RESOLUTION OENO 33/2004

DETERMINATION OF SHIKIMIC ACID IN WINE BY HPLC AND UV-DETECTION

THE GENERAL ASSEMBLY,

CONSIDERING Article 2 paragraph 2 iv of the agreement establishing the International organisation of vine and wine

UPON PROPOSAL of the Sub-Commission of methods of analysis and appraisal of wine,

DECIDES to complete Annex A of the Compendium of international methods of analysis of wines and musts, by the following Type II method:

1. INTRODUCTION

Shikimic acid (3,4,5-Trihydroxy-1-cyclohexene-1-carboxylic acid) is biosynthetically synthesized from chinic acid by dehydration and plays a major role as a precursor of phenylanaline, tyrosine, tryptophan and plant alkaloids [1]. As a minor carboxylic acid shikimic acid is naturally found in a wide range of fruits [2].

Member states are encouraged to continue research in this area to avoid any non scientific evaluation of the results.

This method has been validated in an international collaborative study via the analyses of wine samples with naturally occurring amounts of shikimic acid ranging from about 10 to 150 mg/l. The trueness has been proved by an interlaboratory comparison using HPLC and GC/FID and GC/MS respectively [3].

2. SCOPE

This paper specifies an isocratic routine method for the quantitative determination of shikimic acid in red, rosé and white wine (included sparkling and special wines) at concentration levels ranging from 1 mg/l up to 300 mg/l by high performance liquid chromatography. When the method is applied to sparkling wine the samples must be previously degassed (for instance by sonication).

3. PRINCIPLE

Shikimic acid is determined directly without previous sample preparation by high

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performance liquid chromatography using a coupled column system. In a first step the organic acids in wine are pre-separated with a C_{18} reversed phase column followed by a cation exchange column at 65 °C performing the final separation. By using slightly acidified water as elution solvent a baseline resolution of shikimic acid is achieved without any interferences from the wine matrix . Due to the double bond within the cyclohexene ring system shikimic acid has a strong absorption and can therefore be detected easily with an UV-detector at its absorption maximum at 210 nm.

4. REAGENTS AND MATERIALS

4.1. Shikimic acid (CAS 138-59-0) , at least 98 % pure

4.2. Sulfuric acid 0,5 M

4.3. Bidestilled water

4.4. Preparation of the elution solvent (0,01 M H_2SO_4)

Pipette 20 ml of the 1 N sulfuric acid (4.2) to a 1000 ml volumetric flask, fill up with bidestilled water (4.3) to about 900 ml, shake and adjust to 1000 ml. Filter the elution solvent with a filter of a pore size less than or equal to 0,45 µm and degas.

4.5. Preparation of stock standard solution (500 mg/l shikimic acid)

Weigh exactly 50 mg shikimic acid (4.1), transfer them without loss to a 100 ml volumetric flask, fill up with bidestilled water (4.3) to about 90 ml, shake and adjust to 100 ml. At –18 °C the stock standard solution can be stored for months.

4.6. Preparation of working standard solutions (5 , 25 , 50 , 100, 150 mg/l shikimic acid)

Dilute stock solution 500 mg/l (4.5) appropriately with bidestilled water (4.3) to give five working standards of 5 , 25 , 50 , 100, 150 mg/l shikimic acid. Prepare working standard solutions daily.

5. APPARATUS

Usual laboratory equipment, in particular, the following:

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5.1. HPLC system capable of achieving baseline resolution of shikimic acid

5.1.1. High-performance liquid chromatograph with a six-way injection valve fitted with a 5 µl loop or any other device, either automatic or manual, for a reliable injection of microvolumes

5.1.2. Isocratic pumping system enabling one to achieve and maintain a constant or program¬med rate of flow with great precision.

5.1.3. Column heater enabling one to heat a 300 mm column to 65 °C

5.1.4. UV-VIS detector with a flow cell and wavelength set of 210 nm

5.1.5. Computational integrator or other data collection system

5.2. HPLC column system of stainless steel

5.2.1. Guard column

It is recommended that a suitable pre-column is attached in front of the analytical column system.

5.2.2. Analytical column system

1. Reversed Phase Column (ambient)

Material: stainless steel
Internal diameter: 4 - 4,6 mm
Length : 200 - 250 mm

Stationary phase: spherical C18 reversed phase material, particles 5µ in diameter*[1]) coupled with

2. Cation exchange column (heated up to 65 ° C)

Material: stainless steel
Internal diameter: 4 - 7,8 mm
Length : 300 mm
Stationary phase: Sulfonated sterene-divinylbenzene gel type resin (S-DVB), containing a hydrogen packing, cross linked 8 %**[2])

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6. SAMPLING

Clear samples are filled directly into sample vials and supplied to chromatography without any sample preparation. Cloudy wine samples are filtered through a 0,45 µm membrane filter before injection, while the first fractions of filtrates are rejected.

