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On-line ^{1}H-NMR to facilitate tube preparation in SNIF-NMR analysis

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Abstract The detection of adulteration of wine by SNIF-NMR (site-specific natural isotopic fractionation-nuclear magnetic resonance) analysis of wine alcohol is a well established and widely used method. Quantitative deuterium ([^{2}H]) NMR spectroscopy of defined distillate/tetramethylurea (TMU) mixtures enables the calculation of the site-specific deuterium/hydrogen ([^{2}H]/H) ratios in the ethanol molecule. TMU with a known [^{2}H]/H ratio serves as the internal standard. The comparison of the [^{2}H]/H ratios of unknown samples with values obtained from authentic samples allows conclusions to be drawn, in terms of the origin of the sugar in the must before fermentation. Up to now, the calculation of [^{2}H]/H ratios has required the exact weighing of both the distillate and the quantity of TMU for the tube preparation, as they affect ^{2}H-NMR measurements. The precise quantity of ethanol in the distillates must be determined by Karl-Fischer titration or densitometry. Volatile distillate components are quantified by gas chromatography to ensure a satisfactory purity of the ethanol under study. The quantitative ^{1}H-NMR method described involves determining the TMU/ethanol mass ratios in the tubes prepared for SNIF-NMR measurements. This mass ratio is necessary for the calculation of the [^{2}H]/H ratio. The mass ratios measured using conventional procedures and those determined by on-line ^{1}H-NMR agree 100%. Using this method means that the exact weighing of distillates and TMU for tube preparation and the determination of ethanol content in the distillates are no longer required. At the same time, higher concentrations of volatile components in the distillates, e.g. methanol, can be easily detected.

Key words SNIF-NMR · ^{1}H-NMR · Tube preparation

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Introduction

The measurement of site-specific deuterium ([^{2}H]) content by nuclear magnetic resonance (NMR) spectroscopy has been applied successfully to the authentication of aroma compounds and alcohols [1–4]. The method was developed in the early 1980s and is based on quantitative high-resolution ^{2}H-NMR spectroscopy.

In 1990 site-specific natural isotopic fractionation NMR (SNIF-NMR) analysis of wine alcohols was adopted by the European Community (EC) as an official method [5] for the detection of illegal sugar enrichments of musts before fermentation. The measurement yields [^{2}H]/H ratios at the relevant [^{2}H] positions of the ethanol molecule in the methyl $([^2H]/H)_I$ and the methylene $([^2H]/H)_{II}$ groups. The characteristic [^{2}H]/H distribution of sugars from various biological sources is found in the ethanol molecule after fermentation. The addition of saccharose originating from beet or cane to grape must leads to a significant alteration of $([^2H]/H)_I$ in the resulting ethanol. The $([^2H]/H)_{II}$ ratio is largely dependent on the fermentation medium. With respect to their geo-climatic location vintage and grape variety, the [^{2}H]/H ratios of authentic wines vary within a certain range. Therefore, a comprehensive data bank of authentic wine samples is indispensable for the production of evidence of chaptalisation [6].

The first step for the determination of site-specific [^{2}H] content is the separation of the wine alcohol by distillation. The distillation systems used for this purpose guarantee high alcohol yields and low concentrations of other volatile compounds in the distillates. However, the [^{2}H]/H ratio calculation requires the exact determination of ethanol in the distillates, for which the EC regulatory bodies [5] describe two possible methods. For one method, the water content (% m/m) of the distillates is determined by Karl-Fischer titration; the ethanol concentration (% m/m) can then be calculated, assuming that the water content (%) plus ethanol content (%) equal 100%. For the

other method, measurement of the density of distillates (densitometry) is used. Volatile distillate components such as methanol, higher alcohols, ethyl acetate and acetal are quantified by GC. Increased contents lead to a further correction of the ethanol mass concentration.

The tube preparation for NMR spectroscopy includes the following steps:

1. Defined amounts of distillate and internal standard, i.e. tetramethylurea (TMU) with a known [^{2}H]/H ratio are weighed exactly.
2. After addition of hexafluorobenzene as a lock substance, the mixture is transferred to an NMR tube and is ready to use for the ^{2}H-NMR measurement.

