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Intramolecular Carbon Isotope Distribution of Acetic Acid in Vinegar

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ABSTRACT: Compound-specific carbon isotope analysis of acetic acid is useful for origin discrimination and quality control of vinegar. Intramolecular carbon isotope distributions, which are each carbon isotope ratios of the methyl and carboxyl carbons in the acetic acid molecule, may be required to obtain more detailed information to discriminate such origin. In this study, improved gas chromatography−pyrolysis−gas chromatography−combustion−isotope ratio mass spectrometry (GC-Py-GC-C-IRMS) combined with headspace solid-phase microextraction (HS-SPME) was used to measure the intramolecular carbon isotope distributions of acetic acid in 14 Japanese vinegars. The results demonstrated that the methyl carbons of acetic acid molecules in vinegars produced from plants were mostly isotopically depleted in ^{13}C relative to the carboxyl carbon. Moreover, isotopic differences ($\delta^{13}C_{carboxyl} - \delta^{13}C_{methyl}$) had a wide range from −0.3 to 18.2‰, and these values differed among botanical origins, C3, C4, and CAM plants.

KEYWORDS: intramolecular carbon isotope distribution, acetic acid, SPME, quality control, vinegar

INTRODUCTION

Stable isotope ratio analysis is a useful technique to distinguish botanical and geographical origins for food authenticity.[1−5] In particular, compound-specific isotope analysis is useful to determine the authenticity of food, because molecular isotope ratios provide clear information about precursor molecules and their origins.[6,7] Furthermore, the intramolecular isotope ratio should give more detailed information about the origin because of a large conservation of isotope ratio at a certain molecular site within synthetic pathways and metabolic fluxes.[8,9] By measuring the intramolecular isotope ratio in specific molecules, it may be possible to infer properties of the source that might not be measurable otherwise.

Previously, we reported the availability of compound-specific isotope ratios of acetic acid for discrimination of the raw materials of vinegar.[10] The precursor molecule of acetic acid in vinegar is primarily ethanol. Some of the botanical origins of ethanol are beet, sugar cane, corn, wheat, fruits, and grapes. The methyl and carboxyl carbons of acetic acid are derived from the methyl and methylene carbons in ethanol. Acetic acid in vinegar is generally produced by the oxidation of ethanol with *Acetobacter*. Hence, intramolecular carbon isotope ratios in acetic acid molecules may be based on isotopic distribution in ethanol molecules and the kinetic isotope effect accompanied with oxidation of ethanol, for example, by *Acetobacter*. Therefore, intramolecular carbon isotope ratios may provide a more detailed discrimination than compound-specific isotope ratios alone. This study contributes further to the limited data about the intramolecular carbon isotope distributions in acetic acid samples fermented from a limited species of plants.[11−13]

Intramolecular isotope ratio analysis is generally performed by site-specific natural isotope fractionation by nuclear magnetic resonance (SNIF-NMR) or isotope ratio mass spectrometry (IRMS).[14−20] An important step in the measurement of intramolecular isotope ratios by IRMS is the controlled degradation of the target molecule. The degradation techniques use pyrolysis, hydrolysis, and enzymatic reactions.[21] In this study, we used the pyrolysis technique to degrade acetic acid. Continuous-flow IRMS equipped with a gas chromatograph (GC-IRMS) is widely used to measure the compound-specific isotope ratios of foods.[22−27] In addition, intramolecular isotope ratio analysis using continuous-flow IRMS coupled with an online pyrolysis system has been reported.[28] Dias et al.[29] and Yamada et al.[30] used a GC-pyrolysis-GC-IRMS system to determine the intramolecular carbon isotope ratio of acetic acid. In this study, we improved the GC-Py-GC-IRMS system for online measurements of acetic acid molecules in vinegars by using headspace solid-phase microextraction (HS-SPME) and discuss the difference between the carbon isotope ratios of the methyl and carboxyl carbons in acetic acid in vinegar.

