

Detection of Molasses-Spirit in Rice-Spirits from TK-8 and TCS-10 by SNIF-NMR Method

CHANG-WEI HSIEH¹, HSIU-JU WANG², CHIEH-MING CHANG³ AND WEN-CHING KO^{4*}

¹ Department of Bioresources, Da Yeh University, 112 Shanjiu Rd., Dahtsuen Township, Changhwa County 515, Taiwan, R.O.C.

² Department of Food Science, National Chung Hsing University, 250 Kuokuang Rd., Taichung City 402, Taiwan, R.O.C.

³ Department of Chemical Engineering, National Chung Hsing University, 250 Kuokuang Rd., Taichung City 402, Taiwan, R.O.C.

⁴ Department of Bioindustry Technology, Da Yeh University, 112 Shanjiu Rd., Dahtsuen Township, Changhwa County 515, Taiwan, R.O.C.

(Received: December 6, 2004; Accepted: March 15, 2005)

ABSTRACT

The objective of this study was to identify the origin of edible alcohol usually added in Taiwanese rice-spirits by using deuterium nuclear magnetic resonance (SNIF-NMR method). Rice-spirits labeled TK-8, TCS-10 were made from two rice varieties, Taikeng 8 and Taichung sen 10, whereas molasses-spirit labeled MS was obtained from Taiwan Sugar Corporation. The proportion of ethanol molecules monodeuterated on the methyl site (D/H)_I and methylene site (D/H)_{II}, and the ratio R between the intensities of methyl and methylene signals in deuterium spectra were significantly different among TK-8, TCS-10, and MS. Of the three indices, only (D/H)_I linearly ($R^2 > 0.96$) increased with addition of MS in both TK-8 and TCS-10. Therefore, (D/H)_I was an index for the identification of pure rice-spirits mixed with ethanol originated from molasses.

Key words: rice-spirit, ethyl alcohol, deuterium, SNIF-NMR

INTRODUCTION

In addition to drinking pleasure, rice-spirits are usually used to season food dishes and to prepare tonics for after-pregnancy women in Taiwan. In other words, rice-spirit is one of necessities in Taiwanese daily life. Since joining WTO in 2001, Taiwan government removed monopoly and approved general industries engage in manufacturing and selling alcoholic products.

According to the Administration Law of Tobacco and Wines of Taiwan, pure rice-spirit is defined as an alcoholic product that undergoes thorough processes of cooking, saccharification, fermentation, distillation, and blending with or without edible alcohol⁽¹⁷⁾. Rice is the only ingredient and ethanol source for pure rice-spirits.

In order to increase production and decrease cost, some unscrupulous manufactures often add edible alcohol made from cane molasses and declare "pure rice-spirit" to deceive consumers. Consequently, a suitable and reliable method is required to analyze and detect enrichment of rice-spirits through the addition of edible alcohol to prevent cheating and deceiving consumers.

Martin *et al.*^(11,12,13,14) have proposed a reliable method called site-specific natural isotopic fractionation using ²H nuclear magnetic resonance (SNIF-NMR) for the determination of the enrichment of grape must or wine. In natural organic compounds, there are on average 150 deuterium atoms among 1 million hydrogen atoms. The deuterium content and its distribution in organic materials

is not random nor always the same^(6,12). Therefore, deuterium/hydrogen (D/H) ratios measured at the methyl site (D/H)_I and methylene site (D/H)_{II} of ethanol in wine differ significantly according to the origin of the sugar from which the alcohol is produced^(12,16). Besides ethanol, this has been shown to be the case for other molecules such as glucose or glycerol^(9,19,20). The characteristic site-specific distribution of deuterium within a molecule can be visualized by means of high-resolution ²H nuclear magnetic spectroscopy. Comparison of D/H ratios and ratio R between intensities of methyl and methylene signals in deuterium spectra enables discrimination between natural and enriched wines, and also makes it possible to differentiate wines based on the geographical origin⁽¹⁸⁾. European Union adopted this method for controlling chaptalization in 1990⁽³⁾. Afterwards, a reasonable and representative database of authentic wines from the wine-growing countries in the European Union was established and is constantly growing^(4,5).

