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# Stable carbon isotopic characterization of rice vinegar protein as an intrinsic reference for discriminating the authenticity of brewed rice vinegar

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## Abstract

Rice vinegar plays an important role in daily life. However, some unscrupulous manufacturers may deliberately add synthetic acetic acid in vinegar products to reduce fermentation time and save production costs. To protect the rights and health of consumers, vinegar authenticity must be controlled. The rice vinegar protein was used as an intrinsic reference and its stable carbon isotope ratio ( $\delta^{13}\text{C}_{\text{protein}}$ ) was analyzed by elemental analyzer-isotope ratio mass spectrometry. The stable carbon isotope ratio difference between the acetic acid and the rice vinegar protein ( $\Delta\delta^{13}\text{C}_{\text{acetic acid-protein}}$ ) was calculated to evaluate vinegar authenticity. Sixteen rice vinegar samples were analyzed and a stable carbon isotopic pattern of rice vinegar was established by the 95% confidence interval for  $\Delta\delta^{13}\text{C}_{\text{acetic acid-protein}}$  (0.27‰–2.10‰). An acetic acid adulteration curve of  $\Delta\delta^{13}\text{C}_{\text{acetic acid-protein}}$  was also assumed according to the data from rice vinegar samples, and its validity was confirmed by rice vinegar deliberately blended with acetic acid at different ratios (25, 50, and 75%). The  $\Delta\delta^{13}\text{C}_{\text{acetic acid-protein}}$  values of the adulterated vinegars decreased with increasing amounts blended acetic acid, but the  $\delta^{13}\text{C}_{\text{protein}}$  values did not, showing that rice vinegar protein could be used as an intrinsic reference for identifying the adulterated rice vinegar. The rice vinegar adulterated with acetic acid at higher than approximately 10% could be detected.

**Keywords:** Authenticity, EA-IRMS, Protein, Stable isotope ratio, Vinegar

## 1. Introduction

Food adulteration has been a serious issue world wide in recent years. By illegally replacing food ingredients with less expensive and unnatural substances, it not only reduces the quality of food but increases health risks to consumers. For economic reasons, food adulteration often occurs in high-value food products, such as olive oil, wine and honey [1]. However, some products, such as vinegar, that are inexpensive but sold in large quantities may also be adulterated.

Rice vinegar is a seasoning widely used in Asian cuisine. It can also be used to produce many food products, such as ketchups, mayonnaise and pickled food. Because of its special flavor and health-promoting benefits, it has also recently been increasingly used in functional drinks. The significant

growth in the global market of rice vinegar is expected between 2017 and 2024 [2].

Rice is the predominant material used to rice vinegar fermentation. Sometimes, other grains, such as wheat, oat and barley, can also mix with rice to produce rice vinegar. Rice vinegar is produced through three processes: saccharification, ethanol fermentation, and acetous fermentation. First, the complex carbohydrates are hydrolyzed to fermentable carbohydrates by a culture of *Aspergillus* species, which known as *koji*, during saccharification process [3]. After that, the saccharides are transformed to ethanol during ethanol fermentation process by *Saccharomyces* species. Lastly, the ethanol is oxidized to acetic acid during acetous fermentation by *Acetobacter* species. In many countries, it has been specified that “vinegar” should be obtained by fermentation processes [4]. In Taiwan, only vinegar

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products that are fermented with grain, fruit, alcohol, distiller's grain, molasses or other ingredients and are not mixed with acetic acid or glacial acetic acid can be named “brewed vinegar”. However, some unscrupulous manufacturers may add synthetic acetic acid to reduce fermentation time and save production costs. This synthetic acetic acid may have deleterious impurities and pose potential threat to consumers' health.

Stable isotope ratio analysis has been a promising technique to determine origin and authenticity of food products and applied to olive oil [5], honey [6], juice [7] and wine [8]. Because stable isotope ratios may be altered by plant photosynthesis, environmental factors, geographical factors and climate conditions, they could provide unique isotopic fingerprints, enabling adulterants in foods to be distinguished and the origin of the food to be traced [9]. There are two common stable isotope analysis techniques: bulk stable isotope analysis and compound-specific stable isotope analysis. The former determines the isotopic fingerprints at the bulk level, and elemental analyzer-isotope ratio mass spectrometry (EA-IRMS) is frequently employed. The entire sample is combusted with oxygen under high temperature, and thus the carbon, nitrogen and sulfur present in the sample are converted to carbon dioxide, nitrogen, sulfur dioxide gas. Then the gases released from the sample are delivered to a mass spectrometer through helium gas, and the stable isotopic ratios of the sample are determined. In contrast, the latter determines isotopic fingerprints at the molecular level [10], and liquid chromatography-isotope ratio mass spectrometry (LC-IRMS) or gas chromatography-isotope ratio mass spectrometry (GC-IRMS) is frequently employed. Individual compounds present in the sample are separated by chromatography technology and sequentially enter the reactor, where the compounds are converted to analytical gas. Then the analytical gases evolved from the sample are delivered to a mass spectrometer, and the stable isotopic ratios of the sample are determined. Some past studies have applied these techniques to vinegar for tracing the source of raw materials used in production [11,12].

