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Original Article

Detection limit of molasses spirits mixed in rice spirits using the SNIF-NMR method

Cheng-Hung Lai^a, Chang-Wei Hsieh^b, Wen-Ching Ko^{a,*}^a Department and Graduate Program of BioIndustry Technology, Da-Yeh University, 168 University Road, Dacun, Changhua 51591, Taiwan, ROC^b Department of Medicinal Botanicals and Healthcare, Da-Yeh University, 168 University Road, Dacun, Changhua 51591, Taiwan, ROC

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ABSTRACT

Traditional Taiwanese rice spirits were made from rice, which is their only ingredient and ethanol source. Site-specific natural isotopic fractionation by nuclear magnetic resonance (SNIF-NMR) methods were used to investigate molasses spirits usually mixed in rice spirits. The mean ratios for the parameters in five rice spirits labeled as Taikeng 8, Taikeng 9, Taichung sen 10, Tainan 11, and Tainung 71 were (D/H)_I, 99.0–100.3 ppm; (D/H)_{II}, 121.3–123.0 ppm; whereas for molasses spirits labeled as MS, the mean ratios were 108.7 ppm and 126.8 ppm, respectively. A close and reproducible correlation was found for (D/H)_I values of the mixtures in which MS was mixed with authentic rice spirit samples. The 3.62% MS mixed in Tainung 71 was detected through calculation, whereas the detectable limit for MS mixed in other rice spirits ranged from 8.20% to 11.73%. The SNIF-NMR analysis provides a powerful method for the detection of rice spirits adulteration and can determine the extent to which the rice spirit product is mixed with MS.

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1. Introduction

Rice spirits are commonly used seasonings in Taiwanese cuisine and the unique features of Taiwan dietary culture. According to Chinese National Standards, rice spirits are defined as distilled spirits made only from rice, through processes that include saccharification, fermentation, and distillation [1]. Products blended with less than 50% ethyl alcohol content from other sources may be regarded as cooking rice spirits [2]. The most frequently encountered

adulteration problems include removing or diluting components, adding other components, and product misbranding. The key feature of economic adulteration is the use of an ingredient of lower value or cost than the authentic product. For example, adding edible alcohol made from cane molasses to rice spirits and declaring the product “pure rice spirit” [3]. Similar examples are adding corn syrup in honey, hydrolyzed inulin in apple juice, grapefruit juice in orange juice, and other food oils in olive oil [4–6]. Adulteration is aimed at providing a greater volume of product that is cheaper to produce and can still be sold at almost equivalent prices.

* Corresponding author. Department and Graduate Program of BioIndustry Technology, Da-Yeh University, 168 University Road, Dacun, Changhua 51591, Taiwan, ROC.

E-mail address: wcko@mail.dyu.edu.tw (W.-C. Ko).

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Among the current analytical tools applied to the characterization of foods and beverages, isotope analyses provide powerful authentication criteria [7]. Stable isotope ratio analysis methods provide the most innovative source of chemical information for authenticity assessment and the origin assignment of agro-food products [7]. The isotope ratio mass spectrometry technique (IRMS), which measures overall molecular isotope ratios of various elements, provides additional authenticity indicators [8]. ^{13}C content is especially efficient for distinguishing organic products originating from C_3 and C_4 plants. Thus, addition of sugar syrups can be detected in fruit juices [5] and in honey [6], and carbon isotope ratios can be used for characterizing vinegars [9]. ^2H NMR determination of the site-specific $^2\text{H}/^1\text{H}$ ratios of fermentative ethanol in wine [10] was initially used by the European Union as an official method for identifying sugar addition with C_3 (sugar beet, orange, and grape) and C_4 plants (maize and sugar cane) [11,12]. Subsequently, the procedure was used to detect beet sugar in fruit juices [13] and to determine the authenticity of flavors [14]. With regard to adulteration, the method provides more information than IRMS concerning the chemical pathway of biosynthesis and, in some cases, the geographic origin of the sample [15].

There is no doubt that rice is the main material used for making rice spirits, and edible alcohol made from cane molasses produced by the Taiwan Sugar Corporation is the prevailing non-rice spirit in the final product. If ethyl alcohols originated from rice or molasses and their ratios could be detected, the commercial adulteration and fraud will be restricted. Our previous study found that the site-specific natural isotopic fractionation by nuclear magnetic resonance (SNIF-NMR) method [3] has application potential to distinguish ethyl alcohol obtained from different raw materials because significant differences in $(\text{D}/\text{H})_{\text{I}}$ and $(\text{D}/\text{H})_{\text{II}}$ ratios on ethanol molecules were observed. This study further evaluated whether the method can distinguish spirits made from five representative rice species produced in Taiwan and how much molasses spirit (MS) content mixed in them can be detected. The objective was to establish an effective method for authenticity determination to guarantee the quality of rice spirits for consumers.

