RESOLUTION OENO 11/2008

SIMULTANEOUS DETERMINATION OF L-ASCORBIC ACID AND D-ISO-ASCORBIC ACID (ERYTHORBIC ACID) IN WINE BY HPLC AND UV-DETECTION

THE GENERAL ASSEMBLY

CONSIDERING Article 2 paragraph 2 iv of the Agreement of April 3, 2001 establishing the International Organisation of Vine and Wine,

UPON THE PROPOSAL of the Sub-commission of Methods of Analysis,

DECIDES to complete Annex A of the Compendium of International Methods of Analysis of Wine and Must with the following type II method:

Title	Type of method
Simultaneous determination of L-ascorbic acid and D-iso-ascorbic acid (erythorbic acid) in wine by HPLC and UV-detection	**II**

1. Introduction

Ascorbic acid is an antioxidant that is naturally occurring in a wide range of foods. The natural amount of ascorbic acid in grapes decreases during must and wine production, but it can be added to musts and to wines within certain limits.

The method described has been validated in a collaborative study by the analyses of wine samples with spiked amounts of 30 mg/L to 150 mg/l for L-ascorbic acid and 10 mg/L to 100 mg/l for D-isoascorbic acid respectively.

2. Scope

This method is suitable for the simultaneous determination of L-ascorbic acid and D-iso-ascorbic acid (erythorbic acid) in wine by high performance liquid chromatography and UV-detection in a range of 3 mg/L to 150 mg/l.

For contents above 150 mg/l, sample dilution is necessary.

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3. Principle

The samples are directly injected into the HPLC system after membrane filtration. The analytes are separated on a reversed phase column and UV-detection at 266 nm. The quantification of L-ascorbic acid and D-iso-ascorbic acid is done with reference to an external standard.

Note: The columns and operating conditions are given as example. Other types of columns may also give a good separation.

4. Reagents and Material

4.1. Reagents

4.1.1. N-octylamine, puriss. ≥ 99.0 %

4.1.2. Sodium acetate, 3 H_2O, puriss ≥ 99.0 %

4.1.3. Pure acetic acid, 100 %

4.1.4. Phosphoric acid, approx. 25%

4.1.5. Oxalic acid, puriss. ≥ 99.0 %

4.1.6. Ascorbate oxidase

4.1.7. L-ascorbic acid, ultra ≥ 99.5 %

4.1.8. D-iso-ascorbic acid, puriss. ≥ 99.0 %

4.1.9. Bi-distilled water

4.1.10. Methanol, p.A. 99.8 %

4.2. Preparation of the mobile phase

4.2.1. Solutions for the mobile phase

For the mobile phase prepare the following solutions:

4.1.1.1. 12.93 g n-octylamine in 100 ml methanol

4.1.1.2. 68.05 g sodium acetate, 3 H_2O in 500 ml bi-distilled water

4.1.1.3. 12.01 g pure acetic acid in 200 ml bi-distilled water

4.1.1.4. Buffer solution (pH 5.4) : 430 ml sodium acetate solution (4.2.1.2) and 70 ml acetic acid solution (4.2.1.3)

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4.2.2. Preparation of the mobile phase

Add 5 ml of n-octylamine solution (4.2.1.1) to approximately 400 ml bi-distilled water in a beaker. Adjust this solution to a pH of 5.4 to 5.6 by adding 25% phosphoric acid (4.1.4) drop by drop. Add 50 ml of the buffer solution (4.2.1.4), transfer the composite mix to a 1000 ml volumetric flask and fill up with bi-distilled water. Before use, the mobile phase has to be filtered through a membrane (regenerated cellulose, 0.2 µm) and if possible degassed with helium (approximately 10 minutes) depending on the needs of the HPLC system used.

4.3. Preparation of the standard solution

Note:

All standard solutions (stock solution 4.3.1. and working solutions 4.3.2) have to be prepared daily and preferably stored cold in a refrigerator prior to injection.

4.3.1. Preparation of the stock solution (1 mg/ml)

Prepare a 2% aqueous oxalic acid solution and eliminate dissolved oxygen by blowing through nitrogen.

Weigh exactly 100 mg each of L-ascorbic acid and D-iso-ascorbic acid in a 100 ml volumetric flask and make to the mark with the 2% aqueous oxalic acid solution.