The official methods for detecting the vinegar adulterated with exogenous acetic acid were also proposed. The acetic acid from vinegar was extracted with diethyl ether *via* liquid–liquid extraction and then purified by distillation with a spinning band distillation column. The purified acetic acid was analyzed with site-specific natural isotope fractionation studied by nuclear magnetic resonance to determine the deuterium to hydrogen isotope ratio (D/H) in the methyl site (EN 16466-1) and

analyzed with EA-IRMS to determine the stable carbon isotopic ratio ( $\delta^{13}\text{C}$ ) (EN 16466-2). Combining both results, the origin of acetic acid could be characterized and the adulteration could be identified. These official methods were also shown to be applicable to vinegar produced from alcohol, cider and wine and were extended further to balsamic vinegar which is produced from a mix of grape must and wine vinegar [13,14]. However, most studies using stable isotope techniques to determine the authenticity of vinegar have conducted on balsamic vinegar and wine vinegar while few on rice vinegar.

Recently, the strategy of using intrinsic reference have been employed to stable isotope analysis for food adulteration detection to eliminate variance in isotope ratios that caused by species or individual differences of raw materials.

Generally, the stable isotopic ratios of different ingredients in food should be closely related if they are synthesized from the same source. Hence an ingredient which is naturally present in foods and is unaffected by the adulterant could be used as an intrinsic reference to anchor the isotopic features of food itself. When food is adulterated, the stable isotope ratio of adulterated ingredient would be affected by the adulterant and deviated from the isotope ratio of intrinsic reference. Thus, difference between isotope ratios of the adulterated food ingredient and the intrinsic reference could be used as an index for detecting food adulteration. This strategy has also been applied to honey [15] and juice [16] adulteration detection. In our previous study, acetoin in rice vinegar was extracted and used as an intrinsic reference [17]. The results showed that the  $\delta^{13}\text{C}$  value of acetoin ( $\delta^{13}\text{C}_{\text{acetoin}}$ ) was affected only by the raw materials and not by acetic acid adulteration. The influence of acetic acid addition could be better reflected in the difference between the  $\delta^{13}\text{C}$  values of acetic acid and acetoin ( $\Delta\delta^{13}\text{C}_{\text{acetic acid-acetoin}}$ ) than the  $\delta^{13}\text{C}$  value of acetic acid ( $\delta^{13}\text{C}_{\text{acetic acid}}$ ) alone. The  $\Delta\delta^{13}\text{C}_{\text{acetic acid-acetoin}}$  value was feasible to be an index for discriminating rice vinegar authenticity. However, the contents of acetoin vary over a wide range (1–2 orders of magnitude) in rice vinegar [18]. To prevent amount-dependent stable isotope fractionation occurring during the analysis, the acetoin content needs to be quantitated by GC–MS and controlled within an appropriate concentration range before GC-IRMS analysis. This is laborious, time-consuming and less practical for routine analysis. Compared to GC-IRMS, EA-IRMS encompasses simple sample preparation, fast detection, and easy operation and maintenance and is widely used in many stable isotope laboratories. Therefore, we tried to find another intrinsic

reference that could be analyzed by EA-IRMS in this study. The rice vinegar protein was precipitated by tungstic acid and used as an intrinsic reference. The  $\delta^{13}\text{C}$  value of rice vinegar protein ( $\delta^{13}\text{C}_{\text{protein}}$ ) was measured by EA-IRMS, and the stable carbon isotope ratio difference between the acetic acid and the rice vinegar protein ( $\Delta\delta^{13}\text{C}_{\text{acetic acid-protein}}$ ) was calculated by Eq. (1). The suitability of the rice vinegar protein as an intrinsic reference for determining the authenticity of rice vinegar was evaluated.

$$\Delta\delta^{13}\text{C}_{\text{acetic acid-protein}} = \delta^{13}\text{C}_{\text{acetic acid}} - \delta^{13}\text{C}_{\text{protein}} \quad (1)$$

where  $\delta^{13}\text{C}_{\text{acetic acid}}$  is the  $\delta^{13}\text{C}$  value of acetic acid in vinegar and  $\delta^{13}\text{C}_{\text{protein}}$  is the  $\delta^{13}\text{C}$  value of rice vinegar protein.

## 2. Materials and methods

### 2.1. Materials

#### 2.1.1. Rice vinegar

Sixteen rice vinegar samples were purchased from markets in Taiwan. All rice vinegars were purely brewed and produced from five leading manufacturers. The information about the raw materials used in these vinegar samples is reported in Table 1S.

