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Short Article

Methyl and ethyl chloroformate derivatizations for compound-specific stable isotope analysis of fatty acids-Ⅱ

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Abstract

Methyl (MCF) and ethyl chloroformate (ECF) derivatizations are potentially useful for rapid and simple esterification (i.e., at room temperature for 5 min) prior to compound-specific stable isotope analysis (CSIA) of fatty acids. In this study, we have used acetonitrile as an organic solvent in the esterification process to improve the yields of derivatives, and we evaluated whether the improved esterification is suitable for carbon and hydrogen CSIA. Six saturated (14:0, 16:0, 18:0, 20:0, 22:0, and 24:0) and three unsaturated (18:1 n-9, 18:2 n-6, and 18:3 n-3) fatty acids were used in this study. The yields of derivatives are 67−107% for MCF and 84−89% for ECF derivatizations, which are approximately twice the yields obtained in MCF and ECF derivatizations that did not use acetonitrile. The accuracy of the CSIA is <±0.4‰ (1σ) for carbon and <±11‰ (1σ) for hydrogen, with no significant differences in these uncertainties observed for saturated and unsaturated fatty acids. Thus, MCF and ECF derivatizations using acetonitrile are suitable for the CSIA of fatty acids.

Key words: fatty acids, esterification, carbon isotopic composition, hydrogen isotopic composition

1. Introduction

Fatty acids are frequently found as abundant lipid molecules in biological and geological samples, and have been widely used as biomarkers (e.g., Naraoka and Ishiwatari, 2000; Fang et al., 2006; Hou et al., 2006; Jones et al., 2008). However, gas chromatographic analysis of fatty acids always requires a derivatization (e.g., esterification or silylation) of the carboxyl group, which typically prevents high throughput for compound-specific stable isotope analysis (CSIA) of fatty acids in biological and geological samples. In previous studies, esterification has generally been carried out with BF_3 or HCl in an alcohol medium (e.g., methanol or ethanol) at 70−100℃ for 10−120 min (e.g., Cifuentes and Salata, 2001; Chikaraishi et al., 2004; Osbum et al., 2011). This heating step is a time-consuming process and great care is required to prevent leaks of the derivative reagent by the use of tightly sealed vials as, otherwise, leakage can potentially cause significant isotopic fractionations.

Recently, Goto et al. (2011) suggested that methyl (MCF) and ethyl chloroformate (ECF) derivatizations could be used as a rapid and simple esterification step

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prior to the CSIA of fatty acids. This previous study proposed that esterification of fatty acids occurs at room temperature in a short time period of just 5 min, in both aquatic or organic solutions, and also that the accuracy of the measured isotopic composition for carbon is always < ±0.3‰. However, the yields of derivatives depend on the chemical constituents and pH of the derivative reagent. Quantitative esterification was only achieved for an ethyl esterification with ECF/ethanol/pyridine (2/60/5, v/v, yield = 94−103%), but not for methyl esterifications (maximum yield = 52−95%).

This low yield for methyl esterifications is problematic, because methyl ester derivatives are frequently used as substrates for subsequent reactions, such as dimethyl disulfide (DMDS) and dimethyloxazoline (DOMX) derivatizations to identify of the location of double bonds in unsaturated fatty acids (e.g., Chikaraishi et al., 2004).

On the other hand, Several previous studies have used different chemical constituents as derivative reagents (e.g., acetonitrile as a solvent) during MCF esterification to increase the yields of derivatives (e.g., Hušek, 1993; Hušek et al., 2002). These studies reported the yields of derivatives to be >95% for some fatty acids (14:0, 16:0, 16:1, 18:0, and 18:1). However, derivatization with acetonitrile has not yet been used in the CSIA. Furthermore, quantitative esterification in a highly polar solvent (e.g., acetonitrile) might be inconsistent with the results of Goto et al. (2011), in that MCF and ECF esterifications with relatively high yields (52−103%) were observed under hydrophobic (i.e., less polar) conditions, whereas the yields were dramatically lower under hydrophilic (i.e., highly polar) conditions.

