RESOLUTION OENO 24/2004

DETERMINATION OF PLANT PROTEINS IN WINES AND MUSTS

THE GENERAL ASSEMBLY,

IN VIEW of Article 2 paragraph 2 iv of the Agreement of 3 April 2001 establishing the International Organisation of Vine and Wine,

UPON THE PROPOSAL by the Sub-Commission of methods of analysis and appraisal of wines,

DECIDES to complete Annex A of the Compendium of international methods of analysis of wines and musts, by the following method:

DETERMINATION OF PLANT PROTEINS IN WINES AND MUSTS

The technique developed below enables to determine the quantity of proteins possibly remaining in beverages treated with proteins of plant origin after racking.

1. PRINCIPLE

Wine and must proteins are precipitated with trichloroacetic acid, then they are separated by electrophoresis in polyacrylamide gel in the presence of dodecyl sodium sulphate (DSS). The addition of Coomassie blue colours the proteins. The intensity of the colouration enables to determine the protein content using a calibration curve made beforehand with the known protein concentration solutions. The antigenic capacity of musts and treated wines is determined by immunoblotting testing.

2. PROTOCOL

2.1. Concentration of proteins by precipitation with trichloroacetic acid (TCA)

2.1.1. Reagents

2.1.1.1. Pure trichloroacetic acid (TCA)

2.1.1.2. TCA at 0.1% prepared using 2.1.1.1: 0.1 g in 100 ml of water.

Certified in conformity Vienna, 30th July 2004
The Director General of the OIV
Secretary of the General Assembly
Frederico CASTELLUCCI

© OIV 2004

2.1.1.3. TCA at 100% prepared using 2.1.1.1: 100 g in 100 ml of water.

2.1.1.4. Sodium hydroxide 0.5 M

2.1.1.5. Buffer Tris/HCl 0.25 M pH=6.8

30.27 g of Tris-(hydroxymethyl)aminomethane (Tris) are dissolved in 300 ml of distilled water. The pH is adjusted to 6.8 with concentrated hydrochloric acid for analysis. The volume is completed to 1 l with distilled water. The buffer is stored at 4°C.

2.1.1.6. Pure glycerol

2.1.1.7. Pure dodecyl sodium sulphate (DSS)

2.1.1.8. Pure 2-mercaptoethanol

2.1.1.9. Buffer solution for samples: it is made up of a buffer Tris/HCl 0.25 M, pH=6.8 (2.1.1.5); 7.5% of pure glycerol (2.1.1.6); 2% of dodecyl sodium sulphate (DSS) (2.1.1.7) and 5% of pure 2-mercaptoethanol (2.1.1.8). The percentages of different reagents correspond to the final concentration in the buffer solution.

2.1.2. Procedure

3 ml of trichloroacetic acid at 100% (2.1.1.3) and 24 ml of wine or must (treated or untreated) are successively put in 50 ml centrifuge tubes. The final concentration in TCA thus obtained is 11%.

After 30 minutes at 4°C, the samples are centrifuged at 10,000 rpm for 30 minutes at 4°C. The pellets are washed in an aqueous solution of TCA at 0.1% (2.1.1.2), re-centrifuged and put again in suspension in 0.24 ml mixture (1:1, v/v) of sodium hydroxide 0.5 M (2.1.1.4) and buffer solution (2.1.1.9). The samples are heated at 100°C in a water bath for 10 minutes.

2.2. Electrophoresis in Polyacrylamide Gel in the presence of DSS

2.2.1. Reagents

2.2.1.1. Buffer Tris/HCl 1.5 M pH=8.8

181.6 g of Tris-(hydroxymethyl)aminomethane are dissolved in 300 ml of distilled water. The pH is adjusted at 8.8 with concentrated hydrochloric acid for analysis. The

Certified in conformity Vienna, 30th July 2004
The Director General of the OIV
Secretary of the General Assembly
Frederico CASTELLUCCI

© OIV 2004

volume is completed to 1 l with distilled water. The buffer is stored at 4°C.

