COEI-1-EPLEV Yeast protein extracts (YPE)

1. Object, origin and scope of application

The proteins of yeast protein extracts mainly come from the cytoplasm of Saccharomyces *spp. or non-Saccharomyces* yeasts. These are separated by physical methods after an extraction process that limits their hydrolysis.

The proteins of yeast protein extracts have variable molecular weights and electric charges depending on the manner in which they were obtained and they are capable of flocculation in musts and wines so as to enable clarification and colloidal stabilizing (fining operations).

When the yeast protein extracts come from genetically modified oenological yeasts, they must have been subject to the prior authorisation of the competent authorities.

2. Labelling

The label must include the following:

the scope of application (must and wine fining)

the conditions of safety and conservation as well as their shelf life

possible admixtures

the manufacturing batch number

the indication of whether the protein extracts come from yeasts obtained through genetic modifications and their modified character if that is the case.

3. Analysis

3.1. The YPEs are in the form of powder, generally with micro-granulate, of yellow-to light beige or beige colour, with a slight smell of yeast.

3.2. The YPEs are water-soluble but not ethanol-soluble.

When in aqueous solution, they precipitate if 1 volume of ethanol is added.

3.3. *Protein determination*

3.3.1. Total proteins

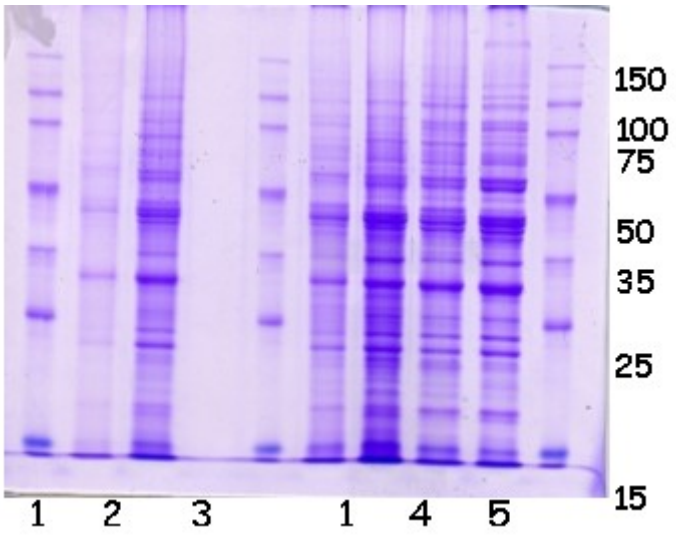
The determination of proteins is to be carried out with the Lowry method as described in appendix 1.

The total protein content of YPEs must be greater than 50% of the dry product.

3.3.2. Size of proteins

The assessment of the proteins' size or weight is carried out by electrophoresis separation technique SDS-PAGE, as described in appendix 3.

Example of different yeast protein extract profiles with Coomassie blue staining:



- 1: Size marker
- 2: Strain 1 Exponential phase
- 3: Strain 1 Stationary phase
- 4: Strain 2 Exponential phase
- 5: Strain 2 Stationary phase
- 6: Strain 3 Exponential phase (Strain without protease A)
- 7: Strain 3 Stationary phase (Strain without protease A)

3. Protein level greater than 15 KDa

This level is assessed using the gel permeation technique described in appendix 4.

At least 50% of the total proteins must have a molecular weight greater than 15 kDa.

3.4. Amine Nitrogen

The amine nitrogen content given as glycine, represents 10 to 20% of the dry product maximum

The determination of amine nitrogen may be carried out by the Dinitrofluorobenzene method (DNFB) described in appendix 2.

4. Testing

4.1. Desiccation-related losses

Put 5g of YPE in a 70mm silica capsule then place in an incubator at 100-105 °C for 5 hours. The loss of weight must not exceed 15.p.100.

The limiting values indicated below are for dry product.

4.2. Ash

Incinerate the dry residue at 550-600°C. The ash content must not exceed 8%.

4.3. Preparation of the solution for testing

Prepare an YPE solution at 10g/l in water.

4.4. Lead

Determine the lead content of the solution prepared for testing purposes (4.3) with the method described in chapter II of the International Oenological Codex.

The lead content must not exceed 2mg/kg.

4.5. Mercury

Determine, without evaporating the solution, the mercury content of the solution prepared for testing purposes (4.3) with the method described in chapter II of the International Oenological Codex.

The mercury content must not exceed 1 mg/kg.

4.6. Arsenic

Determine the arsenic content of the solution prepared for testing purposes (4.3) with the method described in chapter II of the International Oenological Codex.

The arsenic content must not exceed 3 mg/kg.

4.7. Cadmium

Determine the cadmium content of the solution prepared for testing purposes (4.3) with the method described in chapter II of the International Oenological Codex.

The cadmium content must not exceed 1 mg/kg.

4.8. Microbiological analysis

4.8.1. Total aerobic mesophilic flora

Carry out the enumeration with the method described in Chapter II of the International Oenological Codex.

The number must not exceed 10,000 total aerobic mesophilic bacteria in 1g.

4.8.2. Coliforms

Carry out the enumeration with the method described in Chapter II of the International Oenological Codex.

