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Article

Methyl and ethyl chloroformate derivatizations for compound-specific stable isotope analysis (CSIA) of fatty acids

Akiko S. Goto *,a, Takashi Korenaga * and Yoshito Chikaraishi **

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Abstract

We evaluated methyl (MCF) and ethyl chloroformate (ECF) derivatizations as rapid and simple esterification techniques for the compound-specific stable isotope analysis (CSIA) of fatty acids. Although the fatty acids were esterified very rapidly (within 5 min) at room temperature in both derivatizations, the yield of derivatives depended strongly on the chemical constituent and pH of the derivative reagent and the carbon-chain length of fatty acids. In this study, quantitative esterification was observed during ethyl esterification with ECF:ethanol:pyridine (2:60:5, v:v). In this esterification process, the accuracy of the carbon isotope ($\delta^{13}C$) measurements was always better than 0.3‰.

1. Introduction

Fatty acids are frequently found as abundant lipid molecules in biological and geological samples, and have therefore been used as biomarkers in a number of studies, particularly in organic geochemistry (e.g., Naraoka and Ishiwatari, 2000; Hou et al., 2006; Jones et al., 2008). However, because of the low volatility and high polarity of fatty acids, derivatization (e.g., esterification or silylation) of the carbonyl group in fatty acids is required to improve the chromatographic resolution of each fatty acid in gas chromatographic analyses. This is particularly necessary for compound-specific stable isotope analysis (CSIA) by gas chromatography/isotope ratio mass spectrometry (GC/IRMS), because this technique requires true baseline resolution of the compounds (e.g., Meier-Augenstein, 2002; Chikaraishi and Oba, 2008). Esterification is generally achieved with BF_3 or HCl in alcohol (e.g., methanol or ethanol) at 100℃ for 1 h or 75℃ for 8 h, whereas silylation (e.g., trimethyl silylation) is achieved with N,O-bis-trimethylsilyl-acetamide or N,O-bis-trimethylsilyl-trifluoroacetamide at 75℃ for 30 min to several hours (Fig. 1). Although both types of derivatizations are quantitative reactions without carbon isotopic fractionation (Rieley, 1994), they have several disadvantages. First of all, heating at 75–100℃ for 30 min to several hours is time consuming, and also often causes leaks (which allow isotopic fractionation) of the derivative reagents when the reaction is performed in a vial that is not tightly sealed with a cap. Second, BF_3 is inconvenient because it is a very toxic chemical reagent. Third, the lower stability of silyl derivatives always causes problems in the subsequent wet chemistry processes (e.g., silica gel column chromatography to purify the compounds; e.g., Chikaraishi et al., 2004).

Methyl chloroformate (MCF) and ethyl chloroformate (ECF) derivatization techniques are used as rapid

*Department of Chemistry, Tokyo Metropolitan University, 1-1 Minami-Osawa, Hachioji, Tokyo 192-0397 Japan.
**Institute of Biogeosciences, Japan Agency for Marine-Earth Science and Technology 2-15 Natsushima-Cho, Yokosuka 237-0061 Japan.
[a]Corresponding author. e-mail: akigoto@tmu.ac.jp Tel/Fax: +81-42-677-8541

and simple derivatizations for quantitative and isotopic analyses of amino acids (e.g., Hušek, 1991; Huang et al., 1993; Yamaguchi et al., 2009; Kvitvang et al., 2011). In these derivatizations, esterification of the carbonyl groups and acylation of the amino groups in amino acids are simultaneously achieved at room temperature in only 5–10 min. It could be simply expected that the esterification occurs on the carbonyl groups of fatty acids, so fatty acids could be esterified with alcohol following the formation of carboxylic anhydrides (Fig. 1). In this study, we evaluated whether these MCF and ECF derivatizations can be used in the rapid and simple esterification required for the CSIA of fatty acids.

