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Measurement of the Isotope Ratio of Acetic Acid in Vinegar by HS-SPME-GC-TC/C-IRMS

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Acetic acid is the main ingredient of vinegar, and the worth of vinegar often depends on the fermentation of raw materials. In this study, we have developed a simple and rapid method for discriminating the fermentation of the raw materials of vinegar by measuring the hydrogen and carbon isotope ratio of acetic acid using head space solid-phase microextraction (HS-SPME) combined with gas chromatography−high temperature conversion or combustion-isotope ratio mass spectrometry (GC-TC/C-IRMS). The measurement of acetic acid in vinegar by this method was possible with repeatabilities (1σ) of ±5.0‰ for hydrogen and ±0.4‰ for carbon, which are sufficient to discriminate the origin of acetic acid. The fermentation of raw materials of several vinegars was evaluated by this method.

KEYWORDS: Acetic acid; isotope ratios; SPME; authenticity; vinegar

INTRODUCTION

The worth of food generally depends not only on the senses of taste and smell but also on its raw materials or geographical origin. Indeed, the price differs on the basis of the raw materials or geographical origin even for the same foods. Furthermore, adulteration, by illegally adding cheap raw materials to foods and replacing precious materials with cheaper ones, often happens all over the world. Therefore, quality assurance techniques for authenticity verification of foods or monitoring of adulteration are becoming more important, e.g., discrimination of geographical origin by elemental composition (*1*−*3*) and identification of species by DNA analysis (*4*,*5*). Stable isotope ratio analysis is a useful technique for distinguishing the botanical or geographical origin and therefore well suited for authenticity testing of food (*6*−*10*). Moreover, molecular level analysis is useful for determining the authenticity of food since molecular isotope ratios provide clear information of the precursor molecules or origin (*9*, *10*).

Vinegar whose main ingredient is acetic acid is a popular seasoning and is generally made by fermentation. In Europe, grape is generally one of the most used raw materials to produce vinegars. However, vinegars made from grains are common in Japan. The raw materials of vinegar are various, and representative ones are as follows: sugar cane, corn, wheat, fruits, wine, and so on. Vinegar is generally cheap but can often be comparatively expensive because of the fermentation of raw materials. It is difficult to check the fermentation of raw material by smell, taste, or composition because various ingredients such as sugar syrups or fruit juices are sometimes added to vinegar. Isotope ratio analysis of acetic acid in vinegar has been reported as an assurance technique for the authenticity of vinegar (*11*−*13*). It is possible to discriminate the fermentation of raw materials of vinegar by measurements of isotope ratios of acetic acid since the precursor molecule of acetic acid in vinegar is mostly ethanol originated from sugars of various plants. Moreover, acetic acid made by chemical synthesis was discriminated by the hydrogen isotope ratio (*12*). Remaud et al. (*12*) and Hermann (*13*) measured the δD and δ^{13}C values of acetic acid in vinegar by site-specific natural isotope fractionation studied by nuclear magnetic resonance (SNIF-NMR) and isotope ratio mass spectrometer (IRMS) after isolating pure acetic acid using the off-line pretreatment procedures, distillation, or solvent extraction.

In this study, we used head space solid-phase microextraction (HS-SPME) combined with gas chromatography−high temperature conversion or combustion-isotope ratio mass spectrometry (GC-TC/C-IRMS) to develop a simpler and more rapid method for measuring the carbon and hydrogen isotope ratios of acetic acid in vinegars (*14*). The online isotope ratio analysis for acetic acid was done with the use of HS-SPME-GC-IRMS without the off-line pretreatment procedures. The repeatabilities (1σ) of the measurements of acetic acid in vinegars were ±5.0‰ for hydrogen and ±0.4‰ for carbon. We obtained the isotope ratio data for acetic acid in vinegars produced by various raw materials to evaluate the raw material of acetic acid.

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Table 1. Details of 14 Kinds of Vinegar Samples with Isotope Ratios Measured by SPME-GC-TC/C-IRMS