2.2.1.2. Mixture of acrylamide (30%)-bis-acrylamide (0.8%)-glycerol (75%)

Slowly add 300 g of acrylamide and 8 g of bis-acrylamide to 600 ml of a glycerol solution at 75%. After dissolution, adjust the volume to 1 l with glycerol at 75%. The mixture is stored in the dark at room temperature.

2.2.1.3. DSS at 10%

10 g of DSS are dissolved in 100 ml of distilled water. Store at room temperature.

2.2.1.4. N,N,N',N'-tetramethylenediamine (TEMED) for electrophoresis

2.2.1.5. Ammonium persulfate at 10%

1 g of ammonium persulfate is dissolved in 10 ml of distilled water. Store at 4°C.

2.2.1.6. Bromophenol blue solution

10 mg of bromophenol blue for electrophoresis are dissolved in 10 ml of distilled water.

2.2.1.7. Solution for the separation gel (15% of acrylamide)

It is prepared just before use:

- 1.5 ml of Tris/HCl 1.5 M, pH=8.8 (2.2.1.1),
- 1.5 ml of distilled water
- 3 ml of glycerol acrylamide mixture (2.2.1.2),
- 50 µl of DSS 10% (2.2.1.3),
- 10 µl of N,N,N',N'-tetramethylenediamine (TEMED) for electrophoresis (2.2.1.4),
- 20 µl of ammonium persulfate (2.2.1.5).
- 1 drop of bromophenol blue (2.2.1.6)

2.2.1.8. Buffer Tris/HCl 0.5 M pH=6.8

60.4 g of Tris-(hydroxymethyl)aminomethane are dissolved in 400 ml of distilled water. The pH is adjusted to 6.8 with concentrated hydrochloric acid for analysis. The volume is completed to 1 l with distilled water. The buffer is stored at 4°C.

Certified in conformity Vienna, 30th July 2004
The Director General of the OIV
Secretary of the General Assembly
Frederico CASTELLUCCI

© OIV 2004

2.2.1.9. Mixture of acrylamide (30%)-bis-acrylamide (0.8%)-water

Slowly add 300 g of acrylamide and 8 g of bis-acrylamide to 300 ml of water. After dissolution, adjust the volume to 1 l with distilled water. The mixture is stored in the dark at room temperature.

2.2.1.10. Concentration gel at 3.5% of acrylamide

It is prepared just before use:

- 0.5 ml of Tris/HCl 0.5 M pH=6.8 (2.2.1.8),
- 1.27 ml of distilled water
- 0.23 ml of water acrylamide mixture (2.2.1.9),
- 20 µl of DSS 10% (2.2.1.3),
- 5 µl of N,N,N',N'-tetramethylenediamine (TEMED) for electrophoresis (2.2.1.4),
- 25 µl of ammonium persulfate (2.2.1.5),
- 1 drop of bromophenol blue (2.2.1.6).

2.2.1.11. Migration buffer

30.27 g of Tris-(hydroxymethyl)aminomethane, 144 g of glycine and 10 g of DSS are dissolved in 600 ml of distilled water. The pH should be 8.8. If necessary, it is adjusted with concentrated hydrochloric acid for analysis. The volume is completed to 1 l with distilled water. The buffer is stored at 4°C. At the time of use, the solution is diluted to 1/10 in distilled water.

2.2.1.12. Colouring solution

Are successively mixed:

- 16 ml of ultra-pure Coomassie brilliant blue G-250 at 5% (5 g in 100 ml of distilled water),
- 784 ml from a 1 l solution where 100 g of ammonium sulphate and 13.8 ml of orthophosphoric acid at 85% were dissolved for analysis,
- 200 ml of absolute ethanol.

Certified in conformity Vienna, 30th July 2004
The Director General of the OIV
Secretary of the General Assembly
Frederico CASTELLUCCI

© OIV 2004

2.2.1.13. Discolouring solution

Are successively mixed:

- 100 ml of glacial acetic acid 100% for analysis,
- 200 ml of absolute ethanol for analysis.
- 700 ml of distilled water.

2.2.2. Procedure

The separation gel solution (2.2.1.7) is poured between two glass plates of 7x10cm. The upper surface of the gel is levelled by the addition of 2 drops of distilled water.

