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Study of intramolecular distribution of hydrogen isotopes in ethanol depending on deuterium content of water and the origin of carbohydrates

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Abstract. The paper presents the results of consistently developing studies carried out in 2022-2024 on the distribution of deuterium ^{2}H(D) in intracellular water of grapes and wine products, taking into account the influence of natural, climatic and technogenic factors using high-resolution quantitative nuclear magnetic resonance spectroscopy ^{2}H(D)-qNMR. The aim of the current studies was to investigate the intramolecular distribution of hydrogen isotopes on the example of deuterium ^{2}H(D) during alcoholic fermentation carried out under controlled conditions in model systems, the main components of which were water with different content of ^{2}H(D), carbohydrates of different botanical origin (D-glucose, D-fructose, sucrose) and L(+)-tartaric acid from grapes. Taking into account the known scientific data on the effect of the botanical type of carbohydrates on the hydrogen isotopes in the methyl group (CH_3-), the main emphasis in the study was placed on the evaluation of the deuterium content of the methylene groups (-CH_2-) of ethanol molecules depending on the composition of the system components. The artificially deuterated water containing ^{2}H(D) in the amount of 142-143, 175-212 and 212-284 ppm was used for the preparation of 3 series of model systems. The carbohydrate concentration in the model systems was constant at 200 g/L for all sugars - D-glucose (from grapes), D-fructose (from corn) and sucrose (from sugar beets). The concentration of the acidifier L(+)-tartaric acid was 4 g/L. The content of deuterium in water and ethanol, the isotopic composition of carbon ^{13}C/^{12}C in sugars and ethanol by IRMS/SIRA mass spectrometry, the content of ethanol (GC method) and residual sugar (HPLC method) were studied in all model systems at the beginning and end of fermentation. Experimental data were obtained on the effect of the composition of model systems on the intramolecular distribution of deuterium in ethanol formed during fermentation, as well as on changes in the hydrogen isotopy in the water of the studied model systems using high-resolution nuclear magnetic resonance spectroscopy qNMR under the conditions of the developed measurement method. The results of the fundamental study are a consistent development of the approach to solving the applied problem of assessing the presence of technologically unavoidable water in wine, which in turn provides the basis for identifying unacceptable dilution of wine (must) by adding water from underground and surface sources.

1. Introduction

The present study is a continuation 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 2022-2024 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 [1-3]. The purpose of the current part of 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 of different botanical origin - D-glucose from grapes, sucrose from sugar beets, D-fructose from corn. L(+)-Tartaric acid from grapes was used as an acidulant in the model systems.

2. Materials and methods

2.1 Reagents

Analytically pure D-glucose from grapes (NovaSweet Ltd., Russia), sucrose from sugar beets (RusHim Ltd., Russia) and D-fructose from corn (RusHim Ltd., Russia), as well as L(+)-tartaric acid from grapes (Chemical Leaders Ltd., 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. (Russia). The exact values for the deuterium content in experimental water samples and the corresponding model systems are given in Tables 1-3.

2.2. Model systems

The composition and initial parameters of the model systems used in the current study are given in Tables 1-3. The concentration of added L(+)-tartaric acid in all model systems was 4 g/l. After mixing all components, the finished model systems *Cn*, *Dn* & *En* (Note: The model system designations are a continuation of a series of experiments with model systems published in [3]) were kept before fermentation at room temperature for 1 hour for stabilizing of composition. For fermentation, *Saccharomyces cerevisiae* yeast "Classic 48 Turbo Yeast" (Hambleton Bard Ltd., United Kingdom) 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 *Cn*: *D-glucose from grapes* (before fermentation).

No.	Component	Model sytem		
		C1	*C2*	*C3*
1	D-Glucose, g/l	200	200	200
2	Water, ml	1000	1000	1000
Parameters:				
- $(D/H)_a$ of water, ppm		143.36	212.89	284.59
- Soluble solids, ºBrix		15.5	15.5	15.5

Table 2. Composition of the model systems *Dn*: *sucrose from sugar beets* (before fermentation).