7. PROCEDURE

7.1. Operating conditions of HPLC analysis

Inject 5 µL of wine into the chromatographic apparatus by full loop injection system.

Flow rate: 0,4 ml/min (if internal diameter of the cation exchange column is 4 mm)

0,6 ml/min (if internal diameter of the cation exchange column is 7,8 mm)

Mobile Phase: 0,01 M H_2SO_4

Column heater for cation exchange column: 65 °

Run time: 40 min

Equilibration time: 20 min (to ensure that all substances from the wine matrix are completely eluted)

Detection wavelength: 210 nm

Injection volume: 5 µL

Note: Due to the different separation properties of various columns and different dead volumes of various HPLC-equipments the absolute retention time (min) for the shikimic acid peak may vary more or less significantly. Even though shikimic acid can be identified easily by calculating the a relative retention (r) related to a reference peak, here tartaric acid, a major organic acid naturally occurring in wine and the first and dominant peak in the chromatogram . By trying different C18 reversed phase columns and various cation exchange columns a relative retention (r) of 1.33 (± 0.2) has been calculated.

7.2. Detection limit

The detection limit of this method calculated according to the OIV protocol was estimated to 1 mg/l.

8. CALCULATION

Prepare a 5-point calibration curve from the working standard solutions (4.6).

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Following the method of external standard the quantification of shikimic acid is performed by measuring the peak areas at shikimic acid retention time and comparing them with the relevant calibration curve. The results are expressed in mg/l shikimic acid at 1 decimal place.

9. PRECISION

The method was tested in a collaborative study with 19 international laboratories participating. Design and assessment followed O.I.V. Resolution Oeno 8/2000 "Validation Protocol of Analytical Methods". The study included 5 different samples of red and white wines. The samples covered concentration levels from 10 to 120 mg/l (see Annex 3).

The Standard Deviations of Repeatability and Reproducibility correlated with the shikimic acid concentration (see Annex 2). The actual performance parameters can be calculated by

$$s_r = 0{,}0146 \,.\, x + 0{,}2716$$

$$s_R = 0{,}0286 \,.\, x + 1{,}4883$$

x: shikimic acid concentration (mg/l)

Example:

Shikimic acid : 50 mg/l

$$s_r = \pm\, 1{,}0\ mg/l$$

$$s_R = \pm\, 2{,}92\ mg/l$$

10. ANNEX

A typical separation of shikimic acid from other organic acids in wine is given in the Annex 1.

The correlationship of shikimic acid concentration and the standard deviation of repeatability and reproducibility is given in Annex 2.

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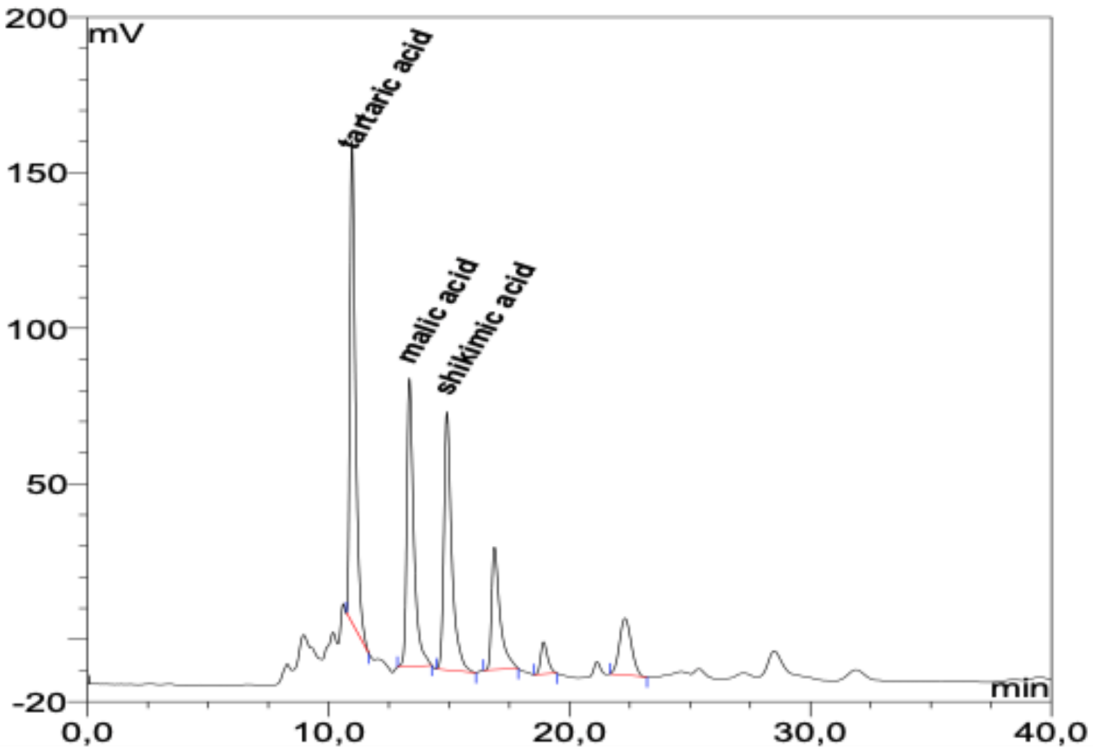
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The statistical data derivated from the results of the interlaboratory study is given in Annex 3.

