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Quantitative nuclear magnetic resonance spectroscopy ^{2}H(D)-qNMR in the study of deuterium distribution in intracellular water and fermentation products of grape carbohydrates using ethyl alcohol as an example

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Abstract. The paper presents results which develop the results of studies carried out in 2022-2023 under the OIV grant on the topic of distribution of deuterium (^{2}H(D)) in the intracellular water of grapes and wines, taking into account the impact of natural, climatic and technogenic factors using quantitative nuclear magnetic resonance spectroscopy (qNMR). The purpose of the current research was to study the mechanism of deuterium distribution during alcoholic fermentation in model systems, the main components of which were water with different ^{2}H(D)-contents, carbohydrates (D-glucose, D-fructose) and L(+)-tartaric acid. To study the distribution of deuterium in the main target fermentation product, ethanol, we used model systems with different ^{2}H(D)-contents in water (from 142 to 247 ppm), as well as systems with a constant ^{2}H(D)-content (235 ppm), but different amounts of carbohydrates (from 15 to 25 %). The qNMR method was used to study the ^{2}H(D)-dynamics in water of the initial model systems before and after fermentation, as well as to study the ^{2}H(D)-distribution in the methyl (CH_3-) and methylene (CH_2-) groups of ethanol molecules formed during fermentation. The results of fundamental study using the ^{2}H(D)-qNMR methodology and the described model systems before and after carbohydrate fermentation are a consistent development of the approach in solving the applied problem of assessing the presence of technologically inevitable water in wine, which in turn provides the basis for identifying unacceptable of wine and/or must extension.

1. Introduction

This study is part of a scientific project to develop and apply a new methodological approach for the rapid analysis of intracellular water in grapes and wine without prior sample preparation, based on quantitative deuterium nuclear magnetic resonance spectroscopy ^{2}H(D)-qNMR. The paper presents results which develop the results of studies carried out in 2022-2023 under the OIV grant on the topic of distribution of deuterium ^{2}H(D) in the intracellular water of grapes and wines, taking into account the impact of natural, climatic and technogenic factors using quantitative ^{2}H(D)-qNMR [1, 2]. The purpose of the current research was to study the mechanism of deuterium distribution during alcoholic fermentation in model systems, the main components of which were water with different ^{2}H(D)-content, carbohydrates (D-glucose, D-fructose) and L(+)-tartaric acid from grapes.

2. Materials and methods

2.1. Reagents

Analytically pure D-glucose and D-fructose from the RusHim Ltd. (Moscow, Russia), as well as L(+)-tartaric acid from grapes (Chemical Leaders Ltd., Moscow, Russia) were used in the study. Waters for model systems were prepared based on tap water from the Moscow city network and standardized fully deuterated water D_2O from the Solvex-D Ltd. (Moscow, Russia). The exact values for the deuterium content in experimental water samples and the corresponding model systems are given in Tables 1 & 2.

2.2. 2.2 Model systems

The composition and initial parameters of the model systems used in experiments are given in Tables 1 & 2. The concentration of added L(+)-tartaric acid in all model systems was 4 g/l. After mixing all components, the finished model systems were kept before fermentation at room temperature for 1 hour for stabilizing of composition. For fermentation, *Saccharomyces cerevisiae* yeast "Turbo Yeast T3" (Hambleton Bard Ltd., UK) was used in a dosage sufficient to completely convert the carbohydrates contained in the model systems. The total duration of fermentation in two experiments was 14 days at the temperature of 20-21° C.

Table 1. Composition of the model systems *An*: constant level of carbohydrates/varying level of deuterium (before fermentation).

No.	Component	Model sytem		
		A1	*A2*	*A3*
1	D-Glucose, g/l	100	100	100
2	D-Fructose, g/l	100	100	100
3	Water, ml	1000	1000	1000
Parameters:				
- $(D/H)_a$ of initial water, ppm		142.1	192.6	246.9
- soluble solids, °Brix		16.3	16.3	16.3

Table 2. Composition of the model systems *Bn:* constant level of deuterium/varying level of carbohydrates (before fermentation).

