OIV-MA-AS316-01 Quantitation of glucose, malic acid, acetic acid, fumaric acid; shikimic acid and sorbic acid in wine using quantitative nuclear magnetic resonance spectrometry (^1H-NMR)

Type IV method

1. Introduction

NMR spectroscopy is a so called primary quantitative analytical technique with broad linear ranges (5-6 orders of magnitude). Multiple suppression of water and ethanol signals can significantly increase the sensitivity for matrices containing water and ethanol (such as wine) during automated measurements. Various compounds in wine can be identified and quantified by specific signals in only one analytical run.

2. Scope

The described method is suitable for the quantitative determination of glucose, malic acid, acetic acid, fumaric acid, shikimic acid at their natural concentrations in wine and in addition for the preserving agent sorbic acid.

Working range mg/L:

Glucose	600-50000
Malic acid	300-5000
Acetic acid	30-2000
Fumaric acid	20-300
Shikimic acid	20-500
Sorbic acid	20-800

3. Abbreviations

NMR: Nuclear Magnetic Resonance

^1H NMR :Proton Nuclear Magnetic Resonance

TSP :3 - (trimethylsilyl) propionic acid sodium salt

PULCON: PULse length based CONcentration determination

4. Principle

The wine sample is diluted with 10% buffer in D_2O. TSP is used as an internal standard to adjust the chemical shift (□ (0 ppm). After that the sample is measured by 1H NMR. At least single water suppression has to be applied. To increase sensitivity, also suppress the signal from the ethanol. The selected wine analytes are evaluated and quantified using appropriate signals (see 11.1 Annex Table 1).

5. Reagents and materials

The reagents used and the water (5.8) must be free from the analytes to be determined. Unless otherwise stated, solution means an aqueous solution.

5.1. D_2O (99.9 atom % D) CAS 7789-20-0

5.2. (trimethylsilyl) propionic acid 2,2,3,3-d4 sodium salt (TSP) (98 atom % D) CAS 24493-21-8

5.3. Buffer-Solution pH 2.9 – 3.3 to adjust the pH of wine

For example: 1 M KH2PO4 (Potassium dihydrogen phosphate, CAS 7778-77-0, 0.1 % TSP (3 - (trimethylsilyl) propionic acid sodium salt) (5.2),

3 mM NaN_3 (sodium azide, CAS 26628-22-8) in D_2C (5.1), pH 3.10

5.4. 1 M hydrochloric acid, CAS 7647-01-0

5.5. 1 M sodium hydroxide, CAS 1310-73-2

5.6. Buffer solutions for the calibration of electrodes

pH 4.00 and pH 2.00

(certified Reference Material, e. g. Certipur®)

5.7. Citric acid monohydrate, CAS 5949-29-1

5.8. Agua ultrapura, ISO 3696.

6. Apparatus

The membrane filters (6.8) must be free from the analytes to be determined.

6.1. 5 mm NMR tubes, max. ± 1 % inner diameter deviation

6.2. Spinner with template for adjustment of NMR tubes

6.3. Device for pH measurement (± 0.01 pH units)

6.4. Automatic titration system (adjustment of the pH to ± 0.01 pH units) alternativ manual adjustment

6.5. NMR spectrometer, for example, 400 MHz with 5 mm probe (z-Gradient) and temperature stabilization ± 0.2K

6.6. 100 - 1000 µl pipettes

6.7. 1.5 ml reaction vials

6.8. Polyvinylidene Fluoride (PVDF) membrane filter, 0.2 µm pore size, 15 mm diameter

7. Sampling

Wine samples must be clear and have to be filtered (6.8) otherwise. For the sample preparation, wines are diluted with 10% buffer in D_2O. TSP is used as an internal standard for referencing the chemical shift to □ 0 ppm. The final pH of the sample solutions should be 3.10 ± 0.02.

