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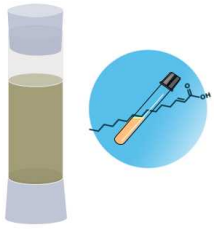
Comparison of Direct Transesterification and Conventional Multi-Step Methods for Fatty Acid Analysis in Marine Sediments



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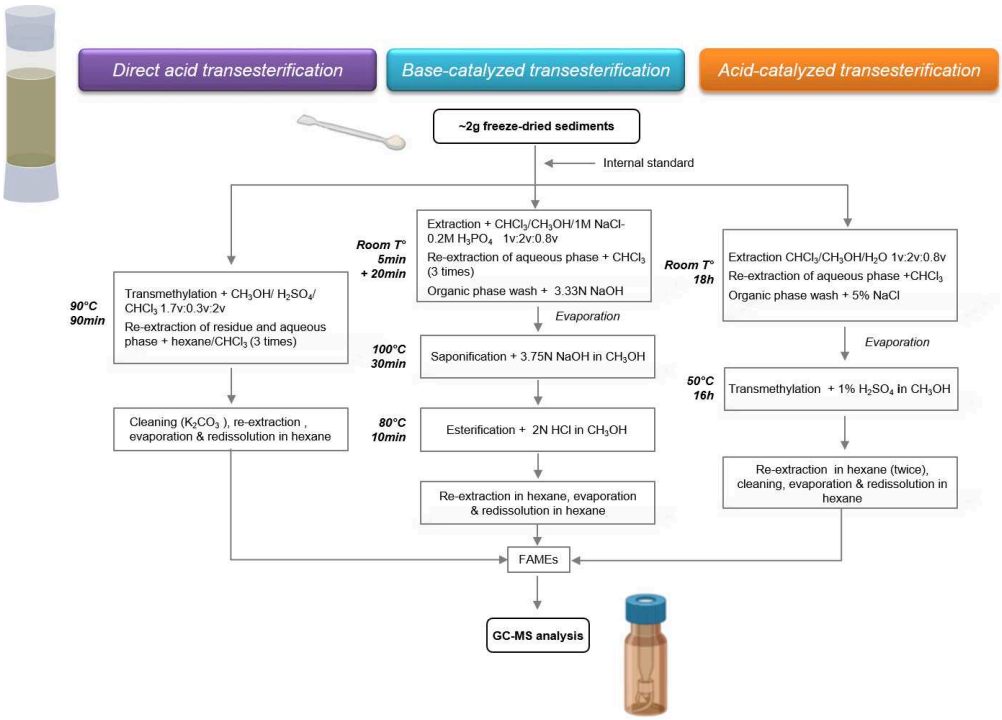
Abstract

This protocol describes three methods for the quantitative determination of total fatty acid methyl esters (FAMEs) in marine sediments:

1. **One-Step Direct Transesterification**
2. **Base-Catalyzed Saponification**
3. **Acid-Catalyzed hydrolysis**

The **one-step method** combines extraction and transesterification into a single step, significantly reducing handling time and minimizing the risk of fatty acid degradation. Compared to the **two conventional multi-step methods**, the one-step protocol yields better recovery and improved repeatability, with no adverse effects on fatty acid profiles. The one-step procedure is an efficient, simple, rapid and economic alternative to multi-step methods for the routine analysis of total sedimentary fatty acids.
Additionally, each protocol was tested with and without the use of **BHT** (butylated hydroxytoluene) as an antioxidant to assess its impact on lipid stability and recovery.