2. Experiments

A standard mixture (30–50 μmol/L in ethyl acetate: methanol, 7:3, v:v) of nine saturated *n*-fatty acids (C_{16}, C_{18}, C_{23}, C_{24}, C_{25}, C_{26}, C_{28}, C_{30}, and C_{31}) with known $\delta^{13}C$ values (ranging from –31.4‰ to –18.1‰) was used for this experiment. Methanol and ethanol with known $\delta^{13}C$ values (–57.4‰ and –14.8‰, respectively) were used as derivative regents. Generally, MCF or ECF derivatization is reacted with an admixture of MCF:H_2O:methanol:pyridine (1:20:10:2.5, v:v) or ECF:H_2O:ethanol:pyridine (1:20:10:2.5, v:v) at room temperature for 5 min (e.g., Yamaguchi et al., 2009). In this study, we used 0.1 M HCl aq. (pH = 1), H_2O (pH = 7), 0.1 M NaOH aq. (pH = 14), or alcohol (i.e., methanol or ethanol) instead of H_2O in the admixture described above for the derivatizations (Table 1), to evaluate the efficiency of derivatizations in terms of the solubility of fatty acids in the derivatization reagents and to identify the optimum conditions for the isotope analysis. Briefly, 450 μl of the standard solution (～150 μmol of total fatty acids) was dried in a 4 ml vial with a gentle stream of N_2, and then the MCF or ECF derivatization was performed at room temperature for 5 min. The fatty acid methyl esters (FAMEs) or ethyl esters (FAEEs) obtained were extracted with *n*-hexane:dichloromethane (2:1, v:v).

The carbon isotopic composition ($\delta^{13}C$, relative to V-PDB; Vienna-PeeDee Belemnite) of the individual FAMEs and FAEEs was determined with GC/IRMS using a Delta XP IRMS interfaced with a gas chromatograph through a GC-C Ⅲ interface (Thermo Fisher Scientific). Representative chromatograms are shown in Fig. 2. The analytical error for the isotope measurements was always better than 0.3‰. We also determined the abundance of individual derivatives by a comparison of the *m/z* 44 peak areas on the chromatograms for the derivatives and external *n*-alkane standards (standard mixture of 16 *n*-alkanes). The accuracy of the quantification was always better than 10%.

The determined isotopic compositions of the FAMEs ($\delta^{13}C_{FAMEs}$) and FAEEs ($\delta^{13}C_{FAEEs}$) are defined below in

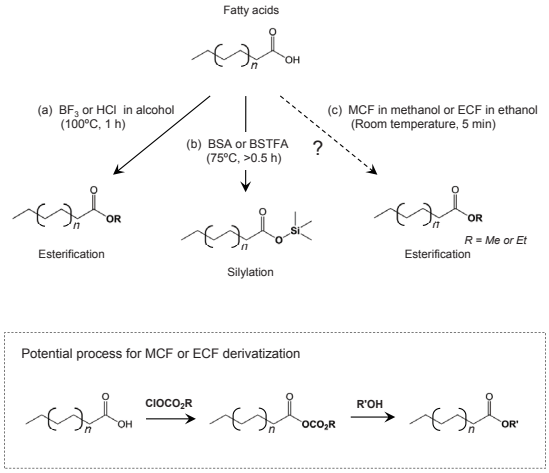

Fig. 1. Derivatizations of fatty acids (a) esterification with BF_3 or HCl in alcohol, (b) trimethyl silylation with N,O-bis-trimethylsilyl-acetamide (BSA) or N,O-bis-trimethylsilyl-trifluoroacetamide (BSTFA), and (c) esterification with methyl (MCF) or ethyl chloroformate (ECF), and potential process for MCF or ECF derivatization.

Table 1. Derivative reagents used in this study

No.	MCF (μl)	ECF (μl)	Water (μl)	Methanol (μl)	Ethanol (μl)	Pyridine (μl)
#1	50	-	1000 (0.1 M HCl aq., pH=1)	500	-	125
#2	50	-	1000 (pH=7)	500	-	125
#3	50	-	1000 (0.1 M NAOH aq., pH=14)	500	-	125
#4	50	-	-	1500	-	125
#5	-	50	1000 (0.1 M HCl aq., pH=1)	-	500	125
#6	-	50	1000 (pH=7)	-	500	125
#7	-	50	1000 (0.1 M NAOH aq., pH=14)	-	500	125
#8	-	50	-	-	1500	125

eqs (1) and (2), respectively, according to the isotopic mass balance with the isotopic composition of the underivatized fatty acids ($\delta^{13}C_{FA}$) and the derivative group ($\delta^{13}C_{Me}$ or $\delta^{13}C_{Et}$):

$$n \times \delta^{13}C_{FA} = (n + 1) \times \delta^{13}C_{FAMEs} - \delta^{13}C_{Me} \quad (1)$$

$$n \times \delta^{13}C_{FA} = (n + 2) \times \delta^{13}C_{FAEEs} - 2 \times \delta^{13}C_{Et} \quad (2)$$