For example, 900 µl of wine is mixed with 100 µl buffer (5.3) and the pH adjusted exactly to 3.10 (+/- 0.02 pH units) with hydrochloric acid (5.4) or sodium hydroxide (5.5) in order to use the quantification parameters given in 11.1 Annex Table 1. For this purpose, suitable automatic titration systems or manual adjustment with a sensitive pH meter should be used. From this mixture 600 µl is transferred in a 5 mm NMR tube and is directly measured.

8. Procedure

8.1. NMR Spectrometer and Measurement

The measurements have to be performed with water and ethanol multipresaturation suppression schemes. Several techniques for suppressing unwanted signals, such as selective multi-presaging and watergate, are available. To properly suppress the water signal, spectrometers are equipped with appropriate pulse sequences.

The signal-to-noise ratio of the selected signals for quantification of the analytes shall be more than 10:1.

In principle, the essential parameters are as follows:

The recycling delay has to be at least 6 sec.

The measurements have to be performed at 300 K (27 °C) with temperature stability of ± 0.2 K without rotation.

Resolution equal or better 16 points/Hz

The sweep width (SW) must be equal or greater than 18 ppm.

The calibration and measurements of wine samples must be carried out under the same pulse angle.

8.2. Quantification of compounds

The quantification is performed on the recommended signal areas/intensities of the analytes (see 11.1 Annex Table 1). It can be done by internal or external (PULCON) standardization and calibration. The signals of appropriate analytes have to be assigned (see 11.1 Annex Table 1). Pure standards in comparable concentrations have to be measured under the same conditions as samples in order to obtain the additional response correction factor for the analyte in question (e.g. by spiking experiments). Suitable signals of the target substances are listed in 11.1 Annex Table 1.

9. Calculations

9.1. Calculation with internal standard

For the quantification, appropriate signals of analytes can be evaluated according to the following formula (internal standard):

$$m_x = \frac{MW_x}{MW_{std}} \cdot \frac{nH_{std}}{nH_x} \cdot \frac{A_x}{A_{std}} \cdot m_{std} \cdot CF$$

m_x and m_{std}	Masses of analyte and standard [g]
MW_x and MW_{std}	Molecular weights of analyte and standard [g/mol]
nH_x and nH_{std}	Numbers of protons of analyte and standard
A_x and A_{std}	Areas for the selected peaks of analyte and standard
x	Analyte
std	Reference standard, internal standard (e.g. TSP 5.2)
CF	Correction factor – see 11.1 Annex Table 1 (if needed, obtained by spiking experiments of the analyte in question)

9.2. Calculation with external standard, PULCON method

For the quantification, appropriate signals of analytes can be evaluated according to the PULCON method by the following formula F1. For quantification an external sample with known concentration of suitable substances for calibration is used each run, Quantref sample (citric acid 20 g/L). The resulting quantification factor is part of the data of each sample. The Quantref sample is used to calculate qf according to formula F2. The PULCON method is based on the following formula:

F. 1
$$\gamma_{An} = \frac{I_{An} \cdot SW_{An} \cdot M_{An} \cdot P_{An} \cdot k_{An}}{SI_{An} \cdot qf \cdot P_{Ref} \cdot N_{H,An} \cdot d_{An}^2 \cdot NS_{An}}$$

with:

- γ_{An} = searched analyte mass concentration (in mg/L)
- I_{An} = absolute integral of analyte in sample

- SW_{An} = spectral width (e.g. 20.55 ppm)
- M_{An} = molar mass of the analyte (g/mol)
- P_{An} = excitation pulse length used for the sample (in µs)
- k_{An} = correction factor (if needed, obtained by spiking experiments of the analyte in question)
- SI_{An} = Size (e.g. 131072)
- qf = mean value quantification factor from QuantRef
- P_{Ref} = excitation pulse length used for the QuantRef (in µs)
- $N_{H,\,An}$ = number of protons per analyte molecule giving this resonance
- d_{An} = inner diameter of the analyte tube
- NS_{An} = number of acquired FIDs for the analyte tube

F. 2 $$qf = \frac{I_{Ref} \cdot SW_{Ref} \cdot M_{Ref}}{SI_{Ref} \cdot \gamma_{Ref} \cdot N_{H,\,Ref} \cdot d_{Ref}^2 \cdot NS_{Ref}} \left(\text{in } \frac{\text{a.u.} \cdot \text{ppm} \cdot \text{l}}{\text{mol}}\right)$$