#### 2.1.2. Chemicals and gases

Sodium tungstate dihydrate, sulfuric acid, arginine, D-(+)-turanose and casein were obtained from

Sigma–Aldrich (St. Louis, Missouri, USA). The ultra-high purity (99.9999%) helium, oxygen and carbon dioxide gases were obtained from Shinn Hwa Gas Inc. (Taoyuan, Taiwan).

### 2.2. Rice vinegar protein preparation

The rice vinegar protein was precipitated by tungstic acid based on procedures described by Folin and Wu [19] but with some modifications. A 45 mL rice vinegar sample was centrifuged (Allegra 25R Centrifuge, Beckman Coulter, Brea, California, USA) at  $5000\times g$  for 2 min. A 40 mL supernatant was loaded into a 50-mL polypropylene centrifuge tube, and an amount of 5 mL 0.333 M sulfuric acid solution and 5 mL 10% (w/v) sodium tungstate dihydrate solution were added successively to the same centrifuge tube. This mixture was incubated in a shaking waterbath at  $80\text{ }^{\circ}\text{C}$  for 30 min and vortexed every 10 min, forming a floc. The mixture was centrifuged ( $5000\times g$ , 2 min), and the supernatant was decanted. Then, the pellet was resuspended in 50 mL of ddH<sub>2</sub>O, vortexed and centrifuged ( $5000\times g$ , 2 min). The supernatant was decanted afterwards. The washing, vortexing and centrifugation steps were repeated five times. The pellet was dried in an oven at  $75\text{ }^{\circ}\text{C}$  and grind with a mortar and pestle. The fine powder was collected as the rice vinegar protein.

### 2.3. Stable carbon isotope ratio analysis

The rice vinegar protein was weighed (0.3–0.5 mg) using a precision balance with an accuracy of 0.00001 g (XS105, Mettler Toledo, Switzerland) into tin capsules (8 mm  $\times$  5 mm, Elemental Microanalysis, Okehampton, UK) and sealed for stable carbon isotope ratio analysis. The  $\text{C}_{13}/\text{C}_{12}$  ratio was measured using an isotope ratio mass spectrometer (Delta V Advantage, Thermo Fisher Scientific, Waltham, Massachusetts, USA) coupled with an elemental analyzer (Flash 2000 HT O/H-NC, Thermo Fisher Scientific, Waltham, Massachusetts, USA) through a continuous flow interface (ConFlo IV, Thermo Fisher Scientific, Waltham, Massachusetts, USA). Data was acquired and analyzed using Isodat 3.0 software (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The elemental analyzer was operated using helium as the carrier gas (100 mL/min). The temperature of combustion reactor and GC oven were respectively set to  $1020\text{ }^{\circ}\text{C}$  and  $70\text{ }^{\circ}\text{C}$ . The oxygen purge (250 mL/min) for flash combustion was 3 s per run. A carbon dioxide reference gas pulse with an intensity of 6000 mV was introduced three times for 20 s at the

Table 1. Stable carbon isotope ratio results of the protein in the rice vinegar samples.

Sample <sup>a</sup>	$\delta^{13}\text{C}_{\text{acetic acid}}$ <sup>b</sup> (‰)	$\delta^{13}\text{C}_{\text{protein}}$ <sup>c</sup> (‰)	$\Delta\delta^{13}\text{C}_{\text{acetic acid-protein}}$ <sup>d</sup> (‰)
A1	$-14.83 \pm 0.16$	$-28.18 \pm 0.05$	13.35
A2	$-15.33 \pm 0.18$	$-28.26 \pm 0.08$	12.93
A3	$-14.66 \pm 0.08$	$-28.24 \pm 0.05$	13.58
A4	$-15.28 \pm 0.05$	$-28.19 \pm 0.02$	12.91
B1	$-24.60 \pm 0.37$	$-27.24 \pm 0.03$	2.64
B2	$-25.91 \pm 0.33$	$-27.93 \pm 0.05$	2.02
B3	$-27.57 \pm 0.11$	$-27.46 \pm 0.07$	-0.11
B4	$-26.52 \pm 0.26$	$-27.79 \pm 0.03$	1.27
B5	$-25.99 \pm 0.09$	$-27.60 \pm 0.04$	1.61
C1	$-25.27 \pm 0.18$	$-27.93 \pm 0.10$	2.66
C2	$-28.07 \pm 0.14$	$-27.68 \pm 0.05$	-0.40
C3	$-27.01 \pm 0.37$	$-27.79 \pm 0.04$	0.78
D1	$-26.36 \pm 0.03$	$-25.99 \pm 0.06$	-0.37
D2	$-26.32 \pm 0.13$	$-25.99 \pm 0.06$	-0.33
D3	$-26.90 \pm 0.28$	$-27.29 \pm 0.04$	0.39
E1	$-23.54 \pm 0.18$	$-27.57 \pm 0.01$	4.03

<sup>a</sup> Samples designated with the same capital letter (from A to E) were produced by the same manufacturer.

<sup>b</sup> Data are from our previous study and presented as the mean  $\pm$  SD (n = 3) [17].