where n represents the carbon number of the underivatized fatty acids. Because we used fatty acid standards with known $\delta^{13}C$ values in this study, we first calculated the $\delta^{13}C_{Me}$ and $\delta^{13}C_{Et}$ values using eqs (1) and (2), respectively. The propagation errors for the $\delta^{13}C_{Me}$ and $\delta^{13}C_{Et}$ values (σ_{Me} and σ_{Et}, respectively) were calculated using eqs (3) and (4), respectively, with the uncertainty of ～0.03‰ (= analytical error on the off-line dual inlet method) for $\delta^{13}C_{FA}$ (σ_{FA}) and 0.3‰ (= analytical error with this GC/IRMS method) for both $\delta^{13}C_{FAMEs}$ (σ_{FAMEs}) and $\delta^{13}C_{FAEEs}$ (σ_{FAEEs}):

$$\sigma_{Me}^2 = \sigma_{FAMEs}^2 \times (n + 1)^2 + \sigma_{FA}^2 \times n^2 \quad (3)$$

$$\sigma_{Et}^2 = \sigma_{FAEEs}^2 \times \{(n + 2)/2\}^2 + \sigma_{FA}^2 \times (n / 2)^2 \quad (4)$$

We then recalculated the $\delta^{13}C_{FA}$ values as unknown using the $\delta^{13}C_{FAMEs}$ or $\delta^{13}C_{FAEEs}$ determined and the mean value for the calculated $\delta^{13}C_{Me}$ or $\delta^{13}C_{Et}$, respectively (Tables 2 and 3; e.g., −53.9‰ for experiment #1), to confirm whether the recalculated $\delta^{13}C_{FA}$ (Re$\delta^{13}C_{FA}$) values were identical to the original $\delta^{13}C_{FA}$ values within the analytical error for all the carbon numbers of fatty acids.

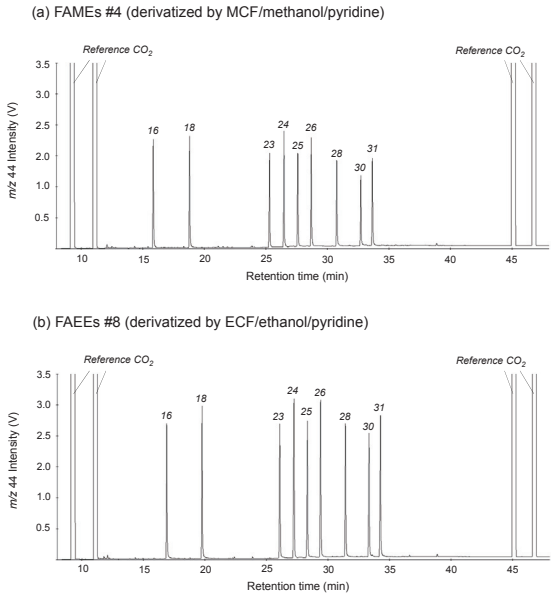

Fig. 2. Representative *m/z* 44 chromatograms of (a) MCF and (b) ECF derivatives. The number on the compound peak indicates carbon number of underivatized fatty acids.

3. Results and discussion

As shown in Tables 2 and 3, the yield of derivatives depended strongly on the chemical constituent and pH of derivative reagent and on the carbon-chain length of fatty acids. For both the MCF and ECF derivatives, relatively high yields (52%−103%) were commonly obtained when water was not used (#4 and #8), whereas relatively low yields were obtained when basic (43%−98% for #3 and #7), natural (11%−70% for #2 and #6), or acidic waters (4%−70% for #1 and #5) were used. Higher yields were observed for short-chain fatty acids (e.g., C_{16} and C_{18}) than for their long-chain homologues (e.g., C_{30} and C_{31}). ECF derivatives also showed higher yields than MCF derivatives under corresponding conditions (e.g., 94%−103% for #8 vs 52%−95% for #4). These results may be consistent with the solubility tendency of the fatty acids in the derivatization reagents, because fatty acids dissolve well in methanol and ethanol but not in acidic water, and this characteristic increases as the length of the hydrophobic groups of fatty acids increase. In this study, quantitative esterification was observed during ethyl esterification with ECF:ethanol:pyridine (2:60:5, v:v).