2. Materials and methods

2.1. Materials

Five varieties of rice, Taikeng 8 (TK-8), Taikeng 9 (TK-9), Taichung sen 10 (TCS-10), Tainan 11 (TN-11), and Tainung 71 (TN-71), were obtained from the District Agricultural Research and Extension Station, Taichung, Taiwan. In addition, 95% edible alcohol (an MS) produced from cane molasses was purchased from the Taiwan Sugar Corporation. *Rhizopus formosaensis* (BCRC 31150) and *Saccharomyces sake* (BCRC 20262) were obtained from the Food Industry Research and Development Institute in Taiwan. Unless otherwise specified, all chemicals were of analytical grade.

2.2. Fermentation of rice into rice spirits

Rice spirits were made with a 1:1 ratio of rice grains (10 kg) and water (10 kg). The rice/water mixture was simmered at 100°C for

approximately 30 minutes and subsequently cooled to room temperature (25°C). *R. formosaensis* (BCRC 31150) was inoculated at 3×10^7 cells/mL into the rice to produce amyloglucosidase, α -amylase, and β -glucuronidase. These enzymes broke down starch polymers into smaller starch oligocarbohydrate polymers and monosaccharides, and also liquefied the rice for further fermentation [16]. Following saccharification for 3 days, 2.8×10^7 cells/mL of *S. sake* (BCRC 20262) was inoculated into the rice with 30 kg of water, with the temperature maintained at 25°C for fermentation. After fermentation was complete (12–14 days, depending on desired alcohol content, which was 40 L 12% rice moromi in our experiment), 9 L of 40% alcohol rice spirits was collected through distillation for the SNIF-NMR and authentication model [17].

2.3. Karl Fischer measurements

The water content in the ethanol samples was determined by the Karl Fischer method (AQV-200, HIRANUMA, Mito, Japan) to calculate the alcohol strength (t_{m}^{D} ; %, w/w) of the ethanol samples.

2.4. NMR measurements

During SNIF-NMR measurement, tetramethylurea (TMU) with known isotopic content was used as an internal standard. The ethanol concentration must be distilled to 95% (v/v) for the NMR analysis. The TMU solution (1.3 mL) was placed into a previously weighed bottle, and weighed to the nearest 0.1 mg (m_{st}). Subsequently, 3.2 mL of the sample was poured into the bottle and weighed to the nearest 0.1 mg (m_{A}), and then homogenized by shaking [18]. ^2H NMR spectra were recorded on a Bruker DMX 600 NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany) equipped with a 10-mm probe, operating at ^1H frequency of 299.9 MHz, and at 92.0 MHz for deuterium. Measurements were obtained according to the AOAC protocol [18]. Transients were accumulated with 16,320 data points over a 1200-Hz spectral bandwidth with a $30\ \mu\text{s}$ (90°) rf pulse. The acquisition time was 1.5 seconds. This set of accumulations was repeated $2000\times$ for each sample. The D/H ratios were measured by recording the intensities of the deuterium signals corresponding to the $(\text{D}/\text{H})_{\text{I}}$ (methyl site) and $(\text{D}/\text{H})_{\text{II}}$ (methylene site) of ethanol and tetramethylurea [19]. Means and standard deviations of isotopic ratios were calculated. The D/H ratios of $(\text{D}/\text{H})_{\text{I}}$ and $(\text{D}/\text{H})_{\text{II}}$ were expressed in ppm. The average precision values for the measurement of ratios were below 0.5 ppm for $(\text{D}/\text{H})_{\text{I}}$ and below 0.7 ppm for $(\text{D}/\text{H})_{\text{II}}$. The parameters $(\text{D}/\text{H})_{\text{I}}$ and $(\text{D}/\text{H})_{\text{II}}$ can be calculated by the following equations:

$$(\text{D}/\text{H})_{\text{I}} = 1.5866 \times T_{\text{I}} \times \frac{m_{\text{st}}}{m_{\text{A}}} \times \frac{(\text{D}/\text{H})_{\text{st}}}{t_{\text{m}}^{\text{D}}}$$

$$(\text{D}/\text{H})_{\text{II}} = 2.3799 \times T_{\text{II}} \times \frac{m_{\text{st}}}{m_{\text{A}}} \times \frac{(\text{D}/\text{H})_{\text{st}}}{t_{\text{m}}^{\text{D}}}$$

$(\text{D}/\text{H})_{\text{I}}$: isotope ratio associated with molecule I; $(\text{D}/\text{H})_{\text{II}}$: isotope ratio associated with molecule II

$$T_I = \frac{\text{height of signal I (CH}_2\text{DCH}_2\text{OH)}}{\text{height of signal of internal standard (TMU)}}$$

$$T_{II} = \frac{\text{height of signal II (CH}_3\text{CHDOH)}}{\text{height of signal of internal standard (TMU)}}$$

mst: weight of TMU; mA: weight of ethyl alcohol sample; t_m^D : alcoholic strength of ethyl alcohol sample; $(D/H)_{st}$: certified deuterium content of TMU provided by the supplier.