Guidelines

- **Storage:** Keep extracted FAMEs at **-20°C** under nitrogen if not analyzed immediately.
- **Sample Handling**: Handle sediments quickly to prevent microbial degradation.
- **Safety:** Handle solvents and acids under appropriate safety conditions (fume hood, PPE).
- **Equipment**: Ensure all glassware and tubes are pre-burnt (450°C) to avoid contamination.
- **Chemicals and solvents:** Use GC-MS or HPLC reagent grade products
- **Internal Standard**: Nonadecanoic acid (C19:0) is used to correct for extraction losses.
- **Temperature Control**: Maintain precise heating (90°C) and cooling (ice bath) conditions to ensure consistent results.
- **GC-MS Settings**: Use an appropriate polar capillary column and high purity helium (Research GR 6.0 99.9999% total purity) for optimal separation of FAMEs.
- **Equipment Calibration:** Ensure GC-MS instruments are calibrated with certified standards.

Materials

Biological samples

- Marine sediment samples (approximately 2 g of freeze-dried material for each analysis)

Chemicals and reagents:

- Chloroform
- Methanol
- Hexane
- Water (ultrapure)
- 1 M NaCl
- 0.2 M H_3PO_4
- 6N HCl
- 1% H_2SO_4 in methanol
- Sodium hydroxide (NaOH)
- Potassium bicarbonate (2%)
- Toluene butylated hydroxytoluene (BHT) 50 mg/l: To be added optionally to chloroform to prevent lipid oxidation during extraction.
- Hydrochloric acid (HCl)
- Commercial FAME standards (Qualmix Fish Synthetic, Ladoran Fine Chemicals, INTERCHIM, France; Supelco 37, PUFA No. 1 and No. 3, SUPELCO France)
- Nonadecanoic acid (C19:0= internal standard)
- Hexacosanoic acid methyl ester (C26:0), octacosanoic acid methyl ester (C28:0) and triacontanoic acid methyl ester (C30:0)

Equipments:

- Ultrasonic bath
- Rotary shaker
- Ultracentrifuge
- Thermo Scientific™ Savant™ Speed-Vac concentrator
- GC-MS system
- Polar capillary column (e.g. VF-Wax ms or ZB-WAX)

Safety warnings

- **Sample Degradation:** Avoid delays between sample collection and conditioning to prevent fatty acid degradation.
- **Temperature Control:** Maintain specified temperatures during extraction and evaporation to avoid loss volatile fatty acids and ensure consistent results.

Before start

Protocol Note on BHT

For each protocol, lipid extraction and transesterification steps can be performed with or without BHT to evaluate its effect on fatty acid recovery and profile stability. Ensure to document which variation (with or without BHT) is used during each run.

1. One-step Direct Transesterification Protocol

1h 45m

1 FAMEs are prepared by a direct transesterification procedure which involves methanolic sulphuric acid as a catalyst for the esterification of fatty acids. The protocol is adapted from Indarti *et al.* [2005] and follows recommandations of Christies [2003].

1.1 **Sample Preparation**

- Condition sediments rapidly after collection to prevent bacterial degradation of fatty acids.
- Freeze-dry sediments and store at -80 °C until analysis.
- Grind freeze-dried sediments to a fine powder using liquid nitrogen and a **TissueLyser II (QIAGEN)**.

1.2 **Lipid extraction and Transesterification**

1h 30m

- **Weigh** approximately 2-3 g of dry sediment into a pre-burnt (450 °C) screw-top tube with a Teflon-lined cap.
- Add 8 mL of a cold monophasic mixture of **Methanol, sulfuric acid, and chloroform** (1.7v:0.3v:2v) with **BHT** (butylated hydroxytoluene) as an antioxidant.
- Add 20 µL of internal standard (**C19:0 nonadecanoic acid,** [M] 1 mg/mL **)**
- Vortex to mix and tightly seal the tube.
- Heat at 90 °C in an oven for 01:30:00 shaking manually **3-4 times** during heating.

1.3 **Phase Separation**

5m

- Cool tubes in an **ice bath**.
- Open slowly

> Note
>
> The mixture can bubble due to the formation of CO_2 in presence of calcium carbonates.

- Add 2 mL of cold ultrapure water and vortex to mix.
- Centrifuge at 3000 rpm, 4°C, 00:05:00
- Transfer the **lower organic phase** (chloroform layer) to a clean screw-top tube.

