OIV-MA-AS315-25 Determination of lysozyme in wine using high-performance liquid chromatography

Type IV method

1. Introduction

This method describes the analytical procedure used to determine lysozyme in red and white wines. The determination can be carried out on the sample directly for white wines, but for red wines a dissociation of the enzyme from the polyphenolic macromolecules by means of rapid alkalinisation must be undertaken, using the principle of the amphoteric nature of the protein.

2. Field of application

This method allows the lysozyme (mg of protein·L^{-1}) content in red and white wines to be quantified independently from enzyme activity. It should be made clear that this method makes it possible to detect lysozyme added to wine, but the limit of detection of the method does not exclude any allergenicity associated with the presence of low levels of lysozyme.

3. Principle

The analysis is carried out using high performance liquid chromatography (HPLC) with a spectrofluorimetric detector. The unknown quantity in the wine sample is calculated according to the chromatographic peak area using the external standard method.

4. Materials and reagents

4.1. Solvents and working solutions

4.1.1. Acetonitrile (CH_3CN), HPLC grade (CAS no. 75-05-8)

4.1.2. Trifluoroacetic acid (TFA) (CAS no. 76-05-1)

4.1.3. Deionised water, HPLC grade (CAS no. 7732-18-5)

4.1.4. Tartaric acid (CAS no. 87-69-4)

4.1.5. Ethanol (CAS no. 64-17-5)

4.1.6. Neutral potassium tartrate (CAS no. 921-53-9)

4.1.7. 28% Ammonium hydroxide (w/w) (CAS no. 1336-21-6)

4.1.8. 0.65 µm cellulose acetate filters

4.2. Stock solution: 1 g·L^{-1} of tartaric acid in 10% ethanol (v/v) adjusted to a pH of 3.2 with neutral potassium tartrate.

4.3. Eluents

A: 1% CH_3CN, 0.2% TFA, 98.8% H_2O

B: 70% CH_3CN, 0.2% TFA, 29.8% H_2O

4.4. Reference solution

Solution containing 250 mg·L^{-1} lysozyme standard dissolved in the stock solution by stirring continuously for 1 hour. It is stored in a refrigerator for a maximum of 4 weeks.

4.5. Preparation of working solutions

For the working solutions, the reference solution is diluted with the stock solution until the desired concentrations have been reached. These solutions are prepared daily.

5. Equipment

5.1. HPLC apparatus equipped with a pumping system suitable for gradient elution

5.2. Thermostatted column compartment (oven)

5.3. Spectrofluorimetric detector

5.4. 20 µL loop injection

5.5. Reverse phase polymeric column with phenyl functional groups (porosity = 1,000 Å, exclusion limit = 1,000,000 Da), Tosoh Bioscience TSK-gel Phenyl 5PW RP, 4.6 mm ID x 7.5 cm, for example.

5.6. Pre-column in the same material as the column: Tosoh Bioscience TSK-gel Phenyl 5PW RP Guardgel, 3.2 mm ID x 1.5 cm, for example.

6. Operating conditions (by way of example)

6.1. Eluent flow rate: 1 mL.min^{-1}

6.2. Elution temperature: 40°C

6.3. Spectrofluorimetric detector: □ ex = 276 nm; □ em = 345 nm; Gain = 4

6.4. Average lysozyme retention time: 7.9 minutes

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	60	40
10.00	0	100
10.20	60	40

12.00	Controller	Stop

7. Sample preparation

7.1. White wines

White wine samples are filtered using cellulose acetate filters with 0.65 µm porosity and then undergo chromatographic analysis. (There is a lower recovery rate if using nylon filters with 0.45 µm porosity.)

7.2. Red wines

Red wine samples (50 mL) are adjusted to a pH of 11.5. The samples are alkalinised using NH_4OH (taking into account the volume of the latter for the final calculation) and are then immediately filtered (after 5 min) using cellulose acetate filters with 0.65 µm porosity and injected into the liquid chromatograph. (There is a lower recovery rate if using nylon filters with 0.45 µm porosity.)

