RESOLUTION OENO 15/2001

LYSOZYME

THE GENERAL ASSEMBLY,

HAVING CONSIDERED Article 5 of the October 13, 1954 international convention for the unification of means of analysis and appreciation of wine,

WITH THE PROPOSAL of the means of analysis and appreciation of wine sub-committee

DECIDES to introduce the following monograph “Lysozyme” in the International Oenological Codex:

LYSOZYME

Muramidase

N°SIN: 1105 (enzyme 3.2.1.17)

1. OBJECT, ORIGIN AND FIELD OF APPLICATION

Lysozyme (Chlorhydrate and Lysozyme) is an edible egg white extract from hens. It is used to inhibit bacterial growth and can be used in musts and wine. Doses are limited in level.

Lysozymes contain no substances, micro-organisms or collateral enzyme activities, which are:

- harmful to the health,
- harmful for the quality of the products treated,
- lead to the formation of undesirable products or favour acts of fraud.

2. LABELLING

The concentration of the product must be indicated on the label, in addition to the security conditions, the preservation and the expiration date.

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3. COMPOSITION

Lysozyme is a natural polypeptide made up of 129 amino acids, out of which there are 21 aspartic acids, 5 glumatic acids, 12 alanines, 11 arginines, 8 cystines, 3 phenylalanines, 12 glycines, 6 isoleucines, 1 histidine, 8 leucines, 6 lysines, 2 prolines, 2 methionines, 10 serines, 3 tyrosines, 7 threonines, 6 tryptophanes and 6 valines.

The molecular mass of lysozyme is 14,700 Daltons.

The water content must be less than or equal to 6%.

4. CHARACTERISTICS

Lysozyme can be in crystal powder form, white, odourless with a mild taste.

5. SOLUBILITY

Lysozyme is soluble in water and insoluble in organic solvents.

6. IDENTIFYING CHARACTERISTICS

A 2% aqueous solution must have a pH between 3.0 and 3.6. An aqueous solution containing 25-mg/100 ml has a maximum absorption of 281 nm and a minimum of 252 nm.

7. ENZYME ACTIVITY

Enzyme activity is capable of hydrolysing a link between N-acetylmuramic acid and N-acetylglucosamine of gram positive bacteria cell walls. The minimum concentration for lysozyme is 95%. There is no secondary enzyme activity.

8. ENZYME ORIGIN AND MEANS OF PRODUCTION

Enzyme is extracted form edible hen egg white by a procedure of separating ion-exchange resin.

The microbiological purity guarantees the security for its usage in food. The egg white used in the preparation of enzymes are compatible with parameters established by inspection agencies and is treated in compliance with hygienic manufacturing procedures.

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9. SUBSTANCES USED AS DILUENTS, PRESERVATIVES, AND ADDITIVES

There are no substances used as preservatives as the crystalline form guarantees the stability.

10. TRIAL TESTS

10.1. Sulphuric ashes

As indicated in the appendix, the sulphuric ash content of lysozyme should not exceed 1.5%.

10.2. Total nitrogen

Evaluated according to the procedure outlined in the appendix, nitrogen content should be between 16.8 and 17.8% on dry matter.

10.3. Preparation of test trials solution

Dissolve 5 g of lysozyme in 100 ml of water.

10.4. Heavy Metals

Add 2 ml of pH 3.5 solution (R) and 1.2 ml of reactive thioacetamide (R) to 10 ml of prepared test trial solution (10.3). There should be no precipitate. If a brown colour is produced, it should be less than the sample produced as indicated in the appendix. (Heavy metal content measured in lead should be under 10 mg/kg).

10.5. Arsenic

Look for arsenic using the procedure in the appendix on 2 ml of test trial solution (10.3). (Arsenic content under 1 mg/kg).

10.6. Lead

Measure out lead following the procedure in the Compendium on the test trial solution (10.3). (Lead content under 5mg/kg.)

10.7. Mercury

Measure out mercury following the procedure in the appendix on the test trial

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solution (10.3). (Mercury content under 1 mg/kg).

10.8. Biological Contaminants

Evaluation carried out according to procedure in the appendix.

Total bacteria	under 10^3 CFU per g of preparation
Coliforms	maximum 10 per g of preparation
Escherichia coli	absence checked on 1 g sample
St. aureus	absence checked on 1 g sample
Salmonella	absence checked on 25 g sample
Yeasts	content limit 10^2 CFU per g of preparation
Total lactic bacteria	content limit : absence checked on a 10 g sample preparation
Acetic bacteria	content limit 10^2 CFU per g of preparation
Mould	content limit 10^2 CFU per g of preparation

11. MEASURING TURBIDITY OF LYSOZYME ACTIVITY IN WINE (Turbidimetric measuring)

11.1. Principle

The analytical procedure was established by FIP (1997) with some modifications made by FORDRAS. The procedure is based on changes in turbidity changes in Micrococcus luteus ATCC 4678 induced by a lytic lysozyme activity.

