ± 2.58
C18:1n9c	98.83 ± 4.37	103.08 ± 2.52
C18:2n6c	104.74 ± 4.23	104.96 ± 1.64
C20:0	88.33 ± 7.21	97.14 ± 5.60
C18:3n6	104.61 ± 5.03	108.24 ± 3.30
C22:1n9	109.93 ± 5.25	103.19 ± 11.95

Data are mean values with standard deviation (± s.d.) (n=3).

temperature, the requirement for tight sealing of the reaction vessel is not as high as the previous method, we used common EP tubes. The comparison of the results of FA methylation by the use of the previous method (reaction at 85°C for 2 h in 10-mL round bottom capped glass tubes) with that of our method (reaction at 64°C for 4 h in the 2-mL EP tube), our new method with a lower reaction temperature (64°C) and a more convenient container (2-mL EP tube) could work well (Table 3). Taking 2-mL EP tubes instead of rotary evaporators or Teflon-lined screw cap glass tubes as reaction containers in FA methylation pretreatment, the number of samples that can be processed is not subject to the conditions of equipment and containers, and specific sample quantity can be changed at any time according to experimental requirements, so the experimental efficiency is

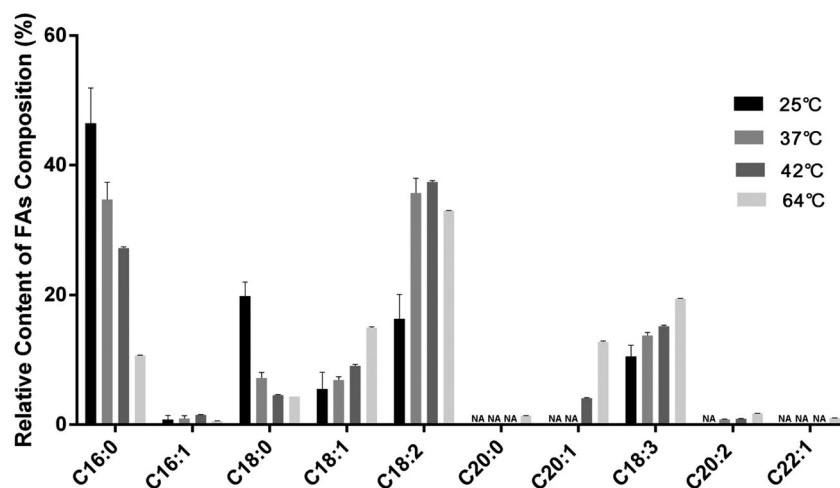


Figure 3. Relative content of FAs composition in *Arabidopsis thaliana* seeds at different methylation temperatures. NA: not available ($n=3$).

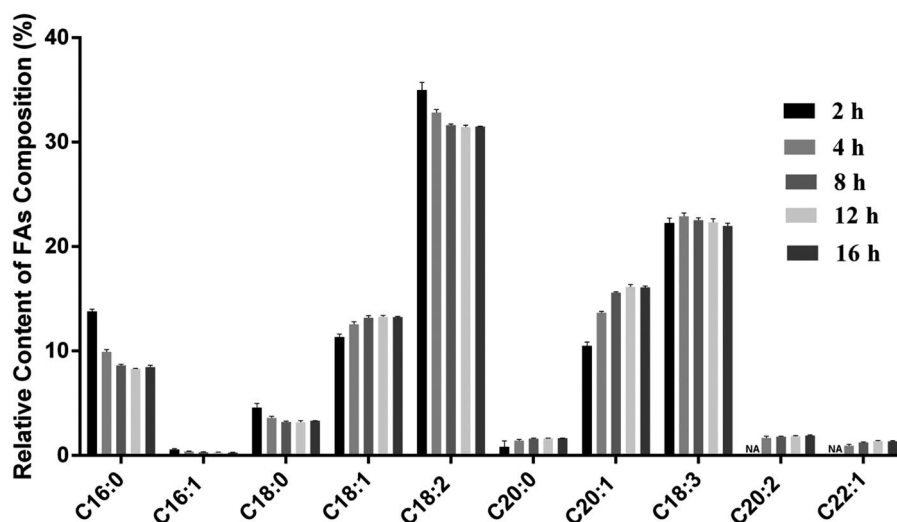


Figure 4. Relative content of FAs composition in *Arabidopsis thaliana* seeds with different methylation time. NA: not available. The error bars represent the standard deviation of the measurements ($n=3$).

improved. In addition, lower reaction temperature means more safety for the operators and the environment (Figure 2B).