In this study, pure rice-spirits made from two rice varieties were mixed with molasses-spirit to imitate market products. SNIF-NMR was used to identify the origin of ethyl alcohol. Our objective was to set up a method that is able to identify if pure rice-spirits contain ethyl alcohol that is not derived from rice.

MATERIALS AND METHODS

I. Microorganisms and Chemicals

Commercial Koji containing mold and yeast was

* Author for correspondence. Tel: +886-4-8511888 ext. 4191; Fax: +886-4-8511324; E-mail: wcko@mail.dyu.edu.tw

purchased from Masahisa Biotechnology Co., Ltd. (Taiwan). Karl Fischer reagent Hydranal[®] Composite 5 was purchased from Sigma-Aldrich. Two varieties of rice, Taikeng 8 (TK-8) and Taichung sen 10 (TCS-10), were obtained from the District Agricultural Research and Extension Station, Taichung, Taiwan. The 95% edible alcohol (a molasses-spirit; MS) produced from cane molasses was purchased from the Taiwan Sugar Corporation. *N,N*-tetramethylurea (TMU) with known isotopic content was supplied by the Institute for Reference Materials and Measurements (IRMM) at Geel, Belgium. Unless otherwise specified, all chemicals were of analytical grade.

II. Fermentation of Rice into Rice-spirit

Fermentation of all rice was done under the control and standard conditions according to the Protocol Procedures assigned by Masahisa Biotechnology Co. in order to obtain full conversion of sugar into ethanol (at least 90% of the theoretical yield). After fermentation, the mashes were carefully distilled and the alcohol concentration was determined by Karl Fischer method. Alcohol grade of the distillate must be greater than 95% (w/w) to guarantee that the isotopic fractionation on distillate is below 0.2 ppm for $(D/H)_I^{(3,7,14)}$.

III. Rice-spirit Authentication Model Test

Different ratios of edible alcohol (MS) were mixed with two varieties of pure rice-spirit. The ratio of MS/total ethyl ethanol was 0%, 20%, 50%, 80% and 100%, respectively. $(D/H)_I$, $(D/H)_{II}$, and *R* value of the spirit mixtures were determined by the SNIF-NMR method.

IV. Karl Fischer Measurements

The water content in the ethanol samples was determined by the Karl Fischer methods (AQV-200, HIRANUMA, Japan) to calculate the alcoholic strength (t_m^D ; %, w/w) of the ethanol samples.

V. NMR Measurements

Tetramethylurea (TMU) with known isotopic content was used as an internal standard. The TMU solution (1.3 mL) was placed into a previously weighed bottle, and weighed to the nearest 0.1 mg (m_{st}). Then 3.2 mL of the ethanol sample was put into the bottle and again weighed to the nearest 0.1 mg (m_A), and then homogenized by shaking⁽¹⁴⁾.

The ²H NMR spectra were recorded on a Bruker DMX 600 NMR spectrometer equipped with a 10 mm probe, operating at ¹H frequency of 299.9 MHz and at 92.0 MHz for deuterium. Measurements were taken according to the AOAC protocol⁽¹⁴⁾. Transients were accumulated with 16,320 data points over a 1,200 Hz spectral bandwidth with

a 30 μ s (90°) rf pulse. The acquisition time was 1.5 sec. This set of accumulations was repeated 2,000 times for each sample. The D/H ratios were measured by recording the intensities of the deuterium signals corresponding to the methylene and methyl sites of ethanol and tetramethylurea⁽¹⁰⁾. Means and standard deviations of isotopic ratios and *R* values were calculated. The D/H ratios of $(D/H)_I$ and $(D/H)_{II}$ were expressed in ppm⁽³⁾. The average precision values of the measurement of ratios were below 0.5 ppm for $(D/H)_I$, and below 0.6 ppm for $(D/H)_{II}$.