2.5. Rice spirit authentication model test

Various ratios of MS were mixed with five varieties of pure rice spirits. The ratio of MS/rice spirits was 0%, 25%, 50%, 75%, and 100%. The $(D/H)_I$ and $(D/H)_{II}$ of spirit mixtures were determined using the SNIF-NMR method.

2.6. Statistical analysis

All experimental combinations were replicated three times. Data were analyzed by analysis of variance with SAS for Windows v.8 (SAS Institute, Cary, NC, USA). Significant differences were analyzed using Duncan's multiple range test ($p < 0.05$).

3. Results and discussion

3.1. SNIF-NMR index of ethyl alcohols from various sources

Stable isotope content and accumulation rates are related to geographic regions [19]. Stable hydrogen isotope ratio $(D/H)_I$ is connected in methyl site(CH_3), whereas $(D/H)_{II}$ is connected in methylene site(CH_2) of ethanol molecules. Stable hydrogen isotope ratio determinations were performed on fermentative ethanol, which must be distilled to 95% (v/v) in concentration. The SNIF-NMR index of ethyl alcohols from TK-8, TK-9, TCS-10, TN-11, TN-71, and MS are presented in Table 1. The values of parameters $(D/H)_I$ and $(D/H)_{II}$ differed markedly for spirits made from the five rice varieties and those of MS. The mean ratios for these parameters in TK-8, TK-9, TCS-10, TN-11, and TN-71 were $(D/H)_I$, 99.0–100.3 ppm (standard deviation = 0.5); $(D/H)_{II}$, 121.3–123.0 ppm (standard deviation = 0.7). For MS, the values were $(D/H)_I$, 108.7 ppm (standard deviation = 0.5); $(D/H)_{II}$, 126.8 ppm (standard

Table 1 – ^2H fingerprints of fermentative ethanol in Taiwanese rice spirits using different varieties of rice.

Rice spirits	$(D/H)_I$ (ppm)	$(D/H)_{II}$ (ppm)
TK-8	99.0 ± 0.5^c	122.8 ± 0.7^c
TK-9	99.1 ± 0.5^c	123.0 ± 0.7^c
TCS-10	$99.5 \pm 0.5^{b,c}$	$121.9 \pm 0.7^{b,c}$
TN-11	99.4 ± 0.5^c	$122.3 \pm 0.7^{b,c}$
TN-71	100.3 ± 0.5^b	121.3 ± 0.7^c
MS	108.7 ± 0.5^a	126.8 ± 0.7^a

Data are presented as mean \pm SD ($n = 3$).

^{a–c}The means in the same column followed by different letters are significantly different ($p < 0.05$).

deviation = 0.7). The values among the five rice varieties were not markedly different; however, they differed from that for MS. Using C_3 (sugar beet, orange, and grape) or C_4 (sugar cane and maize) biomass for ethanol fermentation, the mean values of $(D/H)_I$ ranged from 90.4 ppm to 101.3 ppm or from 103.7 ppm to 110.5 ppm [12,20]. These results were similar to our results in that $(D/H)_I$ on ethanol molecules can be distinguished because different sources have unique proportions of natural isotopes [3].

3.2. Model test for rice spirits mixed with molasses spirit from cane

The capability of the developed method to detect the addition of MS was further confirmed by the analysis of rice spirits with known amounts of MS. Figs. 1 and 2 show the change of $(D/H)_I$ and $(D/H)_{II}$ for rice spirits mixed with various ratios of MS. As shown in Fig. 1, a linear increase of the $(D/H)_I$ ratio occurs when increasing amounts of MS ratio are added to the rice spirits (correlation coefficient $R^2 > 0.96$). The high coefficients of linear correlation indicated that $(D/H)_I$ was an available index for identification. Conversely, addition of MS in rice spirits resulted in irregular change in $(D/H)_{II}$ (correlation coefficient $R^2 = 0.01$ –0.73). The observation that $(D/H)_I$ produced high coefficients may indicate that material sources are a crucial factor in SNIF-NMR determination. Although $(D/H)_{II}$ is also connected to sources of ethanol [12,20,21], its effect on authenticity must be further investigated in the future.