11. BIBLIOGRAPHY

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3. 44th Session SCMA, 23-26 march 2004, Comparison of HPLC-, GC- and GC-MS-Determination of Shikimic Acid in Wine, FV 1193

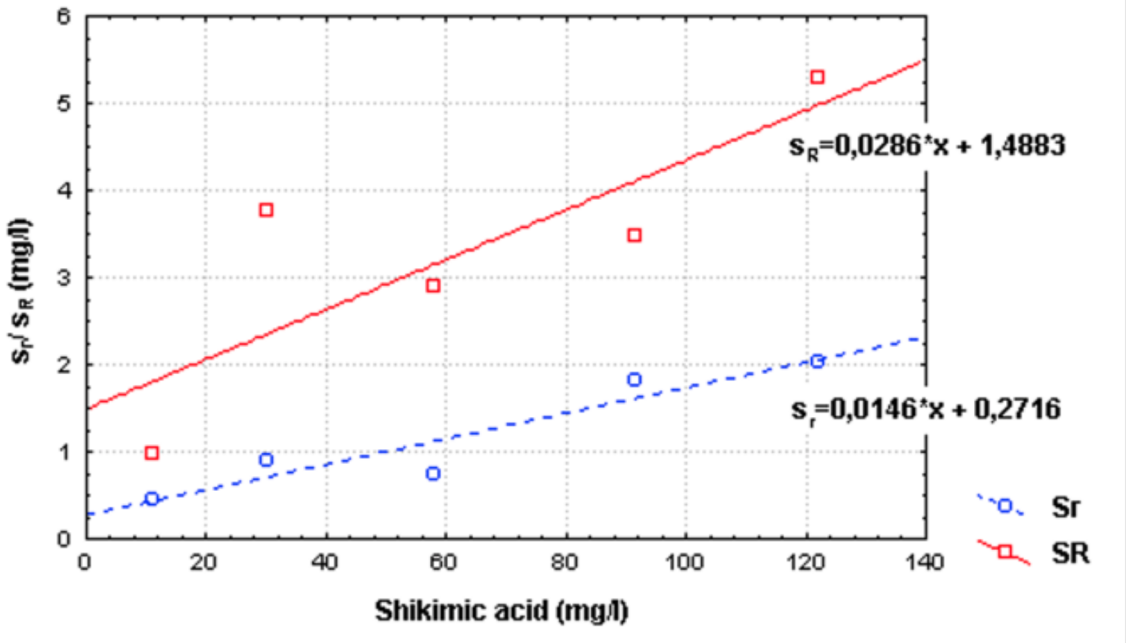
Annex 1 : Chromatogram of organic acids in wine



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Annex 2: Correlationship of shikimic acid concentration and standard deviation of repeatability and reproducibility respectively



Annex 3: Table of method performance parameters

sample identification	A	B	C	D	E
Number of participating laboratories	19	19	19	19	19
Number of accepted laboratories	17	18	17	18	18
mean	58.15	30.05	11.17	122.17	91.20
s_r^2	0.54588	0.84694	0.19353	4.32417	2.67306

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s_r	0.73884	0.92030	0.43992	2.07946	1.63495
RSD_r (%)	1.27	3.06	3.93	1.70	1.79
r	2.07	2.58	1.23	5.82	4.58
s_L^2	8.45221	13.27078	0.73013	24.62737	8.55508
s_R^2	8.99809	14.11773	0.92366	28.95154	11.22814
s_R	2.99968	3.75736	0.96107	5.38066	3.35084
RSD_R (%)	5.16	12.50	8.60	4.40	3.67
R	8.40	10.52	2.69	15.07	9.38

s_r^2 variance of repeatability
s_r standard deviation of repeatability
RSD_r (%) relative standard deviation of repeatability
r repeatability
s_L^2 variance between laboratory
s_R^2 variance of reproducibility
s_R variance of reproducibility
RSD_R (%) relative standard deviation of reproducibility
R reproducibility

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[1] *Lichrospher TM 100 RP-18 , HypersilTM-ODS or OmnichromTM YMC-ODS-A are examples of suitable columns available commercially

[2] **Aminex TM HPX 87-H or RezexTM ROA-Organic Acid are examples of suitable columns available commercially

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