1.4 **Organic Phase Wash**

5m

- Add 2 mL of a cold hexane-chloroform (4v:1v) mixture and vortex.
- Centrifuge at **3000 rpm (1912 g)** for 00:05:00 .
- Transfer the **upper organic layer** to the first organic phase. Repeat this step **twice**.

Note

While performing these steps, phase inversion may occur. This usually occurs when organic phase from the previous extraction remained. Be careful to remove the good phase.
The system may also be triphasic due to organic phase retention within sediments. In this case, vortex and centrifuge at a slightly higher speed.

- Combine all organic layers (~10 ml).

1.5 **Final Purification** 5m

- Rinse the organic phase with 4 mL of cold potassium carbonate solution (2%). Vortex and centrifuge 3000 rpm, 4°C, 00:05:00 .
- Discard the upper polar layer and transfer 6-8 mL of the lower organic phase to a clean screw-cap tube.

1.6 **Drying and Dissolving FAMEs**

- Evaporate solvent in a SpeedVac concentrator at Room temperature .
- Dissolve FAMEs in 50 µL of pure hexane and vortex.
- Centrifuge at 3000 rpm, 4°C .
- Transfer the solution to a GC-MS vial with a **200 µl insert**.

2. Base-Catalyzed Saponification Protocol 1h 38m

2 Total lipids are extracted from sediments with methanol–chloroform following the standard method of Bligh and Dyer [1959] prior to the release and methylation of fatty acids under alkaline conditions (adapted from Zarnowski [2002]).

2.1 **Sample Preparation and Lipid Extraction** 35m

- Weigh ~ 2 g of sediment and add 20 µL of internal standard (C19:0, [M] 1 mg/mL).
- Add 40 mL of a monophasic mixture (chloroform:methanol:water-NaCl 1M-$H_3PO_4$0.2M, 1v:2v:0.8v) with BHT ([M] 50 mg/l).

Note

In this step, water was replaced by 1M NaCl and 0.2M phosphoric acid was added to the extraction reagent to increase the yield of lipids (Hajra 1974).

- Sonicate for 00:05:00 5 minutes On ice .

- Agitate for 00:20:00 at Room temperature .
- Add 5 mL chloroform and 5 mL ultrapure water.
- Shake for 00:05:00 . Centrifuge at 2700 x g, 4°C, 00:05:00 .

2.2 Phase Separation and Saponification

36m

- Collect the lower organic phase.
- Repeat extraction 3 times with 10 mL chloroform.
- Combine organic layers and wash with 5 mL of cold sodium hydroxide solution.
- Shake for 00:01:00
- Centrifuge 2700 x g, 5°C, 00:05:00 (system becomes biphasic)
- Place in a new tube 40 ml of the organic phase
- Evaporate the solvent at 40-43 °C in a speed-vac.
- Add 4 mL of methanolic NaOH solution (made of 45g NaOH in 300 ml of Methanol:H_2O 1v:1v).
- Heat at 100 °C for 00:30:00 in a water bath .

> Note
>
> A red color appears in presence of BHT.

2.3 Methylation

10m

- Add 8 mL of methanol-HCl solution (made of 325 ml HCl 6N and 275 ml methanol).
- Heat at 80 °C for 00:10:00 .
- Cool down rapidly on ice.

2.4 Extraction of FAMEs

17m

- Add 5 mL hexane.
- Agitate for 00:10:00 .
- Centrifuge at 165 x g, 5°C, 00:02:00 .
- Evaporate hexane in a SpeedVac concentrator and dissolve FAMEs in 50 µL hexane.
- Centrifuge at 3000 rpm, 4°C, 00:05:00 .
- Transfer the solution to a GC-MS vial with a 200 µl insert.

3. Acid-Catalyzed Saponification Protocol

1d 10h 20m

3 The protocol of Lewis [2000] was followed with minor modifications to improve extraction and purification (Christie 2003).