With:

- I_{Ref} = absolute integral of the reference signal
- SW_{Ref} = spectral width (e.g. 20.55 ppm)
- M_{Ref} = molar mass of the reference substance (g/mol)
- SI_{Ref} = size (e.g. 131072 = 128k = 2^{17})
- γ_{Ref} = mass concentration of the reference substance
- $N_{H,\,Ref}$ = number of protons per reference molecule giving this resonance
- d_{Ref} = inner diameter of sample tube for the reference tube
- NS_{Ref} = number of acquired FIDs for the reference tube

Furthermore, it is mandatory to acquire both reference and analyte spectra respectively with the same setting for receiver sensitivity (receiver gain). It is also advisable to keep all

measurement parameters the same (temperature, the type of sample tube, SI, SW, NS) when determining qf or y_{An} respectively.

9.3. Expression of results

Results should be expressed in mg/L with one decimal for minor constituents (<1 g/L)

and without decimals for major constituents (>1 g/L).

10. Precision

A ring trial was conducted to provide a realistic indication of the method performance. Details are given in Godelmann et al. 2016.

10.1. Repeatability and reproducibility

Table 1 summarizes the relative standard deviations of the repeatability and reproducibility for the different analytes (Godelmann et al. 2016).

Table 1: Relative standard deviations of the repeatability and reproducibility, taken from the proficiency test (Godelmann et al. 2016) for the different analytes.

	Glucose	Malic acid	Acetic acid	Fumaric acid	Shikimic acid	Sorbic acid
NMRP01 (Model wine)						
Number of laboratories	15	14	15	14	13	14
Mean mg/L	9903	2628	1056	82.1	103.6	126.1*
RSDr %	1.5	1.7	1.4	1.5	1.8	7.5
RSDR %	5.5	4.3	7.5	12.5	5	11.9
NMRP02 (white wine)						
Number of laboratories	15	14	15	14	13	15
Mean mg/L	28304	3528	868	12.1 (<LOQ)	45.8	164.6
RSDr %	1.3	1.7	1.6		4	2.1
RSDR %	4.6	14.3	9		18.3	5.1
NMRP03 (red wine)						

Number of laboratories	14	9	14	13	13	14
Mean mg/L	11505	240 (<LOQ)	544	29.1	34.6	149.7
RSDr %	2.2		3.6	3.1	14.7	2.7
RSDR %	4.3		7.6	9.1	20	5.4
NMRP04 (red wine)						
Number of laboratories	14	11	14		13	5
Mean mg/L	12538	251	627	n.d.	61.8	10.9 (<LOQ)
RSDr %	1.7	6.6	2.7		9.4	
RSDR %	3.9	11.4	5.8		15.3	
NMRP05 (white wine)						
Number of laboratories	14	13	14	13	13	15
Mean mg/L	30303	2615	1011	27.1	29.3	191.8
RSDr %	3.2	4.6	2.7	3	8.8	3.9
RSDR %	5	13.7	7	5.3	15.6	7
NMRP06 (red wine)						
Number of laboratories	14	11	14	4	12	14
Mean mg/L	6090	267	630	n.d.	30	158.5

RSDr %	3	6.1	2.1		9.6	4.3
RSDR %	5.3	10.2	4.9		12.6	6.3
NMRP07 (white wine)						
Number of laboratories	13	10	14	14	13	4
Mean mg/L	5297	8036	794	n.d.	36.2	3.2 (<LOQ)
RSDr %	1.5	3.7	2.6		11.5	
RSDR %	6.3	5.9	7.4		14.7	
NMRP08 (white wine)						
Number of laboratories	14	14	14	13	13	6
Mean mg/L	16518	5254	301	n.d.	68.4	5.4 (<LOQ)
RSDr %	1	2.2	3		4.2	
RSDR %	4.5	8.6	7.1		6.4	
NMRP09 (white wine)						
Number of laboratories	14	12	14	14	13	15
Mean mg/L	2091	2390	176	45.9	44.1	98
RSDr %	3.1	2.6	4.6	2.2	4.3	1.8
RSDR %	6.6	5.7	12	4.6	8.4	5.1