MATERIALS AND METHODS

Chemicals and Vinegar Samples. One acetic acid reagent, represented as AA-1, as a working standard was purchased from Wako Pure Chemical Industries (Osaka, Japan). Another, represented as AA-2, was purchased from Aldrich (Milwaukee, WI) for validation of the analytical method. Fourteen commercial Japanese vinegars obtained from supermarkets in Japan were used for this study. Table 1 shows the global $\delta^{13}C$ values of acetic acid, which have been reported in a previous study, and the main raw materials.[10] The acidities of the vinegar samples

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Table 1. $\delta^{13}C$ Values of Methyl and Carboxyl Carbons of Acetic Acid in Various Vinegars and Difference between These Values

sample	$\delta^{13}C_{acetic\ acid}$ (‰)	$\delta^{13}C_{carboxyl}$ [a] (‰)	$\delta^{13}C_{methyl}$ (‰)	$\Delta\delta$ [b] (‰)	raw materials
1	−27.72 ± 0.16	−25.96 ± 0.25	−29.48	3.52	rice
2	−27.67 ± 0.40	−26.48 ± 0.21	−28.86	2.38	nonglutinous brown rice
3	−27.39 ± 0.09	−25.58 ± 0.29	−29.20	3.62	rice
4	−26.99 ± 0.09	−25.24 ± 0.22	−28.74	3.49	rice
5	−27.27 ± 0.46	−26.22 ± 0.23	−28.32	2.10	tomato
6	−26.81 ± 0.04	−23.96 ± 0.23	−29.66	5.70	apple fruit juice
7	−29.28 ± 0.20	−26.02 ± 0.24	−32.54	6.52	apple fruit juice
8	−28.15 ± 0.12	−24.78 ± 0.07	−31.52	6.74	wheat
9	−23.90 ± 0.40	−24.04 ± 0.25	−23.76	−0.28	lychee vinegar, grapefruit juice, lychee fruit juice
10	−15.81 ± 0.25	−12.44 ± 0.18	−19.18	6.75	apple fruit juice, alcohol
11	−12.81 ± 0.08	−8.11 ± 0.26	−17.51	9.41	wheat, sake lees, rice, cone, alcohol
12	−14.76 ± 0.15	−11.82 ± 0.31	−17.70	5.88	sake lees, rice, alcohol
13	−16.20 ± 0.26	−10.39 ± 0.38	−22.01	11.62	sugar cane
14	−17.47 ± 0.32	−8.37 ± 0.37	−26.57	18.20	pineapple vinegar, pineapple fruit juice

[a] Mean value of three independent determinations. [b] $\Delta\delta = \delta^{13}C_{carboxyl} - \delta^{13}C_{methyl}$.

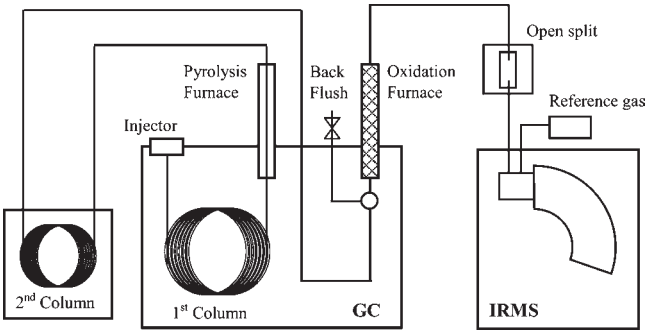

Figure 1. Schematic view of the continuous flow GC-pyrolysis-GC-combustion-IRMS system for online intramolecular carbon isotope ratio analysis of acetic acid.

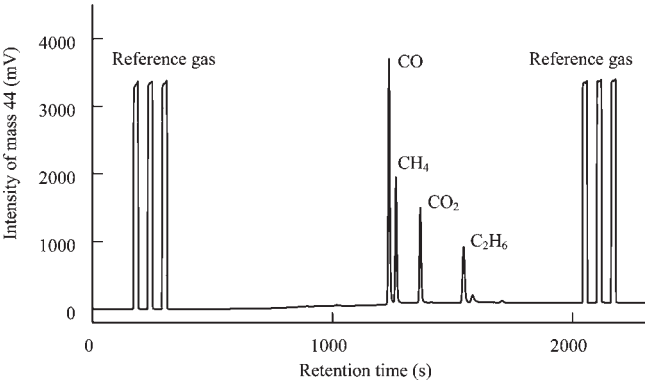

Figure 2. Ion chromatogram (mass 44) of acetic acid standard sample obtained by online measurement system.

ranged from 4.2 to 5% as indicated on the product packages. The working standard and vinegar samples were diluted with saturated saline water to 1.7 mmol/L of acetic acid and adjusted using hydrochloric acid to obtain a pH of 2. The prepared 10 mL sample solutions were enclosed in 20 mL SPME vials for the intramolecular isotope ratio analysis.