3.1 Sample Preparation and Lipid Extraction

18h

- Weigh ~ 2 g of sediment and add 20 µL internal standard (C19:0, [M] 1 mg/mL).
- Add 10 mL chloroform-BHT. Vortex.
- Add 20 mL methanol and 8 mL water-NaCl-H_3PO_4. Vortex.
- Agitate for 18:00:00 at Room temperature .

3.2 Phase Separation

15m

- Add 10 mL chloroform and then 10 mL water. Shake.
- Centrifuge at 167 x g, 5°C, 00:05:00 .
- Collect the lower organic phase.
- Wash the residue with 10 mL chloroform.
- Centrifuge at 2700 x g, 5°C, 00:05:00 .
- Recover the lower organic phase.
- Combine with the first organic phase.
- Rinse the organic phase with 5 mL of salted water (5%)
- Centrifuge 2700 x g, 5°C, 00:05:00 .

3.3 Transesterification

16h

- Recover 25 mL of the organic phase and evaporate it wit a Speedvac concentrator
- Dissolve the dry residue with 1 mL of chloroform:methanol (2v :1v).
- Add 2 mL H_2SO_4-methanol ([M] 1 % volume).
- Heat at 50 °C for 16:00:00 .

3.4 Extraction of FAMEs

5m

- Add 5 mL NaCl solution.
- Extract FAMEs with 5 mL hexane (2 times).
- Recover the organic phase containing the FAMEs.

- Wash the organic phase with 4 mL potassium bicarbonate (2%).
- Evaporate in a SpeedVac concentrator and dissolve in 50 µL hexane.
- Centrifuge at 3000 rpm, 4°C, 00:05:00 .
- Transfer the solution to a GC-MS vial with a 200 µl insert.

4. Analysis of FAMES by GC-MS

4 FAMEs are ready for analysis by gaz chromatography with Mass spectrometry analysis.

Note

- Marine sediments contains a very large diversity of fatty acids, with saturated, monounsaturated and many polyunsaturated (PUFAs) components. Bacterial fatty acids are also presents (odd number, branched, hydroxylated and cyclic fatty acids).
- Use a polar low bleeding capillary column (e.g., **VF-Wax ms** or **ZB-WAX**, 30m × 0.25mm ID, 0.25µm film thickness) for better separation of PUFAs.
- Apply a **ramp temperature program** optimized for effective separation of FAMEs in marine sediments.

In the case of the authors the following analytical conditions were followed (Table 1).

Gas chromatograph	Saturn 2100T ion-trap GC-MS (Varian)
Carrier gas	Helium 1 $ml.min^{-1}$
Split	100:1
Injector temperature	260 °C
Oven temperature	150 °C for 30 min, 2 $°C.min^{-1}$ to 180 °C, 1
Column	Factor Four, VF-Wax ms, 30m x 0.25 mm ID,
Autosampler	
Helium column head pressure	13.2 psi
Injected volume	1 µl
Electronic flow control	Constant, 1 $ml.min^{-1}$
Mass spectrometer	
Transfer-line temperature	230 °C
Manifold	50 °C
Ion-source temperature	230 °C
Mode	EI, full scan, 0.9 $scans.s^{-1}$, 70 EV
m/z Full scan	m/z 40 to m/z 650

Table I. Analytical conditions of the Saturn 2100T ion-trap GC-MS.

5. Results and Comparison of Protocols

5 To assess the efficiency and repeatability of the three extraction protocols (direct transesterification, base-catalyzed saponification, and acid-catalyzed transesterification), we tested each method with and without BHT as an antioxidant. The primary findings are summarized below (Table 2).