4.3.2. Preparation of the working solutions

For the working solutions dilute the stock solution (4.3.1) to the desired concentrations with the 2% oxalic acid solution. Concentrations between 10 mg/l and 120 mg/l are recommended, e.g. 100 µl, 200 µl, 400 µl, 800 µl, 1200µl to 10 ml, corresponding to 10, 20, 40, 80 and 120 mg/l.

5. Apparatus

Usual laboratory equipment, in particular the following:

5.1. HPLC-pump

5.2. Loop injector, 20 µl

5.3. UV-detector

6. Sampling

Wine samples are fltered through a membrane with pore size 0.2 µm before injection.

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For contents above 150 mg/L, it is necessary to dilute the sample.

7. Procedure

7.1. Operating conditions for HPLC

Inject 20 µl of the membrane-filtered sample into the chromatographic apparatus.

Precolumn: e.g. Nucleosil 120 C18 (4cm x 4 mm x 7 µm)

Column: e.g. Nucleosil 120 C18 (25 cm x 4 mm x 7 µm)

Injection Volume: 20 µl

Mobile Phase: see 4.2.2, isocratic

Flow rate: 1ml/min

UV-detection: 266 nm

Rinse cycle: at least 30ml bi-distilled water followed by 30ml methanol and 30ml acetonitrile

7.2. Identification/Confirmation

Identification of peaks is done by the comparison of retention times between standards and samples. With the chromatographic system described as an example, the retention times are: for L-ascorbic acid 7.7 min. and for D-iso-ascorbic 8.3 min. respectively. (See figure 1, chromatogram A).

For further confirmation of positive findings these samples should be treated with a spatula of ascorbate oxidase and measured again (see figure 1, chromatogram B).

As a result of the degradation of L-ascorbic acid and D-iso-ascorbic acid caused by the ascorbate oxidase, no signal should be found at the retention time of L-ascorbic acid and D-iso-ascorbic acid. If interfering peaks are detected, their peak area should be taken into account for the calculation of the concentration of the analytes.

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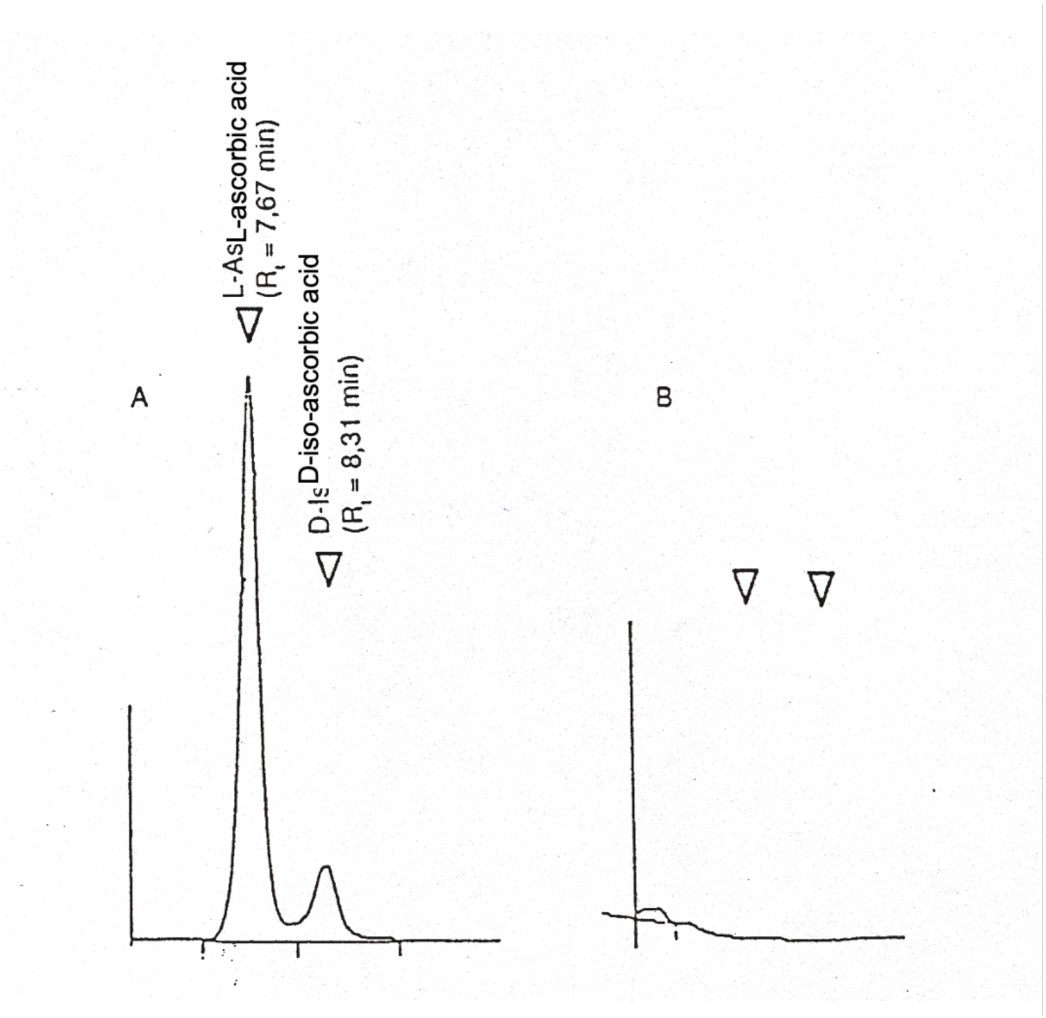