In this study, we evaluate whether acetonitrile is suitable for quantitative MCF and ECF esterification, and test whether this esterification process is suitable for the CSIA of fatty acids. While Goto et al. (2011) only evaluated carbon CSIA, herein we also extend this approach to hydrogen CSIA.

2. Samples and methods

All chemicals were purchased from Wako Pure Chemical Industries Ltd. Six authentic saturated (14:0, 16:0, 18:0, 20:0, 22:0, and 24:0) and three unsaturated (18:1 n-9, 18:2 n-6, and 18:3 n-3) fatty acids were used in this study. The carbon and hydrogen isotopic compositions of these fatty acids were determined by conventional methods using a Thermo Fisher Scientific Flash Element Analyzer (1112HT) coupled to a Delta V Advantage Isotope Ratio Mass Spectrometer (IRMS) *via* a ConFlo Ⅲ interface.

These fatty acids were esterified with the following four solutions at room temperature for 5 min using the procedure of Goto et al. (2011):
(1) MCF/acetonitrile/pyridine/methanol (1/22/2/1, v/v),
(2) ECF/acetonitrile/pyridine/ethanol (1/22/2/1, v/v),
(3) MCF/pyridine/methanol (1/2.5/30, v/v),
(4) ECF/pyridine/ethanol (1/2.5/30, v/v).
After derivatization, a saturated $NaHCO_3$ solution was added to the reaction products, and then the fatty acid methyl esters (FAMEs) and ethyl esters (FAEEs) were extracted with 2 mL of *n*-hexane by 3 times.

The yields and stable carbon ($\delta^{13}C$, ‰ relative to V-PDB; Vienna-PeeDee Belemnite) and hydrogen isotopic compositions (δD, ‰ relative to V-SMOW; Vienna-Standard Mean Ocean Water) of the FAMEs and FAEEs were measured with a gas chromatograph/isotope ratio mass spectrometer (GC/IRMS) using a Delta V Advantage IRMS interfaced with a gas chromatograph through a GC-C Ⅲ interface (Thermo Fisher Scientific). The yields were measured for some fatty acids (14:0, 16:0, 18:0, 20:0, and 22:0 for FAMEs; 14:0, 16:0, and 18:0 for FAEEs) by comparison with the *m/z* 44 peak areas on the chromatograms for the derivatives and external standards of authentic FAMEs or FAEEs. The accuracy of the yield quantification is estimated to be approximately ±10%. We do not show the yields for other fatty acids, because of a lack of availability of standard authentic FAMEs and FAEEs for those fatty acids. The isotopic compositions of the fatty acids were obtained *via* an isotopic mass balance calculation from the measured isotopic compositions of

the FAMEs and FAEEs (Goto et al., 2011). Standard deviations of the isotope measurements were always < ± 0.3‰ (1σ; ±0.2‰ on average) for carbon and < ±7‰ (1σ; ±4‰ on average) for hydrogen.

3. Results and discussion

3.1. Yield of derivatives

The yields of derivatives were 67−107% and 84−89% for derivatizations (1) and (2), respectively (Fig. 1). These yields are approximately twice those obtained in derivatizations (3) and (4) that did not use acetonitrile as an organic solvent in the esterifications. These results are consistent with the results reported in Hušek (1993). Clearly, MCF and ECF derivatizations using acetonitrile significantly improve the yield of derivatives as compared with esterifications that do not use acetonitrile.