NMRP10 (red wine)						
Number of laboratories	9	7	14	15	13	15
Mean mg/L	442 (<LOQ)	178 (<LOQ)	502	90.6	58	200.3
RSDr %			2.4	2.5	5.2	2.9
RSDR %			11.7	7.7	16.4	6.8

10.2. Limit of detection and limit of quantification

The limit of detection (LOD) and the limit of quantification (LOQ) need to be estimated individually by the laboratories and depend on the NMR system used (particularly on the field strength). Nevertheless to give an indication the LODs and LOQs listed in Table 2 were calculated according to the instructions of the resolution OENO 7-2000 (E-AS1-10-LIMDET) on basis of sample preparation and parameters of NMR measurement previously (400 MHz, and using ethanol suppression).

Table 2: Limit of detection and limit of quantification

Analyte	LOD mg/L	LOQ mg/L
Glucose	150	600
Malic acid	90	300
Acetic acid	10	30
Fumaric acid	5	20
Shikimic acid	5	20
Sorbic acid	5	20

11. Annex

11.1. Table 1 Quantification Parameters, according 400 MHz, pH 3.10

Analyte	Molarity (g/mol)	Signal used for Quantification	n of Protons	Description	Typical Region (example) ppm	Correction Factor	Comment for Correction
Malic Acid	134	H3B, 4 Signals @ approx. 2.88ppm	1	Region: Left Signal + 4.5Hz, Right Signal -4.5Hz Local Baseline-Correction (typically zero order)	2.8465-2.9216	1.05	Due to truncated region
Sorbic Acid	112	Half of H2, Left signal of Doublet @ approx. 5.85 ppm	0.5	Region: Left Signal of Doublet ± 7Hz Local Baseline-Correction (typically zero order)	5.8250-5.8600	0.95	Due to roof-effect of doublet
Fumaric Acid	116	H2+H3, Singlet @ approx. 6.75 ppm	2	Region: Signal ± 3Hz Local Baseline-Correction (zero/first order)	6.7350-6.7600	1.00	-
Acetic Acid	60	H2A + H2B + H2C, Singlet @ approx. 2.07 ppm	3	Region: Signal ± 3Hz Local Baseline-Correction (zero order)	2.0720-2.0830	1.28 (combined factor)	Due to T1 (factor 1.16) and truncated region (factor 1.1)
Glucose	180	H1-alpha, Doublet @ approx. 5.21 ppm	1	Region: Center of Doublet ± 5Hz Local Baseline-Correction (zero order)	5.1900-5.2300	2.50 (combined factor)	alpha/beta-glucose (factor 2.33), truncated region (factor 1.07)
Shikimic Acid	174	H2, Higher Order (5) @ approx. 6.8 ppm	1	Region: Center of Multiplet ± 6Hz Local Baseline-Correction (zero/first order)	6.7850-6.8200	1.00	-

11.2. Trueness/Recovery

The recovery was determined for the ring test samples by comparing the mean values of the NMR method with respective analysis of the relevant parameter by the OIV method (for acetic acid enzymatic/HPLC methods and fumaric acid HPLC method were used)

The calculated recoveries are for glucose 100.3 % (n=9, 95.2 – 106.4), for acetic acid 18.9

% (n=10, 99.9 – 123), for malic acid 104.1 % (n=7, 91.4 – 124.1), for shikimic acid 105.2 % (n=10, 91.2 – 122.3), for sorbic acid 10.2 % (n=8, 97.9 – 102.5) and for fumaric acid 96.8 % (n=6, 80.5 – 104.1). Only for the parameter acetic acid were yielded always findings of above

100% with a total mean of 109%. For these findings, the correction factor of 1.28 could be the reason – see 11.1 Annex Table 1. The recoveries are calculated on the basis of analysis of reference values originating from OIV methods.

