OIV-MA-AS315-15 Determination of 3-methoxypropane-1,2-diol and cyclic diglycerols (by products of technical glycerol) in wine by GC-MS- Description of the method and collaborative study

Type II method

1. Introduction

This is an internationally validated method for the determination of 3-methoxypropane-1,2-diol (3-MPD) and cyclic diglycerols (CycDs) - both being recognised as impurities of technical glycerol - in different types of wine. It is known that glycerol produced by transesterification of plant and animal triglycerides using methanol contains considerable amounts of 3-MPD. The synthesis of glycerol from petrochemicals leads to impurities of CycDs. One of the published methods [1, 2, 3[i]] was adopted, modified and tested in an collaborative study. Here we present the optimized method and report the results of the collaborative study [2]. Design and assessment of the validation study followed the O.I.V. Resolution 8/2000 “Validation Protocol of Analytical Methods”.

2. Scope

The described method is suitable for the determination of 3-MPD and 6 cyclic diglycerols (cis-, trans-2,6-bis(hydroxymethyl) 1,4-dioxane; cis-, trans-2,5-bis(hydroxymethyl) 1,4-dioxane; cis-, trans-2,-hydroxymethyl-6-hydroxy-1,4-dioxepane) in white, red, sweet and dry wines. The study described covers the concentration range of 0.1 to 0.8 mg/L for 3-MPD and 0.5 to 1.5 mg/L for the CycDs.

3. Definitions

3-MPD	3-methoxypropane-1,2-diol
ANOVA	Analysis of Variance
C	Concentration
CycDs	Cyclic diglycerols
GC-MS	Gas chromatography – mass spectrometry
H_2	Hydrogen

IS	Internal standard
m/z	mass/charge ratio
ML	Matrix calibration level
S0	Standard dilution 1000 ng/ µL
S1	Standard dilution 100 ng/ µL
S2	Standard dilution 10 ng/ µL

4. Principle

The analytes and the internal standard are salted-out by addition of K_2CO_3, and extracted using diethyl ether. Extracts are analyzed directly by GC-MS on a polar column. Detection is then carried out in selected ion monitoring mode.

5. Reagents and Materials

5.1. Chemicals

5.1.1. K_2CO_3 p.A .

5.1.2. Diethyl ether Uvasol for spectroscopy

5.1.3. Molecular sieve (2 mm diameter, pore size 0.5 nm)

5.1.4. Ethanol (Absolute)

5.2. Standards

5.2.1. Cyclic diglycerol mixture (6 components) Solvay Alkali GmbH[1], 89.3 %
cis-, trans-2,6-bis(hydroxymethyl) 1,4-dioxane; cis-, trans-2,5-bis(hydroxymethyl) 1,4-dioxane; cis-, trans-2,-hydroxymethyl-6-hydroxy-1,4-dioxepane

5.2.2. 3-Methoxypropane-1,2-diol (3-MPD) 98% (CAS 623-39-2)

5.2.3. Butane-1,4 -diol-1,1,2,2,3,3,4,4-$(^2H)_8$ 98% (CAS 74829-49-5)

5.3. Preparation of standard solution

5.3.1. S0 stock solutions

Accurately weigh 10.0 mg $\pm$ 0.05 mg of each standard substance (11.2 mg are weighed for the CycDs, corresponding to 89.3 % purity) and transfer them to a 10 mL volumetric flask (one for each). Add exactly 10 mL of ethanol and mix thoroughly. The

concentration of this solution is 1000 ng/ µL.

5.3.2. S1 working solutions

Volumetrically transfer 1000 µL of the S0 stock solution (6.3.1) to a 10 mL volumetric flask, dilute the contents to volume with ethanol, thoroughly stopper the flask and invert to mix. The concentration of this solution is 100 ng/ µL.

5.3.3. S2 working solutions

Volumetrically transfer 100 µL of the S0 stock solution (6.3.1) to a 10 mL volumetric flask, dilute the content to volume with ethanol, thoroughly stopper the flask and invert to mix. The concentration of this solution is 10 ng/ µL.