Under conditions of quantitative [^{2}H] acquisition and in proton decoupling mode, spectra of each tube are acquired repeatedly. The signal intensities obtained from the NMR spectra are proportional to the number of the monodeuterated molecules in the measuring cell.

Materials and methods

The calculation of the [^{2}H]/H ratio in position i of the ethanol molecule by internal comparison with the standard TMU has been described by Eq. 1 by Martin et al. [7].

$$([^2H]/H)_i^{Eth} = \frac{P^{TMU}}{P_i^{Eth}} \times \frac{M^{Eth}}{M^{TMU}} \times \frac{m^{TMU} \times t_m^{TMU\%}}{m^D \times t_m^{D\%}} \times \frac{Sig_i^{Eth}}{Sig^{TMU}} \times ([^2H]/H)^{TMU} \quad \text{Eq. 1}$$

with,

$$K = \frac{m^{TMU} \times t_m^{TMU\%}}{m^D \times t_m^{D\%}} \quad \text{Eq. 2}$$

where, P^{TMU} = number of H atoms of the TMU molecule, P_i^{Eth} = number of H atoms in position i of the ethanol molecule, M^{Eth} = molecular mass of ethanol, M^{TMU} = molecular mass of TMU, m^D = mass of distillate, m^{TMU} = mass of TMU, Sig_i^{Eth} = signal height of ethanol in position i, Sig^{TMU} = signal height of TMU, $t_m^{D\%}$ = alcoholic grade in weight of distillate, $t_m^{TMU\%}$ = purity grade in weight of TMU, $([^2H]/H)^{TMU}$ = [^{2}H]/H ratio in position i of TMU (known), K = mass quotient.

TMU (1.3 ml) and distillate (3.2 ml) are weighed exactly and mixed for the preparation of a 10-mm NMR tube. After addition of 50 µl hexafluorobenzene as lock substance (400 MHz spectrometer) the mixture is measured by ^{2}H-NMR. With quantitative ^{2}H-NMR conditions and proton decoupling mode, ten spectra of each tube are acquired. The [^{2}H]/H ratios are calculated according to Eq. 1. After elimination of outliers the mean is calculated. A detailed description of the method is found in the EC regulation [5]. For the calculation of the [^{2}H]/H ratio the mass of the water content in the distillate is substrated from the mass of the distillate. A further correction is only performed in cases of high concentrations of volatile components determined by GC. Volatile components of the distillate other than ethanol fall into two categories. First, the so-called non-resonating impurities, giving NMR signals which do not interfere with the important methyl group signal of ethanol, and, second, the resonating impurities, which exhibit signals which do interfere. For the latter (2-methyl-1-butanol, acetal and ethyl acetate) a correction is not required, for the non-resonating volatiles a correction is only necessary when the sum of their concentrations exceeds 0.3% (m/m) [8].

Usually, a correction for the TMU weight is not necessary. The certified TMU, recommended for this measurement, and distributed by the Institute of Reference Materials and Measurements (IRMM) in Geel (Belgium), is of a highly pure quality.

For quantitative ^{1}H-NMR acquisition conditions Eq. 3 is given.

$$\frac{m_c^{TMU}}{m_c^{Eth}} = \frac{S_{TMU} \times \frac{5}{M^{Eth}}}{\sum S_{Eth} \times \frac{12}{M^{TMU}}} \quad \text{Eq. 3}$$

with,

$$\frac{m_c^{TMU}}{m_c^{Eth}} = K = \frac{m^{TMU} \times t_m^{TMU\%}}{m^D \times t_m^{D\%}} \quad \text{Eq. 4}$$