No.	Component	Model sytem		
		B1	B2	B3
1	D-Glucose, g/l	75	100	125
2	D-Fructose, g/l	75	100	125
3	Water, ml	1000	1000	1000
Parameters:				
- (D/H)a of initial water, ppm		234.7	234.7	234.7
- soluble solids, oBrix		13.0	16.6	19.6

2.3. Instrumentation

2.3.1. ^{2}H(D)-qNMR-Measurements in water of model systems

The determination of the deuterium content $(D/H)_a$ in water (e.g. intracellular grape water, wine water etc.) is based on the direct dependence of the areas of NMR signals on the number of nuclei responsible for them. We have developed a method using an internal standard, by analogy with the well-known OIV-SNIF-NMR method [3]. Dimethyl sulfoxide (DMSO) was chosen as an internal standard, the signals of which in the NMR spectrum do not overlap with the signals of the main components of wine, and also due to the possibility to change the deuterium content using the available solvent for NMR spectroscopy DMSO-d_6. The deuterium content of the internal standard DMSO is increased by adding DMSO-d_6 to such an extent that the signal from 10-15 % of the standard is commensurate with the analyte water signal. The exact content of deuterium in the internal standard is determined by comparing the integrated signal intensities in the ^{2}H(D)-qNMR spectrum of the "Vienna Standard Mean Ocean Water" (VSMOW) with a known content of deuterium. The deuterium content of the internal DMSO standard is calibrated before starting the study. After calibration, the amount of deuterium according to the ^{2}H(D)-qNMR spectrum of the sample should provide an integral signal intensity comparable to the water signal of the unfermented or fermented object when 50 µl of DMSO is added to 550 µl of sample. The ratio (D/H) in the prepared solution is calculated after providing the necessary amount of deuterium in the standard by comparing in the ^{2}H(D)-qNMR spectrum the integral signal intensities of DMSO and an IAEA standard water sample VSMOW with known deuterium content. For this purpose, a solution consisting of 550 µl of the VSMOW standard water sample and 50 µl of DMSO is prepared. Then a ^{2}H(D)-qNMR spectrum is recorded under standard measurement conditions: 90° pulse, 3 s delay, O1 - 5 ppm, sweep - 25 ppm, 8K points per spectrum, acquisition time - 2.5 s, 1000 scans. The spectrum was processed using Bruker TopSpin 4.1.3 software. Automatic baseline correction, manual phase correction, exponential multiplication for 2.0 Hz were used to process the spectrum. An acceptable measurement accuracy is provided by the technical characteristics of the used high-resolution NMR spectrometer Bruker Avance™ NEO 700 (Germany) equipped with a cryoprobe, and by the applied measurement conditions (Fig. 1). Before NMR measurements in the studied samples of model systems, the determination of the water content was carried out. In grape musts, soluble solids content was determined on a digital laser refractometer RM-40 (Mettler Toledo, Switzerland). In samples the content of water was determined by ^{1}H-qNMR spectroscopy by subtraction of the main components - ethanol and glycerol - in the obtained NMR spectrum. Typical ^{1}H- and ^{2}H(D)-qNMR spectra of the wine materials are shown in Figures 2 & 3. Isotope ratio $(D/H)_a$ of water in model systems was calculated by equation:

$$(D/H)a = \frac{N_{st} * M_{H2O} * m_{st} * I_{H2O}}{N_{H2O} * M_{st} * m_a * I_{st} * \omega_{H2O}} * (D/H)st \qquad (1)$$

where :

- Nst is stoichiometric number of hydrogen atoms in DMSO;
- NH2O – stoichiometric number of hydrogen atoms in water;
- MH2O – molar mass of water, g/mol;
- Mst – molar mass of DMSO, g/mol;
- ma – mass of test portion, g;
- mst – mass of added DMSO, g;
- IH2O – integrated signal intensity of water sample;
- Ist – integrated signal intensity of DMSO;
- ωH2O – the water content in sample;
- (D/H)st – isotope ratio of DMSO, ppm.

2.3.2. ^{2}H(D)-qNMR-Measurements in ethanol of model systems

The distribution of deuterium in the methyl (CH_3-) and methylene (CH_2-) groups of ethanol molecules formed in model systems during fermentation in experiments *A* & *B* was studied using the analytical complex presented above in section 2.3.1, according to the OIV method [3], as well as based on methodological approach that take into account the configuration of NMR equipment [4, 5].