3.3. Detectable limit of molasses spirit mixed in rice spirits

The $(D/H)_I$ values obtained from various authentications of samples were used to determine the limits of detection. The equations of the regression lines of $(D/H)_I$ for rice spirits mixed with MS, as shown in Fig. 1, were as follows:

$$y = 0.0962x + 98.711 \text{ [TK – 8]},$$

$$y = 0.0865x + 98.856 \text{ [TK – 9]},$$

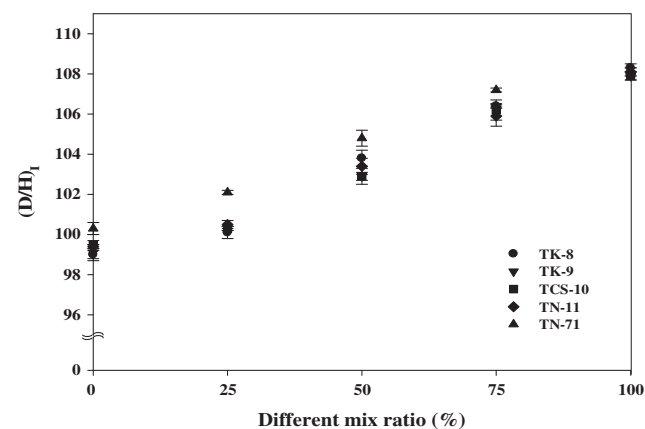


Fig. 1 – Changes in $(D/H)_I$ values of rice spirits mixed with molasses spirit.

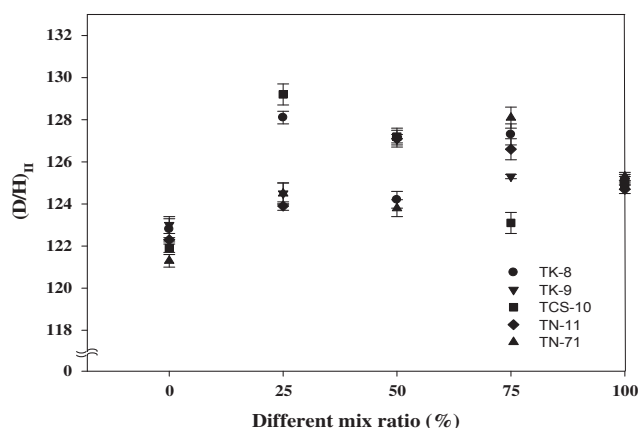


Fig. 2 – Changes in $(D/H)_{II}$ values of rice spirits mixed with molasses spirit.

$$y = 0.0878x + 98.97 \text{ [TCS - 10]},$$

$$y = 0.0841x + 99.154 \text{ [TN - 11]}$$

and

$$y = 0.0799x + 100.51 \text{ [TN - 71]}.$$

Experimental data on the detectable limit for rice spirits mixed with MS were calculated as shown in Table 2. Taking TN-71 as an example, $(D/H)_I$ value became 100.8 ppm (i.e., y in the equation) after adding the average precision value of the NMR measurement 0.5 ppm to it. Calculation with these equations, TN-71 exhibited 3.62% in detectable limit, whereas the limits ranged from 8.20% to 11.73% for the other four rice varieties. We have confirmed that the detection limit at a confidence level of 95% reaches 12% when MS was added to the five known origins of rice varieties. This may verify applicability of the detection model. Nevertheless, it is not easy to detect all other unknown sources and content ratios of ethyl alcohol, even the SNIF-NMR method also has detectable restriction. Only 20% additions of C_4 syrup can be estimated in authentications of honey [6]. For lemon juices, Gonzalez et al [22] also indicated that the detection limit is more difficult to appraise in the case of using unknown origin materials.

4. Conclusions

The positional $^2H/^1H$ ratios allow a satisfactory discrimination between MS and rice spirits. Ethanol obtained from various raw materials revealed significant differences in the D/H ratio of the methyl group. Our model test demonstrated that SNIF-NMR is a potential method for the identification of pure rice

spirits [alcohol produced from rice has a $(D/H)_I$ ratio of approximately 99.0–100.3 ppm, and alcohol derived from molasses 108.7 ppm] and for distinguishing the amount of MS mixed with rice spirits. The same approach may be applicable to other types of rice spirits that contain MS. However, further studies are necessary to establish a database and a more exact and relevant detection method for specific items.

Conflicts of interest

All contributing authors declare no conflicts of interest.

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Table 2 – Lower limit of detectability for molasses spirit (MS) mixed in rice spirits.

	TK-8	TK-9	TCS-10	TN-11	TN-71
Rice spirit with MS (%)	8.20	8.60	11.73	8.87	3.62

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