Overview of required standard solutions:

CycDs mixture (6 components)

Solution	Concentration	
S0	1000	ng/ µL
S1	100	ng/ µL

3-Methoxypropane-1,2-diol (3-MPD)

Solution	Concentration	
S0	1000	ng/ µL
S1	100	ng/ µL
S2	10	ng/ µL

1,4 Butane-1,4-$(^2H)_8$ (internal standard IS)

Solution	Concentration	
S0	1000	ng/ µL
S1	100	ng/ µL

5.4. Preparation of the matrix calibration curve

Matrix-matched calibration solutions are prepared in an uncontaminated wine. It is necessary to analyze this wine first to check that it is not contaminated with 3-MPD or CycDs. If the concentrations of the analytes in the sample are outside the range of

the calibration curve, additional levels must be prepared. To ensure that the internal standard does not interfere with any wine components, a blank should be included.

Table 1. Pipetting scheme of matrix calibration

Matrix calibration level				Volume Wine	C Wine	C Wine
		Spike µl		ml	µg/L	mg/L
Blank	IS	-		10	0	0
	3-MPD	-				
	CycDs	-				
ML0	IS	100	S1	10	1000	1.00
	3-MPD	-				
	CycDs	-				
ML1	IS	100	S1	10	1000	1.00
	3-MPD	100	S2		100	0.10
	CycDs	50	S1		500	0.50
ML2	IS	100	S1	10	1000	1.00
	3-MPD	25	S1		250	0.25
	CycDs	100	S1		1000	1.00
ML3	IS	100	S1	10	1000	1.00
	3-MPD	50	S1		500	0.50
	CycDs	20	S0		2000	2.00
ML4	IS	100	S1	10	1000	1.00

	3-MPD	100	S1		1000	1.00
	CycDs	30	S0		3000	3.00
ML5	IS	100	S1	10	1000	1.00
	3-MPD	200	S1		2000	2.00
	CycDs	40	S0		4000	4.00

6. Apparatus

6.1. Analytical balance. ± 0.0001 g readability.
6.2. Lab centrifuge (at least 4000 rpm/min)
6.3. Gas chromatograph.-With mass spectrometric detector, split-splitless injector,
6.4. Diverse precision pipettes and volumetric flasks
6.5. Pasteur pipettes
6.6. 40 mL centrifugation vials
6.7. GC-vials (1.5 –2.0 mL)
6.8. Thermostat
6.9. Shaking machine

7. Sampling

Wine samples for the analysis should be taken in a sufficient size. Volume needed for one test sample is 10 mL. The wine used for the preparation of the matrix-calibration (5.4) shall be free of analyte.

8. Procedure

8.1. Extraction

Add 100 µL internal standard solution S1 (6.3.2) to 10 mL wine to a suitable centrifugation vial e.g. 40 mL. (This corresponds to a concentration of 1 mg/L butane-1,4-$(^2H)_8$). Carefully add 10 g of K_2CO_3 and mix. Take care during this addition as heat is produced due to the release of CO_2. After cooling the solution to approximately 20 °C in a water bath, add 1 mL diethyl ether. Homogenise the mixture for 5 minutes using a vertical-shaking machine. Centrifuge the vials at 4000 rpm for 5 min. For better removal of the organic phase, the extract can be partially transferred

into a vial with a smaller diameter. Using a Pasteur pipette, transfer the upper organic phase, composed of diethyl ether and ethanol, into a GC vial. Add approximately 120 mg of molecular sieve into the vial. Close the vial, leave for at least 2 h and shake well from time to time. The clear supernatant is transferred to a second GC vial for the GC-MS analysis.

8.2. GC-MS Analysis

Specific parameters for the GC-MS analysis are provided below. Alternative systems may be used, if they provide a similar chromatographic performance and adequate sensitivity. The chromatographic system must be able to separate the internal standard from phenylethanol, a potential interference.

Typical GC conditions

Gas chromatograph: HP 5890 or equivalent

DB-Wax (J&W) column 60 m, 0.32 mm internal diameter, 0.25 µm film thickness, 2 m capillary containment same dimensions or equivalent

Carrier gas: H_2

Flow: Pressure 60 k Pa column head

Temperature program:

90° C, 2 min., ramp at 10°C/min. up until 165° C, held for 6 min., ramp at 4° C/min to 250°C, held for 5 min.

Injection temperature: 250° C; Injected volume; 2 µL, 90 sec splitless for 90 s.

Specificl MS conditions

Mass spectrometer: Finnigan SSQ 710 or equivalent

Transfer line: 280° C

Source: 150° C

MS detection:

window1.: 0-25 min.:

14.3 min. 3-MPD: *m/z* 75, *m/z* 61

16.7 min IS: *m/z* 78, *m/z* 61

Acquisition time for each mass is 250 µs (dwell time).