Table 2 Recoveries of analytes

Analyte	Mean recovery % n: number of ring test samples calculated	Variation %
Glucose	100.0 (n=9)	95.2 – 106.4
Malic acid	104.1 (n=7)	91.4 – 124.1
Acetic acid	108.9 (n=10)	99.9 123
Fumaric acid	96.8 (n=6)	80.5 – 104.1

Shikimic acid	105.2 (n=10)	91.2 – 122.3
Sorbic acid	100.2 (n=8)	97.9 – 102.5

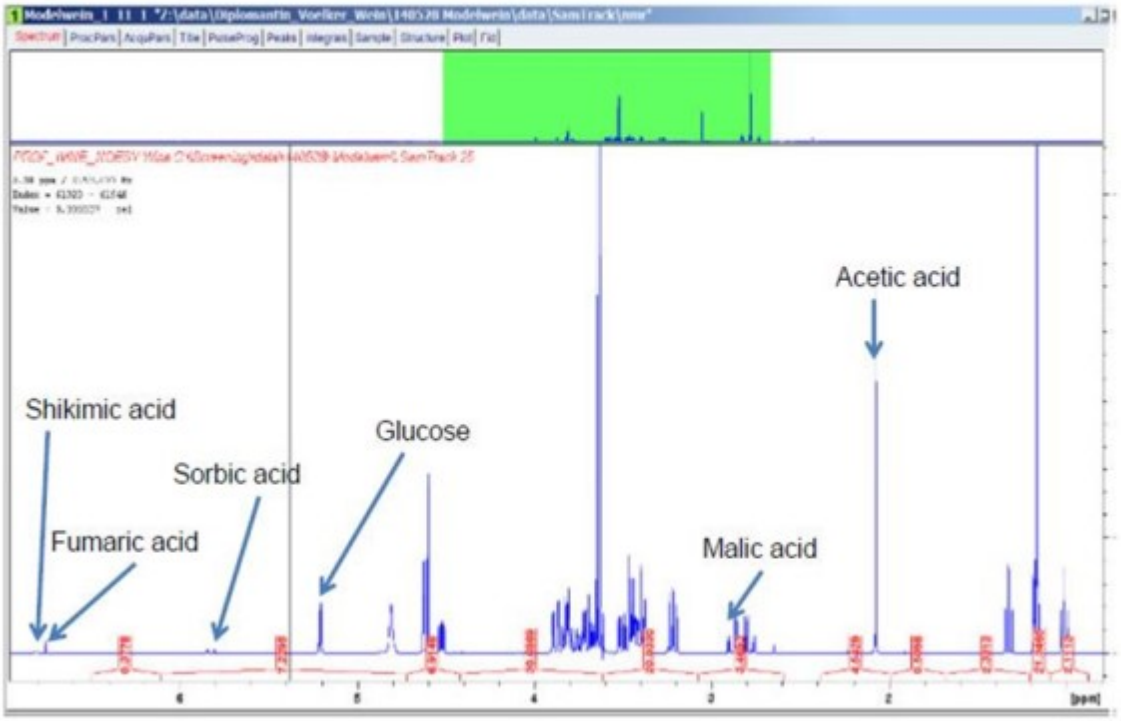
11.3. Horrat values of all compounds

Table 4

Parameter		Horrat value		Samples <LOQ
0,5-1,5		1,6-2,0	>2,0	
Acetic acid	7 (0,8-1,3)	3 (1,6;1,6;1,9)	0	0
Malic acid	4 (0,9-1,5)	2 (1,6;2,0)	2 (2,8;3,1)	2
Glucose	9 (1,0-1,5)	0	0	1
Sorbic acid	7 (0,6-1,5)	0	0	3
Fumaric acid	6 (0,5-1,5)	0	0	4
Shikimic acid	5 (0,6-1,3)	5 (1.6:1.6;1.8;1.9;2,0	1 (2,1)	0

11.4. Example for an NMR spectra

Figure 1. NMR spectra of model wine with selected signals for quantification



12. Literature

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