OIV-MA-AS315-29 Detection of chitinase and thaumatin-like proteins in white wines

Type IV method

1. Introduction

For the detection of unstable proteins and risks of protein *casse* in white wines, many tests are heat- or precipitation-based, the latter using a chemical agent. These tests give very different, unreliable and even contradictory results. This immunological method of semi-quantitative immunoprinting makes it possible to determine the presence or absence of unstable proteins in wines. Therefore, chitinase and thaumatin-like proteins can be detected from a total concentration of as low as 1 mg/L in wines. This value is taken from the comparison of results with the SDS electrophoresis method described in the *Compendium of Methods of Analysis* (OIV-MAA-AS315-12), for which the limit of detection is 1 mg/L.

2. Scope of application

This immunological method of immunoprinting applies to white wines.

3. Principle

The immunological method of immunoprinting is conducted in 3 steps:

3.1. Application of the wine sample to a nitrocellulose membrane

3.2. Detection of unstable proteins

3.3. Revelation of the presence of unstable proteins

The intensity of the coloured spots observed on the membrane is proportional to the quantity of unstable proteins and to the risk of protein *casse* in wine.

4. Reagents and products

4.1. List of reagents and products

Unless otherwise indicated, use the products as marketed.

4.1.1. Ultra-pure water: resistivity ≥ 18 MΩ.cm at 25°C

4.1.2. A wine very rich in proteins and a wine containing no proteins following treatment with bentonite. These wines are used for the positive and negative controls respectively: verification and quantification of proteins present in these wines may be conducted using SDS-PAGE electrophoresis (Method OIV-MA-AS315-12)

4.1.3. Rabbit polyclonal antibodies directed against unstable proteins in wine: see the protocol in the Annex

4.1.4. Goat anti-rabbit IgA polyclonal antibodies conjugated to horseradish peroxidase (hereinafter referred to as: goat anti-rabbit-HRP antibodies)

4.1.5. Anhydrous sodium chloride (NaCl): CAS No. 7647-14-5

4.1.6. Anhydrous Tris-HCl: CAS No. 1185-53-1

4.1.7. Concentrated HCl in solution; purity ≥ 36.5%: CAS No. 7647-01-0

4.1.8. Tween 20: CAS No. 9005-64-5

4.1.9. Lyophilised Bovine Serum Albumin (BSA) powder; purity ≥ 96%: CAS No. 9048-46-8

4.1.10. 4-Chloro-1-naphthol; purity ≥ 99%: CAS No. 604-44-4

4.1.11. Methanol; purity ≥ 99.8%: CAS No. 67-56-1

4.1.12. Hydrogen peroxide in solution (H_2O_2); purity ≥ 30% : CAS No. 7722-84-1

4.2. Preparation of working solutions

All of the solutions may be stored for 1 year at 4 °C.

4.2.1. TBS buffer (tris-buffered saline)

Dissolve 29.22 g of sodium chloride (4.1.5) and 2.42 g of anhydrous Tris-HCl (4.1.6) in 1 litre of ultra-pure water (4.1.1). Adjust the pH to 7.5 using a concentrated HCl solution (4.1.7).

4.2.2. TBS-Tween 20 buffer

Add 0.05% of Tween 20 (4.1.8) to the TBS buffer (4.2.1).

4.2.3. Blocking solution

Add 4% of BSA (4.1.9) to the TBS buffer (4.2.1).

4.2.4. Polyclonal antibody solution (available on the market or according to the protocol described in the Annex)

4.2.4.1. Dilute the unstable anti-protein polyclonal antibodies (primaries) according to commercial recommendations or to their concentrations in the TBS buffer (4.2.1).

4.2.4.2. Dilute the goat anti-rabbit-HRP polyclonal antibodies (secondaries) according to commercial recommendations or to their concentrations in the TBS buffer (4.2.1).

4.2.5. Solutions for revelation of unstable proteins

4.2.5.1. Dissolve 30 mg of 4-chloro-1-naphthol (4.1.10) in 10 mL of methanol (4.1.11). Place this solution in the dark at -20 °C until needed.

4.2.5.2. Add 30 µL of 30% H_2O_2 (4.1.12) to 50 mL of TBS (4.2.1) just before use.

5. Materials

5.1. List of materials for the immunoprinting reaction:

- nitrocellulose membrane with 0.2 µm pores for conducting immunoprinting;
- 0.5-10 µL and 100-1000 µL automatic pipettes, corresponding cone filters;
- tubes, tube rack for dilutions of antibodies;
- class-A graduated cylinders;
- absorbent paper;
- tweezers;
- laboratory glassware to carry out the reaction: small crystalliser, Petri dish, tubes, stoppers, etc.;
- platform shaker (for a reaction in a dish) or vortex mixer (for a reaction in a tube) with a maximum speed of 20 RPM.

Equipment required to prepare the solutions:

- class-A calibrated flasks;
- pH meter;
- precision weighing balance with an accuracy of 0.1 mg;
- 3000-g centrifuge and centrifuge tubes.