VI. Statistical Analysis

All experiments were performed on triplicate samples. The statistical analysis systems (SAS) software package was used for statistical analysis⁽¹⁾. Analysis of variance was performed by ANOVA procedures. Significant differences between means were determined using Duncan's multiple range test ($p < 0.05$).

RESULTS AND DISCUSSION

I. Isotopic Ratios and *R* Values of Individual Spirit

Figure 1 shows the typical ²H NMR spectra of TK-8 (similar patterns not shown for TCS-10 and MS) with TMU certified as a reference standard. As shown, the signals I, II, III, and IV referred to the working standard CH₂DCH₂OH, CH₃CHDOH, CH₃CH₂OD, and TMU, respectively. According to the EU and AOAC protocol^(3,14), the parameters $(D/H)_I$, $(D/H)_{II}$, and *R* could be calculated by the following equations:

$$(D/H)_I = 1.5866 \times T_I \times \frac{m_{st}}{m_A} \times \frac{(D/H)_{st}}{t_m^D}$$

$$(D/H)_{II} = 2.3799 \times T_{II} \times \frac{m_{st}}{m_A} \times \frac{(D/H)_{st}}{t_m^D}$$

$$R = 2(D/H)_{II}/(D/H)_I$$

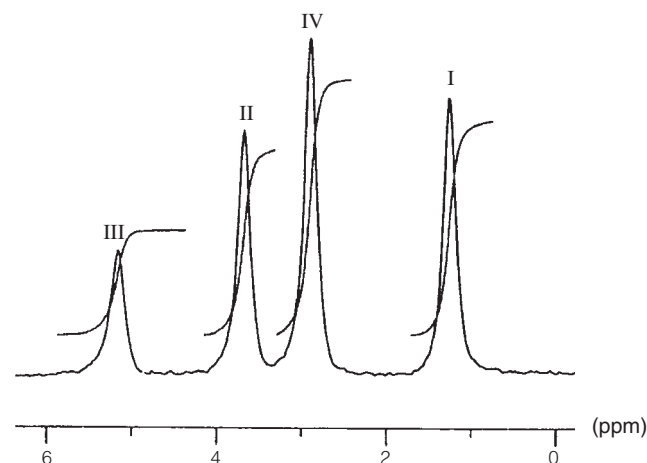


Figure 1. The typical ²H NMR spectra of natural ethanol (TK-8). The signals I, II, III, and IV refer to the working standard CH₂DCH₂OH, CH₃CHDOH, CH₃CH₂OD, and TMU, respectively.

where

(D/H)_I: isotope ratio associated with molecule I

(D/H)_{II}: isotope ratio associated with molecule II

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

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

m_{st}: weight of TMU

m_A: weight of ethyl alcohol sample

*i*_m^D: alcoholic strength of ethyl alcohol sample

(D/H)_{st}: isotope ratio of internal standard (TMU) supplied by the Community Bureau of References

Table 1 shows the isotopic ratios and R values of ethyl alcohol from the three individual spirits, TK-8, TCS-10, and MS, based on their typical NMR spectra. Three parameters (D/H)_I, (D/H)_{II}, and R value were notably different for spirits made from the two rice varieties and cane molasses. Among all the sources that can be used to brew alcohol drinks, Asian people prefer grains as the sources for producing spirits compared with fruit. In Taiwan, TK-8 that represents the Japonica type-rice and TCS-10 that represents the Indica type-rice are prevalent for making rice-spirit. But for the reasons of adjusting flavor and decreasing cost, some manufacturers often add molasses-spirit to rice-spirit and illegally declared the product "pure rice-spirit". Though in terms of individual material, ethyl alcohols could be distinguished due to different compounds having their own unique proportion of natural isotopes^(2,3). SNIF-NMR has been adopted as an effective method for the detection of added sugar in fruit juices⁽¹⁴⁾. It is necessary to confirm whether the method is still sensitive for distinguishing rice-spirits which were mixed with molasses-spirit.

II. Isotopic Ratios and R Values of Mixed Spirits

Figure 2 shows the change of (D/H)_I for rice-spirits mixed with various ratios of molasses-spirit. As shown, (D/H)_I increased with the addition content of MS for both TK-8 and TCS-10. High coefficients (R² > 0.96) of linear correlation indicated that (D/H)_I was an available index for identification.