No.	Component	Model sytem		
		D1	*D2*	*D3*
1	Sucrose, g/l	200	200	200
2	Water, ml	1000	1000	1000
Parameters:				
- $(D/H)_a$ of water, ppm		143.36	212.89	284.59
- Soluble solids, ºBrix		17.1	17.1	17.1

Table 3. Composition of the model systems *En: D-fructose from corn* (before fermentation)

No.	Component	Model sytem		
		E1	*E2*	*E3*
1	D-Fructose, g/l	200	100	125
2	Water, ml	1000	1000	1000
Parameters:				
- $(D/H)_a$ of water, ppm		142.13	175.16	212.04
- Soluble solids, ºBrix		16.1	16.8	16.8

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 [4]. Dimethyl sulfoxide (DMSO) was chosen as an internal standard, the signals of which in the qNMR 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 qNMR spectrometer Bruker Avance^{TM} 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. Isotope ratio $(D/H)_{st}$ of DMSO and $(D/H)_{a}$ of water in model systems was calculated by equation (1-2):

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

where *Nst* is stoichiometric number of hydrogen atoms in DMSO;

N_{H2O} – stoichiometric number of hydrogen atoms in water;

M_{H2O} – molar mass of water, g/mol;

M_{st} – molar mass of DMSO, g/mol;

m_{H2O} – weight of a sample of VSMOW, g;

m_{st} – weight of DMSO, g;

I_{H2O} – integrated signal intensity (area) of VSMOW water;

I_{st} – integrated signal intensity (area) of DMSO;

$(D/H)_{H2O}$ – isotope ratio of DMSO, ppm

$$(D/H)a = \frac{N_{st}*M_{H2O}*m_{st}*I_{H2O}}{N_{H2O}*M_{st}*m_{a}*I_{st}*\omega_{H2O}} *(D/H)st \qquad (2)$$

where *Nst* is stoichiometric number of hydrogen atoms in DMSO;

N_{H2O} – stoichiometric number of hydrogen atoms in water;

M_{H2O} – molar mass of water, g/mol;

M_{st} – molar mass of DMSO, g/mol;

m_{a} – weight of a sample, g;

m_{st} – weight of DMSO, g;

I_{H2O} – integrated signal intensity (area) of water sample;

I_{st} – integrated signal intensity (area) of DMSO;

ω_{H2O} – 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 *C* - *E* was studied using the analytical complex presented above in section 2.3.1, according to the OIV method [4], as well as based on methodological approach that take into account the configuration of qNMR equipment [5, 6].

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

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

The determination of carbon isotopes composition (δ$^{13}C_{VPDB}$ value) in ethanol of model systems after fermentation was performed according to the OIV method [7] by using of the following laboratory facilities for stable isotopes measurements (Fig. 2):

- 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 2. 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, sucrose and D-fructose after fermentation of model systems in experiments *C-E* - was determined on an LC-20 Prominence HPLC chromatograph (Shimadzu, Japan) in accordance with the OIV method [8].

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 4. 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 °C/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 [9].

3. Results and discussion

The results of studies of model systems in experiments *C-E* after carbohydrate fermentation are presented below in sections 3.1, 3.2 and 3.3.

3.1. Model systems *C1-C3*: D-glucose from grapes

The composition of model systems *C1*, *C2* and *C3* after fermentation is presented in Table 5.

Table 5. Model systems *C1-C3*: main components.

No.	Component	Model sytem		
		C1	C2	C3
1	D-Glucose, g/l	f.f.	f.f.	f.f.
2	Ethanol, g/l	8.4	8.4	8.4
Parameters:				
δ13CVPDB of ethanol, ‰		-27.05	-27.53	-27.22

Notes:
1) f.f. = fully fermented.
2) $\delta^{13}C_{VPDB}$ = typical quantitative values for ethanol from grapes carbohydrates (C3 photosynthetic pathway).

