Method OIV-MA-VI-24 : R2015
Type IV method

Determination of the distribution of deuterium in acetic acid extracted from wine vinegar using nuclear magnetic resonance (NMR)

(OIV-OENO 527-2015)

1. Scope of application

This method makes it possible to analyse the isotope ratio of hydrogen $(D/H)_{CH3}$ at the methyl site of acetic acid extracted from wine vinegar according to the procedure described in the method OIV-OENO 510-2013. The analysis is carried out by ^{1}H-NMR and ^{2}H-SNIF-NMR using a composite NMR experiment (Fauhl et al., 1996; Hermann, 2001). The ^{1}H-NMR experiment is used to determine the ratio between tetramethylurea (TMU) and acetic acid, which is then used, together with the results from the ^{2}H-SNIF-NMR experiment, to calculate the $(D/H)_{CH3}$ isotope ratio. If the acetic acid extracted is pure (> 99%) or if the exact content of acetic acid in the extract is known, the ^{1}H-NMR experiment is not necessary.

2. Definition

$(D/H)_{CH3}$: isotope ratio associated with the molecule CH_2D COOH

3. Principle

The deuterium contained in the sugars and the water in grape must will be redistributed after alcoholic fermentation in molecules I, II, III and IV of the wine ethanol and after acetic fermentation in molecules V and VI of the acetic acid:

$CH_2D\ CH_2$ OH	$CH_3\ CHD$ OH	$CH_3\ CH_2$ OD	HOD	CH_2D COOH	CH_3 COOD
I	II	III	IV	V	VI

The parameter $(D/H)_{CH3}$ expresses the D/H ratio of the sugars in the must.

4. Reagents and products

- Hexafluorobenzene (C_6F_6), used as a field-frequency stabilisation substance (lock),
- or, alternatively trifluoroacetic acid (TFA, CAS: 76-05-1) or trifluoroacetic anhydride (TFAA, CAS: 407-25-0), standard N,N-tetramethylurea (TMU); standard TMU with a calibrated D/H isotope ratio,
- 10 mm NMR tubes (probes),
- 0.45 µm filter.

5. Apparatus

NMR spectrometer fitted with a specific deuterium probe tuned to a frequency □0, characteristic of the field B0 (e.g. where B0 = 7.05 T, □0 = 46.05 MHz and where B0 = 9.4 T, □0 = 61.4 MHz) with a proton decoupling channel (B2) and a field-frequency stabilization channel (lock) at the fluorine frequency. The second channel, B2, is also used for the 1H-NMR experiment. The resolution measured on the spectrum, transformed without exponential multiplication (i.e. LB = 0) and expressed as the width at half height of the methyl signal of acetic acid and the methyl signal of TMU, must be less than 0.5 Hz. The sensitivity (signal-to-noise ratio), measured with an exponential multiplying factor LB equal to 2, must be greater than or equal to 150 for the methyl signal of acetic acid containing less than 25% water. For example, using an NMR spectrometer of field B0 = 7.05 T, 200 scans are required to reach this value.

- balance with a precision of 0.1 mg or greater,
- balance with a precision of 0.1 g or greater.

The laboratory apparatus and consumables set out in sections 5 and 6 are examples and may be replaced by other materials with equivalent performance.

6. Preparation of samples

Acetic acid must be extracted from the vinegar and purified in order to be analysed by SNIF-NMR. At least 6 mL of pure acetic acid must be recovered at the end of the extraction.

The extraction and purification procedure is described in the method OIV 510-2013.

7. Procedure

Preliminary note: the amount and type of reagents and the instrumental conditions depend on the type of apparatus used. The procedure described here is merely an example.

All the preparatory stages must be carried out without any significant evaporation of acetic acid, which would change the isotopic composition of the sample.

7.1. Preparation of the acetic acid sample for NMR measurement

NMR tube 10 mm in diameter: in a pre-weighed glass vial, collect 3.25 g of the solution containing acetic acid obtained in step 6 and weigh to the nearest 0.1 mg (m_A); then add 1.1 g of internal standard TMU and reweigh the flask to the nearest 0.1 mg (m_{ST}).