Figures 3 and 4 show the (D/H)_{II} and R values for rice-spirits mixed with different ratios of molasses-spirit. Addition of MS in rice-spirits resulted irregular change in (D/H)_{II} and almost no change in R values for the mixed spirits. These two parameters were appropriate for identification of the origin of ethyl alcohols in rice-spirits.

III. Adopted Index for Rice-spirit by SNIF-NMR

Ethyl alcohol produced from grapes shows (D/H)_I ratio at 96-102 ppm^(6,10,13). The slight difference may be connected with the geographical origin, vintage, grape species, and even the harvest years. However, addition of

extraneous sugar, beet or cane to grape must lead to significant alterations in the (D/H)_I of the resulted ethyl alcohol^(10,14). Fauhl and Wittkowski⁽⁶⁾ indicated that (D/H)_I is the most indicative parameter for the determination of the addition of extraneous sugar to wine due to none of the parameters such as yeast strain, fermentation temperature, or wine fining may affect it⁽⁸⁾. This method can also be used to detect sugar addition to fruit juices and concentrates or to identify the origin of ethyl alcohol^(12,15).

Table 1. The ²H fingerprints of fermentative ethanol in Taiwanese rice-spirits using different varieties of rice [Mean values ± confidence intervals for the means (p ≤ 0.05)]

Varieties of rice	Isotopic variable		R*
	(D/H) _I (ppm)	(D/H) _{II} (ppm)	
TK-8	98.9 ± 0.3 ^c	122.1 ± 0.2 ^b	2.5 ^a
TCS-10	99.7 ± 0.3 ^b	119.0 ± 0.3 ^c	2.4 ^b
MS	108.7 ± 0.3 ^a	126.8 ± 0.3 ^a	2.3 ^c

*Relative intramolecular deuterium distribution.

^{a,b,c}Superscript letters indicate the order from large to small values.

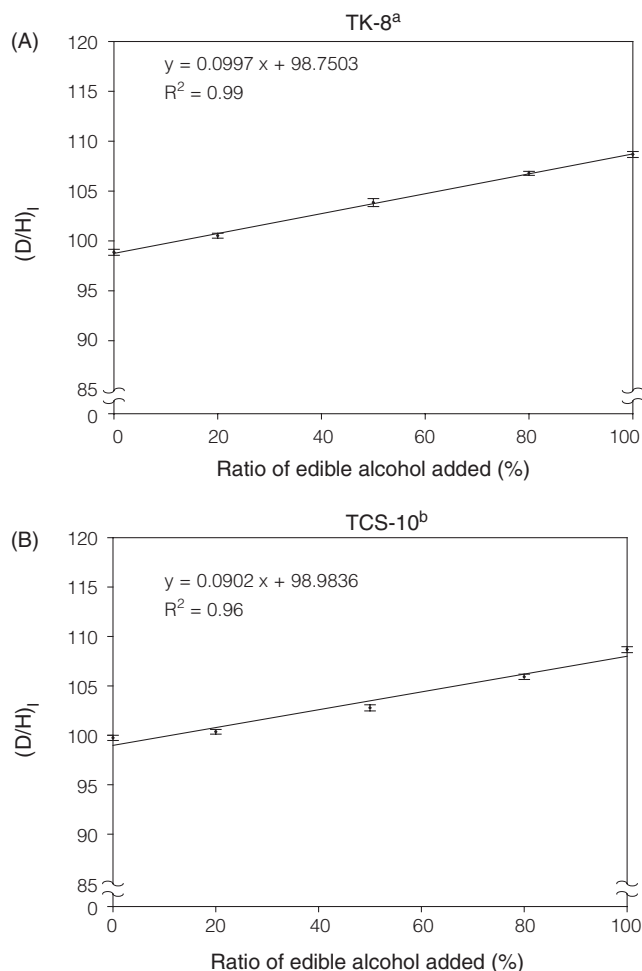


Figure 2. Changes in (D/H)_I values for rice-spirits with different ratios of edible alcohol derived from molasses spirit (MS) as determined by SNIF-NMR method. (a) and (b) were ethyl alcohols derived from rice varieties Taikeng 8 (TK-8) and Taichung sen 10 (TCS-10), respectively.