6. Sampling

The samples should be taken and stored at 4 °C so as not to modify the proteins naturally present in the wine.

6.1. Sample preparation

The samples (or laboratory samples) of wines are applied directly to the nitrocellulose membrane (5.1.1) using the pipette (5.1.2), without prior preparation.

7. Procedure

Analysis may be conducted on unfiltered wines on the sole condition that these wines do not contain bentonite in suspension. If this is the case, carry out centrifugation at

3000 g (5.2.4) for 10 min at room temperature.

As indicated in point 3, the immunological method of immunoprinting takes place in 3 steps, and the reactions are conducted at a room temperature of between 18 °C and 25°C.

7.1. Application of the wine sample

Apply 5 µL (5.1.2) of test portion from the samples and standard colorimetric solutions to the nitrocellulose membrane (5.1.1).

Leave to dry for 15-20 min at room temperature.

7.2. Addition of monoclonal antibodies

7.2.1. Place the membrane in the dish or tube (5.1.7). The volume of the solutions will be dependent on the container and the size of the membrane. This membrane should be covered.

The volumes specified below are for a small Petri dish-type container (5.1.7).

Add the blocking solution (4.2.3). Mix for at least 30 minutes (5.1.8).

7.2.2. Wash by draining off the solution, holding down the membrane if necessary with tweezers, before adding 20 mL of TBS (4.2.1) and mixing for several minutes (5.1.8).

Wash a second time as described above and drain off the solution.

7.2.3. Add 20 mL of primary antibody solution (4.2.4.1).

Mix for one hour (5.1.8).

Wash 3 times with the TBS-Tween 20 solution (4.2.2).

7.2.4. Add 20 mL of goat anti-rabbit-HRP secondary antibody solution (4.2.4.2).

Mix for one hour.

7.2.5. Wash with the TBS-Tween 20 solution (4.2.2) as described above for 5 min.

Wash 2 times with the TBS solution (4.2.1) as described above for 15 min.

Drain off the solution.

7.3. Revelation of the presence of unstable proteins

7.3.1. Mix the two solutions to reveal the unstable proteins (4.2.5.1 and 4.2.5.2) and place in contact with the membrane (5.1.1) prepared according to protocols 7.1 and 7.2, to which the wine has been applied, and stir.

A black-dark purple to mauve precipitate appears on the membrane where the unstable proteins are present.

The colour intensity is dependent upon the concentration of unstable proteins and therefore the risk of protein *casse*.

After 20-30 min, when the spot corresponding with the application of the positive standard colorimetric solution (4.1.2) is very intense, stop the colouration by washing the nitrocellulose membrane (5.1.1) in water.

Place the membrane to be dried between 2 sheets of absorbent paper (5.1.5).

The results can be interpreted when the membrane is dry.

8. Results

For the results to be interpretable:

- the place of application of the positive standard colorimetric solution should show a spot of high colour intensity (dark purple-black),
- the place of application of the negative standard colorimetric solution should show no spots,
- the background "noise" (place on the membrane where no sample has been applied) should be very light, even white.

A semi-quantitative result may be obtained by making a calibration curve based on a wine naturally rich in proteins, for which a dilution range will be used. This calibration curve will be dependent on the surface areas obtained through integration of the colour intensity of the spots corresponding to the formation of immunocomplexes. Analysis may be carried out with the same equipment as that used to analyse the electrophoresis gels described in the Method OIV-MA-AS315-12.

Interpretation of the results may also be carried out visually.

8.1. For direct control over the presence or absence of unstable proteins in wine

Proteins are present in the laboratory sample if the colour intensity of the spot obtained is higher than that of the spot for the negative standard colorimetric solution.

The colour intensity of the spot obtained after the reaction is proportional to the quantity of unstable proteins and, consequently, proportional to the risk of protein *casse* in this wine.

8.2. To verify the absence of proteins after treatment (in particular, with bentonite)

Proteins are present in the sample if the colour intensity of the spot obtained for the test portion without bentonite treatment is higher than that of the spot for the negative standard colorimetric solution.

In the case of application of a "range of treatment products (bentonite)" in a laboratory test, the colour intensity of the spots in each test portion should decrease as the treatment product concentration increases. Where this intensity is null or minimal for

one spot but consistent in relation to the other samples in the range, the dose of the treatment product corresponding to the spot in question is applied to achieve protein stability in the tested wine.

9. Annexes

Production of polyclonal antibodies directed against unstable proteins

The antibodies directed against unstable proteins in white and rosé wines may be prepared in rabbits. It is their specificity which makes the method reliable and precise.