For all eight conditions tested (#1–8), the calculated $\delta^{13}C_{Me}$ and $\delta^{13}C_{Et}$ values fell between −69.7‰ and −47.2‰, and between −20.2‰ and −4.9‰, respectively (Tables 2 and 3). Although these values have large variations, the values for 1σ (3.7‰−6.8‰ for $\delta^{13}C_{Me}$ and 2.4‰−6.7‰ for $\delta^{13}C_{Et}$) are considered adequate variations of the propagation errors in eqs (3) and (4) (i.e., ～7.7‰ and ～4.0‰, respectively). Furthermore, considering both the measured and propagated errors, the mean values for the calculated $\delta^{13}C_{Me}$ (−59.8‰ to −53.9‰) and $\delta^{13}C_{Et}$ (−14.9‰ to −13.9‰) seem to be identical to the known $\delta^{13}C$ values for methanol

Table 2. Summary for the $\delta^{13}C_{Me}$ and reculculated $\delta^{13}C_{FA}$ values in MCF derivatization

Cn	$\delta^{13}C_{Dual\ Inlet}$[a]	#1				#2				#3				#4			
		$\delta^{13}C_{Me}$[b]	Re$\delta^{13}C_{FA}$[c]	Δ[d]	Yeild (%)	$\delta^{13}C_{Me}$[b]	Re$\delta^{13}C_{FA}$[c]	Δ[d]	Yeild (%)	$\delta^{13}C_{Me}$[b]	Re$\delta^{13}C_{FA}$[c]	Δ[d]	Yeild (%)	$\delta^{13}C_{Me}$[b]	Re$\delta^{13}C_{FA}$[c]	Δ[d]	Yeild (%)
16	-28.43	-50.5	-28.2	0.2	70	-53.4	-28.2	0.2	70	-54.7	-28.3	0.1	98	-50.6	-27.9	0.6	95
18	-18.09	-62.0	-18.5	-0.4	46	-52.9	-17.9	0.2	47	-64.5	-18.5	-0.4	93	-66.5	-18.5	-0.4	84
23	-28.94	-47.2	-28.6	0.3	8	-61.2	-29.1	-0.2	11	-54.3	-28.8	0.1	53	-58.6	-28.9	0.1	76
24	-28.01	-56.2	-28.1	-0.1	6	-53.5	-27.9	0.1	11	-58.8	-28.1	-0.1	43	-62.1	-28.1	-0.1	77
25	-26.76	-	-	-	4	-56.6	-26.7	0.0	11	-66.3	-27.1	-0.4	43	-58.8	-26.7	0.0	68
26	-28.11	-	-	-	5	-57.1	-28.1	0.0	13	-49.7	-27.8	0.3	48	-60.2	-28.1	0.0	72
28	-26.65	-	-	-	5	-64.1	-26.9	-0.3	14	-49.4	-26.4	0.3	48	-69.7	-27.0	-0.4	63
30	-26.16	-	-	-	5	-56.4	-26.1	0.0	15	-64.3	-26.4	-0.2	49	-61.8	-26.2	-0.1	52
31	-31.35	-	-	-	5	-58.0	-31.4	0.0	16	-50.5	-31.1	0.2	49	-49.6	-31.0	0.3	58
mean		-53.9		0.0		-57.0		0.0		-56.9		0.0		-59.8		0.0	
SD(1σ)		6.5		0.3		3.7		0.2		6.8		0.3		6.6		0.3	

[a] $\delta^{13}C$ value of standard fatty acids was determined by traditional dual inlet method.
[b] $\delta^{13}C_{Me}$ value was calculated by isotpic mass balance with the $\delta^{13}C_{Dual\ Inlet}$ and detemined $\delta^{13}C_{FAME}$ values (see text).
[c] Re$\delta^{13}C_{FA}$ value was calculated by isotpic mass balance with the determined $\delta^{13}C_{FAME}$ value and a mean value of the observed $\delta^{13}C_{Me}$ (see text).
[d] Δ value means difference between the Re$\delta^{13}C_{FA}$ and $\delta^{13}C_{Dual\ Inlet}$ values (Δ = Re$\delta^{13}C_{FA}$ - $\delta^{13}C_{Dual\ Inlet}$).