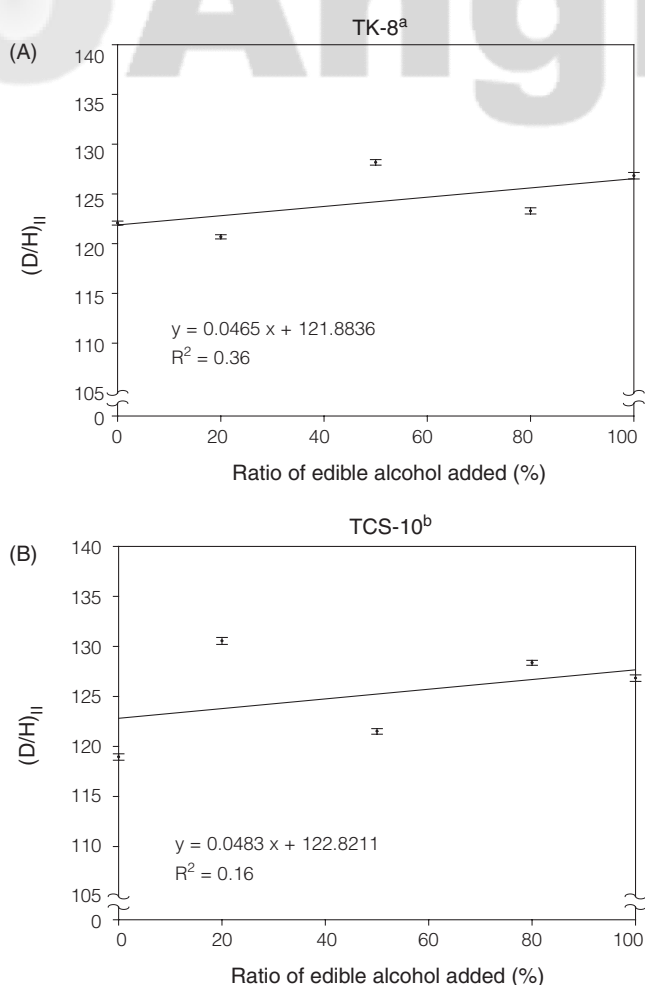


Figure 3. $(D/H)_{II}$ values for rice-spirits with different ratios of edible alcohol derived from molasses spirit (MS) as determined by SNIF-NMR method. (a) and (b) were ethyl alcohols derived from rice varieties Taikeng 8 (TK-8) and Taichung sen 10 (TCS-10), respectively.

In our study, $(D/H)_I$ of ethyl alcohol derived from rices was 98.9 ppm for TK-8 and 99.7 ppm for TCS-10. The $(D/H)_I$ showed regular increases in proportion to the additional content of ethyl alcohol produced from cane molasses. From the results obtained, we primarily concluded that SNIF-NMR method is an effective method for the identification of pure rice-spirits and $(D/H)_I$ is an available index to estimate the percentage of molasses-spirit added. Taking TK-8 as an example, $(D/H)_I$ value of TK-8 and MS are 98.9 ± 0.3 and 108.7 ± 0.3 ppm, respectively. The average precision value of the NMR measurement is 0.5 ppm for $(D/H)_I$. Therefore, as the difference of $(D/H)_I$ value is over 0.5 ppm [$(D/H)_I$ value ~ 99.4 ppm], we can infer that the sample was likely to be mixed with extra molasses-spirit (the additive content $\sim 6.5\%$).

Several studies indicated that $(D/H)_{II}$ and R values are useful parameters for detecting origin of ethyl alcohols^(10,12,18). Our study showed the distribution of $(D/H)_{II}$ was indiscriminate and R value was not related to the content of molasses-spirit in rice-spirit.