Table 1 Acquisition conditions for ^{1}H-NMR measurements

Parameter	Detail
Spectrometer	Bruker ARX 400
Hardware	Aspect 3000/X32
Software	UXNMR version 930303
Probe	10 mm selective deuterium (61.42 MHz), proton decoupling, fluorine lock
Acquisition time	4.1 s
Spectral width	20.2 ppm
Time domain	65536 data points
Flip angle	10°; 3.8 µs
Temperature	306 K
Spin rate	15 Hz
Carrier frequency	400.137009 MHz, between CH_3-TMU and CH_3 ethanol, decoupler coil
Lock	^{19}F, hexafluorobenzene
Number of dummy scans	4
Number of scans	16
Relaxation delay	5 s
Line broadening factor (LB)	0.2
Integration	CH_2 group ethanol (quartet): 3.90–3.20 ppm TMU (singlet; exactly calibrated to 2.80 ppm): 3.15–2.35 ppm CH_3 group ethanol (triplet): 1.65–0.6 ppm

where, m_c^{TMU} = mass of pure TMU in measuring cell, m_c^{Eth} = mass of ethanol in measuring cell, S_{TMU} = signal area of CH_3 groups TMU (12 H), $\sum S_{Eth}$ = sum of signal areas,CH_2 and CH_3 groups of ethanol (5 H).

The mass ratio of TMU and ethanol calculated from the ^{1}H-NMR signal areas (Eq. 3) is identical with the quotient K in Eq. 2. Conventionally, K is obtained by the weight of the distillate and TMU, combined with the mass reduction for the secondary ingredients determined by Karl-Fischer titration and GC.

The following conditions were used for the acquisition of ^{1}H-NMR spectra of distillate/TMU mixtures, which were prepared for the ^{2}H-SNIF-NMR measurement (Table 1).

Either five or ten spectra were acquired for each tube on the decoupling coil [^{1}H] of the selective [^{2}H] probe, which is indicated for the ^{2}H-SNIF-NMR measurement. The Fourier transformation was performed with zero filling and a line broadening factor (LB) of 0.2. The automatic routines of the NMR software were used for both the phase and base line corrections. The integration intervals were kept constant for all spectra. The ^{13}C-satellites have to be included in the integration range. Likewise, other small signals have to be integrated too, because a correction for volatile components is the exception rather than the rule.

Results and discussion

The spin-lattice relaxation times (t_1) of the proton signals were determined by the inversion recovery method (Table 2).

Due to the sensitive measurement and the high analyte concentrations, the 10° pulse angle was chosen. Quantitative acquisition conditions are guaranteed for a pulse interval of 9.1 s (4.1 s acquisition time, 5 s relaxation delay). The exclusion of the first four free induction decays (FID) is necessary to ensure steady-state measurement conditions. The short pulse angle is beneficial to the thermal equilibrium during the acquisition. The application of usual conditions for quantitative ^{1}H-NMR measurements (90° pulse, relaxation delay $> 5 \times t_1$) to these kinds of samples (pure analyte) can easily lead to a FID truncation combined with a poor integration of the resulting spectra. Furthermore, problems in the adjustment of the receiver gain can occur, expressed as saturated FID signals.

During the defined conditions (400 MHz spectrometer, 16 scans, line broadening factor 0.2) and for an appropriate homogeneous magnetic field, a signal-to-noise ratio of $> 20\,000$ can be obtained, estimated for the highest signal of the ethanol CH_3 triplet. The splitting between the two central lines of the ethanol methylene quartet was 5–8% (LB = 0). Typical half-line widths (LB = 0) for the highest signals are estimated as follows: ethanol CH_2: 1.1 Hz; TMU CH_3: 1.7 Hz; ethanol CH_3: 1.3 Hz.

Several tubes containing different distillates were prepared by the conventional method [5] for the ^{2}H-SNIF-NMR measurement. Table 3 shows the mass quotient, K, determined by ^{1}H-NMR, compared to the K determined by the conventional procedure. The mass quotent K (conventional) was calculated by including a weight correction for the water content of the distillates determined by Karl-Fischer titration. The standard deviation of the quotient K (^{1}H-NMR) for (n) acquired spectra is included. For the conventional tube preparation procedure no standard deviation can be presented.