Monitor for *m/z* 91 the separation of the internal standard (IS) peak from phenylethanol, which also produces a fragment m/z 78.

window 2. 25-40 min.:

32-34.5 min. CycDs: *m/z* 57, *m/z* 117

Acquisition time for each mass is 250 µs (dwell time).

It has been observed that the analysis may degrade chromatographiccolumn. In particular, the injection of the high boiling CycDs mixture is suspected to cause irreversible damage. Injections of reference standard solutions should be avoided; analysis should be restricted to salted-out solutions with low analyte concentrations. In addition it is recommended to use a 1-2 m pre column in order to protect the analytical column. Nevertheless, the analytical column has to be considered as a consumable and must be replaced quite regularly.

9. Evaluation

9.1. Identification

Record the relative retention time of each analyte to the IS. Calculate the mean relative retention time of the analytes in the calibration standards. The relative retention time of the analyte should be the same as that of the standard within a margin of ± 0.5 %. As a confirmation criterion, an ion ratio can be calculated for each analyte from the selected ion monitoring. This ratio is 117/57 for CycDs, 75/61 for 3-MPD and 78/61 for the IS. The ratio should be within ± 20 % of that which is found in the spiked sample. Confirmation of the identity of substances by full scan using ionsn can also be used.

9.2. Quantification

The quantification is done by a matrix calibration curve prepared according to appropriate section. The analyte/IS area ratios of the indicated mass ratios are correlated by linear regression against the concentration of the analyte. Quantification of the CycDs is achieved by summing the peak area of all six peaks and calculating the total content, to allow for other distributions of the six characteristic CycDs than in the standard. The following m/z values are used for quantification:

3-MPD: m/z 75

IS:m/z 78

CycDs: m/z 117

9.3. Expression of results

Results should be expressed in mg/L for 3-MPD and CycDs with two decimals (e.g. 0.85 mg/L).

9.4. Limit of Detection and limit of quantification

The limit of detection (LD) and the limit of quantification (LQ) depend on the individual measurement conditions of the chemical analysis and are to be determined by the user of the method.

The limit of detection (LD) and the limit of quantification (LQ) were estimated using

the instrumentation and conditions mentioned exemplarily above (s. 8) following the instructions in the resolution OENO 7-2000 (E-AS1-10-LIMDET) "Estimation of the Detection and Quantification Limits of a Method of Analysis". Along the line of the „Logic Diagram for Decision-Making" in N° 3 the graph approach has to be applied following paragraph 4.2.2. For this purpose a part of the ion trace (m/z) chromatogram is drawn extendedly enclosing a range of a tenfold peak width at mid-height ($w_{½}$) of an analyte peak in a relevant part of the chromatogram. Furthermore two parallel lines are drawn which just enclose the maximum amplitude of the signal window.

The distance of these two lines gives h_{max}, expressed in abundance units is multiplied by 3 for LD, by 10 for LQ and finally converted into concentration units by implementing the individual response factor.

3-MPD:

LD: 0,02 mg/l

LQ: 0,06 mg/l

CycDs (sum):

LD: 0,08 mg/l

LQ: 0,25 mg/l

(Note: Since the CD are a mixture of six single compounds with the same response factor - due to their chemical equality - and with h_{max} constant in the relevant part of the chromatogram the LD and LQ for each single compound are one sixth of the figures above)

10. Precision (interlaboratory validation)

Eleven laboratories participated in the collaborative study. The participating laboratories have proven experience in the analysis of the by-products. All of them participated in the pre-trial.

Repeatability (r) and reproducibility (R) and the respective standard deviations (S_r and S_R) were found to be correlated statistically significantly with the concentration of the analytes (ANNEX: Figures 1 and 2), r with more than 95% probability and R with more than 99% probability for each of the analytes using the linear regression model.