After polymerisation of the separation gel and the elimination of water, 1 ml of concentration gel (2.2.1.10) is deposited on the separation gel using a 1 ml pipette. Then the comb is set up whose imprints will create deposit wells.

The samples necessary for the calibration range are prepared in a mixture (1:1), v/v, 0.5% M sodium hydroxide (2.1.1.4) and the buffer solution (2.1.1.9) in order for the calibration range be between 5 µg/ml and 50 µg/ml.

20 to 30 µl of wine and calibration solution are deposited in the wells.

After migration (at a constant voltage of 90 V) at room temperature for about 3-4 hours, the gels are removed from the mould. They are immediately plunged into 50 ml of an aqueous solution of TCA 20% for 30 minutes then in 50 ml of the colouring solution (2.2.1.12).

The proteins appear in the form of blue coloured bands. The gel is then discoloured with 50 ml of discolouring solution (2.2.1.13). When the bottom of the gel is transparent, it is placed in distilled water for storage.

3. QUANTITATIVE ANALYSIS

The intensity of each spot is evaluated by using a scanner for gel with an image analyser software. The quantity of protein on the gel is determined by the calculation of the average density of the pixels of the band and by integration of the band width. The protein content of each sample is obtained using a calibration curve. The points of this curve are obtained by tracing the known concentration values of plant proteins deposited on the gel depending on the corresponding integration area

The detection and quantification limit is about 0.030 ppm for peas and at 0.36 ppm for

Certified in conformity Vienna, 30th July 2004
The Director General of the OIV
Secretary of the General Assembly
Frederico CASTELLUCCI

© OIV 2004

gluten, in an environment concentrated 100 times. The coefficient of variation is always below 5%.

4. SEARCH BY IMMUNOBLOTTING OF THE ANTIGENIC POTENTIAL OF WINES AND MUSTS TREATED

The antigenic capacity of proteins that could remain in the beverages treated after racking is then evaluated.

4.1. PRINCIPLE

After electrophoresis, the gels are submitted to the immunoblotting technique. The proteins are transferred to a membrane where they are adsorbed. An antigen–antibody complex is formed by the addition of a plant anti-protein antibody (for example anti–gliadin antibodies if the plant protein is gluten). The method is revealed by the addition of an antibody directed against the plant anti-protein antibodies coupled with phosphatase. In the presence of the chromogenic substrate of the enzyme, a colouration whose intensity will be proportional to the quantity of immunocomplexes will develop. This immunoreactivity will be quantified using a calibration curve made with known concentration plant proteins solutions.

4.2. PROTOCOL

4.2.1. Reagents

4.2.1.1. Transfer buffer

3.03 g of Tris, 14.4 g of glycine (R), 200 ml of methanol (R) are mixed and completed to 1 l with distilled water.

4.2.1.2. Gelatine 1%

8.77 g of sodium chloride (R), 18.6 g of ethylenediaminetetraacetic acid (EDTA) for analysis, 6.06 g of Tris and 0.5 ml of Triton X are dissolved in 800 ml of distilled water. The pH is adjusted to 7.5 with concentrated hydrochloric acid for analysis. 10 g of gelatine are added and the volume is completed to 1 l.

4.2.1.3. Gelatine 0.25%

8.77 g of sodium chloride (R), 18.6 g of ethylenediaminetetraacetic acid (EDTA) for analysis, 6.06 g of Tris and 0.5 ml of Triton X are dissolved in 800 ml of distilled water. The pH is adjusted to 7.5 with concentrated hydrochloric acid for analysis. 2.5 g of

Certified in conformity Vienna, 30th July 2004
The Director General of the OIV
Secretary of the General Assembly
Frederico CASTELLUCCI

© OIV 2004

gelatine are added and the volume is completed to 1 l.

4.2.1.4. Polyclonal antibody solution (marketed or described in the annex)

- 10 µl of polyclonal plant anti-protein antibodies
- q.s.f. 10 ml with gelatine at 0.25% (4.2.1.3).

4.2.1.5. TBS buffer

29.22 g of sodium chloride for analysis and 2.42 g of tris are dissolved in 1 l of distilled water.

