

## Accurate determination of ethanol in water by qNMR: Validation and uncertainty assessment

İlker Ün\* and Ahmet C Goren

TUBITAK, UME, P.Box:54 41470 Gebze-Kocaeli, Türkiye

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**Abstract:** A rapid and simple method, having high reproducibility and low uncertainty for determination of low concentration of ethanol in water has been developed by using qNMR. Method validation was performed applying CRM and calibration solutions. The correlation coefficient for the analyte in the calibration interval was 1. At a concentration range of 0.5 mg/g to 5 mg/g, the recovery was determined to be 99.71%. The relative uncertainties for ethanol solutions of 0.8123 mg/g and 3.8029 mg/g in water were found to be 0.0053 mg/g and 0.0027 mg/g, respectively.

**Keywords:** qNMR; ethanol; traceability; method validation; uncertainty. © 2017 ACG Publications. All rights reserved.

### 1. Introduction

qNMR applications is now widely available in various fields, such as drug industry [1], manufacturing of reference materials [2], food analyses [3, 4] and metabolite determination in human body fluids [5-11]. Moreover, applications of qNMR involve determination of purity of a compound and amount and concentration of a sample inside a matrix. NMR is the sole method, which can determine, with one analysis, a small molecule having a single proton or an oligosaccharide possessing multiple protons. In addition to its easy and quick sample preparation procedure, recovering the sample after the analysis is possible especially in case of using external standard method, which is not possible with chromatographic techniques. While the reference material is used as independent of the sample in qNMR, it must have a structural similarity to the sample in chromatographic analyses Also, the chromatographic techniques require calibration curve, but it is up to the operator in qNMR. Due to its practical and reliable results, it looks, qNMR will attract even more interest in near future with upcoming cost reductions.

Following quantitative determinations of active substances in drug and food supplements by qNMR [12], a rapid measure of alcohol concentration in food samples, with high accuracy and lower uncertainty is possible. qNMR can be used as an alternative and practical method for determination the amount of ethanol instead of GC-MS in food samples [13]. This method is particularly applicable for liquid samples such as wine [14] and alcohols in frozen fruit extracts [15]. In European pharmacopeia, the limits for the presence of organic solvents in drug samples have already been established. Ethanol has a concentration limit of 5000 ppm per day as it displays a lower toxicity [16].

The aim of this study is to determine the amount of ethanol in water samples at high and low concentration levels. The developed method will be fully validated and measurement uncertainty will be evaluated.

\* Corresponding author: E-Mail: [ilker.un@tubitak.gov.tr](mailto:ilker.un@tubitak.gov.tr), Tel.:(+90) 262 679 50 00 - 6109

## 2. Experimental

### 2.1. Chemicals

Sample for applications; Ethanol-Water Solution (0.5% and 0.05%). Sample for trueness studies; Standard Reference Material 2894 NIST. Internal standard for ethanol concentration; 3-(Trimethylsilyl)propionic-2,2,3,3-d<sub>4</sub> acid sodium salt (TSP-d<sub>4</sub>) Sigma Aldrich. NMR Solvent; Deuterium oxide 99.9 % Merck. Internal standard for TSP-d<sub>4</sub> purity; Benzoic acid Standard Reference Material NIST 350b. Certified reference material for traceability; Chloramphenicol Primary Calibrant UME CRM 1301 (certified value 99.58% ± 0.15%).

### 2.2. qNMR Conditions

All NMR experiments were performed at 298.15 K on a Varian VNMRs 600 spectrometer (Varian, San Francisco, CA, USA) operating at 599.747 MHz for proton (<sup>1</sup>H) resonance frequency equipped with a 5 mm One NMR probe using 5 mm sample tubes (5 mm diameter, 178 mm length, Duran Group, Mainz, Germany). The VnmrJ 4.2 (Agilent Technologies, Santa Clara, CA, USA) and MestReNova 11.0.0 (Mestrelab Research S.L., Santiago de Compostela, Spain) softwares were used for data acquisition and data processing, respectively. More detailed phase and baseline correction can be done with MestReNova software. For this reason, MestReNova software was used for data processing.