The actual performance parameters can be calculated by:

3-MPD

Sr=0,060x x=concentration of 3-MPD [mg/L]

SR=0,257x

r = 0,169 x x=concentration of 3-MPD [mg/L]

R = 0,720 x

CycDs

Sr = 0,082 x x=concentration of CycDs [mg/L]

SR = 0,092 x + 0,070

r = 0,230 x x=concentration of CycDs [mg/L]

R = 0,257 x + 0,197

Annex (Interlaboratory Study)

Participants

11 international laboratories participated in the collaborative study (5). The participating laboratories have proven experience in the analysis of the by-products. All of them participated in the pre-trial:

- CSL, York, UK
- Unione Italiana Vini, Verona, Italy
- BfR, Berlin, Germany
- BLGL, Würzburg, Germany
- Istituto Sperimentale per l'enologia, Asti, Italy
- LUA, Speyer, Germany
- Labor Dr. Haase-Aschoff, Bad Kreuznach, Germany
- CLUA, Münster, Germany
- Kantonales Laboratorium, Füllinsdorf, Switzerland
- LUA, Koblenz, Germany

- ISMAA, S. Michele all Adige, Italy

Samples

In November 2002, participating laboratories were sent 11 wine samples consisting of five sets of blind duplicates and one further single test material. Dry white wines, dry red wines and a sweet red wine were used for test materials. The samples were subjected to homogeneity testing previously ([ii]).

Data analysis

Statistical analysis was carried out according to the "Protocol for the Design, Conduct and Interpretation of Method Performance Studies" ([iii]) using a blind duplicate model.

Determination of outliers was assessed by Cochran, Grubbs and paired Grubbs tests.

Statistical analysis was performed to obtain repeatability and reproducibility data.

Horrat values were calculated.

Table 2. Results for 3-MPD

	Sample A White wine	Sample B Red wine [a]	Sample C White wine	Sample F Sweet red wine	Sample G White wine
Mean mg/L	0.30	0.145	0.25	0.48	0.73
Spiked mg/L	0.30	0.12	-	-	0.80
Recovery %	100	121	-	-	91
n	10	10 [a]	10	10	10
nc	1	1 [a]	1	1	1
outliers	2	0	0	1	1
n1	7	9 [a]	9	8	8
r	0.03	-	0.05	0.08	0.13
sr	0.01	-	0.02	0.03	0.05
RSDr %	3.20	-	7.20	5.80	6.57

Hor	0.30	-	0.60	0.50	0.59
R	0.13	0.13	0.15	0.31	0.59
sR	0.05	0.05	0.05	0.11	0.21
RSDr %	15.50	32.67	21.20	22.70	28.91
HoR	0.80	1.53	1.10	1.30	1.72

[a] Single test sample; n, nc and n1 are single results

mean: arithmetic mean of the data used in the statistical analysis

n: total number of sets of data submitted

nc: number of results (laboratories) excluded due to non-compliance

outliers: number of results (laboratories) excluded due to determination as outliers by either Cochran's or Grubbs' tests

n1: number of results (laboratories) retained in statistical analysis

S_r: the standard deviation of the repeatability

RSD_r: the relative standard deviation of the repeatability (S_rx100/mean)

r: repeatability (2.8 x S_r)

Ho_r: the Horrat value for repeatability is the observed RSD_r divided by the RSD_r value estimated from the Horwitz equation using the assumption r = 0.66R

R: reproducibility (between laboratory variation) (2.8 x S_R)

S_R: the standard deviation of the reproducibility

RSD_R: the relative standard deviation of the reproducibility (S_Rx100/mean)

Ho_R: the Horrat value for reproducibility is the observed RSD_R value divided by the RSD_R value calculated from the Horwitz equation

Figure 1. Correlation between 3-MPD concentration and r and R.