Table 6 shows data on the distribution of deuterium in the molecules of the components of model systems *C1*, *C2* and *C3* after fermentation.

Table 6. Model systems *C1-C3*: deuterium distribution.

No.	Water		Ethanol		
	$(D/H)_{a\text{-inc}}$ before fermentation, ppm	$(D/H)_a$ after fermentation, ppm	$(D/H)_I$, ppm	$(D/H)_{II}$, ppm	R
C1	150.38	146.45	94.56	121.72	2.57
C2	212.97	210.18	106.55	164.33	3.08
C3	286.50	276.08	116.92	209.31	3.58

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 *D1-D3*: sucrose from sugar beets

The composition of model systems *D1*, *D2* and *D3* after fermentation is presented in Table 7.

Table 7. Model systems *D1-D3*: main components.

No.	Component	Model sytem		
		D1	*D2*	*D3*
1	Sucrose, g/l	f.f.	f.f.	0.7
2	Ethanol, g/l	9.8	9.8	9.8
Parameters:				
$\delta^{13}C_{VPDB}$ of ethanol, ‰		-26.62	-27.05	-26.70

Notes:
1) f.f. = fully fermented.
2) $\delta^{13}C$ = typical quantitative values for ethanol from sugar beets carbohydrates (C3 photosynthetic pathway).

Table 8 shows data on the distribution of deuterium in the molecules of the components of model systems *D1*, *D2* and *D3* after fermentation.

Table 8. Model systems *D1-D3*: deuterium distribution.

No.	Water		Ethanol		
	(D/H)a-inc before fermentation, ppm	(D/H)a after fermentation, ppm	(D/H)I, ppm	(D/H)II, ppm	R
D1	149.83	147.30	89.39	124.75	2.79
D2	219.23	211.85	100.92	174.28	3.45
D3	289.83	280.81	111.41	220.30	3.95

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. Model systems *E1-E3*: D-fructose from corn

The composition of model systems *E1*, *E2* and *E3* after fermentation is presented in Table 9.

Table 9. Model systems *E1-E3*: main components.

No.	Component	Model sytem		
		E1	E2	E3
1	D-Fructose, g/l	f.f.	f.f.	f.f.
2	Ethanol, g/l	8.3	8.3	8.3
Parameters:				
δ13CVPDB of ethanol, ‰		-12.00	-11.41	-11.44

Notes:
1) f.f. = fully fermented.
2) $\delta^{13}C_{VPDB}$ = typical quantitative values for ethanol from corn carbohydrates (C4 photosynthetic pathway).

Table 10 shows data on the distribution of deuterium in the molecules of the components of model systems *E1*, *E2* and *E3* after fermentation.

Table 10. Model systems *E1-E3*: deuterium distribution.

No.	Water		Ethanol		
	(D/H)a-inc before fermentation, ppm	(D/H)a after fermentation, ppm	(D/H)I, ppm	(D/H)II, ppm	R
E1	144.80	146.44	110.08	122.84	2.23
E2	176.56	176.59	113.59	145.70	2.57
E3	209.82	211.94	120.11	175.32	2.92

4. Discussion

The results of the performed experiments are in good agreement with the 2024 results published in [3]. They demonstrate in all model systems the equivalence of the level of deuterium saturation of water after fermentation (Tab. 6, 8 & 10) with the quantitative level in the initial deuterated water (Tab. 1-3), which was used to prepare the fermentation mixture $(D/H)_a$. Minor discrepancies should be attributed to the qNMR-measurement uncertainty. The initial increase in the level of deuterium in water $(D/H)_{a\text{-inc}}$ is associated with the introduction of carbohydrates (D-glucose, sucrose & 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 all experiments, correlates well with the results of a study of deuterium dynamics in grape processing products - must and wine, published earlier [1-3].