Figure 1. Magnet unit of the BRUKER NMR spectrometer Avance™ NEO 700 (Germany) at the RUDN University (Moscow).

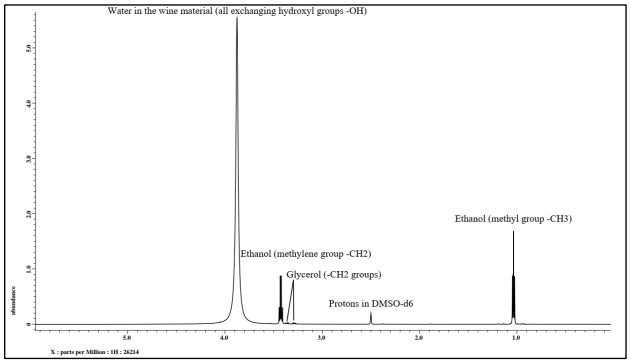

Figure 2. Typical ^{1}H-qNMR spectrum of wine.

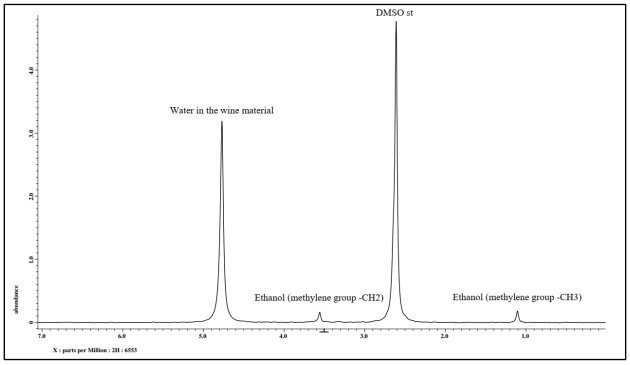

Figure 3. Typical ^{2}H(D)-qNMR spectrum of wine.

2.3.3. IRMS/SIRA Measurements of $\delta^{13}C$ in ethanol of model systems after fermentation

The determination of carbon isotopes composition ($\delta^{13}C$ value) in ethanol of model systems after fermentation was performed according to the OIV method [6] by using of the following laboratory facilities for stable isotopes measurements (Fig. 4):

- an elemental analyzer FlashEA1112® with oxidation & reduction reactors (Thermo Fisher Scientific, Germany);
- an isotopic interface Conflo III® (Thermo Fisher Scientific, Germany);
- an IRMS/SIRA isotopic mass spectrometer Delta V Advantage® (Thermo Fisher Scientific, Germany);
- a gas system Sigm-Plus® for the supply of the analytical devices with highly purified gases (Sigm-Plus Ltd., Russia);
- a PC workstation Optiplex 745® (Dell, USA) for data registration and processing by the Isodat NT 2.5® software (Thermo Fisher Scientific, Germany).

The reference substance BCR-656 “Ethanol” (European Commission, Community Bureau of Reference BCR, Individual Identification № 00425, $\delta^{13}C_{VPDB}$ = -26,91 ± 0,07 ‰) was used for the calibration of the working reference gas (WRG) - carbon dioxide 99.999 % (NIIKM, Russia). The high purity helium 99.9999 % (NIIKM, Russia) was used in the study as carrier gas.

Figure 4. IRMS/SIRA Laboratory facilities for stable isotopes measurements at the RUDN University (Moscow).

2.3.4. Residual carbohydrates in model systems after fermentation

The residual content of carbohydrates – D-glucose and D-fructose after fermentation of model systems in experiments *A* & *B* - was determined on the LC-20 Prominence HPLC chromatograph (Shimadzu, Japan) according to the OIV method [7].

2.3.5. Ethanol in model systems after fermentation

Ethanol measurements in model systems after fermentation were performed by the GC-FID- method by using of the GS-system GC 2014 (Shimadzu, Japan) with flame ionization detector (FID). Carrier gas: nitrogen. Column flow: 1 ml/min. Make-up flow: 30 ml/min. Column: fused silica capillary column 30 m length, 0.25 mm internal diameter coated with CP Wax 52 CB (Varian, Netherlands), film thickness 0.15 µm. Sample solutions were prepared by dilution of exact volume of model system with water in volumetric flask with dilution factor 10 followed by filtration through Nylon membrane filter with 0.45 µm pore size. Quantitative determination was performed by external standard calibration using the series of ethanol solutions with concentration range 1.0-20.0 vol. %. Injection: split 200, injection volume 1 µl.