<sup>c</sup> Data are presented as the mean  $\pm$  SD (n = 3).

<sup>d</sup> Difference between the mean  $\delta^{13}\text{C}$  values of acetic acid and rice vinegar protein.

beginning and end of each run. The dilution of sample gas was set to 65%. The isotope ratio mass spectrometer was operated at 3.0 kV accelerating voltage. The ion source was held at  $1.2 \times 10^{-6}$  mbar, and ions were generated by electron ionization (70 eV). Ions were collected at  $m/z$  values of 44, 45 and 46. Zero enrichment tests were performed daily by the carbon dioxide reference gas prior to analysis. The  $\delta^{13}\text{C}$  values of ten carbon dioxide pulses (20 s each) were measured, and the standard deviation should be less than 0.06‰.

The  $C_{13}/C_{12}$  ratio is denoted in delta notation ( $\delta^{13}\text{C}$ ) in relation to the international standard VPDB (Vienna PeeDee Belemnite) according to Eq. (2)

$$\delta^{13}\text{C}_{\text{sample}} (\text{‰}) = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 1000 \quad (2)$$

where  $R_{\text{sample}}$  is the  $C_{13}/C_{12}$  ratio of the sample and  $R_{\text{standard}}$  is the  $C_{13}/C_{12}$  ratio of the international standard used. The measured  $\delta^{13}\text{C}$  value related to the in-house reference carbon dioxide gas was calibrated to the VPDB scale against five international reference materials (International Atomic Energy Agency, Vienna, Austria): glycine (USGS64,  $\delta^{13}\text{C}_{\text{VPDB}} = -40.81 \pm 0.04\text{‰}$ ), L-glutamic acid (USGS40,  $\delta^{13}\text{C}_{\text{VPDB}} = -26.39 \pm 0.04\text{‰}$ ), cellulose (IAEA-CH-3,  $\delta^{13}\text{C}_{\text{VPDB}} = -24.72 \pm 0.04\text{‰}$ ), graphite (USGS24,  $\delta^{13}\text{C}_{\text{VPDB}} = -16.05 \pm 0.04\text{‰}$ ), sucrose (IAEA-CH-6,  $\delta^{13}\text{C}_{\text{VPDB}} = -10.45 \pm 0.03\text{‰}$ ) by multiple point linear normalization [20]. All samples were measured in triplicate, and  $\delta^{13}\text{C}$  values are expressed as the mean  $\pm$  standard deviation (SD).

#### 2.4. Quality control

Two in-house working standards: arginine and D-(+)-turanose were chosen as the quality control samples for  $\delta^{13}\text{C}$  analysis. Quality control samples were analyzed before each batch (less than ten measurements) to verify the accuracy of the stable isotope ratio measurement. The  $\delta^{13}\text{C}$  values of arginine and D-(+)-turanose were  $-13.49 \pm 0.17\text{‰}$  (mean  $\pm$  SD,  $n = 199$ ) and  $-25.48 \pm 0.10\text{‰}$  (mean  $\pm$  SD,  $n = 186$ ), respectively. The  $\delta^{13}\text{C}$  values of the quality control samples measured should neither consecutively fall over the warning limit range (mean  $\pm 2 \times$  SD) nor fall over the control limit range (mean  $\pm 3 \times$  SD).

#### 2.5. Statistical analysis

The statistical analysis was performed using SPSS software version 13.0 (SPSS Inc., Chicago, Illinois, USA). The  $\delta^{13}\text{C}$  values of bulk casein and recovered

casein were compared with the two-sample T-test to evaluate the accuracy of this method. The  $\delta^{13}\text{C}_{\text{protein}}$  values between adulterated vinegars with different adulteration ratios were compared with one-way analysis of variance (ANOVA). Two-tailed P values below 0.05 were considered significant.

### 3. Results and discussion

#### 3.1. Multiple point linear normalization of measured $\delta^{13}\text{C}$ values

The plot of measured  $\delta^{13}\text{C}$  values for five international reference materials versus true  $\delta^{13}\text{C}$  values expressed in VPDB international scale is shown in Fig. 1. The normalization curve was fitted as  $y = 1.0326x + 0.8055$  with determination coefficient ( $R^2$ ) = 1.0000 and was in the range from  $-10.45\text{‰}$  to  $-40.81\text{‰}$ . In this study, all the EA-IRMS measured  $\delta^{13}\text{C}$  values related to the in-house reference carbon dioxide gas were calibrated to VPDB scale by this normalization curve.