sample	vinegar type	raw materials[a]	$\delta^{13}C$ (‰)[b]	δD (‰)[b]
A	rice vinegar	rice	−27.72 ± 0.16	−203.7 ± 3.8
B	rice vinegar	nonglutinous brown rice	−27.67 ± 0.40	−210.9 ± 1.3
C	rice vinegar	rice	−27.39 ± 0.09	−193.2 ± 3.8
D	rice vinegar	rice	−26.99 ± 0.09	−209.5 ± 2.4
E	tomato vinegar	tomato	−27.27 ± 0.46	−187.6 ± 5.3
F	apple vinegar	apple fruit juice, alcohol	−15.81 ± 0.25	−156.0 ± 3.5
G	apple vinegar	apple fruit juice	−26.81 ± 0.04	−189.8 ± 0.9
H	apple vinegar	apple fruit juice	−29.28 ± 0.20	−187.1 ± 3.3
I	pineapple vinegar	pineapple vinegar, pineapple fruit juice	−18.47 ± 0.32	−187.9 ± 1.3
J	lychee vinegar	lychee vinegar, grape and lychee fruit juice	−23.90 ± 0.40	−228.8 ± 6.5
K	grain vinegar	wheat, sake lees, rice, corn, alcohol	−12.81 ± 0.08	−166.6 ± 0.3
L	grain vinegar	sake lees, rice, alcohol	−14.76 ± 0.15	−164.7 ± 2.4
M	wheat vinegar	wheat	−27.15 ± 0.12	−186.9 ± 3.5
N	grain vinegar	sugar cane	−16.20 ± 0.26	−164.8 ± 3.1

[a] Raw materials are as indicated on the vinegar product package. [b] $n = 3$.

MATERIALS AND METHODS

Isotopic Standardization. Isotopic standardization was accomplished by comparison with reference H_2 gas (99.9999%; Taiyo Nippon Sanso Co. Ltd., Tokyo, Japan) and CO_2 gas (99.9999%; Taiyo Nippon Sanso Corp.) that were introduced before the acetic acid peak on the chromatogram. The hydrogen and carbon isotope ratios were expressed in conventional δ-notation calculated from the following equation:

$$\delta = (R_{sample}/R_{standard} - 1) \times 1000(‰)$$

where R_{sample} and $R_{standard}$ denoted the stable isotope ratios (D/H and $^{13}C/^{12}C$) of sample and international standard materials, respectively. The international standard materials were Vienna Standard Mean Ocean Water (VSMOW) for hydrogen and Vienna PeeDee Belemnite (VPDB) for carbon. International standardization to the VSMOW and VPDB scales was performed by comparison with acetic acid and sodium acetate standard reagents. The δD and $\delta^{13}C$ values of these standard reagents ($\delta_{certified}$) were measured beforehand using conventional sealed-tube combustion techniques. These reagents were standardized using IAEA-CH-6 (sucrose) with a $\delta^{13}C$ value of −10.45‰ for VPDB scaling (*15*) and VSMOW and Standard Light Arctic Precipitation (SLAP) with δD values of 0‰ and −428‰, respectively, for VSMOW scaling.

Chemicals and Vinegar Samples. One acetic acid reagent, Std-AA1, used as a standard reagent for hydrogen isotope ratio analysis was obtained from Nacalai Tesque (Kyoto, Japan). The $\delta D_{certified}$ value for Std-AA1 was −66.8 ± 1.3‰. Four sodium acetate reagents, Std-SA1, Std-SA2, Std-SA3, and Std-SA4, used as standard reagents for carbon isotope ratio analysis were obtained from Nacalai Tesque, Kanto Chemical (Tokyo, Japan), Wako Pure Chemical Industries (Osaka, Japan), and Aldrich (WI, USA), respectively. The $\delta^{13}C_{certified}$ values were as follows: −31.38 ± 0.02‰ for Std-SA1, −34.59 ± 0.09‰ for Std-SA2, −31.54 ± 0.11‰ for Std-SA3, and −10.94 ± 0.03‰ for Std-SA4.

Fourteen commercial vinegars were obtained from supermarkets in Japan. The main raw materials of each vinegar as indicated on the bottle labels are listed in **Table 1**. The concentrations of acetic acid in the vinegars ranged from 42 to 50 g/L. All vinegar samples were diluted to 10 mg/L and 100 mg/L of acetic acid with saturated saline water and adjusted to pH 2 by hydrochloric acid for isotope ratio analysis by HS-SPME-GC-IRMS as described below. In hydrogen isotope ratio analysis, the same water was utilized for dilution with standards and vinegar samples because the hydrogen atom of the hydroxyl group of the ethanol molecule was easily substituted by the hydrogen atom of water used for dilution. The 10 mL prepared solutions were injected in 20 mL SPME vials (Gerstel, Mülheim, Germany). Standard samples were prepared by the same method.