FAMEs (% area)	Direct without BHT		Direct with BHT		Basic without BHT		Basic with BHT		Acidic without BHT		Acidic with BHT	
	Mean	RSD (%)	Mean	RSD (%)	Mean	RSD (%)	Mean	RSD (%)	Mean	RSD (%)	Mean	RSD (%)
12:0	1.27	8.6	1.10	3.4	0.89	9.2	0.80	7.7	0.72	8.9	0.68	2.2
13:0	0.33	12.0	0.30	1.8	0.30	7.8	0.32	6.6	0.35	9.0	0.33	6.9
14:0	6.76	8.5	5.85	1.9	5.50	2.2	11.76	23.4	4.62	0.2	4.49	2.1
15:0	3.33	6.6	2.87	2.6	3.89	3.0	3.79	4.4	3.66	1.7	3.43	1.4
16:0	22.07	6.9	19.63	2.1	19.73	1.9	19.42	5.2	17.44	1.8	17.49	1.0
17:0	t		t		3.17	3.0	t		t		t	
18:0	4.76	5.7	4.34	2.1	4.31	5.1	4.95	5.9	3.85	6.6	3.99	6.7
20:0	2.88	3.9	2.78	3.3	1.91	8.7	1.87	7.5	1.89	6.5	1.80	3.9
21:0	0.49	8.4	0.48	5.8	0.43	14.6	0.46	15.5	0.42	3.1	0.39	9.3
22:0	3.69	9.9	4.10	3.0	2.85	18.2	2.74	8.2	3.02	2.5	3.08	4.5
23:0	0.70	18.1	0.94	8.0	0.68	21.4	0.78	14.4	0.63	12.1	0.67	7.6
24:0	3.92	25.0	5.46	3.5	2.73	19.3	2.47	10.1	4.12	5.6	4.26	4.3
26:0	2.59	49.0	4.46	6.0	1.34	12.7	1.46	17.0	3.59	7.7	3.81	5.2
28:0	2.11	63.8	3.96	11.8	nd		nd		4.79	21.1	4.37	14.8
30:0	t		2.93	29.3	nd		nd		t		t	
16:1ω7	10.93	7.2	9.86	1.8	14.97	4.4	13.73	8.1	13.66	1.1	13.71	1.1
18:1ω9 c	3.52	5.7	3.16	1.8	4.01	3.6	3.81	5.7	3.63	4.4	3.76	3.4
18:1ω7	5.95	5.9	5.43	2.1	8.04	1.3	8.11	3.2	8.05	0.7	8.15	1.9
20:1ω9	0.50	8.6	0.44	7.0	0.45	29.9	0.52	7.0	0.60	11.9	0.56	4.6
16:2ω4	0.55	8.9	0.49	4.6	0.65	5.9	0.58	5.3	0.81	3.5	0.79	6.1
16:3ω4	0.68	11.4	0.60	6.0	0.89	7.4	0.77	6.2	0.85	4.4	0.89	5.7
18:2ω6 c	1.00	6.3	0.94	4.1	1.05	37.9	0.85	4.9	0.89	6.3	0.93	2.6
18:4ω3	0.62	6.6	0.58	5.8	0.56	6.2	0.49	8.5	0.60	6.3	0.60	3.4
20:2ω6	0.54	8.9	0.53	5.2	0.63	8.3	0.55	14.9	0.67	5.6	0.61	11.3
20:4ω6	2.88	8.0	2.58	1.6	3.48	6.5	3.27	7.3	3.80	2.8	3.91	5.0
20:5ω3	4.27	6.0	3.90	5.2	4.61	5.9	4.40	5.1	5.60	4.4	5.82	2.7
22:6ω3	1.81	9.5	1.53	4.4	1.44	11.5	1.35	14.6	1.96	10.7	2.01	8.7
i 15:0	3.83	6.7	3.40	2.6	4.10	3.3	3.73	6.4	3.41	3.5	3.27	3.2
ai 15:0	4.27	6.5	3.82	1.8	4.65	3.3	4.23	6.9	4.01	2.0	3.94	2.0
i 16:0	1.53	6.9	1.38	2.1	1.55	5.1	1.63	4.0	1.30	12.2	1.21	6.5
i 17:0	1.65	7.0	1.48	2.0	1.20	6.2	1.15	6.3	1.09	1.8	1.03	4.0
3-OH-14	0.74	3.7	0.68	9.2	t		t		t		t	
Σ FA (µg.g^{-1} DW)* **	**45.70**	**3.68**	**48.28**	**3.55**	**35.27**	**8.59**	**33.19**	**3.58**	**33.24**	**4.13**	**34.38**	**3.54**