Table 2 Spin-lattice relaxation time (t_1) of the proton signals

	t_1(s)
CH_2-Ethanol	3.1
CH_3-TMU	2.7
CH_3-Ethanol	3.4

Table 3 Comparison of the mass quotients K (conventional) and K (^{1}H-NMR)

Tube	K (conventional)	K (^{1}H-NMR)	Standard deviation (n)	Percentage fit
1	0.5212	0.5216	0.0003 (10)	100.08
2	0.5127	0.5144	0.0038 (10)	100.33
3	0.5199	0.5207	0.0004 (10)	100.15
4	0.5185	0.5187	0.0004 (10)	100.04
5	0.5039	0.5040	0.0010 (10)	100.02
6	0.5166	0.5165	0.0008 (5)	99.98
7	0.5162	0.5160	0.0006 (5)	99.97
8	0.5007	0.5012	0.0005 (5)	100.10
9	0.4798	0.4800	0.0005 (5)	100.04
10	0.5196	0.5218	0.0005 (5)	100.43
11	0.5193	0.5189	0.0003 (5)	99.91
12	0.5178	0.5169	0.0007 (5)	99.82
13	0.5186	0.5175	0.0005 (5)	99.80
14	0.5236	0.5235	0.0003 (5)	99.99
15	0.5335	0.5329	0.0003 (5)	99.88
16	0.5208	0.5201	0.0007 (5)	99.85

Both K values agree 100%. It must be noted that the quotient K (conventional) in Table 3 was set to a 100% but may involve a deviation within the precision of the procedure.

Differences in the mass quotient K proportionally affect the calculation of the [^{2}H]/H ratios (Eq. 1). Therefore, the exact determination of the mass quotient K is required for a reliable [^{2}H]/H calculation. Table 3 clearly demonstrates that both the conventional and the ^{1}H-NMR procedures lead to comparable results in this matter.

In order to check the linearity of the determination of K using ^{1}H-NMR, tubes 17, 18 and 19 were prepared. The volume portions of TMU and distillate were varied. The [^{2}H]/H ratios measured by the ^{2}H-SNIF-NMR method are shown in Table 4. The recalculation of the [^{2}H]/H ratios with the same [^{2}H] signal intensities and the mass quotient K (conventional) for all three tubes was performed and these are also depicted in Table 4.

The determination of K by ^{1}H-NMR is independent of the amounts of distillate and TMU under study. The usual volumes (1.3 ml TMU, 3.2 ml distillate) are recommended since the [^{2}H] signal proportions should be comparable.

Table 4 Mass quotient, K, determined for different TMU/distillate ratios. (TMU Tetramethylurea)

Tube	$([^2H]/H)_I$ ppm	$([^2H]/H)_{II}$ ppm	R value	Volume TMU (ml)	Volume distillate (ml)	K(^{1}H-NMR)
17	102.46	123.84	2.417	1.3	3.2	0.5074
18	102.14	123.84	2.425	1.0	3.4	0.3876
19	102.34	123.60	2.416	1.5	3.0	0.6403
Recalculation						K(conventional)
17	102.47	123.86	2.417	1.3	3.2	0.5075
18	102.18	123.89	2.425	1.0	3.4	0.3878
19	102.38	123.65	2.416	1.5	3.0	0.6405

Table 5 Non-resonating impurities

Impurity	Molecular mass	Group	Multiplicity of NMR signal	Chemical shift (TMU singlet exactly calibrated to 2.80 ppm)
Acetic aldehyde	44.05	CH_3	Doublet	2.155 ppm
Methanol	32.04	CH_3	Singlet	3.325 ppm
3-Methyl-1 butanol	88.15	CH_2	Quartet	1.425 ppm
2-Methyl-1 propanol	74.12	CH_2	Doublet	3.295 ppm
1-Propanol	60.10	CH_2	Sixtet	1.535 ppm

As mentioned above, a further correction of ethanol contents for the distillates has to be performed if the "non-resonating impurities" determined by GC exhibit a sum concentration exceeding 0.3 mass%.

In the case of the ^{1}H-NMR method, greater contents of volatile components can be estimated from the spectrum. Table 5 outlines the most typical non-resonating impurities in distillates. The chemical shifts included correspond to characteristic non-overlapping ^{1}H-NMR signals for each of the compounds.

The described integral intervals for the calculation of K(^{1}H-NMR) must be reduced for the analysis of distillates containing larger amounts of certain volatile compounds. If a mass correction is necessary, the following integration intervals are recommended: TMU (CH_3 group, singlet, exactly calibrated to 2.80 ppm): 3.02–2.57 ppm; ethanol (CH_3 group, triplet): 1.38–0.95 ppm.