<sup>1</sup>H NMR experiments were recorded with 32 scans without sample spinning. The measurements were conducted with the following parameters optimized for <sup>1</sup>H NMR: pulse angles of 90°, 64 k data points, acquisition time of 4 s and auto gain function.  $T_1$  was approximately 3.48 s for ethanol -CH<sub>3</sub> resonance, and 6.50 s for TSP-d<sub>4</sub> -CH<sub>3</sub> resonance. 15xT<sub>1</sub> was used to ensure a complete relaxation and reducing the influence of relaxation delay. Therefore the relaxation delay between two scans was set to 98 s. Spectral width of 9615.4 Hz and transmitter offset at 0.59 ppm were applied. Fourier transformation was performed after zero filling the data to 64 k time domain points. Prior to Fourier transformation, an exponential line-broadening function of 0.2 Hz was applied to FID (Free Induction Decay) in all NMR experiments. During the data processing, phase and baseline were corrected manually and the signals were also integrated manually for the same region. Chemical shift was referenced to the TSP-d<sub>4</sub> signal at 0.00 ppm in D<sub>2</sub>O. Each sample was measured once due to the long analysis period, and the concentrations were calculated with the mean of the parallel detection results.

### 2.3. Sample preparation

The weighing processes were performed on a micro balance (XP56, Mettler-Toledo GmbH, Switzerland) with a readability of 0.001 mg, certified by UME (TÜBİTAK National Metrology Institute) and checked with the class E2 weights. The balance is positioned on a 500 kg stone table, with a U-electrode in place to remove potential static charge. Screw cap clear glass vials with the capacity of 4 ml were used for sample preparation. Firstly, an empty vial was weighed and then TSP-d<sub>4</sub> was added as an internal reference (3-8 mg) into the vial. The weighing was repeated 3 times for the empty vial and internal standard. Finally, water sample which contains ethanol was weighed (0.4 mL) quickly in the same vial. 0.6 mL D<sub>2</sub>O was added into the vial and screw cap was tightly closed. The solutions were mixed via vortex until a clear solution was obtained. Then, 0.7 mL of solution was transferred into a NMR tube.

## 3. Results and discussion

### 3.1. Theory

Quantitative determination of ethanol concentration by qNMR was conducted by dissolving the sample with known quantity together with the internal standard in completely deuterated water.

The quantitative value was calculated using integral values of ethanol and the internal standard signals in the spectrum. In order to determine the correct integral values, phase and baseline corrections of the spectrum were made manually. The calculation equation of qNMR for the analyte purity is as follows:

$$C_{EtOH} = \frac{I_{EtOH}}{I_{Std}} \frac{N_{Std}}{N_{EtOH}} \frac{M_{EtOH}}{M_{Std}} \frac{m_{Std}}{m_{Sample}} P_{Std} \quad (1)$$

$I_{Std}$ ,  $N_{Std}$ ,  $M_{Std}$ ,  $m_{Std}$  and  $P_{Std}$  are the peak area, number of proton, molecular weight, weighed mass and purity of the internal standard, respectively. The terms  $I_{EtOH}$ ,  $N_{EtOH}$ ,  $M_{EtOH}$ , and  $C_{EtOH}$  indicate the peak area, number of proton, molecular weight and concentration of the analyte (ethanol),  $m_{Sample}$  weighed mass for ethanol in water sample respectively.

### 3.2. Method Validation

Trueness, linearity, limit of detection (LOD), limit of quantification (LOQ), specificity and selectivity parameters were evaluated to validate the method. Additionally, traceability was established to obtain comparable results and measurement uncertainty was calculated.

#### 3.2.1. Trueness

In order to assess trueness, two different ways were followed, in the first method the NIST 2894 standard reference material, in which ethanol concentration was reported as  $1.0084 \text{ mg/g} \pm 0.0083$ , was used. The results of the analysis conducted with 5 different samples are given in Table 1. When the obtained results were evaluated, it was determined that the ethanol concentration found by qNMR analysis and the certificate value of ethanol solution in water were very close to each other in terms of both concentration and uncertainty.

**Table 1.** qNMR results of NIST 2894 SRM

NIST 2894	qNMR Results (mg/g)
Sample 1	1.0063
Sample 2	1.0203
Sample 3	0.9996
Sample 4	1.0167
Sample 5	1.0032
Mean	1.0092
Standard Deviation	0.0089

In the second validation method, qNMR analyzes were performed by preparing ethanol water solution in 6 different concentrations between 0.5 mg/g and 5 mg/g. Recovery studies were conducted through dilution of the standard stock solution to different concentrations. The average recovery value for linearity samples was found to be 99.71% and the relative standard deviation value was 1.52 (Table 2). Recovery was calculated according to the following formula.