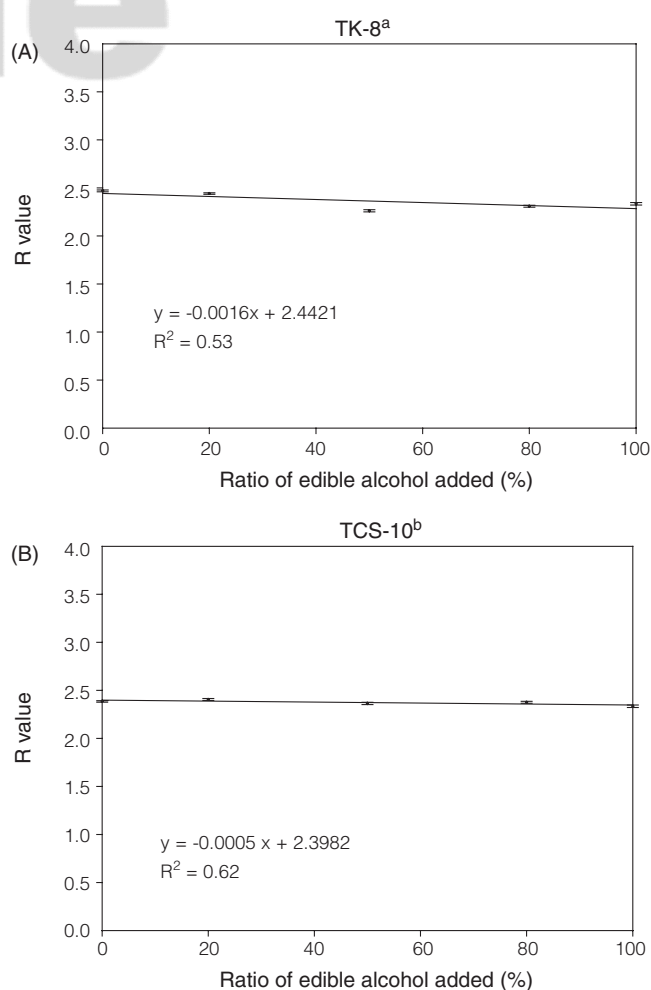


Figure 4. R values for rice-spirits with different ratios of edible alcohol derived from molasses spirit (MS) as determined by SNIF-NMR method. (a) and (b) were ethyl alcohols derived from rice varieties Taikeng 8 (TK-8) and Taichung sen 10 (TCS-10), respectively.

CONCLUSIONS

Ethyl alcohol obtained from different raw materials reveals significant differences in the D/H ratio of the methyl group. Our model test showed that SNIF-NMR is not only a potential method for the identification of pure rice-spirits [alcohol produced from rice has a $(D/H)_I$ ratio ~ 99 ppm, and alcohol derived from molasses ~ 108 ppm], but also for distinguishing of rice-spirits mixed with molasses-spirit. Nevertheless, further works are needed to build up database for rice varieties that are probably used to make rice-spirits in Taiwan.

REFERENCES

1. SAS Institute, Inc. 1997. SAS User's Guide: Applied Statistics and the SAS Programming Language. 4th ed. SAS Inst. Cary, NC, U. S. A.
2. Cross, J. L., Gallaher, T. N., Leary, J. J. and Schreiner,