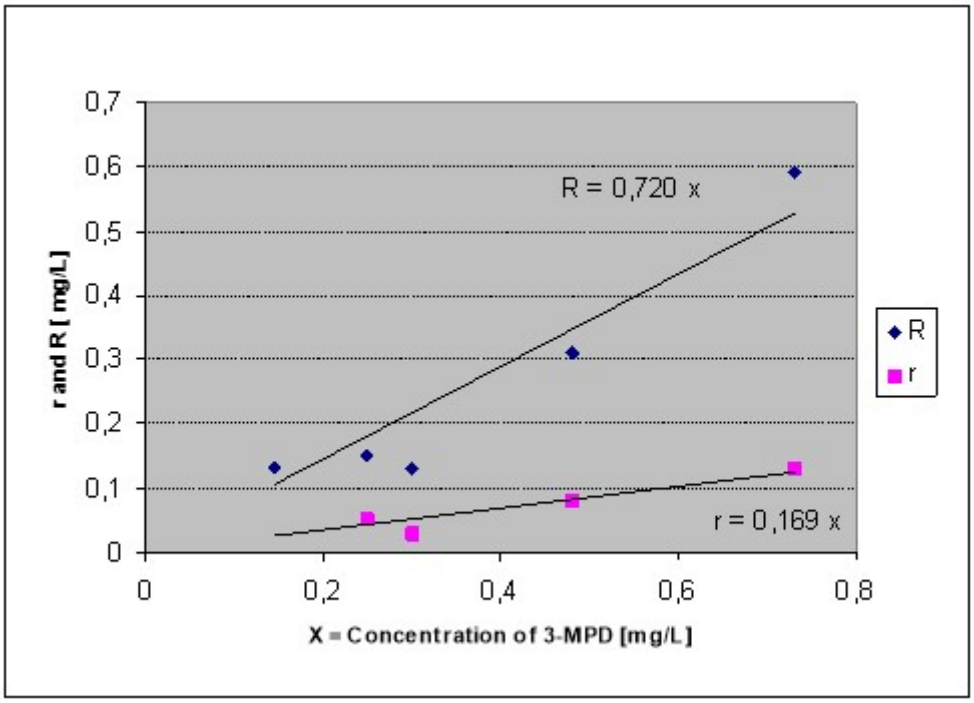


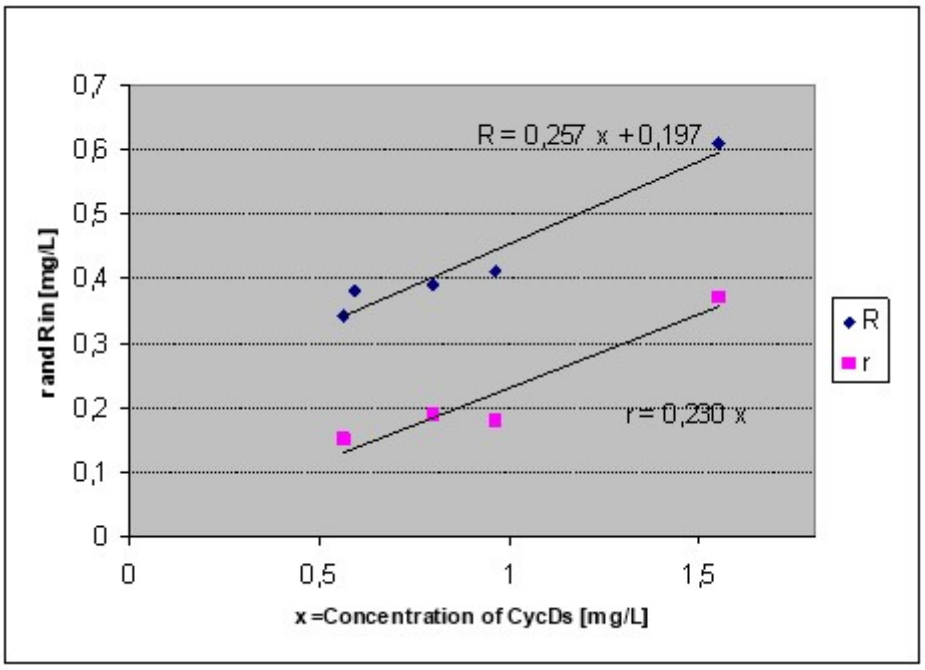
Table 3. Results for cyclic dyglycerols

	Sample A White wine	Sample B Red wine[a]	Sample D Red wine	Sample F Sweet red wine	Sample G White wine
Mean mg/L	1.55	0.593	0.80	0.96	0.56
Spiked mg/L	1.50	0.53			0.50
Recovery %	103	113			112
n	11	11[a]	11	11	11
nc	0	0	0	0	0
outliers	2	0	1	2	1
n1	9	11[a]	10	9	10
r	0.37	-	0.19	0.18	0.15

sr	0.13	-	0.07	0.07	0.05
RSDr %	8.50	-	8.60	6.70	9.30
Hor	0.90	-	0.80	0.60	0.80
R	0.61	0.379	0.39	0.41	0.34
sR	0.22	0.135	0.13	0.15	0.12
RSDR %	14.00	22.827	17.30	15.20	21.50
HoR	0.90	1.319	1.00	0.90	1.20

[a] Single test sample; n and nc are single results

Figure 2. Correlation between CycDs concentration and r and R.



[1] Solvay Alkali GmbH no longer provides the standard mixture; solutions of the mixture may be obtained from the BfR. Federal Institute for Risk Assessment, Thielallee 88-92, D-14195 Berlin. www.bfr.bund.de; poststelle@bfr.bund.de

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