Instruments. The intramolecular carbon isotope distributions were measured using the GC-Py-GC-C-IRMS system (Figure 1). The system consisted of two GC capillary columns, two high-temperature furnaces, and an isotope ratio mass spectrometer (ThermoQuest Delta^{plus}XL; Thermo Fisher Scientific, Bremen, Germany). The first GC (HP6890; Agilent Technologies, Palo Alto, CA) was equipped with one capillary column (Nukol, 30 m × 0.32 mm i.d., 1 μm film thickness; Supelco, Bellefonte, PA) to separate the injected compounds, and the pyrolysis furnace, operating at 1000 °C, consisted of a ceramic tube (25 cm × 0.5 mm i.d.) for the pyrolysis of acetic acid. The second capillary column (PoraPLOT Q, 30 m × 0.32 mm i.d., 10 μm film thickness; Varian, Harbor City, CA) was operated at 30 °C for separating the pyrolysis gases. A deactivated fused-silica capillary (0.32 mm i.d.) was used as transfer line from GC columns to the pyrolysis furnaces. The oxidation furnace consisted of a ceramic tube packed with CuO, NiO, and Pt wires and operated at 960 °C. The first GC conditions were as follows: splitless injection mode, 5 min; injector temperature, 220 °C; oven temperature program, 60 °C for 5 min, raised from 60 to 190 °C at 15 °C min^{-1}, maintained at 190 °C for 20 min, then raised to 200 °C at 10 °C min^{-1}, and maintained at 200 °C for 5 min; constant flow mode at 0.8 mL min^{-1}.

The carboxyl carbon of acetic acid was the measurement target in this online pyrolysis method. This aim was achieved by the measurement of CO_2 gas produced by pyrolysis at 1000 °C. The carbon isotope ratio of CO_2 gas derived from the carboxyl carbon of acetic acid was equal to that of the carboxyl carbon.[30] Therefore, this system could measure the $\delta^{13}C$ value of the carboxyl carbon ($\delta^{13}C_{carboxyl}$), and the $\delta^{13}C$ value of the methyl carbon ($\delta^{13}C_{methyl}$) could be calculated from the $\delta^{13}C$ value of acetic acid ($\delta^{13}C_{acetic\ acid}$) and $\delta^{13}C_{carboxyl}$ by isotopic mass balance.[30]

The extraction procedure in the gas phase of the SPME vial was carried out by using an 85 μm SPME fiber coated with carboxen/polydimethylsiloxane (Carboxen/PDMS StableFlex; Supelco). The SPME conditions were as follows: extraction temperature, 30 °C; extraction time, 60 min.

Standardization. Isotopic standardization was achieved by comparison with the reference CO_2 gas that was introduced before and after the pyrolysis gas peak on the chromatogram (Figure 2). The carbon isotope ratio of the reference CO_2 gas was calibrated against the Vienna PeeDee Belemnite (VPDB) scale. The following equation was used to calculate the carbon isotope ratio expressed in δ notation:

$$\delta^{13}C\ (‰) = [(^{13}C/^{12}C)_{sample}/(^{13}C/^{12}C)_{standard} - 1] \times 1000$$

An acetic acid reagent was used as the working standard for international standardization to the VPDB scale and for the correction of the isotopic fractionation. The $\delta^{13}C$ value of acetic acid reagent, represented as $\delta^{13}C_{acetic\ acid}$, was standardized by a conventional offline sealed-tube

combustion method[31] using IAEA-CH-6 (sucrose) having a $\delta^{13}C$ value of −10.45‰ for the VPDB scaling. In addition, $\delta^{13}C_{methyl}$ was determined using a conventional offline pyrolysis method,[30,32] and $\delta^{13}C_{carboxyl}$ was calculated from $\delta^{13}C_{acetic\ acid}$ and $\delta^{13}C_{methyl}$ by isotopic mass balance. The values of standard reagents were as follows: $\delta^{13}C_{acetic\ acid} = -32.26 \pm 0.12$‰, $\delta^{13}C_{methyl} = -38.23 \pm 0.25$‰, and $\delta^{13}C_{carboxyl} = -26.29 \pm 0.25$‰.