Table 3. Column temperature program.

Time since injection, min	Duration of program step, min	Initial temperature °C	Final temperature °C	Speed of temperature program
0	4,5	35	35	isothermal
4,5	6,5	35	100	10 oC/min
11	2	100	100	isothermal

2.3.6. Soluble solids

Soluble solids in model systems were measured using a digital laser refractometer RM-40 with temperature compensation up to 20° C (Mettler Toledo, Switzerland) according to the OIV method [8].

3. Results and discussion

The results of studies of model systems in experiments *A* & *B* after carbohydrate fermentation are presented below in sections 4.1 and 4.2.

3.1. Model systems *A1-A3*

The composition of model systems *A1*, *A2* and *A3* after fermentation is presented in Table 4.

Table 4. Model systems *A1-A3*: main components.

No.	Component	Model sytem		
		A1	A2	A3
1	D-Glucose, g/l	f.f.	f.f.	f.f.
2	D-Fructose, g/l	10.4	2.1	1.8
4	Ethanol, g/l	84.0	89.0	88.0
Parameters:				
δ13C of ethanol, ‰		-11.12	-11.17	-11.17

Notes:

[1)] f.f. = fully fermented; [2)] $\delta^{13}C$ = typical quantitative values for ethanol from plant carbohydrates of C4 photosynthetic pathway.

Table 5 shows data on the distribution of deuterium in the molecules of the components of model systems *A1*, *A2* and *A3* after fermentation.

Table 5. Model systems *A1-A3*: deuterium distribution.

No.	Water		Ethanol		
	$(D/H)_{a\text{-}inc}$ before fermen-tation, ppm	$(D/H)_a$ after fermen-tation, ppm	$(D/H)_I$, ppm	$(D/H)_{II}$, ppm	R
A1	150.3	146.2	109.4	124.3	2.3
A2	200.5	197.4	117.9	158.6	2.7
A3	254.1	247.7	127.6	199.2	3.1

Notes:

[1)] $(D/H)_I$ = deuterium in methyl groups of ethanol molecules; [2)] $(D/H)_{II}$ = deuterium in methylene groups of ethanol molecules; [3)] $R = 2(D/H)_{II}/(D/H)$.

3.2. Model systems *B1-B3*

The composition of model systems *B1*, *B2* and *B3* after fermentation is presented in Table 6.

Table 6. Model systems *B1-B3*: main components.

No.	Component	Model sytem		
		B1	B2	B3
1	D-Glucose, g/l	f.f.	f.f.	0.7
2	D-Fructose, g/l	0.41	0.91	8.3
4	Ethanol, g/l	72.0	93.0	105.0
Parameters:				
δ13C of ethanol, ‰		-11.71	-11.48	-11.71

Notes:

[1)] f.f. = fully fermented; [2)] $\delta^{13}C$ = typical quantitative values for ethanol from plant carbohydrates of C4 photosynthetic pathway.

Table 7 shows data on the distribution of deuterium in the molecules of the components of model systems *B1*, *B2* and *B3* after fermentation.

Table 7. Model systems *B1-B3*: deuterium distribution.

No.	Water		Ethanol		
	(D/H)a-inc before fermen-tation, ppm	(D/H)a after fermen-tation, ppm	(D/H)I, ppm	(D/H)II, ppm	R
B1	244.2	231.2	125.1	189.1	3.02
B2	243.5	235.4	124.4	192.2	3.09
B3	247.6	234.1	123.6	186.9	3.03

Notes:

[1)] $(D/H)_I$ = deuterium in methyl groups of ethanol molecules; [2)] $(D/H)_{II}$ = deuterium in methylene groups of ethanol molecules; [3)] $R = 2(D/H)_{II}/(D/H)$.