Depending on the type of spectrometer and probe used, add a sufficient amount of hexafluorobenzene as a field-frequency stabilization substance (lock):

Spectrometer	10 mm probe
7.05 T	150 µL
9.4 T	35 µL

These figures are indicative and the actual volume to be used should be adjusted according to the sensitivity of the NMR apparatus. During the probe preparation

operations and until the NMR measurement is carried out, the operator must ensure that the acetic acid and TMU do not evaporate, as this would result in isotope fractionation.

The sample is then poured into the NMR probe and, if necessary, filtered using a syringe fitted with a 0.45 μm filter.

N.B.: It is recommended that the NMR probe be prepared under a fume hood, for which gloves and protective goggles should be worn.

7.2. Acquisition of the NMR spectrum

The following conditions are recommended when acquiring the ^{2}H-SNIF-NMR spectrum:

- probe temperature must be constant (e.g. 303 K),
- the sample should be rotated (e.g. 15-20 Hz,)
- acquisition time of at least 5.5 s at a spectral width of 1200 Hz (memory 16 K) (i.e. about 20 ppm at 61.4 MHz or 27 ppm at 46.1 MHz),
- 90° pulse angle,
- adjustment of acquisition time: its value must be of the same order as the dwell time,
- parabolic detection: fix the offset 01 between the OD and CH2D reference signals for acetic acid,
- determine the value of the decoupling offset 02 from the proton spectrum measured by the decoupling coil on the same tube. Good decoupling is obtained when 02 is located in the middle of the frequency interval between the CH3- and TMU groups. Use the wide band decoupling mode or composite pulse sequences (e.g. WALTZ16) to ensure uniform decoupling across the entire spectrum.

For each spectrum, carry out a set of NS accumulations sufficient to obtain the signal-to-noise ratio indicated in step 6, and repeat the set of NS accumulations five times. The NS values will depend on the type of spectrometer and probe used.

The conditions suggested for obtaining the ^{1}H-NMR spectrum are as follows:

- probe temperature must be constant (e.g. 303 K),

- the sample should be rotated (e.g. 15-20 Hz),
- acquisition time of at least 4.1 s for an 8000 Hz spectral width (16 K memory),
- pulse angle of 30° or lower,
- D1=7s at least,
- parabolic detection: fix the offset 01 between the OH and CH3 reference signals for acetic acid,
- absence of decoupling.

For ^{1}H-NMR, 16 scans and 4 tests are sufficient to get an appropriate signal-to-noise ratio. 5 repetitions will also be needed.

8. Calculation of results

Appropriate software based on a complex signal processing algorithm determined by the method of least squares must be adopted to determine the area of the signal (phase and baseline are sensitive parameters that must be properly adjusted). If there are no significant phase and baseline errors (e.g. using appropriate corrections), then other software may be adopted.

For each of the ^{1}H-NMR spectra, calculate the RH ratio as follows:

$$RH = S_{TMU}/S_{acetic\ acid}$$

where S is the area of the ^{1}H-NMR signal, provided by the data processing software according to the Fourier-transformed Free Induction Decay, with a bandwidth equal to 0.5 Hz.

Calculate the mean number of repetitions and standard deviation.

Determine for each of the ^{2}H-SNIF-NMR spectra:

the RD ratio as follows:

$$RD = S_{acetic\ acid}/S_{TMU}$$

Where S' is the area of the ^{2}H-SNIF-NMR signal, provided by the data processing software according to the Fourier-transformed Free Induction Decay, with a bandwidth equal to 2 Hz;

the D/H_{CH3} (ppm) as follows:

$$(D/H)_{CH3} = RH \times RD \times D/H_{TMU}$$

where $(D/H)_{TMU}$ is the isotope ratio of the internal standard (TMU) indicated on the certificate issued by IRMM.

Calculate the mean number of repetitions and standard deviation.