Although 10 mg *A. thaliana* seeds were usually recommended for seed oil detection in previous studies [10, 24], a microscale sample preparation method would be advantageous for the small sized *A. thaliana* seeds. The data showed that both 5 mg and 10 mg seeds were used as a sample amount for GC-MS analysis, and 5 mg could be suitable for FAs analysis of seeds. In addition, the method in this study was applied to detect the FAs composition of *Brassica juncea* seeds, *Arachis hypogaea*, *Sesamum indicum*, *Helianthus annuus* and *Glycine max* successfully (Table 4), which suggests that this method also can be introduced to detect other plants.

In order to illustrate the difference and the advantage of our method, the reported methods based on acid catalysis are summarized in Table 5 focusing on comparing the methylation temperature, time, reaction device and sample size. As shown in Table 5, the method in our work with a simpler vessel, lower temperature and smaller sample size would improve the efficiency of the experiment.

Conclusions

This study proposed a simplified and high throughput methylation method followed by GC-MS analysis for analysis of FAs in oilseeds based on one-step acid-catalyzed methylation. The proposed method is

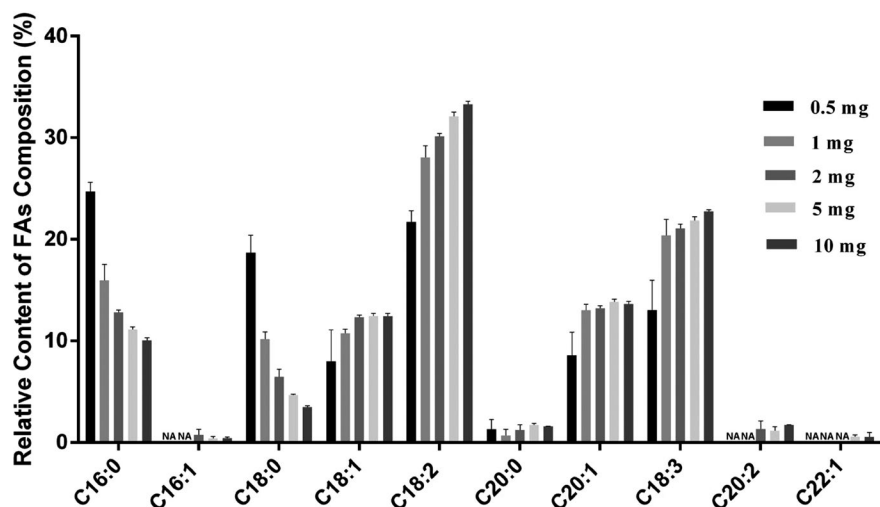


Figure 5. Relative contents of FAs composition with different *Arabidopsis thaliana* seeds usage. NA: not available. The error bars represent the standard deviation of the measurements ($n=3$).

Table 4. FAs composition and total FAs contents in different materials.

Species	<i>Brassica juncea</i>	<i>Arachis hypogaea</i>	<i>Sesamum indicum</i>	<i>Helianthus annuus</i>	<i>Glycine max</i>
Sum ($\mu\text{g}/\text{mg}$)	192.18 ± 18.55	367.87 ± 15.05	412.42 ± 7.28	300.05 ± 3.76	102.76 ± 3.06
Fatty acids (%)					
C16:0	7.54 ± 0.30	18.17 ± 0.07	15.12 ± 0.03	20.33 ± 0.12	21.48 ± 0.18
C16:1	0.28 ± 0.03	0.05 ± 0.01	0.14 ± 0.01	0.06 ± 0.01	0.10 ± 0.01
C18:0	2.23 ± 0.06	2.33 ± 0.02	5.90 ± 0.06	3.79 ± 0.03	4.76 ± 0.09
C18:1	16.75 ± 0.28	42.10 ± 0.14	38.85 ± 0.14	23.32 ± 0.15	28.70 ± 0.20
C18:2	19.17 ± 0.29	35.12 ± 0.16	39.08 ± 0.09	52.22 ± 0.31	40.70 ± 0.15
C20:0	0.70 ± 0.02	1.03 ± 0.01	0.54 ± 0.01	0.18 ± 0.01	0.26 ± 0.01
C20:1	7.76 ± 0.38	1.10 ± 0.02	0.15 ± 0.01	0.08 ± 0.01	0.22 ± 0.01
C18:3	12.93 ± 0.31	0.11 ± 0.08	0.23 ± 0.08	0.02 ± 0.01	3.77 ± 0.09
C20:2	0.08 ± 0.01	NA	NA	NA	NA
C22:1	32.57 ± 0.46	NA	NA	NA	NA

Data are mean values with Standard Error (\pm S.E.) ($n=3$). NA: not available.