#### 3.2. Validation of the method for measuring the $\delta^{13}\text{C}_{\text{protein}}$ values in rice vinegar

It is known that stable isotope ratio measurement shows an amount dependency. The stable isotope ratio value measured may shift when the sample amount introduced into the instrument was different and thus cause decrease in precision and accuracy [21]. To avoid amount dependent isotope fractionation, the linearity range, in which the stable isotope ratio value measured is stable [22], was determined by measuring eight levels (0.05–0.5 mg) of rice vinegar protein in five replicates. The plot of

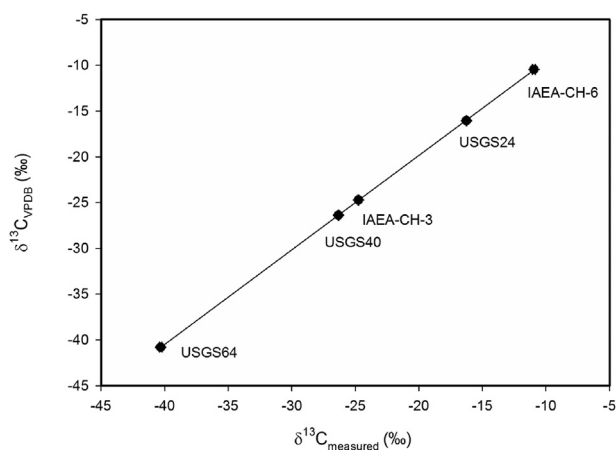


Fig. 1. The  $\delta^{13}\text{C}$  normalization curve obtained by measuring five international reference materials using EA-IRMS. The normalization curve fitted is  $y = 1.0326x + 0.8055$  with determination coefficient ( $R^2$ ) = 1.0000.



instrument amplitude of  $m/z$  44 versus  $\delta^{13}\text{C}$  values is shown in Fig. 2. The linearity range was given when the slope (absolute value) of fitted regression line was below  $0.1\text{‰}/\text{V}$  [23] and was found to be 1.4–8.9 V, which was corresponding to rice vinegar protein amounts of 0.05–0.5 mg. In addition, the  $\delta^{13}\text{C}$  values in the range of 5.2–8.9 V, which was corresponding to rice vinegar protein amounts of 0.3–0.5 mg, had smaller SDs; thus, the amount of rice vinegar protein encapsulated for EA-IRMS analysis was controlled between 0.3 to 0.5 mg to obtain stable  $\delta^{13}\text{C}$  values.

To assess the precision of the method for analyzing the  $\delta^{13}\text{C}_{\text{protein}}$  value in rice vinegar, the rice vinegar sample (B5) was independently analyzed for seven times under reproducible conditions on three days. The  $\delta^{13}\text{C}_{\text{protein}}$  values were  $-27.67 \pm 0.03\text{‰}$  (mean  $\pm$  SD,  $n = 7$ ) with RSD of 0.12% for intra-day and  $-27.67 \pm 0.04$  (mean  $\pm$  SD,  $n = 21$  on 3 different days) with RSD of 0.15% for inter-day. These results demonstrated that the developed method provide good precision for analyzing  $\delta^{13}\text{C}_{\text{protein}}$  value in rice vinegar.

Moreover, to confirm whether the isotope fractionation occurred during sample preparation, the accuracy of this method was evaluated. An aqueous solution of 1000 mg/L casein and 5% acetic acid was prepared to simulate the protein in rice vinegar and then was subjected to the vinegar protein preparation procedure to recover the spiked casein. Finally, both the bulk casein and the recovered casein were subject to EA-IRMS analysis. The  $\delta^{13}\text{C}_{\text{protein}}$  values of bulk casein and recovered casein were  $-20.63 \pm 0.07$  and  $-20.65 \pm 0.06$  (mean  $\pm$  SD,  $n = 15$ ),

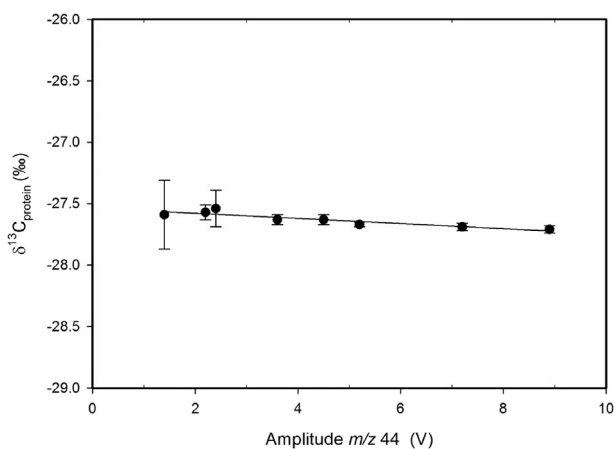


Fig. 2. The linearity range for analyzing the  $\delta^{13}\text{C}$  value of the rice vinegar protein by EA-IRMS. The filled circles indicate the mean of five replicates, and the solid line indicates the fitted regression line with slope =  $0.0218\text{‰}/\text{V}$  (absolute value). The linearity range was found to be 1.4–8.9 V, corresponding to rice vinegar protein amounts of 0.05–0.5 mg.

respectively. The results showed no significant difference between the bulk casein and the recovered casein and demonstrated that the absence of isotope fractionation during the sample preparation.