Instrumentation and HS-SPME-GC-TC/C-IRMS Measurements. An isotope ratio mass spectrometer (ThermoQuest Delta^{plus}XL; Thermo Fisher Scientific, Bremen, Germany) was coupled with a GC-conversion/combustion interface (ThermoQuest GC Combustion III; Thermo Fisher Scientific). The GC-conversion interface was used for hydrogen measurements, and the GC-combustion interface was used for carbon measurements. The GC-conversion/combustion interface consisted of a gas chromatograph (HP6890, Agilent Technologies, CA, USA) equipped with a capillary column (Nukol, 30 m × 0.32 mm i.d., 1.0 μm film; Supelco, PA, USA). GC was used under the following conditions: splitless injection mode, 5 min; injector temperature, 220 °C; oven temperature program, 35 °C for 5 min, raised from 35 to 120 °C at 15 °C min^{-1}, maintained at 120 °C for 10 min, then raised to 190 at 50 °C min^{-1}, and maintained at 190 °C for 8 min; and constant flow mode at 1.5 mL min^{-1}. The combustion furnace for carbon isotope ratio analysis was a microvolume oxidation reactor consisting of a ceramic tube (Al_2O_3) packed with CuO, NiO, and Pt wires and operating at 960 °C to produce CO_2. The conversion furnace for hydrogen isotope ratio analysis was a microvolume high temperature conversion reactor consisting of a cavitary ceramic tube and operating at 1450 °C to produce H_2.

A 85 μm SPME fiber coated with carboxen/polydimethylsiloxane (Carboxen/PDMS StableFlex; Supelco) was used, and the extraction procedure was carried out in the gas phase of the SPME vial without immersion in the sample solution, i.e., by HS-SPME method. During isotope ratio analysis of acetic acid by SPME, 10 mL of the sample solution was adjusted with hydrochloric acid to less than pH 2 for good extraction efficiency and injected into a 20 mL SPME vial. The SPME efficiency and isotope fractionation were evaluated using the following parameters: (a) extraction temperature, 30, 40, 50, 60, 70, and 80 °C; (b) extraction time, 5, 10, 20, 30, 40, 50, 60, and 100 min. In the analysis of the vinegar samples, the extraction temperature was 30 °C, and the time was 60 min. Additionally, we evaluated the optimized concentration of acetic acid for HS-SPME-GC-IRMS with good precision and acetic acid concentrations, 1, 2.5, 3, 4, 5, 10, 15, 20, 25, and 50 mg/L.

RESULTS AND DISCUSSION

Optimal Conditions by HS-SPME-GC-TC/C-IRMS. One of the noted points of isotope ratio analysis of SPME is isotopic fractionation. Isotopic fractionation results vary depending upon the parameters used, e.g., immersion or head space method, the type of the SPME fiber, extraction temperature and time, and analysis compounds (*14*, *16*−*18*). In this study, we evaluated the extraction temperature and time, and the optimal concentration range of acetic acid by HS-SPME-GC-TC/C-IRMS.

Figure 1a shows the relationship between extraction temperature and the $\delta^{13}C$ value of the Std-SA1 solution, and **Figure 1b** shows the relationship of $\delta^{13}C$ to extraction time. The relationships of **Figure 1a** and **b** were evaluated with the following conditions: (a) extraction time, 60 min; acetic acid concentration, 25 mg/L; (b) extraction temperature, 30 °C; acetic acid concentration, 25 mg/L. As can be seen from **Figure 1a**, the extraction efficiency was best at 60 °C. However, the $\delta^{13}C$ value was −33.89‰ at 60 °C and did not agree with the certified value ($\delta^{13}C_{certified}$ = −31.38‰). From 30 to 80 °C, the $\delta^{13}C$ value was