The results of studying the dynamics of deuterium in an experiment with the model systems show that at a constant content of studied carbohydrates – D-glucose, D-fructose & sucrose (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 data array obtained in 3 series of experiments in this study allows us to draw 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 equations (3-5):

For D-glucose from grapes:

$$(D/H)_I = 0.1583(D/H)_a + 72.205 \quad (3)$$

at $R^2 = 0.9974$

For D-fructose from corn:

$$(D/H)_I = 0.1441(D/H)_a + 89.165 \quad (4)$$

at $R^2 = 0.9806$

For sucrose from sugar beet:

$$(D/H)_I = 0.1772(D/H)_a + 63.717 \quad (5)$$

at $R^2 = 0.9987$

Quantitative contribution of water deuterium $(D/H)_a$ in the formation of the total hydrogen fingerprint of methyl groups $(D/H)_I$ in ethanol molecules, according to the results of the research, is equal to an average coefficient of *0.16*. This value shows the minor role of water deuterium in the mechanism of formation of the total hydrogen fingerprint of methyl groups of ethanol molecules. A more significant contribution to the formation of the total hydrogen fingerprint of methyl groups $(D/H)_I$ in ethanol molecules is made by the deuterium coming from carbohydrates. At the same time, the level of saturation directly depends on the botanical origin of carbohydrates.

A similar correlation can be observed also for the methylene group $(D/H)_{II}$. The equations (6-8) in this case take the form:

For D-glucose from grapes:

$$(D/H)_{II} = 0.6203(D/H)_a + 32.630 \quad (6)$$

at $R^2 = 0.9999$

For D-fructose from corn:

$$(D/H)_{II} = 0.9295(D/H)_a + 13.055 \quad (7)$$

at $R^2 = 0.9996$

For sucrose from sugar beet:

$$(D/H)_{II} = 0.6764(D/H)_a + 28.627 \quad (8)$$

at $R^2 = 0.9990$

Quantitative contribution of water deuterium $(D/H)_a$ in the formation of the total hydrogen fingerprint of methylene groups $(D/H)_{II}$ in ethanol molecules, according to the results of the current research, is much more pronounced than in the case of methyl groups $(D/H)_I$. At the same time, the average coefficient was 0.74, which is more than 4 times higher than the quantitative level characterizing the contribution of water deuterium $(D/H)_a$ by saturation of methyl groups. The results of the current study also show that the botanical nature of carbohydrates (e.g., D-glucose from grapes vs. D-fructose from corn) influences the formation of deuterium ^{2}H(D) saturation level of methylene groups $(D/H)_{II}$ of ethanol molecules formed during fermentation in aqueous medium with the same deuterium ^{2}H(D) content.

According to the research results, we can make the assumption that the role of deuterium ^{2}H(D) associated with carbohydrate molecules is reduced to achieving a certain basic level in the methyl and methylene groups of ethanol molecules. The 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 ^{2}H(D) coming from carbohydrates and with deuterium in water, which forms the basis of the fermentation system.

5. Conclusions

The results obtained by using model systems and a new methodological approach of high-resolution quantitative nuclear magnetic resonance spectroscopy ^{2}H(D)-qNMR correlate with previously published data [10-12] and complement them in terms of studying the mechanisms of carbohydrate fermentation in aqueous environments characterized by different levels of deuterium saturation. 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 [1-3]. The toolkit for the practical implementation of the proposed approach is the developed high-resolution quantitative ^{2}H(D)-qNMR method [1-3, 5, 6].

In contrast to existing NMR methods [13, 14] 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. In addition, the results obtained in this study and in the 2024 work, characterizing the relationships between deuterium levels in system water $(D/H)_a$ and fermentation products, provide a basis for predictive modeling and evaluating of, for example, deuterium levels in ethanol $(D/H)_I$ & $(D/H)_{II}$ without performing grape must fermentation. In turn, this may have applied value, for example, when evaluating the geographical and botanical origin as well as general authenticity of wines and other grape processing products. Research on the topic of the presented study will be continued.

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8. Conflict of Interest

The authors declare no conflict of interest.

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