### 3.3. Isotopic pattern of rice vinegar

The  $\delta^{13}\text{C}_{\text{protein}}$  values in the rice vinegar samples were from  $-25.99\text{‰}$  to  $-28.26\text{‰}$  (Table 1) and fell within the typical  $\delta^{13}\text{C}$  range of  $-23\text{‰}$  to  $-30\text{‰}$  for C3 plants [24] due to the C3 plant derived raw materials of these samples, such as rice, waxy rice, wheat, or malt. Our previous study [17] showed that the  $\delta^{13}\text{C}_{\text{acetic acid}}$  values in the A1 to A4 samples using the C4 plant derived ethanol as the main raw material shifted to the typical  $\delta^{13}\text{C}$  range of  $-9\text{‰}$  to  $-15\text{‰}$  for C4 plants [25]. On the contrary, the  $\delta^{13}\text{C}_{\text{protein}}$  values in the A1 to A4 samples were not influenced by the ethanol and still fell within the C3 plant range, which showed that the rice vinegar protein was also a suitable intrinsic reference for vinegar authentication.

We calculated the  $\Delta\delta^{13}\text{C}_{\text{acetic acid-protein}}$  value and evaluated its validity for vinegar adulteration detection (Table 1). The  $\Delta\delta^{13}\text{C}_{\text{acetic acid-protein}}$  values in the A1 to A4 samples were from 12.91‰ to 13.58‰ with mean  $\pm$  SD of  $13.19 \pm 0.33\text{‰}$ , and those in the B1 to E1 samples were from  $-0.40\text{‰}$  to 4.03‰ with mean  $\pm$  SD of  $1.18\text{‰} \pm 1.44\text{‰}$ . Because the  $\delta^{13}\text{C}_{\text{acetic acid}}$  values in the A1 to A4 samples increased by using C4 plant derived ethanol as raw materials, their  $\Delta\delta^{13}\text{C}_{\text{acetic acid-protein}}$  values increased accordingly. Considering that the  $\Delta\delta^{13}\text{C}_{\text{acetic acid-protein}}$  values of the A1 to A4 samples were very different from those of the B1 to E1 samples, we eliminated the results of the A1 to A4 samples. A stable carbon isotopic pattern of rice vinegar was established by the 95% confidence interval for  $\Delta\delta^{13}\text{C}_{\text{acetic acid-protein}}$  based on the results in the B1 to E1 rice vinegar samples and was from 0.27‰ to 2.10‰. The 95% confidence interval for  $\Delta\delta^{13}\text{C}_{\text{acetic acid-protein}}$  was calculated by the Eq. (3).

$$\text{CI} = \bar{X} \pm t_{N-1} \times \frac{\text{SD}}{\sqrt{N}} \quad (3)$$

where CI is the 95% confidence interval for  $\Delta\delta^{13}\text{C}_{\text{acetic acid-protein}}$ ,  $\bar{X}$  is the mean  $\Delta\delta^{13}\text{C}_{\text{acetic acid-protein}}$  value in the B1 to E1 rice vinegar samples,  $t_{n-1}$  is the critical t-value with a degree of freedom of  $N-1$  at 95% confidence level. SD is the standard deviation of  $\Delta\delta^{13}\text{C}_{\text{acetic acid-protein}}$  value in the B1 to E1 rice vinegar samples and  $N$  is sample size.

In our previous study, we used acetoin as an intrinsic reference to identify vinegar adulteration

[17]. The  $\delta^{13}\text{C}_{\text{acetoin}}$  and  $\delta^{13}\text{C}_{\text{acetic acid}}$  values could be simultaneously determined by GC-IRMS. However, the content of acetoin in the rice vinegar must be quantitated by GC-MS prior to GC-IRMS analysis to control the amount of acetoin injected within the linearity range of GC-IRMS due to the large fluctuation of acetoin content between various rice vinegar products. In this study, although both EA-IRMS and GC-IRMS are required for determining the  $\delta^{13}\text{C}_{\text{protein}}$  and  $\delta^{13}\text{C}_{\text{acetic acid}}$  values, the amount of vinegar protein injected could be easily controlled within the linearity range of EA-IRMS by weighing. Besides, the  $\delta^{13}\text{C}_{\text{acetic acid}}$  value could also be simply obtained by appropriately diluting the rice vinegar with acetone and directly analyzing with GC-IRMS. Moreover, the reagents (sodium tungstate and sulfuric acid) used for vinegar protein precipitation cost much less than those used for acetoin extraction (especially primary secondary amine powder). For the aforementioned reasons, choosing vinegar protein as an intrinsic reference could save more time and cost than using acetoin.