Optional software enables these calculations to be performed online.

9. Quality control of the analyses

The sensitivity and resolution of the spectrometer must be checked in accordance with the specifications of the apparatus (5).

10. Method characteristics

A preliminary study was carried out to characterize the method.

The standard deviation of the repeatability (Sr) averages 0.4 ppm and the mean repeatability limit (r) is 1.0 ppm.

The standard deviation of the reproducibility (SR) averages 0.5 ppm and the mean reproducibility limit (R) is 1.4 ppm.

The details and results of the study are presented in Annex A.

The mean repeatability limit (r) and the mean reproducibility limit (R) are comparable to those observed for alcohol and wine (OIV 426-2011).

11. Bibliography

1. Hermann ,A. (2001) Determination of D/H isotope ratio in acetic acid from vinegars and pickled products by H-2-NMR spectroscopy. Eur Food Res Techn, 212 683-686
2. Fauhl, C., Wittkowski , R. (1996) On-line 1H-NMR to facilitate tube preparation in

SNIF-NMR analysis. Z Lebensm Unters Forsch (1996), 541-545

3. Thomas, F., Jamin, E. (2009) (2)H NMR and (13)C-IRMS analyses of acetic acid from vinegar, (18)O-IRMS analysis of water in vinegar: International collaborative study report. *Analytica Chimica Acta*, 649, 98-105

4. Method OIV-OENO 510-2013: Method for $^{13}C/^{12}C$ isotope ratio determination of acetic acid in wine vinegar by isotopic mass spectrometry

5. Method OIV-OENO 426-2011: Determination of the deuterium distribution in ethanol derived from fermentation of grape musts, concentrated grape musts, grape sugar (rectified concentrated grape musts) and wines by application of nuclear magnetic resonance (SNIF-NMR/).

Annex A: Results of the method characterisation

This document sets out the results of the method characterisation study into the method of analysis of the $(D/H)_{CH3}$ ratio in acetic acid extracted from wine vinegar.

An internal repeatability study was performed on 2 samples of vinegar. The results are reported in the table below.

Internal repeatability of 2 samples extracted and analysed 10 times

Repetition	Sample 1	Sample 2
	$(D/H)_{CH3}$	$(D/H)_{CH3}$
1	100.8	104.9
2	101.7	104.9
3	100.9	104.7
4	101.3	104.4
5	101.0	104.9
6	102.1	104.8

7	101.8	104.5
8	101.2	104.6
9	101.6	104.3
10	101.0	104.8
Sr	0.4	0.2
Repeatability limit r (2.8 × S_r)	1.2	0.6

An international collaborative study was carried out in 2012 involving 3 laboratories.

1. Participating laboratories

The study involved three laboratories:

Eurofins, Nantes	France
FEM-IASMA, San Michele all'Adige, Trento	Italy
Landesuntersuchungsamt, Speyer	Germany

2. Samples

The study included 4 samples of wine vinegar (Nos. 1-4) and 6 (Nos. 5-10) samples taken from a wine supplemented with increasing percentages (10, 14, 20, 33, 40, 42%) of sugar cane alcohol and then subjected to acetic fermentation. The acetic acid was extracted from each sample and the $(D/H)_{CH3}$ ratio analysed. The results are shown in the table below.

	$(D/H)_{CH3}$	SR	Reproducibility limit R (2.8 × S_R)

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SAMPLE	lab1	lab2	lab3		
1	104.8	105.4	104.8	0.4	1.1
2	105.0	105.0	104.5	0.3	0.8
3	106.0	106.0	104.8	0.7	2.0
4	106.2	106.5	106.9	0.3	0.8
5	106.9	106.6	106.6	0.2	0.6
6	107.7	108.2	107.6	0.3	0.8
7	107.3	107.9	107.3	0.3	0.8
8	108.5	107.5	108.6	0.6	1.7
9	109.2	109.2	107.9	0.8	2.2
10	108.9	109.5	107.5	0.1	2.8
mean SR				0.5	1.4