8. Control sample preparation

The reference standard solution (4.4) is added to the sample and it is prepared as described in point 7. The percentage recovery is determined.

9. Expression of results

Adequate resolution was observed for the chromatographic profile of lysozyme standard for the analyte tested, with the below chromatographic conditions (Fig. 1 and Fig.4). Analysis of the lysozyme-free sample enabled the wine profile to be observed without finding any interferences in the enzyme detection (Fig. 2 and Fig. 5).

In white wines, more than 95% of the enzyme was recovered (Fig. 3), while in red wines an enzyme recovery of between 70 and 95% was observed using this method, depending on the polyphenol concentration present in the wine sample (Fig. 6). The result is expressed in milligrams per litre ($mg \cdot L^{-1}$).

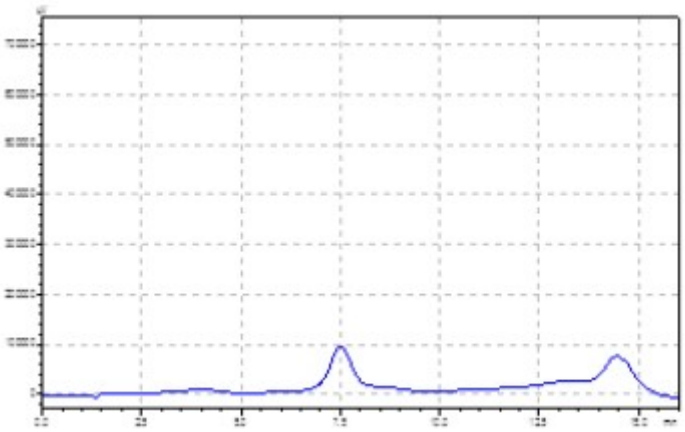

Fig. 1 Chromatogram of the 10 mg·L-1 lysozyme standard

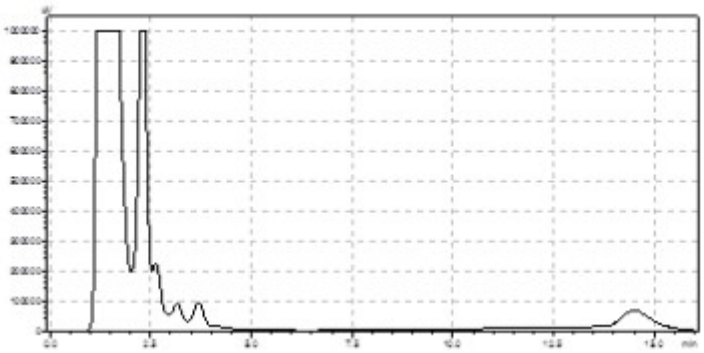

Fig. 2 Chromatogram of a white wine sample without lysozyme

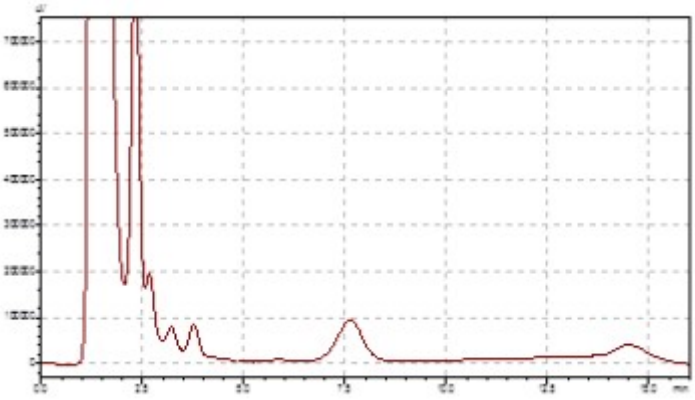

Fig. 3 Chromatogram of a white wine sample with 10 mg·L-1 lysozyme

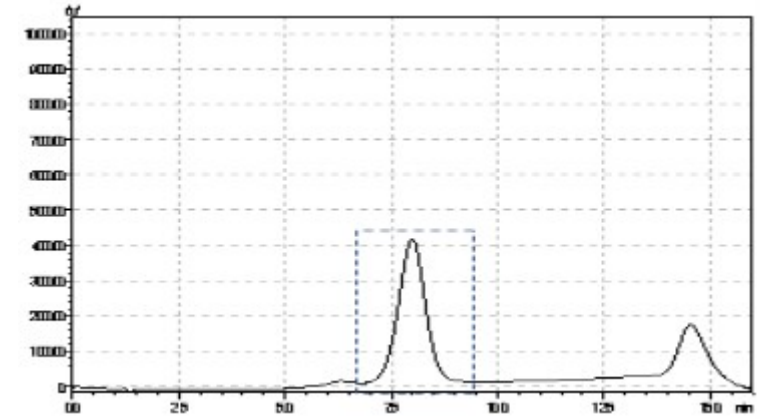

Fig. 4 Chromatogram of the 50 mg·L-1 lysozyme standard