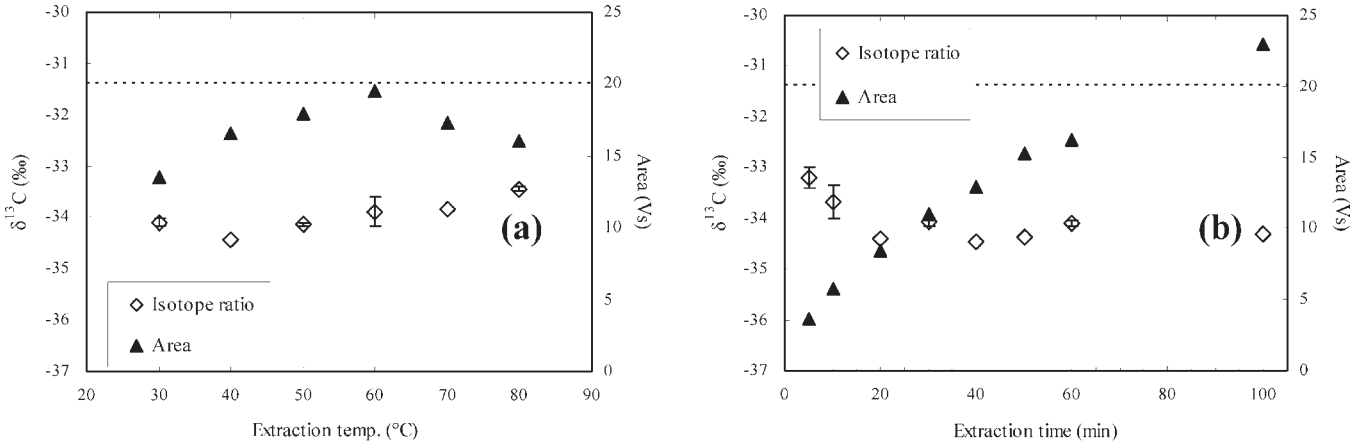

Figure 1. (**a**,**b**). Relationship between $\delta^{13}C$ values or chromatogram area and (**a**) incubation temperature and (**b**) extraction time by the SPME procedure. The open diamonds represent the $\delta^{13}C$ values in per mil, error bars indicate the standard deviations (1σ), and the solid triangles show the chromatogram areas in Vs. The horizontal broken line represents the certified value of sodium acetate standard reagent Std-SA1 ($\delta^{13}C = -31.38 \pm 0.02‰$).

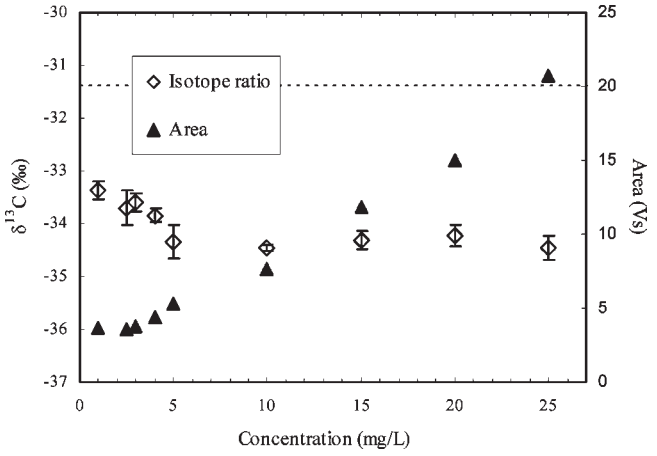

Figure 2. Evaluation of optimal concentration range of acetic acid with the SPME method. The open diamonds represent the $\delta^{13}C$ values in per mil, error bars indicate the standard deviations (1σ), and the solid triangles show the chromatogram areas in Vs. The horizontal broken line represents the certified value of sodium acetate standard reagent Std-SA1 ($\delta^{13}C = -31.38 \pm 0.02‰$).

nearly constant at $-33.97 \pm 0.33‰$, and the precision was within $\pm 0.3‰$ at each experimental temperature. In **Figure 1b**, when the extraction time increased, the extraction efficiency improved. However, when the extraction time exceeded 20 min, the $\delta^{13}C$ value was constant at $-34.28 \pm 0.16‰$, again not in agreement with the certified value. **Figure 2** shows the investigation of the optimal concentration range of acetic acid by the HS-SPME method, with the following SPME conditions: extraction temperature, 30 °C; extraction time, 60 min; pH of sample solution, 2. The repeatability was within $\pm 0.3‰$ in samples with more than 10 mg/L. Moreover, the isotope ratio was $-34.31 \pm 0.15‰$ and was constant in samples with more than 5 mg/L (more than 5 Vs as the chromatogram area).

The isotopic difference between the $\delta^{13}C$ value measured by HS-SPME-GC-IRMS ($\delta^{13}C_{SPME}$) and $\delta^{13}C_{certified}$ was expressed as Δ_{SPME}, which was assigned as the isotopic fractionation, and was calculated from the following equation:

$$\Delta_{SPME}(‰) = \{(\delta^{13}C_{SPME}/1000 + 1)/(\delta^{13}C_{certified}/1000 + 1) - 1\} \times 1000$$

Δ_{SPME} is determined by measuring standard reagents by HS-SPME-GC-C-IRMS. Four kinds of sodium acetate standard

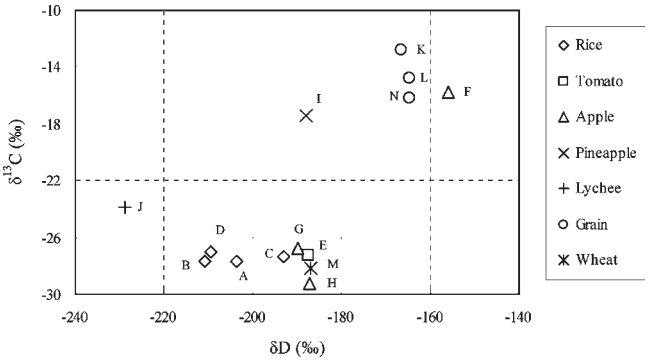

Figure 3. δD and $\delta^{13}C$ values of acetic acid in vinegars of different raw materials.