4.2.1.6. Alkaline phosphatase buffer

5.84 g of sodium chloride (R), 1.02 g of magnesium chloride (R) and 12.11 g of Tris are dissolved in 800 ml of distilled water. The pH is adjusted to 9.5 with concentrated hydrochloric acid and the volume is completed to 1 l.

4.2.1.7. Developer

15 g of bromochloroindol phosphate (BICP) and 30 g of nitro blue tetrazolium (NBT) are dissolved in 100 ml of alkaline phosphatase buffer (4.2.1.6).

4.2.2. Procedure

After electrophoresis, the proteins are transferred from the gel to a membrane of polyvinylidene difluoride by electrophoretic elution: 16 hours at 4°C at 30 V in the transfer buffer (4.2.1.1). The membranes are saturated with gelatine at 1% (4.2.1.2) and washed 3 times with gelatine at 0.25% (4.2.1.3). The gelatine becomes set on free sites and inhibits non specific adsorption of immunological reagents. The membrane is then plunged into 10 ml of the plant anti-protein polyclonal antibody solution (4.2.1.4). For gluten, the anti-gliadin antibodies are purchased. The other antibody types are prepared according to the method provided for in the annex. The IgG-antigen complex is detected by the addition of 10 µl of anti-IgG rabbit antibodies marked with alkaline phosphatase. The membranes are washed twice with gelatine 0.25% (4.2.1.3) and once with the TBS buffer (4.2.1.5). After incubation in the developer (4.2.1.7), a dark purple precipitate is formed in the spot where the enzyme is attached.

4.3. QUANTITATIVE ANALYSIS

In order to calculate the quantity of residual immunoreactivity of a marketed wine, a calibration curve is traced out: known concentrations of plant proteins deposited on

Certified in conformity Vienna, 30th July 2004
The Director General of the OIV
Secretary of the General Assembly
Frederico CASTELLUCCI

© OIV 2004

the gel (and transferred to a membrane) depending on the areas obtained by integration of the intensity of the spots corresponding to the formation of immune-complex. The analysis is done with the same equipment as for analysing electrophoresis gels.

ANNEX Production of polyclonal anti-peas

Anti-peas polyclonal antibodies necessary for the determination of antigenic capacity of pea proteins in wine and musts treated are being carried out on animals.

1. PRINCIPLE

Serums containing polyclonal antibodies are obtained from New Zealand rabbits after an intradermal injection of antigen.

2. PROTOCOL

2.1. Reagents

2.1.1. PBS pH=7.4 phosphate buffer: 8 g of NaCl, 200 mg of KCl, 1.73 of $Na_2HPO_4\ H_2O$ and 200 mg of KH_2PO_4 are dissolved in 300 ml of distilled water. pH is adjusted to 7.4 with sodium hydrate 1 M. The volume is brought to 1 l with distilled water.

2.1.2. Antigens:

10 mg of pea protein is dissolved in 5 ml of PBS phosphate buffer (2.1.1). The solution is then filtered under sterile conditions through 0.2 µm and stored at –20°C until the day of immunization.

2.2. Procedure

1 ml of 2.1.2. solution is mixed with 1 ml of Freund complete adjuvant. 1 ml of this mixture is injected intradermically to a New Zealand rabbit weighing approximately 3 kg. This injection is repeated on day 15, day 30 and day 45.

60 days after the first injection, 100µl of blood were withdrawn from the auricular vein which was then tested for its capacity to react to antigens. Immunoblotting was used for this evaluation as described in Chapter 4.2 of the analysis method using a gel with a pea protein which migrated on the gel.

After checking the formation of an antigen-antibody complex, 15 ml of blood were

Certified in conformity Vienna, 30th July 2004
The Director General of the OIV
Secretary of the General Assembly
Frederico CASTELLUCCI

© OIV 2004

withdrawn from the auricular vein. The blood is placed at 37°C for 30 minutes. The serum containing the anti-pea polyclonal antibodies is withdrawn after centrifuging the blood at 3000 rpm for 5 minutes.

Certified in conformity Vienna, 30th July 2004
The Director General of the OIV
Secretary of the General Assembly
Frederico CASTELLUCCI

© OIV 2004