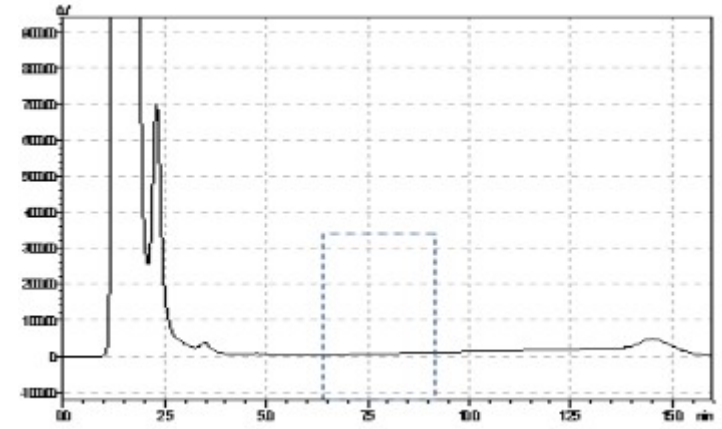

Fig. 5 Chromatogram of a red wine sample without lysozyme

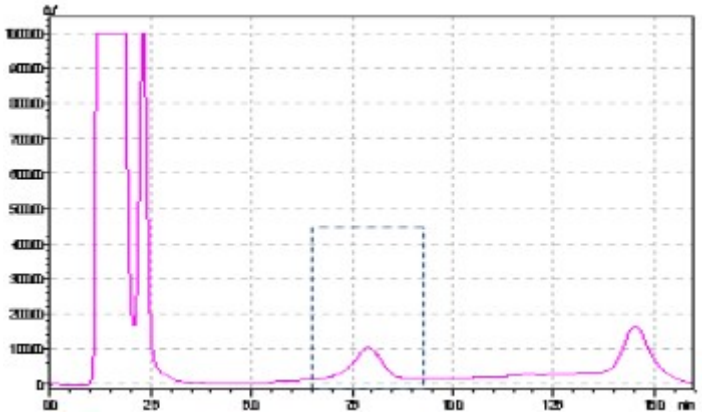

Fig. 6 Chromatogram of a red wine sample with 50 mg·L-1 lysozyme

10. Analytical procedure for white wines

10.1. Internal validation parameters

10.1.1. Repeatability

The repeatability of the method was studied for the interval between 2 $mg \cdot L^{-1}$ and 25 $mg \cdot L^{-1}$ in white wine. 17 samples of white wine enriched with various lysozyme concentrations were analysed in duplicate.

The repeatability results obtained at a probability level of 95% were as follows:

Concentration measured in $mg \cdot L^{-1}$	Sr $mg \cdot L^{-1}$	r (2.8xSr) $mg \cdot L^{-1}$
2	0.25	0.7
5	0.30	0.82
10	0.42	1.1
15	0.61	1.7
25	0.40	1.12

The average repeatability limit (r) is 1.2 $mg \cdot L^{-1}$

10.1.2. Linearity

For the calculation of linearity, 30 peak area measurements of 6 different concentrations of lysozyme in white wine were conducted, these being: an analytical blank without lysozyme, and concentrations of 2 $mg \cdot L^{-1}$, 5 $m \cdot gL^{-1}$, 10 $mg \cdot L^{-1}$, 15 $mg \cdot L^{-1}$, and 25 $mg \cdot L^{-1}$. From these measurements, the y-intercept, the gradient and the coefficient of correlation were calculated.

