Method OIV-MA-AS313-17 Type II method

Determination of shikimic acid in wine by HPLC and UV-detection
(Resolution Oeno 33/2004)

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 performance liquid chromatography using a coupled column system. In a first step the organic acids in wine are pre-separated with a C18 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\textsubscript{2}SO\textsubscript{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:

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 programmed 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 C\textsubscript{18} reversed phase material, particles 5\textmu m in diameter\textsuperscript{)*}

2. Cation exchange column (heated up to 65 °C)
   Material: stainless steel
   Internal diameter: 4 - 7.8 mm
   Length: 300 mm
   Stationary phase: Sulfonated styrene-divinylbenzene gel type resin (S-DVB), containing a hydrogen packing, cross linked 8\% \textsuperscript{**)}

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\textsubscript{2}SO\textsubscript{4}
   Column heater for cation exchange column: 65 °
   Run time: 40 min

\textsuperscript{)*} Lichrospher\textsuperscript{TM} 100 RP-18, Hypersil\textsuperscript{TM}-ODS or Omnichrom\textsuperscript{TM} YMC-ODS-A are examples of suitable columns available commercially

\textsuperscript{**) Aminex\textsuperscript{TM} HPX 87-H or Rezex\textsuperscript{TM} ROA-Orgnic Acid are examples of suitable columns available commercially
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 $C_{18}$ 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).

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

\[ s_r = 0.0146 \cdot x + 0.2716 \]
\[ s_R = 0.0286 \cdot x + 1.4883 \]

x: shikimic acid concentration (mg/l)

Example:

shikimic acid: 50 mg/l

\[ s_r = \pm 1.0 \text{ mg/l} \]
\[ s_R = \pm 2.92 \text{ mg/l} \]

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.

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

11. BIBLIOGRAPHY


Annex 1: Chromatogram of organic acids in wine

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

\[
s_\text{SR} = 0.0146x + 0.2716\]

\[
s_\text{SR} = 0.0296x + 1.4883\]
Annex 3: Table of method performance parameters

<table>
<thead>
<tr>
<th>Sample identification</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
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<tbody>
<tr>
<td>Number of participating laboratories</td>
<td>19</td>
<td>19</td>
<td>19</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>Number of accepted laboratories</td>
<td>17</td>
<td>18</td>
<td>17</td>
<td>18</td>
<td>18</td>
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<tr>
<td>mean</td>
<td>58.15</td>
<td>30.05</td>
<td>11.17</td>
<td>122.17</td>
<td>91.20</td>
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<tr>
<td>( s_r^2 )</td>
<td>0.54588</td>
<td>0.84694</td>
<td>0.19353</td>
<td>4.32417</td>
<td>2.67306</td>
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<tr>
<td>( s_r )</td>
<td>0.73884</td>
<td>0.92030</td>
<td>0.43992</td>
<td>2.07946</td>
<td>1.63495</td>
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<tr>
<td>RSD_r (%)</td>
<td>1.27</td>
<td>3.06</td>
<td>3.93</td>
<td>1.70</td>
<td>1.79</td>
</tr>
<tr>
<td>( r )</td>
<td>2.07</td>
<td>2.58</td>
<td>1.23</td>
<td>5.82</td>
<td>4.58</td>
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<tr>
<td>( s_l^2 )</td>
<td>8.45221</td>
<td>13.27078</td>
<td>0.73013</td>
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<tr>
<td>( s_R^2 )</td>
<td>8.99809</td>
<td>14.11773</td>
<td>0.92366</td>
<td>28.95154</td>
<td>11.22814</td>
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<tr>
<td>( s_R )</td>
<td>2.99968</td>
<td>3.75736</td>
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<tr>
<td>RSD_R (%)</td>
<td>5.16</td>
<td>12.50</td>
<td>8.60</td>
<td>4.40</td>
<td>3.67</td>
</tr>
<tr>
<td>R</td>
<td>8.40</td>
<td>10.52</td>
<td>2.69</td>
<td>15.07</td>
<td>9.38</td>
</tr>
</tbody>
</table>

- \( s_r^2 \): 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