3.3. Discussion

The results of both experiments demonstrate in all model systems the equivalence of the level of deuterium saturation of water after fermentation (Tab. 5 & 7) with the quantitative level in the initial deuterated water (Tab. 1 & 2), which was used to prepare the fermentation mixture (D/H_a). Minor discrepancies should be attributed to the NMR-measurement uncertainty. The initial increase in the level of deuterium in water $(D/H)_{a\text{-}inc}$ is associated with the introduction of D-glucose & D-fructose and the corresponding intensive incorporation of hydrogen from carbohydrates into model systems before fermentation. A further decrease in the deuterium content during fermentation to the level of the initial deuterated water $(D/H)_a$, which was observed in both experiments, correlates well with the results of a study of deuterium dynamics in grape processing products - must and wine, published earlier [1, 2].

The results of studying the dynamics of deuterium in the experiment with the model systems *A1*, *A2* & *A3* show that at a constant content of carbohydrates (equivalent to a constant level of deuterium from carbohydrates) and an increasing content of deuterium in the fermentation medium, an increase in the values of the $(D/H)_I$ & $(D/H)_{II}$ parameters is observed, which characterize the level of deuterium in the methyl and methylene groups of ethanol molecules. This increase indicates the participation of water deuterium in the mechanism of deuterium distribution during the fermentation process between the components of the system.

The limited data array obtained allows us to draw only a preliminary conclusion about the presence of a linear relationship between the amount of deuterium in water of the fermentation system $(D/H)_a$ and the content of deuterium in methyl groups of ethanol molecules $(D/H)_I$, formed during fermentation from carbohydrates contained in this system. This relationship can be expressed as the following equation:

$$(D/H)_I = 0.1798(D/H)_a + 82.864 \quad (2)$$

$$at\ R^2 = 0.9978$$

A similar correlation can be observed also for the methylene group $(D/H)_{II}$. The equation in this case takes the form:

$$(D/H)_{II} = 0.7388(D/H)_a + 15.074 \quad (3)$$

$$at\ R^2 = 0.9971$$

Because small data array of results obtained allows us to draw only preliminary conclusions, the necessary clarifications can be obtained by increasing the amount of experimental data.

The results of studying the dynamics of deuterium in the experiment with the model systems *B1*, *B2* & *B3* show that under conditions of a constant level of deuterium in water of fermentation systems and an increasing content of carbohydrates in it, the $(D/H)_I$ & $(D/H)_{II}$ values do not tend to increase, having reached a certain "saturation point" (equivalent values of the R parameter of the *B1*, *B2* & *B3* model systems)). Thus, we can make the assumption that the role of deuterium associated with carbohydrate molecules is reduced to achieving a certain basic level in the methyl and methylene groups of ethanol molecules. In this case, isotopic effects in relation to the methyl group $(D/H)_I$ of ethanol molecules are more complex in nature, because they are associated both with deuterium coming from carbohydrates and with deuterium in water, which forms the basis of the fermentation system.

4. Conclusions

The results of studies of isotope effects using model systems and a new methodological approach of high-resolution quantitative nuclear magnetic resonance spectroscopy $^2H(D)$-qNMR made it possible to obtain knowledge about the role of deuterium contained in aqueous-alcoholic systems in the processes of fermentation and transformation of the compounds involved in it. The results obtained correlate with previously published data [9-11] and complement them in terms of studying the mechanisms of carbohydrate fermentation in aqueous environments characterized by different levels of deuterium saturation.

The practical significance of the results obtained in this study is determined by a methodological approach based on the use of the isotopic composition of hydrogen (deuterium) as a basic value for assessing grape musts, wines and other wine products in terms of the authenticity of their main components - water and wine. In this part, the knowledge about the correlation between the levels of deuterium in the water and the ethanol contained in the same fermentation system expands the capabilities and reliability of the method for detecting added water, based

on the use of a cut-off value for the $(D/H)_a$ of ≥ 157 ppm and proposed earlier by the authors [1, 2].

The toolkit for the practical implementation of the proposed approach is the developed high-resolution quantitative ^{2}H(D)-qNMR method [1-3, 5]. In contrast to existing NMR methods [12-13] the new methodological approach allows the use of spectrometers not equipped with fluorine lock and self-made standards enriched with deuterium. The developed ^{2}H(D)-qNMR method, in contrast, for example, to the IRMS/SIRA method of mass spectrometry, does not require preliminary extraction of searched compounds from sample matrix before measurement that not only provides selectivity of determination, but also essentially increases reliability and accuracy of its results. Research on the topic of the presented study will be continued.

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7. Conflict of interest

The authors declare no conflict of interest.

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