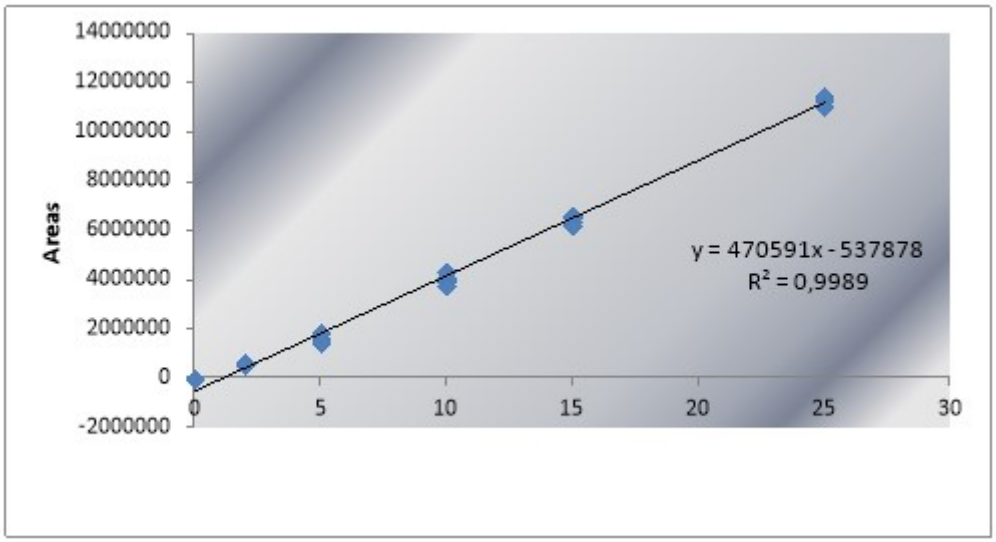

Fig. 7 Dynamic range of lysozyme in white wines up to 25 mg·L-1

10.1.3. Limit of detection and limit of quantification

The limit of detection obtained for this method was calculated using the graphic procedure derived from the background noise of the recording.

The values obtained were as follows:

LOD: 0.49 $mg \cdot L^{-1}$

LOQ: 1.62 $mg \cdot L^{-1}$

10.1.4. Intralaboratory reproducibility

The method intralaboratory reproducibility was studied for the interval between 2 $mg \cdot L^{-1}$ and 25 $mg \cdot L^{-1}$ in a white wine sample for a 30-day period. It should be pointed out that, due to the instability of the analyte, the wine sample was spiked with the lysozyme reference solution (4.4) on the same day as its analysis. 16 measurements were conducted at regular intervals.

The results obtained were as follows:

Concentration measured in $mg \cdot L^{-1}$	SR $mg \cdot L^{-1}$	R (2.8xSR) $mg \cdot L^{-1}$
2	0.19	0.53
5	0.36	1.0
10	0.48	1.3
15	0.64	1.8
25	0.93	2.6

The average reproducibility limit (R) is 1.45 $mgL \cdot^{-1}$

11. Analytical procedure for red wines

1. Internal validation parameters

1. Repeatability

The repeatability of the method was studied for the interval between 5 $mg \cdot L^{-1}$ and 25 $mg \cdot L^{-1}$ in red wine. 21 samples of red wine enriched with various lysozyme concentrations were analysed in duplicate.