- S. 1998. The application of site-specific natural isotope fractionation-nuclear magnetic resonance (SNIF-NMR) to the analysis of alcoholic beverages. *Chem. Educ.* 3: S 1430-4171 (98) 05252-7.
3. EC Regulation 2676/90. 1990. Detecting enrichment of grape musts, concentrated grape musts, rectified concentrated grape musts and wines by application of nuclear magnetic resonance of deuterium (SNIF-NMR/RMN-FINS). *Off. J. Eur. Communities (L272)* 33: 1-192.
 4. EC Regulation 2347/91. 1991. On the collection of samples of wine products for the purposes of cooperation between Member States and for analysis by nuclear magnetic resonance, including analysis for the purposes of the Community databank. *Off. J. Eur. Communities (L214)*: 32-36.
 5. EC Regulation 2348/91. 1991. Establishing a databank for the results of analyses of wine products by nuclear magnetic resonance of deuterium. *Off. J. Eur. Communities (L214)*: 39-43.
 6. Faulstich, C. and Wittkowski, R. 2000. Oenological influences on the D/H ratios of wine ethanol. *J. Agric. Food Chem.* 48: 3979-3984.
 7. Giménez-Miralles, J. E., Salazar, D. M. and Solana, I. 1999. Regional origin assignment of red wines from Valencia (Spain) by ^2H NMR and ^{13}C IRMS stable isotope analysis of fermentative ethanol. *J. Agric. Food Chem.* 47: 2645-2652.
 8. González, J., Remaud, G., Jamin, E., Naulet, N. and Martin, G. G. 1999. Specific natural isotope profile studied by isotope ratio mass spectrometry (SNIP-IRMS): $^{13}\text{C}/^{12}\text{C}$ ratios of fructose, glucose, and sucrose for improved detection of sugar addition to pineapple juices and concentrations. *J. Agric. Food Chem.* 47: 2316-2321.
 9. Hermann, A. 1999. Determination of site-specific D/H isotope ratios of glycerol from different sources by ^2H -NMR spectroscopy. *Z. Lebensm. Unters. Forsch. A.* 208: 194-197.
 10. Košir, I. J., Kocjančič, M., Ogrinc, N. and Kidrič, J. 2001. Use of SNIF-NMR and IRMS in combination with chemometric methods for the determination of chaptalisation and geographical origin of wines (the example of Slovenian wines). *Anal. Chim. Acta* 429: 195-206.
 11. Martin, G. J. and Martin, M. L. 1981. Deuterium labelling at the natural abundance level as studied by high field quantitative ^2H NMR. *Tetrahedron Lett.* 22: 3525-3528.
 12. Martin, G. J., Martin, M. L., Mabon, F. and Michon, M. J. 1983. A new method for the identification of the origin of ethanols in grain and fruit spirits: high-field quantitative deuterium nuclear magnetic resonance at the natural abundance level. *J. Agric. Food Chem.* 31: 311-315.
 13. Martin, G. J., Zhang, B. L., Naulet, N. and Martin, M. L. 1986. Deuterium transfer in the bioconversion of glucose to ethanol studied by specific isotope labeling at the natural abundance level. *J. Am. Chem. Soc.* 108: 5116-5122.
 14. Martin, G. G., Wood, R. and Martin, G. J. 1996. Detection of added beet sugar in concentrated and single strength fruit juices by deuterium nuclear magnetic resonance (SNIF-NMR¹ method): collaborative study. *J. AOAC Int.* 79: 917-928.
 15. Martin, G. G., Guillou, C. and Caisso, M. 1991. Detection of beet sugar addition in fruit juices and concentrates. *Fruit Processing* 1: 18-22.
 16. Masud, Z., Vallet, C. and Martin, G. L. 1999. Stable isotope characterization of milk components and whey ethanol. *J. Agric. Food Chem.* 47: 4693-4699.
 17. National Treasury Agency. 2001. Compilation of Tobacco and Alcohol Administration Law. National Treasury Agency. Taipei, Taiwan, R. O. C.
 18. Ogrinc, N., Košir, I. J., Kocjančič, M. and Kidrič, J. 2001. Determination of authenticity, regional origin, and vintage of Slovenian wines using a combination of IRMS and SNIF-NMR analyses. *J. Agric. Food Chem.* 49: 1432-1440.
 19. Pionnier, S., Robins, R. J. and Zhang, B. L. 2003. Natural abundance hydrogen isotope affiliation between the reactants and the products in glucose fermentation with yeast. *J. Agric. Food Chem.* 51: 2076-2082.
 20. Zhang, B. L., Buddrus, S. and Martin, M. L. 2000. Site-specific hydrogen isotope fractionation in the biosynthesis of glycerol. *Bioorg. Chem.* 28: 1-15.