(Std-SA 1−4) were measured under the following constant conditions: acetic acid concentration, 10 mg/L; extraction temperature, 30 °C; extraction time, 60 min. The Δ_{SPME} average of all standards was $-2.67 \pm 0.57‰$. In view of the 0.3‰ repeatability (1σ) of acetic acid by HS-SPME-GC-C-IRMS, the isotopic fractionation under fixed SPME conditions was considered nearly constant. Therefore, the $\delta^{13}C_{certified}$ of a sample can be calculated from the Δ_{SPME} of a standard reagent and the $\delta^{13}C_{SPME}$ of the sample, which is measured under constant SPME conditions. In the measurement of hydrogen, the Δ_{SPME} of hydrogen was calculated and corrected by measuring the acetic acid standard (Std-AA1) as well.

Measurement of Acetic Acid in Vinegars. The HS-SPME method was applied to the vinegar samples, Std-SA1 was used as the external standard for standardization and calculating isotopic fractionation of carbon, and Std-AA1 was used for hydrogen. The results of the measurements of δD and $\delta^{13}C$ values of acetic acid in vinegar are shown in **Table 1** and are plotted in **Figure 3**. These values were corrected for Δ_{SPME}. The repeatability (1σ) was within $\pm 5.0‰$ for δD and $\pm 0.4‰$ for $\delta^{13}C$. **Figure 3** shows that this repeatability was adequate to discriminate the origin of acetic acid. As can be seen from **Figure 3**, the hydrogen and carbon isotope ratios were good parameters to discriminate the botanical origins of the acetic acid. In particular, the difference between C_3 and C_4 plants was clearly ascertained.

The main raw materials of vinegar F were indicated as apple juice and alcohol. According to the stable isotope result, it was assumed that the raw material of acetic acid is not apple juice but also alcohol originated from C_4 plants. The $\delta^{13}C$ values of C_3

plants (which vary from about −22 to −35‰) are lower than those of C_4 plants (which vary from about −8 to −20‰) (*19*, *20*). If, for example, the predominant raw material of vinegar F was an apple juice, the $\delta^{13}C$ value would be expected in the range from −22 to −35‰. Correspondingly, the raw materials of vinegars K and L were corn and alcohol originating from C_4 plants. It is well known that $\delta^{13}C$ values are a powerful tool for discriminating plant origins (*19*, *20*). In **Figure 3**, the δD values of vinegars fermented from C_3 or crassulacean acid metabolism (CAM) plants were less than −180‰, while vinegars fermented from C_4 plants have values of more than −180‰. This shows that next to the $\delta^{13}C$ values, the δD values of acetic acid in vinegars too could be used for tracing the metabolical pathways of the plant raw materials, as proposed by Martin et al. (*21*). In the measurement of the δD value of acetic acid, the exchangeable hydrogen isotope ratio in the carboxyl group is related to the fermentation of water and has nothing to do with the botanical precursor, but it will certainly contribute to the overall δD value. Therefore, the effect of exchangeable hydrogen was corrected, and these δD values only measured the hydrogen isotope ratio in the methyl group by using the same water for dilution with the standard and samples. The three hydrogen atoms of the methyl group of acetic acid were derived from the methyl group of ethanol; therefore, the δD value of the methyl group of acetic acid is related to the hydrogen isotopic information of sugar (glucose) as well as of ethanol (*21*). Hence, it may be possible to discriminate the geographical origin of the fermentation of raw materials by hydrogen isotope ratio analysis of acetic acid. Moreover, Remaud et al. reported that the δD values enable one to differentiate synthetic and fermented acetic acid (*12*). Acetic acid can also be obtained from wood and is called pyroligneous acid. Moreover, pyroligneous acid includes various compounds, e.g., acetic acid, alcohol, and carbonyl and aromatic compounds. By the use of the SPME-GC-TC/C-IRMS method, the isotope ratios of acetic acid in pyroligneous acid may be measured. Furthermore, the SPME-GC-TC/C-IRMS technique is expected to adapt not only for acetic acid and vinegar but also for various molecules in foods and to be useful for authenticity testing.

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