# OIV-MA-AS315-14 Measurement of lysozyme in wine by high performance liquid chromatography

**Type IV method**

## 1. Introduction

It is preferable to have an analysis method available for lysozyme which is not based on enzyme activity.

## 2. Scope

The method allows the quantification of lysozyme (mg of protein per l) present in red and white wines independently of the enzyme activity (which could be inhibited by partial denaturation or by complex formation or coprecipitation phenomena) found in the test solution.

## 3. Definition

HPLC provides an analytical approach based on steric, polar or adsorptive interactions betwen the stationary phase and the analyte, and is therefore not linked to the actual enzyme activity exhibited by the protein.

## 4. Principle

The analysis is carried out using HPLC with a spectrophotometric detector combined with a spectrofluorimetric detector. The unknown quantity in the wine sample is calculated on the chromatographic peak areas, using the external calibration method.

## 5. Reagents

5.1. Solvents and working solutions

HPLC analysis on Acetonitrile ($CH_3CN$)

Pure trifluoroacetic acid (TFA)

deionised water for HPLC analysis

Standard solution: Tartaric acid 1g/L, Ethyl alcohol 10% v/v, adjusted to pH 3.2 with neutral potassium tartrate.

5.2. Eluents

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

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

5.3. Reference solutions

Quantities from 1 to 250 mg/L standard lysozyme, dissolved in standard solution by stirring continuously for at least 12 hours.

**6. Equipment**

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

6.2. Thermostated column compartment (oven)

6.3. Spectrophotometer combined with spectrofluorimeter

6.4. 20 µL loop injection

6.5. Column: polymer in reverse phase with phenyl functional groups (diameter of pores = 1000 Å, exclusion limit = 1000000 Da) Toso Bioscience TSK-gel Phenyl 5PW RP, 7.5 cm x 4.6 mm ID as an example

6.6. Pre-column in the same material as the column: Toso Bioscience TSK-gel Phenyl 5PW RP Guardgel, 1.5 cm 3.2mm ID as an example

**7. Preparation of the sample**

The wine samples are acidified with HCl (10M) diluted 1/10 and filtered using a polyamide with 0.22 µm diameter pores filter, 5 minutes after the addition. The chromatography analysis is carried out immediately after filtering.

8. Operating conditions

1. Eluent flow-rate: 1mL/min
2. Temperature of column: 30°C
3. Spectrophotometric detection: 280 nm
4. Spectrofluorimetric detection:

- λ ex = 276 nm;
- λ em = 345 nm;
- Gain = 10

5. Gradient elution sequence

| Time (min) | A% | B% | gradient |
|---|---|---|---|
| 0 | 100 | 0 | |

| | | | |
|---|---|---|---|
| | | | isocratic |
| 3 | 100 | 0 | |
| | | | linear |
| 10 | 65 | 35 | |
| | | | isocratic |
| 15 | 65 | 35 | |
| | | | linear |
| 27 | 40.5 | 59.5 | |
| | | | linear |
| 29 | 0 | 100 | |
| | | | isocratic |
| 34 | 0 | 100 | |
| | | | linear |
| 36 | 100 | 0 | |
| | | | isocratic |
| 40 | 100 | 0 | |

8.6. Average retention time of lysozyme: 25.50 minutes

**9. Calculation**

The reference solutions containing the following concentrations of lysozyme: 1; 5; 10; 50; 100; 200; 250 mg/L are analysed in triplicate. For each chromatogram, the peak areas corresponding to the lysozyme are plotted according to the respective concentrations, in order to obtain the linear regresssion lines expressed by the

formula Y= ax+b. The correlation coefficient $r^2$ must be > 0.999

## 10. Characteristics of the method

A validation study was carried out for the purpose of assessing the suitability of the method for the purpose in question, taking into account linearity, limits of detection and quantification and the accuracy of the method. The latter parameter was determined by defining the levels of precision and trueness of the method.