The repeatability results obtained at a probability level of 95% were as follows:

Concentration measured in $mg \cdot L^{-1}$	Sr $mg \cdot L^{-1}$	r (2.8xSr) $mg \cdot L^{-1}$
5	0.38	1.06
10	0.64	1.79
15	0.59	1.65
25	0.30	0.8

The average repeatability limit (r) is 1.4 $mg \cdot L^{-1}$

11.1.2. Linearity

For the calculation of linearity, 25 peak area measurements of 5 different

concentrations of lysozyme in red wine were conducted, these being: an analytical blank without lysozyme, and concentrations of 5 $mg \cdot L^{-1}$, 10 $mg \cdot L^{-1}$, 15 $mg \cdot L^{-1}$, and 25 $mg \cdot L^{-1}$. From these measurements, the y-intercept, the gradient and the coefficient of correlation were calculated.

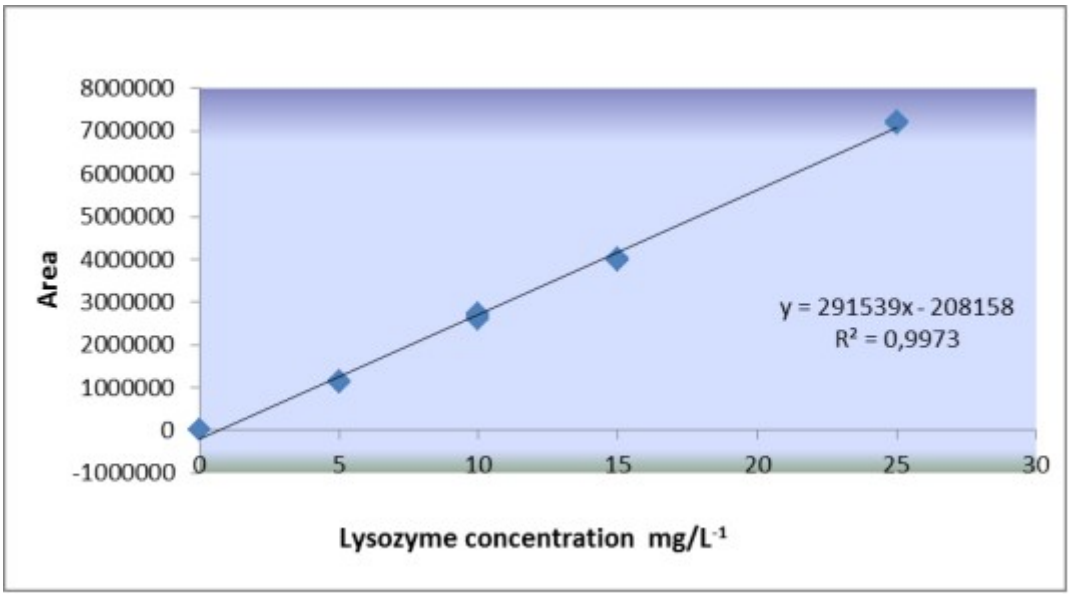

Fig. 8 Dynamic range of lysozyme in red wines up to 25 mg·L -1

11.1.3. Limit of detection and limit of quantification

The limit of detection was calculated using the graphic procedure derived from the background noise of the recording.

The values obtained were as follows:

LOD: 0.88 $mg \cdot L^{-1}$

LOQ: 2.90 $mg \cdot L^{-1}$

11.1.4. Intralaboratory reproducibility

The method intralaboratory reproducibility was studied for the interval between 5 $mg \cdot L^{-1}$ and 25 $mg \cdot L^{-1}$ in a red wine sample for a 30-day period. It should be pointed out that, due to the instability of the analyte, the wine sample was spiked with the lysozyme reference solution (4.4) on the same day as its analysis. 16 measurements were conducted at regular intervals.

The results obtained were as follows:

Concentration measured in $mg \cdot L^{-1}$	SR $mg \cdot L^{-1}$	R (2.8xSR) $mg \cdot L^{-1}$
5	0.4	1.12
10	0.91	2.54
15	0.54	1.5
25	0.53	1.5

The average reproducibility limit (R) is 1.7 $mg \cdot L^{-1}$

12. Bibliography

- Resolution OENO 8/2007 "Measurement of lysozyme in wine by high performance liquid chromatography" (2007).
- Resolution OENO 10/2005 “A practical guide for the validation, quality control, and uncertainty assessment of an alternative oenological analysis method”.