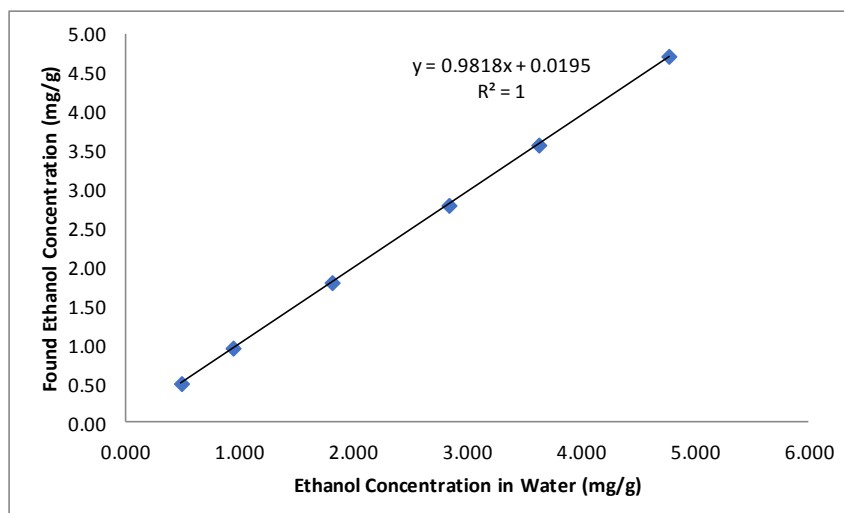
$$\text{Recovery (100\%)} = \frac{\text{Measured Concentration} - \text{endogeneous concentration}}{\text{spiked concentration}} \times 100 \quad (2)$$

**Table 2.** Recovery evaluation by qNMR

Sample	Spiked (mg/g)	Found (mg/g)	Expanded Uncertainty (mg/g)	Recovery %
Ethanol in Water 0,5 mg/g	0.483	0.492	0.001	101.86
Ethanol in Water 1 mg/g	0.937	0.950	0.008	101.39
Ethanol in Water 2 mg/g	1.809	1.796	0.022	99.28
Ethanol in Water 3 mg/g	2.838	2.794	0.012	98.45
Ethanol in Water 4 mg/g	3.629	3.573	0.019	98.46
Ethanol in Water 5 mg/g	4.774	4.719	0.022	98.85
			Mean	99.71
			SD	1.52
			% RSD	1.52

### 3.2.2. Linearity

The linearity was evaluated by preparing three different samples from each concentration and drawing a linearity pilot using the average values of these samples (Figure 1).

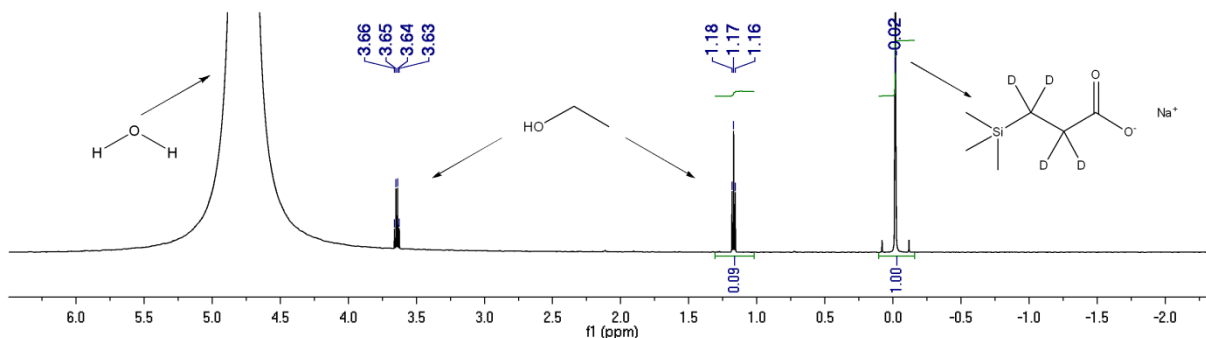
**Figure 1.** Linearity study pilot for ethanol concentration in water

### 3.2.3. LOD and LOQ

LOD and LOQ values were studied with different samples by lowering ethanol concentration in water. The limit of detection (LOD) of the method was found to be 0.005 mg/g for ethanol. The limits of quantification (LOQ) was calculated to be 0.09 mg/g, (for S/N ratio >100 and the above concentrations).

### 3.2.4. Specificity and selectivity

Figure 3 shows  $^1\text{H}$  NMR spectra of ethanol in water and TSP- $\text{d}_4$  in  $\text{D}_2\text{O}$ . It was obvious that the signals obtained at 1.17(t) ppm for ethanol [17] and 0.02 ppm for TSP- $\text{d}_4$  were not disturbed by water and excipients. Moreover, the signals of ethanol and TSP- $\text{d}_4$  were well separated from each other in sample preparations. A representative qNMR spectrum of ethanol in water sample is shown in Figure 2.