The methylene group of the ethanol is omitted for this integration, because interfering NMR signals arise from 3-methyl-1-butanol and 1-propanol. For calculation of K (^{1}H-NMR) the number of protons for ethanol has to be set from five to three in Eq. 3.

Figure 1 shows the ^{1}H-NMR spectrum of a stone fruit distillate containing 0.76% (m/m) methanol. The signal for the methyl group of methanol at 3.325 ppm is clearly detected. The application of reduced integration intervals leads to a mass quotient fit of 99.91% for this tube. Several other tubes were tested with the reduced integration and a suitable agreement between the mass quotient values was achieved. For this comparison, K (conventional) was adjusted for the water content and the amount of "non-resonating impurities" determined by GC.

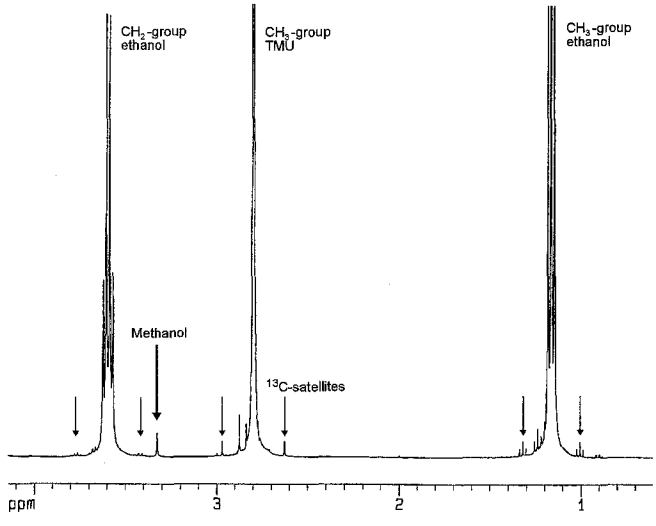

Fig. 1 ^{1}H-NMR spectrum of a distillate containing 0.76% (m/m) methanol

The method presented for the determination of the mass quotient K obtains results which are completely comparable with the established procedure. The tube preparation for ^{2}H-SNIF-NMR is much easier because the exact weighing of the distillate and TMU is not necessary. Errors in weighing each of the masses can be avoided. In addition, the prerequisite of a precise ethanol determination by Karl-Fischer titration or densitometry is no longer required. The observation of small ^{1}H-NMR signals from volatiles substances enables their inclusion in the analysis.

The use of an NMR sample changer allows (the analysis of up to six samples per 24 h ($\approx$ 4 h each) using the SNIF-NMR method. This automation includes the lock and shim procedure, the acquisition of the ^{2}H-NMR signals and the processing of the FIDs for each sample. The ^{1}H-NMR method can be implemented in the automation programme, prior to the ^{2}H-NMR acquisition. The observation channel is changed by the software. The number of spectra can be limited to five in routine analysis, corresponding to a time of 15 min.

The ^{1}H-NMR method is a universal tool for the determination of mass ratios used for the SNIF-NMR analysis of different analytes, including substance mixtures with separate resonance signals.

References

1. Martin GJ, Martin ML (1989) In: Linkens HF, Jackson JF (eds) Modern methods of plant analysis. New series 6: Wine analysis. Springer, Berlin Heidelberg New York, pp 258–272
2. Guillou C, Remaud G, Martin GJ (1991) Trends Food Sci Technol 4: 85–89
3. Hanneguelle S, Thibault JN, Naulet N, Martin GJ (1992) J Agric Food Chem 40: 81–87
4. Hagedorn ML (1992) J Agric Food Chem 40: 634–637
5. EC-Regulation No. 2676/90 3rd October 1990
6. EC Regulation Nos. 2347/91 and 2348/91 29th July 1991
7. Martin GJ, Sun XY, Guillou C, Martin ML (1985) Tetrahedron 41: 3285–3296
8. Lees M, Guillou C, Martin GJ (1994) Office International de la Vigne et du Vin F.V. No. 963, Paris