The isotopic difference between the $\delta^{13}C$ values measured using HS-SPME ($\delta_{HS\text{-}SPME}$) and a conventional offline method ($\delta_{offline}$), expressed as $\Delta_{HS\text{-}SPME}$, was regarded as the isotopic fractionation and was calculated from the following equation:

$$\Delta_{HS\text{-}SPME} = \{(\delta_{HS\text{-}SPME}/1000 + 1)/(\delta_{offline}/1000 + 1) - 1\} \times 1000$$

After the determination of $\Delta_{HS\text{-}SPME}$ under the HS-SPME conditions, the $\delta^{13}C$ value can be corrected from the measured $\delta_{HS\text{-}SPME}$ using this equation.

RESULTS AND DISCUSSION

Intramolecular Isotopic Fractionation by SPME. The SPME procedure was useful in extracting and concentrating the target compounds.[33] However, the SPME procedure induces isotopic fractionation, and the magnitude of the fractionation depends on the type of SPME fiber, the target compound, and the SPME conditions used.[10,34−37] In our previous study, the isotopic fractionation of global acetic acid by HS-SPME-GC-IRMS was −2.67 ± 0.57‰ under the following conditions: extraction time, 60 min; incubation temperature, 30 °C.[10] In this study, the isotopic fractionation of each carbon atom in acetic acid by HS-SPME was investigated using the acetic acid standard reagent, AA-1, the intramolecular value of which was measured by the conventional offline pyrolysis method. An ion chromatogram (mass 44) of AA-1 obtained by this online pyrolysis method at 1000 °C is shown in Figure 2. We measured the CO_2 peak as the carboxyl carbon of acetic acid.

The influence of the extraction time of the HS-SPME method on the isotope ratio of the carboxyl carbon was examined with extraction times from 10 to 60 min. As a result, the $\delta^{13}C_{carboxyl}$ value was constant at all times, likewise, the global isotope ratio measured by HS-SPME.[10] In addition, the magnitude of intramolecular isotopic fractionation was confirmed by comparing $\delta^{13}C_{carboxyl}$ values measured by different injection methods. One method was a direct injection by a microvolume syringe, and another was injection by HS-SPME. The results of injections by microsyringe and HS-SPME were −26.01 ± 0.23 and −28.78 ± 0.28‰, respectively. The differences in $\delta^{13}C_{carboxyl}$ values by syringe and SPME indicate isotopic fractionation of the carboxyl carbon by HS-SPME. Therefore, the isotopic fractionation of the carboxyl carbon was estimated as −2.77‰, and it was nearly equal to the global isotopic fractionation, −2.67 ± 0.57‰. These results suggested that intramolecular isotopic exchange may not have occurred within the HS-SPME process.

The validation of this method by HS-SPME was accomplished by measurement of AA-2, the $\delta^{13}C_{carboxyl}$ value of which was −9.75 ± 0.52‰. The result by HS-SPME was −12.67 ± 0.26‰, and after correction for the isotopic fractionation ($\Delta_{HS\text{-}SPME}$ = −2.77‰) the $\delta^{13}C_{carboxyl}$ was −9.91 ± 0.26‰, which was in good agreement with the offline value.

Application to Vinegar for Intramolecular Carbon Isotope Distribution of Acetic Acid. Table 1 shows the measurement results of intramolecular isotopic ratio of acetic acid in vinegar. The precision of measurement of the carboxyl carbon was within ±0.4‰, which is the same level as that for the global measurement. In all vinegars, irrespective of type of botanical origins, the methyl carbon was depleted in ^{13}C relative to the carboxyl carbon except for sample 9, lychee vinegar. This isotopic trend was in agreement with that of previous research regarding intramolecular isotopic distribution of acetic acid in vinegar by the offline pyrolysis method.[10−13] Moreover, Caer et al.[19] reported intramolecular isotopic distribution of acetic acid by SNIF-NMR. They, too, found that biogenic acetic acid was characterized by ^{13}C depletion in the methyl carbon.