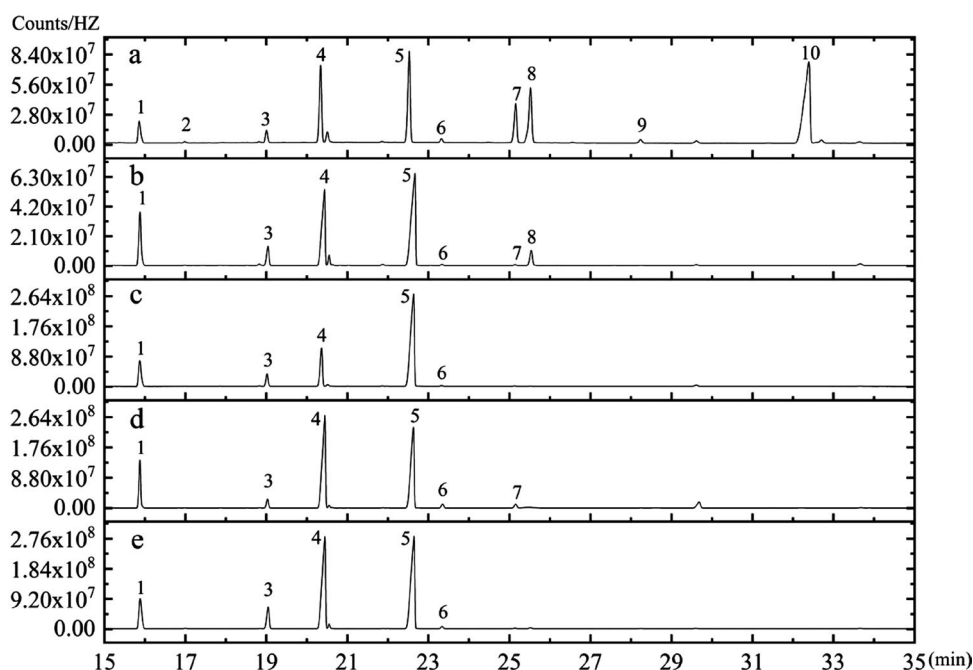


Figure 6. GC-MS spectrum for the FAMES of five plant materials: *Brassica juncea* (a); *Glycine max* (b); *Helianthus annuus* (c); *Arachis hypogaea* (d); *Sesamum indicum* (e). FAMES: (1) C16:0; (2) C16:1; (3) C18:0; (4) C18:1; (5) C18:2; (6) C20:0; (7) C20:1; (8) C18:3; (9) C20:2; (10) C22:1.

Table 5. Comparison of acid catalysis methylation methods for FAs in plant samples.

Plant samples	Derivatization (temperature and time)	Reaction container	Seed weight	Ref.
<i>Arabidopsis thaliana</i> seeds	64°C, 4 h	2-mL EP tube	5 mg	This study [10, 11] [13, 20, 21]
	80°C, 2 h	–	10 mg	
	90–95°C, 1.5 h	Teflon-lined screw cap glass tube	10 mg	
Sunflower seeds (<i>Helianthus annuus</i>)	100°C, 4 h	Soxhlet apparatus	2–4 g	[14, 15] [22]
Tobacco seeds (<i>Nicotiana tabacum</i>)	80°C, 1 h	Screw cap glass tube	–	
<i>Oenothera hookeri</i>	90°C, 1.5 h	–	20 mg	[23]
<i>Jatropha curcas</i>	90–100°C, 4 h	Teflon-lined screw cap glass tube	50–100 mg	[24]
Pomegranate seeds (<i>Punica granatum</i>)	70°C, 2.5 h	Separating funnel and vacuum rotary evaporator	5 g	[35]
<i>Rhodobryum ontariense</i>	70°C, 1 h	Screw cap glass tube and conical bottom tube	300 mg	[36]
	80°C, 4 h	–	1 g	[28]

–: not available.

a successful approach to increase the efficiency of sample preparation and analysis for large-scale experiments.

Disclosure statement

No potential conflict of interest was reported by the authors.

Data availability

All data that support the findings reported in this study are available from the corresponding author upon reasonable request.

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