9.1. Purification of Chitinase and Thaumatin-like proteins

9.1.1. List of products and equipment

9.1.1.1. Ultra-pure water: resistivity ≥ 18 MΩ.cm

9.1.1.2. Wine grape harvested at technological maturity (Chardonnay or Sauvignon blanc vine variety, for example

9.1.1.3. Anhydrous sodium acetate: CAS No. 127-09-3

9.1.1.4. Triton X-100: CAS No. 9002-93-1

9.1.1.5. Anhydrous ammonium sulphate: CAS No. 127-09-3

9.1.1.6. Anhydrous sodium chloride (NaCl): CAS No. 7647-14-5

9.1.1.7. Anhydrous Tris-HCl: CAS No. 1185-53-1

9.1.1.8. 37% Pure hydrochloric acid: CAS No. 7647-01-0

9.1.1.9. 1M NaoH sodium hydroxide solution: CAS No. 1310-73-2

9.1.1.10. Laboratory glassware, including Class-A calibrated flasks and pipettes

9.1.1.11. Tweezers

9.1.1.12. 10,000-g Centrifuge

9.1.1.13. Laboratory weighing balance with an accuracy of 0.1 mg

9.1.1.14. pH meter

9.1.1.15. Strong anionic resin

9.1.1.16. Anionic resin

9.1.1.17. Membrane with cut off of 10 kDa

9.1.1.18. Low-pressure liquid chromatography apparatus with concentration-gradient pump

9.1.1.19. Detector measuring the absorbance at à 280 nm

9.1.1.20. Conductivity detector

9.1.2. Preparation of sodium acetate buffer (9.1.1.3) diluted to 50 mM, 0.25% Triton

X-100 (9.1.1.4) at pH 5

Place the following successively in a 1-L calibrated flask (9.1.1.10):

- 4.1 g sodium acetate (9.1.1.3),
- 2.5 g Triton X-100 (9.1.1.4),
- make up to 1 L with ultra-pure water (9.1.1.1) and stir; adjust the pH to 5 using 37% HCl (9.1.1.8) in order to avoid having a basic environment that could have a harmful effect on the proteins to be extracted or impede their extraction.

3. Preparation of the 50-mM Tris-HCl buffer, pH 8.0

Place the following successively in a 1-L calibrated flask (9.1.1.10):

- 7.9 g anhydrous Tris-HCl (9.1.1.7)

Make up to 1 L with ultra-pure water (9.1.1.1); adjust the pH to 8 using a 1M NaOH solution (9.1.1.9).

9.1.4. Preparation of the 50 mM Tris-HCl buffer, 100 mM NaCl

Place the following successively in a 1-L calibrated flask (9.1.1.10):

- 7.9 g anhydrous Tris-HCl (9.1.1.7),
- 5.8 g anhydrous sodium chloride (9.1.1.6),
- make up to 1 L with ultra-pure water (9.1.1.1) and mix.

5. Preparation of 100 mM sodium chloride solution

- Place the following successively in 1-L calibrated flask (9.1.1.10)
- 5.8 g anhydrous sodium chloride (9.1.1.6),
- make up to 1 L with ultra-pure water (9.1.1.1) and mix.

2. Procedure

Grapes from the Pinot noir or Chardonnay vine varieties are harvested at maturity and frozen at -20 °C. The seeds are removed from the frozen grapes before crushing. 3 g of seeded grapes are crushed into 10 mL of sodium acetate buffer (9.1.2), diluted to 50 mM, pH 5, containing 0.25% Triton X-100 (9.1.1.4). The insoluble material is removed by centrifugation (5 min at 3000 g) (9.1.1.12). The supernatant (2 mL) is then frozen overnight at -20 °C for clarification purposes. The extract is then centrifuged at 10,000 g for 15 min to remove the insoluble material.

Ammonium sulphate (9.1.1.5) is added to the supernatant up to a concentration of 30%. The mixture is mixed for 1 hour at 4 °C then centrifuged once more as described above.

Ammonium sulphate (9.1.1.5) is again added to the supernatant up to a final concentration of 60%. The mixture is mixed for 2 hours at 4 °C then centrifuged once more as described above.

The protein precipitate is collected then re-dissolved in 1 mL of 50 mM Tris-HCl buffer, pH 8.0 (9.1.3). The proteins are bound to a column containing a strong anionic resin (5 x 30 cm) (9.1.1.15). The column is washed with the Tris-HCl buffer described above. Chitinase and Thaumatin-like proteins are then extracted using a Tris-HCl buffer containing 100 mM NaCl. All the fractions are collected then desalinated on a membrane with a MWCO of 10 kDa (9.1.1.17) using the 50 mM Tris-HCl buffer, pH 8.0 (9.1.4.3). The desalinated protein fractions are then loaded onto a low-pressure chromatography column (9.1.1.18) containing an anionic resin (9.1.1.16). Elution is carried out with 120 mL of a NaCl (9.1.5) gradient ranging from 0 to 100 mM by means of a concentration-gradient pump using a solution A of ultra-pure water (9.1.1.1) and a solution B of sodium chloride, 100 mM (9.1.5). The protein and salt concentrations are estimated respectively by measuring the absorbance at 280 nm and the conductivity of the fluids exiting the column using detectors (9.1.1.19 and 9.1.1.20). The Chitinase and Thaumatin-like protein fractions thus purified and separated are used for the production of antibodies.

9.3. Production of anti-Chitinase and Thaumatin-like polyclonal antibodies in rabbits

The protocol used is identical to that described in the Method OIV-MA-AS315-12.

10. Bibliography

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