Figure 1: Example of a chromatogram of white wine: A: prior to treatment with ascorbate oxidase; B: after treatment

Note: It is recommended to analyse the ascorbate oxidase treated samples at the end of a sequence, followed by the rinse cycle for removing remaining ascorbate oxidase from the column. Otherwise the L-ascorbic acid and the D-iso-ascorbic acid may be converted by the remaining ascorbate oxidase during the HPLC-measurement and the result may be altered.

8. Calculation

Prepare a calibration curve from the working solutions (4.3.2). Following the method

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of external standard the quantification of L-ascorbic acid and D-isoascorbic acid is performed by measuring the peak areas and comparing them with the relevant concentration in the calibration curve.

Expression of results

The results are expressed in mg/l L-ascorbic acid and D-isoascorbic acid respectively with one decimal (e.g. 51,3 mg/l).

For contents above 150 mg/L, take into account the dilution.

9. Precision

The method was tested in a collaborative study with 27 laboratories participating, organised by the former Bundesgesundheitsamt (Germany) in 1994. The design of the collaborative trial followed the § 35 of the German Food Law that has been accepted by the O.I.V until the new protocol (OENO 6/2000) was introduced.

The study included four different samples of wine - two white wines and two red wines - of which five repetitions of each were requested. Due to the fact that it was not possible to prepare samples with a sufficient stability of the analytes (different degradation rates) it was decided to send defined amounts of pure standard substances together with the wine samples to the participants. The laboratories were advised to transfer the standards quantitatively to the wine samples and to analyse them immediately. Amounts of 30 to 150 mg/l for L-ascorbic acid and 10 to 100 mg/l for D-iso-ascorbic acid were analysed. In the Annex the detailed study results are presented. Evaluation was done following the DIN/ISO 5725 (Version 1988) standard.

The standard deviations of repeatability (s_r) and reproducibility (s_R) were coherent with the L-ascorbic acid and D-iso-ascorbic acid concentrations. The actual precision parameters can be calculated by the following equations:

L-ascorbic acid

- $s_r = 0.011\ x + 0.31$
- $s_R = 0.064\ x + 1.39$
- x: L-ascorbic acid concentration (mg/l)

D-iso-ascorbic acid

- $sr = 0.014\ x + 0.31$

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- sR = 0.079 x + 1.29
- x: D-iso-ascorbic acid concentration (mg/l)

Example:
D-iso-ascorbic acid 50 mg/l

- sr = 1.0 mg/l
- sR = 5.2 mg/l

10. Other characteristics of the analysis

10.1. Limit of detection

The limit of detection of this method was estimated at 3mg/l for L-ascorbic acid and D-iso-ascorbic acid

10.2. Trueness

The mean recovery calculated from the collaborative trial over four samples (see Annex) was:

- 100.6 % for L-ascorbic acid
- 103.3 % for D-iso-ascorbic acid

11. ANNEX: Collaborative Trial

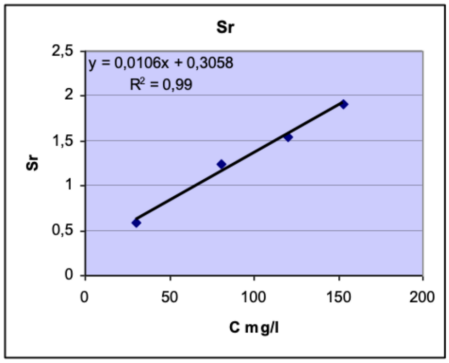
L-Ascorbic Acid

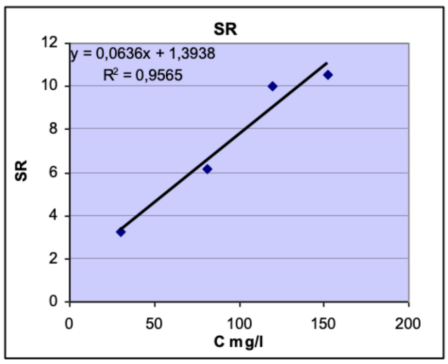
		Red Wine I	White Wine II	Red Wine III	White Wine IV
X	mg/l	**152.7**	**119.8**	**81.0**	**29.9**
Amount spiked	mg/l	150	120	80	30

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Recovery	%	101.8	99.8	101.3	99.7
n		25	23	25	23
Outliers		1	3	1	3
Repeatability s_r	mg/l	1.92	1.55	1.25	0.58
RSD_r	%	1.3	1.3	1.5	1.9
HorRat		**0.17**	**0.17**	**0.19**	**0.20**
r	mg/l	5.4	4.3	3.5	1.6
Reproducibility S_R	mg/l	10.52	10.03	6.14	3.26
RSD_R	%	6.9	8.4	7.6	10.9
Horwitz RSD_R	%	7.5	7.8	8.3	9.6
HorRat		**0.92**	**1.08**	**0.92**	**1.14**
R	mg/l	29.5	28.1	17.2	9.1





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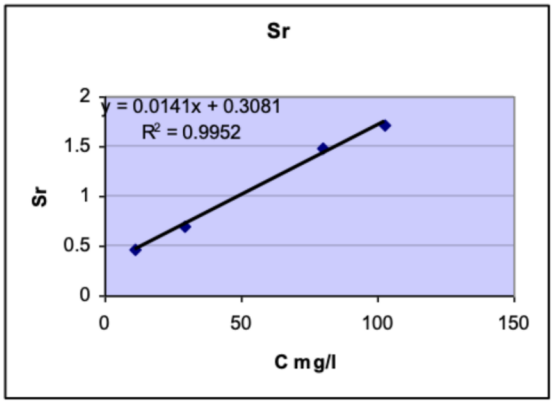
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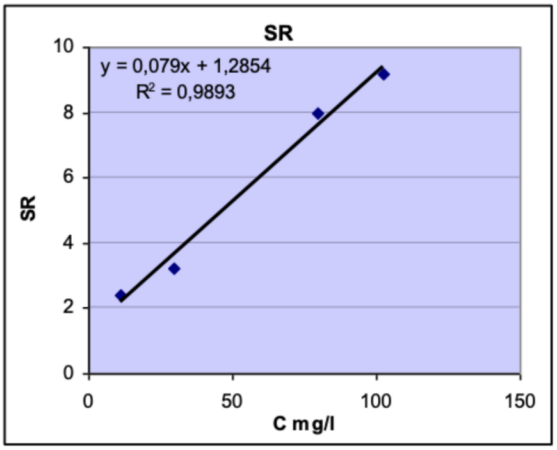
D-Isoascorbic Acid

		Red Wine I	White Wine II	Red Wine III	White Wine IV
X	mg/l	**102.4**	**79.8**	**11.3**	**29.4**
Amount Spiked	mg/l	100	80	10	30
Recovery	%	102.4	99.8	113.0	98.0
n		25	23	24	22
Outliers		1	3	2	4
Repeatability s_r	mg/l	1.71	1.49	0.47	0.70
RSD_r	%	1.7	1.9	4.1	2.4
HorRat		**0.21**	**0.23**	**0.37**	**0.25**
r	mg/l	4.8	4.2	1.3	2.0
Reproducibility S_R	mg/l	9.18	7.96	2.394	3.23
RSD_R	%	9.0	10.0	21.2	11.0
Horwitz RSD_R	%	8.0	8.3	11.1	9.6
HorRat		**1.12**	**1.21**	**1.91**	**1.14**
R	mg/l	25.7	22.3	6.7	9.0

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12. Bibliography

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