10.1. Linearity of the method

Based on the results obtained from the linear regression analysis, the method proved to be linear within the ranges shown in the table below:

| | Linearity range (mg/L) | Line gradient | Correlation coefficient ($r^2$) | LD (mg/L) | LQ (mg/L) | Repeatability (n=5) RSD% | | | Reproducibility (n=5) RSD% |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Std[1] | V.R.[2] | V.B.[3] | Std[1] |
| UV | 5-250 | 3 786 | 0,9993 | 1,86 | 6,20 | 4,67 | 5,54 | 0,62 | 1,93 |
| FLD | 1-250 | 52 037 | 0,9990 | 0,18 | 0,59 | 2,61 | 2,37 | 0,68 | 2,30 |

Table 1: Data related to characteristics of the method: standard solution (Std 1); red wine (V.R 2); white wine (V.B 3)

10.2. Limit of detection and limit of quantification

The detection limit (LD) and limit of quantification (LQ) were calculated as the signal equivalent to respectively 3 times and 10 times the background chromatography noise under working conditions on an actual test solution (table 1),

10.3. Precision of the method

The parameters taken into account were repeatability and reproducibility. Table 1 shows the values of these parameters (expressed as %age St.dv. of measurements repeated in different concentrations) found for standard solution, red wine and white wine

10.4. Trueness of the method

The percentage recovery was calculated on the standard solutions containing 5 and 50 mg/L of lysozyme, with known quantities of lysozyme added, as shown in the table below.

| | Nominal initial [C] (mg/L) | Quantity added (mg/L) | Theoretical [C] (mg/L) | [C] found | Std.Dev. | %age recovery |
|---|---|---|---|---|---|---|
| UV 280 nm | 50 | 13.1 | 63.1 | 62.3 | 3.86 | 99 |
| FD | 50 | 13.1 | 63.1 | 64.5 | 5.36 | 102 |
| UV 280 nm | 5 | 14.4 | 19.4 | 17.9 | 1.49 | 92.1 |
| FD | 5 | 14.4 | 19.4 | 19.0 | 1.61 | 97.7 |

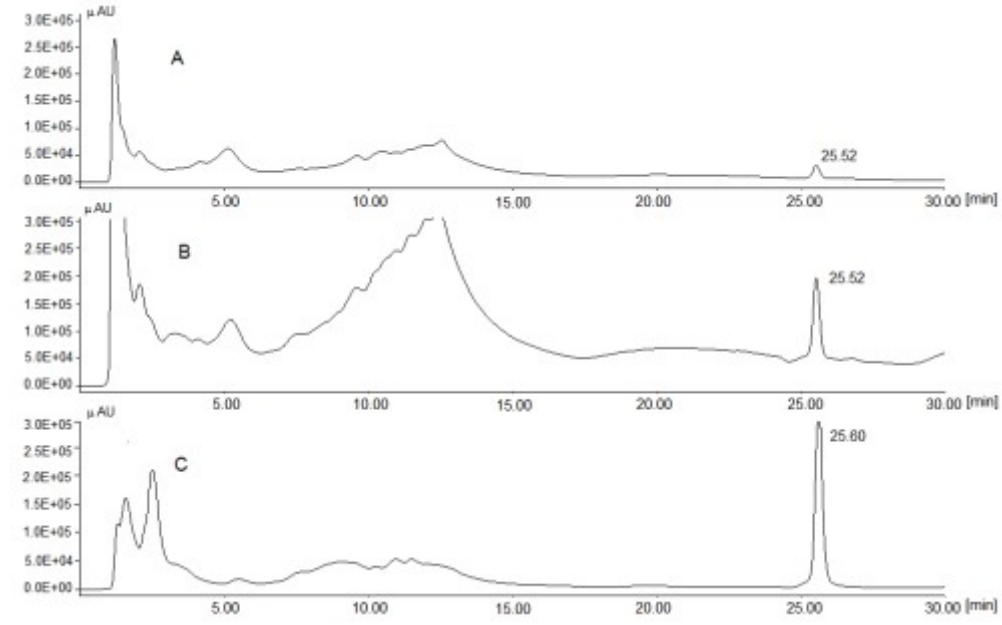


Fig.1 Chromatogram of red wine containing pure lysozyme (standard solution containing 1 000 mg/L of lysozyme was added to wine to obtain a final concentration of 125 mg/L of lysozyme). A: UV detector at 280 nm; B: UV detector at 225 nm; C: FLD detector (□ ex 276 nm; □ em 345 nm).

## 11. Bibliography

- Claudio Riponi; Nadia Natali; Fabio Chinnici. Quantitation of hen’s egg white lysozyme in wines by an improved HPLC-FLD analytical method. Am. J. Enol. Vit., in press.