#### 3.4. Detection model of acetic acid adulteration for rice vinegar

An acetic acid adulteration curve of the  $\Delta\delta^{13}\text{C}_{\text{acetic acid-protein}}$  (Fig. 3) was assumed according to the results in the B1 to E1 rice vinegar samples. The  $\Delta\delta^{13}\text{C}_{\text{acetic acid-protein}}$  values in adulterated vinegar were calculated by Eq. (4), and SD were calculated

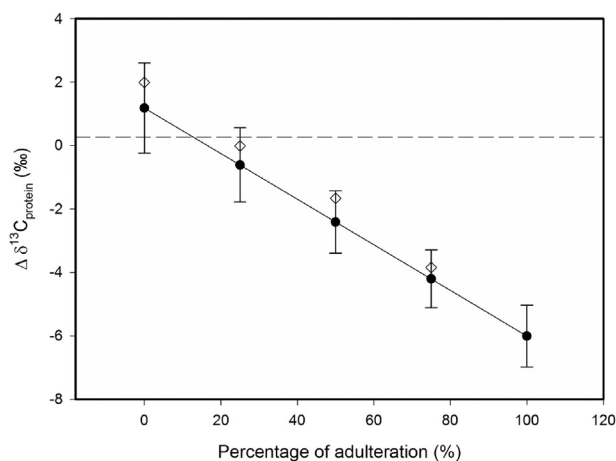


Fig. 3. Variations of the  $\Delta\delta^{13}\text{C}_{\text{acetic acid-protein}}$  value with the adulteration of acetic acid. The solid line represents the acetic acid adulteration curve ( $y = -0.0719x + 1.1833$ ), which was derived from Eq. (4). The full circle represents the calculated  $\Delta\delta^{13}\text{C}_{\text{acetic acid-protein}}$  values with different percentage of adulteration (0, 25, 50, 75, 100%). The dashed line represents the lower bound of the 95% confidence interval for  $\Delta\delta^{13}\text{C}_{\text{acetic acid-protein}}$  in rice vinegar. The diamonds represent the  $\Delta\delta^{13}\text{C}_{\text{acetic acid-protein}}$  value in adulterated vinegar that blended with different ratios of the acetic acid.

by the rules of error propagation [26]. The mean  $\delta^{13}\text{C}_{\text{acetic acid}}$  values in the rice vinegar ( $\overline{\delta^{13}\text{C}_{\text{acetic acid, vinegar}}}$ ) and the mean  $\delta^{13}\text{C}_{\text{acetic acid}}$  values in the acetic acid samples ( $\overline{\delta^{13}\text{C}_{\text{acetic acid, glacial}}}$ ) were obtained from our previous study [17].  $\overline{\delta^{13}\text{C}_{\text{protein}}}$  is the mean  $\delta^{13}\text{C}_{\text{protein}}$  value in the B1 to E1 rice vinegar samples and R is the percent addition of acetic acid.

$$\Delta\delta^{13}\text{C}_{\text{acetic acid-protein}} = \left[ \overline{\delta^{13}\text{C}_{\text{acetic acid, vinegar}}} \times (1 - R) + \overline{\delta^{13}\text{C}_{\text{acetic acid, glacial}}} \times (R) \right] - \overline{\delta^{13}\text{C}_{\text{protein}}} \quad (4)$$

To confirm the validity of the assumed curve for detecting addition of acetic acid, the adulterated vinegars were prepared and analyzed by EA-IRMS and GC-IRMS. An acetic acid sample ( $\delta^{13}\text{C}_{\text{acetic acid}} = -33.35\text{‰}$ ) was first diluted with ddH<sub>2</sub>O to 4.7% acetic acid solution. After that, a vinegar sample (B2, acetic acid content = 4.7%,  $\delta^{13}\text{C}_{\text{acetic acid}} = -25.91\text{‰}$ ,  $\delta^{13}\text{C}_{\text{protein}} = -27.93\text{‰}$ ) was blended with the acetic acid solution at different ratios (0%–75%) to obtain adulterated vinegars, in which the content of acetic acid was kept constant. The  $\delta^{13}\text{C}_{\text{protein}}$  values of the adulterated vinegars were analyzed by EA-IRMS according to the procedures described above. On the other hand, the adulterated vinegars were appropriately diluted with acetone and analyzed directly by GC-IRMS under the conditions described in our previous study to obtain  $\delta^{13}\text{C}_{\text{acetic acid}}$ . The  $\delta^{13}\text{C}_{\text{acetic acid}}$  and  $\Delta\delta^{13}\text{C}_{\text{acetic acid-protein}}$  values of the adulterated vinegars decreased with increasing amount of blended acetic acid, whereas the  $\delta^{13}\text{C}_{\text{protein}}$  values did not (Table 2). The  $\delta^{13}\text{C}_{\text{protein}}$  values were not significantly different

Table 2. Stable carbon isotope ratio results of the acetic acid and protein in the rice vinegar blended with acetic acid at different ratios.