**Figure 2.** Representative qNMR spectrum of ethanol in water sample solved in D<sub>2</sub>O

### 3.3. Traceability

In quantitative NMR analyzes, the TSP-d<sub>4</sub> reference was used as the internal standard. TSP-d<sub>4</sub> purity was determined by qNMR using the benzoic acid internal standard in the traceability chain of UME-CRM-1301 (chloramphenicol primary calibrator). Analyzes carried out on this basis were conducted by establishing a calibration chain that reaches the primary national or international standards, i.e. the International System of Units (SI).

### 3.4. Estimation of Uncertainty

The combined uncertainty  $u(C_x)$  containing additional quantities were calculated via equation (3), according to the literature [18] for determination of ethanol concentrations (Table 4). The letter X represents the ethanol.

$$u(C_x) = C_x \sqrt{\left(\frac{u(I_x/I_{std})}{I_x/I_{std}}\right)^2 + \left(\frac{u(M_x)}{M_x}\right)^2 + \left(\frac{u(M_{std})}{M_{std}}\right)^2 + \left(\frac{u(m_{sample})}{m_{sample}}\right)^2 + \left(\frac{u(m_{std})}{m_{std}}\right)^2 + \left(\frac{u(P_{std})}{P_{std}}\right)^2} \quad (3)$$

Equation (4) was used for the calculation of uncertainty component of molar masses  $u(M)$ . Where,  $N_j$  indicates the number of atoms of the element j (e.g. C, H, etc.) and  $u(j)$  indicates the uncertainties of the atom masses.

$$u(M) = \sqrt{\sum_{j=1}^n (N_j u(j))^2} \quad (4)$$

The uncertainties of the initial weighing  $u(m)$ , given by the uncertainty parameters of balance, were calculated using equation (5).

$$u(m_i) = \sqrt{u_w^2(m) + 2u_{non-linear}^2(m)} \quad (5)$$

**Table 4.** Results from uncertainty evaluation of the <sup>1</sup>qNMR method

	Value (x)	u(x)	u(x)/x
Ethanol Concentration (%)	0.081232	0.000192	0.002362
Reference Purity (%)	96.120000	0.107500	0.001118
M <sub>w</sub> Ethanol (g/mol)	46.068440	0.000971	2.11E-05
M <sub>w</sub> TSP-d <sub>4</sub> (g/mol)	172.266129	0.002831	1.64E-05
m Sample (mg)	399.987211	0.001155	2.89E-06
m Reference (mg)	7.309667	0.002309	0.000316
			0.002633
Ethanol Concentration in water %	0.08123		
Combined uncertainty, u	0.00021		
Expanded uncertainty, U (k=2)	0.00043		

### 3.5. Application of two different concentrations for ethanol in water sample by qNMR

Ethanol concentration was determined at two different levels in water by qNMR. Five different sub-samples were prepared per day for each solution and analyzed on three different days. A total of fifteen NMR sub-samples were prepared from each concentration. The results for each day are given in Table 2 for low and high concentrations.

**Table 3.** qNMR results for two different concentrations of ethanol samples

Ethanol in Water	Low Level Concentration (mg/g)			High Level Concentration (mg/g)		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Sample 1	0.8053	0.8180	0.8256	3.8038	3.7973	3.8134
Sample 2	0.8116	0.8088	0.8128	3.8155	3.7993	3.8009
Sample 3	0.8114	0.7999	0.8231	3.8261	3.8050	3.7978
Sample 4	0.8149	0.8071	0.8050	3.7936	3.8069	3.7997
Sample 5	0.8068	0.8230	0.8117	3.7926	3.7912	3.8005
Mean	0.8100	0.8113	0.8156	3.8063	3.7999	3.8025
SD	0.0039	0.0092	0.0085	0.0144	0.0063	0.0062
Com. Unc. u	0.0020	0.0042	0.0039	0.0078	0.0052	0.0052
Exp. Unc. U	0.0040	0.0084	0.0079	0.0156	0.0105	0.0104

Averages and uncertainty values of 15 sub-samples for each concentration were found as follows. The value for low concentration was found to be 0.8123 mg/g and the expanded uncertainty was 0.0043 mg/g. The value for high concentration was found to be 3.8029 mg/g and the expanded uncertainty was 0.0101 mg/g.

## 4. Conclusion

The qNMR provides significant advantages over other chromatographic, spectroscopic and titrimetric methods, considering lesser analysis time, faster method development, easier data analysis and readiness of the instrument all the time. In this study, two different ethanol solutions, having lower and higher concentrations were studied. At both concentrations, the amount of ethanol was determined and reported with all the uncertainty components. It has been shown that the NMR instrument can be used as an important analytical technique for quantitative calculations of low concentrations of alcohol or metabolites in liquid solutions with high reproducibility and low uncertainty.

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