Table 2: Reproductibility of fatty acid extraction in sediments by the three methods withouth and with
addition of BHT as an antioxidant.
Results obtained from five separated extractions are expressed as mean % areas and relative
standard deviations (RSD in %).
BHT = butylated hydroxytoluene, nd: non detected, t: trace amount, S FA: total fatty acid concentration .
***: significantly different with the direct method in comparison to the basic or acid-catalyzed
transesterification protocols ($p<0.0001$), no statistical difference of BHT treatment on Sum FA.

5.1 Effect of BHT on Repeatability (RSD)

The addition of BHT significantly improved the repeatability of fatty acid extraction, particularly for the one-step direct transesterification protocol. This positive effect on the relative standard deviation (RSD) underscores the value of BHT in maintaining consistency during lipid extraction.

5.2 Fatty Acid Concentrations Across Protocols

We compared the fatty acid concentrations obtained under six conditions (three protocols × with/without BHT). The results clearly demonstrate that the one-step direct transesterification protocol outperformed the other two methods in terms of extraction efficiency and yield.

Extraction Efficiency:

- The direct transesterification method was significantly more efficient (ANOVA, $p < 0.0001$), achieving extraction efficiencies ~40-45% higher than the acid- and base-catalyzed saponification methods.

Fatty Acid Yields:

- Concentrations of individual fatty acids were, on average, 1.7 times higher than with the acid-catalyzed protocol.
- Yields were 2.2 times higher than those obtained with the base-catalyzed protocol.

5.3 Specific Observations

- Polyunsaturated Fatty Acids (PUFAs): The base-catalyzed method significantly underestimated PUFA concentrations.
- Long-Chain Saturated Fatty Acids (LC-SAFAs, ≥ C22): Recovery of LC-SAFAs was 3 to 5 times lower with both base and acid-catalysed protocols compared to the direct transesterification method.

5.4 Conclusions

The optimized one-step procedure described here has three significant advantages over traditional methods for quantifying total fatty acids in marine sediments.

- Higher extraction efficiency
- Improved repeatability (lower RSD)
- Superior recovery of PUFAs and LC-SAFAs

This makes it the most reliable method for analyzing fatty acids in marine sediments, particularly in environmental studies focusing on organic matter inputs.

Note

Among the many advantages of one-step protocols are the simplicity of the procedure which also reduces the risk of contamination or loss of material, the general improved performance, as well as considerable savings of time and reagents.

Protocol references

Christie, W.W., 2003. Lipid analysis: isolation, separation, identification and structural analysis of lipids. The Oily Press, Bridgawater, England.
Hajra, A.K., 1974. Lipids **9**, 502–505.
Indarti, E., Majid, M.I.A., Hashim, R., Chong, A., 2005. Direct FAME synthesis for rapid total lipid analysis from fish oil and cod liver oil. J. Food Compos. Anal. 18, 161–170. https://doi.org/10.1016/j.jfca.2003.12.007
Lewis, T., Nichols, P.D., McMeekin, T.A., 2000. Evaluation of extraction methods for recovery of fatty acids from lipid-producing microheterotrophs. J. Microbiol. Methods 43, 107–116.
Zarnowski, R., 2002. Fatty acid profiling: its usefulness in the evaluation of microbial associations with the green microalga *Apatococcus constipatus*. Cell. Mol. Biol. Lett. 7, 61–67.