For the ^{13}C depletion in methyl carbon, the large isotopic fractionation induced selectively in methyl carbon during acetic acid production from ethanol by *Acetobacter* should be considered. In the culture experiment by Rinaldi et al., they have shown that the methyl carbon of ethanol was preferentially utilized at oxidation by *Acetobacter suboxydans* strain 8.3, and finally the methyl carbon of acetic acid was depleted in ^{13}C relative to the carboxyl carbon.[11] We calculated the isotopic fractionation at the methyl and carboxyl carbons by oxidation on the basis of their cultural results, and α_{methyl} was 1.011 and $\alpha_{carboxyl}$ was 1.001, although we would not adopt directly these values to our study because the strain, fermentative conditions, and the substrate were not same.

We compared our intramolecular isotopic trend of acetic acid with those of ethanol measured by Thomas et al. using SNIF-NMR.[38] For a C3 plant, Thomas et al. reported almost no difference between the methyl and methylene carbons of ethanol, whereas the methyl carbon of acetic acid was depleted in ^{13}C by from 2 to 6‰ relative to the carboxyl carbon. For a CAM plant (pineapple), they reported that the methyl carbon of ethanol was depleted in ^{13}C by from 9 to 14‰ relative to the methylene carbon. The trend of ^{13}C depletion in the methyl carbon for acetic acid was the same, but the difference between methyl and carboxyl carbon was extended to 18‰. They observed that ethanol from a C4 plant (sugar cane) showed the reverse isotope distribution; that is, the methyl carbon was enriched in ^{13}C by from 3 to 6‰ relative to the methylene carbon, whereas the methyl carbon of acetic acid was depleted in ^{13}C by 12‰ relative to the carboxyl carbon. As the result of the comparison with isotopic trend, we found that the methyl carbon of acetic acid was depleted in ^{13}C relative to the carboxyl carbon for all plants. These could be caused by a strong kinetic isotopic effect for an incomplete conversion of ethanol to acetic acid in the methyl carbon by *Acetobacter* as described above. Therefore, the $\delta^{13}C_{methyl}$ might have the information of degree of fermentation from ethanol. On the other hand, in oxidation by *Acetobacter*, the isotopic effect in the carboxyl carbon is not so large, and the $\delta^{13}C_{carboxyl}$ would preserve the closer information of the $\delta^{13}C_{methylene}$ in ethanol.

The intramolecular isotope distribution of sample 9, lychee vinegar, indicated an irregular isotopic trend and was different from the isotope distribution of acetic acid derived from biological reactions. Hence, this could not be regarded as a pure acetic acid fermented by *Acetobacter*, and it is feared that abiotic acetic acids might have been added, for example, a synthetic acetic acid. It has been reported that the isotopic distribution of synthetic acetic acid depended on the synthesis method and that the acetic acid oxidized from ethylene was remarkably different from the biogenic acetic acid in vinegar.[12,13]

The range of isotopic differences ($\Delta\delta$, $\delta^{13}C_{carboxyl} - \delta^{13}C_{methyl}$) in these samples (except sample 9) was about 18‰, from 2.1 to

18.2‰. These differences had an isotopic trend depending on the botanical origins. In detail, the $\Delta\delta$ of the C3 plants ranged from 2.1 to 6.7‰; that of the C4 plant was 11.6‰, and that of the CAM plant was 18.2‰. Moreover, in same C3 plants, the average of $\Delta\delta$ of four rice vinegars was 3.25 ± 0.58‰ and that of two apple vinegars was 6.11 ± 0.58‰. Although the factor of discrimination power for botanical origin remains unexplained, it raised the possibility that the intramolecular carbon isotope ratio enables a more detailed characterization of botanical origins relative to molecular level analysis. Therefore, the intramolecular isotope data of acetic acid are actually still insufficient, and accumulation of isotope data of acetic acid generated from various botanical origins, especially C4 and CAM plants, is necessary.

It is expected that low concentrations of acetic acid in complex media such as food products, for example, fruit juices and alcoholic beverages, should be measurable by the SPME technique. This method may be useful to identify the origin of unintentionally generated acetic acid in food as endogenous or exogenous. Therefore, it is expected that intramolecular carbon isotope distribution will be used for quality assessment and control.

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