Under normal test conditions, the above-mentioned changes are in proportion to the quantity of lysozyme.

11.2. Substrate

Do not use an electromagnetic mixer when suspending between 40 – 60 mg of

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Micrococcus luteus ATCC 4698 (Boehringer) in powder form in phosphate solution M/15 pH 6.6 (± 0.1), when obtaining a homogeneous suspension and complete it with 100 ml with the same buffer. Use a hand mixer or an ultrasound bath.

The exact quantity of Micrococcus luteus to be taken depends on the spectrophotometer used.

Prepare a control sample with 5 ml of buffer and 5 ml of Micrococcus luteus and measure the absorbency with the aid of a 540 nm spectrophotometers compared to control sample of phosphate buffer. Absorbency should not be under 0.800.

If reading the measurement doesn't correspond, the content of Micrococcus luteus must be adapted in the suspension and then measure the desired absorbency.

Note: With a sensitive spectrophotometer, the absorbency levels of above-mentioned solutions are 0.800 to 0.900. Equipment that are not as sensitive may give readings for the absorbency for this same suspension of 0.500 to 0.600.

In this case, we should not increase the amount of substrate to obtain initial absorbency rates of 0.800 to 0.900, because reproducing the measurement linearity are not very dependable.

11.3. Preparation of standard solution

11.3.1. Dissolve exactly 50 mg of lysozyme chlorhydrate in water, and fill up to 100 ml in a graduated flask.

11.3.2. Dilute 5 ml of solution in 11.3.1 with water up to 50 ml.

11.3.3. Dilute 2 ml of this solution with a M/15 phosphate buffer up to 100 ml to obtain a 1 mg/1 of lysozyme (standard solution).

11.4. A solution to analyse

Dilute the sample of wine with m/15 phosphate buffer to obtain the same concentration of standard solution (1 mg/l) in relation with the concentration of lysozyme.

11.5. Procedure

Prepare the following solutions in 180 x 80 mm test tubes

Standard solution to analyse	Buffer M/15	Lysozyme concentration
2.0 ml	3.0 ml	0.4 mg/l

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2.8 ml	2.2 ml	0.56 mg/l
4.0 ml	1.0 ml	0.8 mg/l

It is recommended to repeat each dilution 3 times for the standard solution and for the solution to be tested.

Prepare two test tubes with 5 ml of buffer as a suspension control sample for Micrococcus luteus. Use the first control sample in the beginning and the second one at the end of the trial.

After exactly 30 seconds, add 5 ml of Micrococcus luteus suspension. This must be mixed mannually to avoid over spilling. Mix with a Vortex and keep the tubes in 37°C (± 5°C) water for exactly 12 minutes.

The final quantity of lysozyme in the tubes will be 0.2 – 0.28 – 0.4 mg/l.

After incubation, remove the tubes in the same order they were placed in, with an interval of 30 seconds.

Mix and take a reading of the absorbency with the 540 nm spectrophotometer for white wine and 740nm for the red wine against the control buffer.

Under normal circumstances, the test trial is acceptable when the difference between the absorbency rates for the control samples is under 5%.

11.6. Calculation

Prepare a standard curve indicating the average values of absorbency obtained for each standard solution on the y-axis. On the x-axis put the concentrations of lysozyme on a logarithm scale.

Carry over the results obtained for the dilutions to be analysed.

Draw two straight lines: one between the points obtained from the standard solution and the other between the points of the solution to be analysed. The two lines must be parallel, if not the dose is incorrect.

Then draw a line parallel to the x-axis so that the two right lines are cut about halfway the extreme limit for dosing.

In the two intersection points, which correspond to two concentrations on the x-axis (Cst concentration of the standard curve and Cx concentration of the curve for the solution to be analysed). The activity is calculated as follows:

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$$Concentrartion\ of\ lysozyme\ (\mu g/ml) = \frac{C_{st} \times D}{C_x}$$

Where

- C_{st} = concentration of the standard solution
- C_x = concentration of the solution to be analysed
- D = dilution factor

12. DETERMINATION OF LYSOZYME IN WINE (Determination by HPLC)

The lysozyme residue can be determined by HPLC according to the method described in the Compendium on International Analysis Methods of Wines and Musts.

13. PRESERVATION

Lysozyme must be stored at room temperature in a closed sealed container, away from humidity.

14. BIBLIOGRAPHY

1. FIP (1997), Pharmaceutical Enzymes, A. Lowers and S. Scharpe ed. 1997, vol.84 pages 375-379.

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