Table 3. Summary for the $\delta^{13}C_{Et}$ and reculculated $\delta^{13}C_{FA}$ values in ECF derivatization

Cn	$\delta^{13}C_{Dual\ Inlet}$[a]	#5				#6				#7				#8			
		$\delta^{13}C_{Et}$[b]	Re$\delta^{13}C_{FA}$[c]	Δ[d]	Yeild (%)	$\delta^{13}C_{Et}$[b]	Re$\delta^{13}C_{FA}$[c]	Δ[d]	Yeild (%)	$\delta^{13}C_{Et}$[b]	Re$\delta^{13}C_{FA}$[c]	Δ[d]	Yeild (%)	$\delta^{13}C_{Et}$[b]	Re$\delta^{13}C_{FA}$[c]	Δ[d]	Yeild (%)
16	-28.43	-19.5	-29.1	-0.7	55	-11.7	-28.2	0.3	56	-14.8	-28.4	0.0	90	-16.1	-28.7	-0.3	101
18	-18.09	-13.7	-18.0	0.1	37	-13.6	-18.1	0.0	38	-14.8	-18.1	0.0	70	-16.3	-18.4	-0.3	100
23	-28.94	-18.7	-29.3	-0.4	9	-16.1	-29.1	-0.2	15	-19.8	-29.4	-0.4	59	-17.6	-29.3	-0.3	102
24	-28.01	-4.9	-27.2	0.8	7	-16.2	-28.2	-0.2	11	-10.7	-27.7	0.3	56	-12.8	-27.9	0.1	103
25	-26.76	-	-	-	10	-7.8	-26.3	0.5	11	-13.6	-26.7	0.1	52	-13.2	-26.7	0.1	100
26	-28.11	-	-	-	9	-12.0	-28.0	0.1	14	-15.0	-28.1	0.0	53	-12.8	-28.0	0.1	102
28	-26.65	-	-	-	7	-12.7	-26.6	0.1	19	-6.9	-26.1	0.6	45	-14.3	-26.7	0.0	98
30	-26.16	-	-	-	6	-15.0	-26.2	-0.1	23	-19.4	-26.5	-0.3	45	-12.7	-26.1	0.1	94
31	-31.35	-	-	-	6	-20.2	-31.8	-0.4	25	-18.9	-31.6	-0.3	49	-9.8	-31.1	0.3	94
mean		-14.2		-0.1		-13.9		0.0		-14.9		0.0		-14.0		0.0	
SD(1σ)		6.7		0.6		3.5		0.3		4.2		0.3		2.4		0.2	

[a] $\delta^{13}C$ value of standard fatty acids was determined by traditional dual inlet method.
[b] $\delta^{13}C_{Et}$ value was calculated by isotpic mass balance with the $\delta^{13}C_{Dual\ Inlet}$ and detemined $\delta^{13}C_{FAEE}$ values (see text).
[c] Re$\delta^{13}C_{FA}$ value was calculated by isotpic mass balance with the determined $\delta^{13}C_{FAEE}$ value and a mean value of the observed $\delta^{13}C_{Et}$ (see text).
[d] Δ value means difference between the Re$\delta^{13}C_{FA}$ and $\delta^{13}C_{Dual\ Inlet}$ values (Δ = Re$\delta^{13}C_{FA}$ - $\delta^{13}C_{Dual\ Inlet}$).

(–57.4‰) and ethanol (–14.8‰), respectively, which were used in the corresponding derivatizations. These results indicate that fatty acids were esterified with alcohol in both the MCF and ECF derivatization processes without substantial isotopic fractionation, and suggest that the yield of the derivatives had little effect on the observed $\delta^{13}C$ values. In fact, the recalculated $\delta^{13}C_{FA}$ values were practically identical to the original $\delta^{13}C_{FA}$ values ($\delta^{13}C_{Dual\ Inlet}$ in Tables 2 and 3) for all cases, with a small difference of less than 0.8‰ (Δ values in Tables 2 and 3). This implies that both methyl and ethyl esterification using MCF and ECF derivatizations, respectively, are potentially useful as alternative esterification techniques in the CSIA of fatty acids.

Considering both the yield and isotope data, we conclude that ECF derivatization with ECF:ethanol: pyridine (2:60:5, v:v) is suitable for the rapid and simple esterification required for the CSIA of fatty acids. The most important advantages of this derivatization are that esterification occurs at room temperature for only

5 min, and that both the yield of the derivatives and the quality (i.e., accuracy and precision) of the isotope data are almost equal to those for traditional derivatizations.

4. Summary

In this study, we evaluated MCF and ECF derivatizations as rapid and simple esterification techniques for the CSIA of fatty acids. Quantitative esterification was observed during ethyl esterification with ECF:ethanol:pyridine (2:60:5, v:v) at room temperature for 5 min. Under this conditions, the accuracy of the carbon isotope measurements was better than 0.3‰. We suggest that ECF derivatization overcomes several issues that limit traditional derivatizations (e.g., it is time consuming, heating entails a risk of isotopic fractionation, and the toxicity BF_3 is inconvenient), and is potentially suitable for the rapid and simple esterification for the CSIA of fatty acids.

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