Adulteration ratio <sup>a</sup> (%)	$\delta^{13}\text{C}_{\text{acetic acid}}$ <sup>b</sup> (‰)	$\delta^{13}\text{C}_{\text{protein}}$ <sup>b</sup> (‰)	$\Delta\delta^{13}\text{C}_{\text{acetic acid-protein}}$ <sup>c</sup> (‰)
0	$-25.91 \pm 0.13$	$-27.90 \pm 0.08$	1.99
25	$-27.89 \pm 0.23$	$-27.87 \pm 0.02$	-0.02
50	$-29.59 \pm 0.19$	$-27.93 \pm 0.04$	-1.67
75	$-31.80 \pm 0.30$	$-27.95 \pm 0.03$	-3.85
100	$-33.68 \pm 0.35$	–	–

<sup>a</sup> The rice vinegar sample (B2,  $\delta^{13}\text{C}_{\text{acetic acid}} = -25.91\text{‰}$ ,  $\delta^{13}\text{C}_{\text{protein}} = -27.93\text{‰}$ ) was deliberately blended with the acetic acid ( $\delta^{13}\text{C}_{\text{acetic acid}} = -33.35\text{‰}$ ) at different ratios.

<sup>b</sup> Data are presented as the mean  $\pm$  SD ( $n = 3$ ). The  $\delta^{13}\text{C}_{\text{protein}}$  values show no significant difference between all the adulterated vinegars with different adulteration ratios.

<sup>c</sup> Difference between the mean  $\delta^{13}\text{C}$  values for acetic acid and the protein in the adulterated vinegars.

between the adulterated vinegars with different adulteration ratios ( $P > 0.05$ ), which showed that the rice vinegar protein could be an alternative intrinsic reference for identifying adulterated rice vinegar.

Our previous study [17] indicated that the  $\delta^{13}\text{C}_{\text{acetic acid}}$  value was influenced by both raw materials and blended acetic acid. Conversely, this study showed that the  $\delta^{13}\text{C}_{\text{protein}}$  value was not influenced by blended acetic acid but was mainly influenced by the raw materials. By calculating  $\Delta\delta^{13}\text{C}_{\text{acetic acid-protein}}$  value ( $\delta^{13}\text{C}_{\text{acetic acid}} - \delta^{13}\text{C}_{\text{protein}}$ ), the influence on  $\delta^{13}\text{C}_{\text{acetic acid}}$  value caused by raw materials could be eliminated and that caused by blended acetic acid could be better reflected in the  $\Delta\delta^{13}\text{C}_{\text{acetic acid-protein}}$  value. Thus, the adulterated vinegar could be more easily identified by using  $\Delta\delta^{13}\text{C}_{\text{acetic acid-protein}}$  value than using  $\delta^{13}\text{C}_{\text{acetic acid}}$  value alone.

Besides, the amount of vinegar protein precipitated from the adulterated vinegars was found to decrease with increasing amount of blended acetic acid, and no protein precipitated from the 100% adulterated vinegar at all. The amount of precipitated protein might provide additional information to assist in the identification.

Since the  $\delta^{13}\text{C}_{\text{acetic acid}}$  values of acetic acid samples were much lower than that of rice vinegar samples according to our previous study [17], the  $\Delta\delta^{13}\text{C}_{\text{acetic acid-protein}}$  value of the vinegar sample would decrease when the vinegar was blended with acetic acid. It is assumed that the vinegar might be blended with acetic acid when the  $\Delta\delta^{13}\text{C}_{\text{acetic acid-protein}}$  value was below the lower bound of the 95% confidence calculated. We substituted the lower bound of the 95% confidence interval into the acetic acid adulteration curve of the  $\Delta\delta^{13}\text{C}_{\text{acetic acid-protein}}$  (Fig. 3) and then the adulteration ratio could be calculated. It was indicated that the rice vinegar blended with acetic acid at higher than approximately 10% could possibly be identified.

In the past, European Committee for Standardization had issued isotopic methods to detect adulteration of vinegar with exogenous acetic acid. However, it would take much time to complete the overall extraction and purification processes, and the distillation process should be carefully controlled to prevent isotopic fractionation of acetic acid. In this study, we provide a more efficient and practical procedure to achieve the rice vinegar adulteration identification using the rice vinegar protein as an intrinsic reference. The rice vinegar protein was precipitated by tungstic acid and analyzed by EA-IRMS. Meanwhile, the rice vinegar was diluted with acetone and analyzed directly by GC-IRMS. The  $\delta^{13}\text{C}_{\text{protein}}$  and  $\delta^{13}\text{C}_{\text{acetic acid}}$  values

obtained separately were used to calculate  $\Delta\delta^{13}\text{C}_{\text{acetic acid-protein}}$  value. The fluctuation of  $\delta^{13}\text{C}_{\text{acetic acid}}$  caused by individual differences of raw materials could be eliminated and the deviation of  $\delta^{13}\text{C}_{\text{acetic acid}}$  caused by adulterated acetic acid could be detected efficiently. The  $\Delta\delta^{13}\text{C}_{\text{acetic acid-protein}}$  could be used as an index for discriminating rice vinegar adulteration.

## Conflicts of interest

All authors declare that they have no conflicts of interest.

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