3.2. Carbon and hydrogen isotopic compositions

For derivatizations (1) and (2), the carbon and hydrogen isotopic compositions of all six saturated and three unsaturated fatty acids were determined by GC/IRMS. The δ^{13}C and δD values of fatty acids determined by GC/IRMS are consistent with those independently determined by EA/IRMS (Fig. 2; R^2 = 0.98 and 0.97 for δ^{13}C and δD, respectively). The accuracies of the GC/IRMS measurements on the fatty acids is < ±0.4‰ for carbon and < ±11‰ for hydrogen (both 1σ). These differences are almost identical or only slightly larger than the precision of the isotope measurements. Given these results, it is apparent that MCF and ECF derivatizations with acetonitrile do not result in any substantial carbon or hydrogen isotopic fractionations during the esterification process. The absence of any carbon isotopic fractionation associated with MCF and ECF derivatizations is consistent with the results of Goto et al. (2011), and with carbon and hydrogen during traditional esterification using BF_3 or HCl in

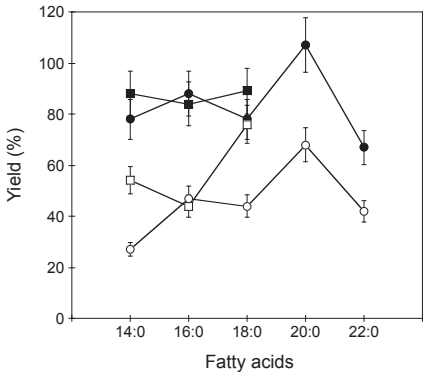

Fig. 1. Yield of derivatives for four derivatizations with (1) MCF/acetonitrile/pyridine/methanol (1/22/2/1, v/v, filled circle), (2) ECF/acetonitrile/pyridine/ethanol (1/22/2/1, v/v, filled square), (3) MCF/pyridine/methanol (1/2.5/30, v/v, open circle), and (4) ECF/pyridine /methanol (1/2.5/30, v/v, open square).

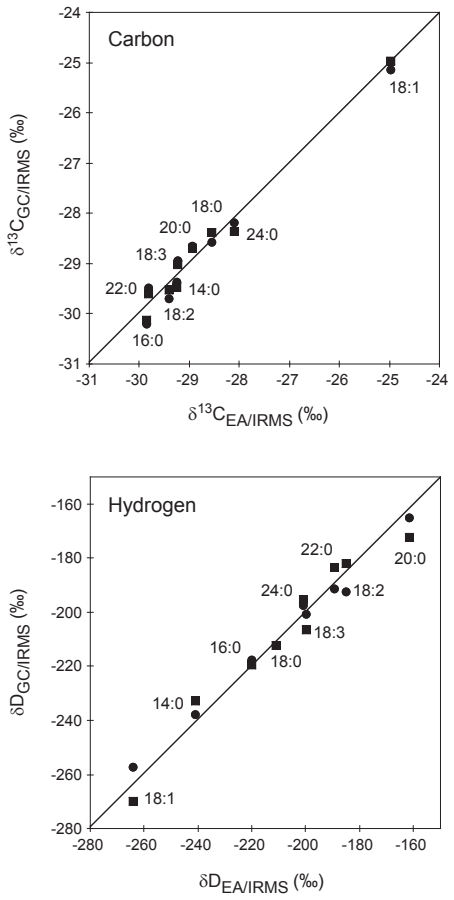

Fig. 2. Carbon and hydrogen isotopic compositions of fatty acids determined by GC/IRMS for MCF (filled circle) and ECF (filled square) derivatizations with acetonitrile and independently determined by EA/IRMS.

alcohol at 70−100℃ (e.g., Cifuentes and Salata, 2001; Chikaraishi et al., 2004; Osbum et al., 2011).

4. Summary

We have shown that MCF and ECF derivatizations using acetonitrile (Hušek, 1993) can improve the yields of derivatives during fatty acid esterification (i.e., formation of FAMEs and FAEEs). Furthermore, these derivatizations do not produce any significant carbon or hydrogen isotopic fractionations. Although these derivatizations are not completely quantitative under the conditions used in this study, further improvement and optimization, such as changing the derivative reagent and reaction time, might lead to 100% yields. We conclude that MCF or ECF derivatization with acetonitrile holds considerable promise for high-throughput CSIA of fatty acids in the fields of organic geochemistry, biology, and ecology.

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