The number must not exceed 10CFU/g of dry matter.

4.8.3. Staphylococcus

Carry out the enumeration with a method that is to be described in Chapter II of the International Oenological Codex. Controlled absence of Staphylococcus aureus in a 1 g sample of dry matter.

4.8.4. Salmonella

Carry out the enumeration with the method described in Chapter II of the International Oenological Codex.

Controlled absence of salmonella in a 25 g sample of dry matter.

4.8.5. *Escherichia coli*

Carry out the enumeration with the method described in Chapter II of the International Oenological Codex.

Controlled absence of Escherichia coli in a 25 g sample of dry matter.

4.8.6. Lactic bacteria

Carry out the enumeration with the method described in Chapter II of the International Oenological Codex.

The number must not exceed 10^3 CFU/g of dry matter.

4.8.7. Mould

Carry out the enumeration with the method described in Chapter II of the International Oenological Codex.

The number must not exceed 50 CFU/g of dry matter.

4.8.8. Yeasts

Carry out the enumeration with the method described in Chapter II of the International Oenological Codex..

The number must not exceed 10^2 CFU/g of preparation.

4.9. Efficiency test of types for the fining of musts and wines

4.9.1. Principle

The aim is to determine the most compatible quantity needed to achieve fast clarification and colloidal stability of the wine.

4.9.2. Product:

Musts or wines to undergo fining

4.9.3. Protocol:

4.9.3.1. YPE solution at 2%

Dissolve 2 g of YPE in 100 ml of distilled water.

4.9.4. Fining test

Place 100 ml of must or wine in as many 100 ml tubes as determinations selected. In practice, the comparison of 4 determinations is sufficient, i.e. five 100 ml tubes, including the control.

Add 0 ml (control), 1.5 ml, 2 ml, 2.5 ml of the YPE solution (4.9.3.1) for a red wine and 0.5 ml, 1 ml, 1.5 ml of YPE solution for a white or rosé must or for a white or rosé wine. These quantities correspond respectively to the final determinations of 0 mg/l, 200 mg/l, 300 mg/l, 400mg/l and 500 mg/l for a red wine and to 0 mg/l, 100 mg/l, 200 mg/l and 300 mg/l for a white or rosé must or for a white or rosé wine.

- Homogenise each tube after admixture of the YPE solution (cover the tubes with a film and shake 2 to 3 times by hand).

Note the turbidity increase speed and the apparition of flakes every 10 minutes for 30 minutes. After 8 hours compare each test and monitor:

- Turbidity
- the volume of lees,
- the colouring intensities,
- the organoleptic quality,
- the colloidal stability by heating to 80°C for 20 minutes in a water bath or incubator at 100 °C and rapid cooling under a stream of cold water.

5. Conservation

The yeast protein extracts have a 3 year shelf-life in closed packaging if stored in temperate facilities and kept away from humidity.

6. Bibliography

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- Charpentier C. (2000). "Yeast autolysis and yeast macromolecules? Their contribution to wine flavor and stability." Am. J. Enol.Vitic. 51: 271-277.
- Charpentier C., Caillet M.M, Feuillat M. (2006) Essais de collage de moûts blancs et de vins rouges avec un extrait protéique levurien : comparaison avec les colles traditionnelles. Rev. Œnologues 120, juillet 2006.

Appendix 1: Lowry Method

1. Introduction

The proposed method is that of LOWRY (LOWRY et al. 1951) but can be replaced by other methods such as that of BRADFORD (1976).

Bradford, M. M. (1976) A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Anal. Biochem. 72:248-254.

2. Scope

The LOWRY method is derived from that of Biuret: in an alkaline medium, copper ions form with proteins a pink-purple copper complex characteristic of peptide bonds. The method is 100 times more sensitive than the Biuret method.

3. Definition

The LOWRY method consists in copper complexing, in an alkaline medium, approximately a quarter of the amino acids constituting the proteins. The Folin Ciocalteu reagent (phosphomolybdic reagent) reacts with the aromatic amino acids of the proteins.

The absorbance of the complex thus formed is determined spectrophotometrically at 750 nm. The main drawback of this method is the interference of the Folin reagent with other compounds (EDTA, dithioerythritol, oxidized glutathione, etc.).

The determination of the water-soluble proteins is performed by comparison with a standard curve plotted on the basis of protein solutions of known concentrations. (BSA type).

4. Reagents and Products

Solution A: solution of 2% Na_2CO_3 in 0.1 M NaOH containing 0.02% (500 mL) of sodium tartrate (or potassium).

Solution B: solution of 5% $CuSO_4.5\ H_2O$ (100 mL).

Reagent C: produce extemporaneously 50 mL of solution A + 1 mL of solution B.
FOLIN-CIOCALTEU reagent: commercial solution.
Standard solution of Bovine Serum Albumin (BSA).

5. Equipment

Test tubes
Class A pipettes
Micropipettes
Plastic film for sealing
Visible Spectrophotometer

6. Procedure

6.1. Standard range of proteins: preparation and determination

The standard range is made using a standard solution of 0.5 $mg.mL^{-1}$ of BSA.
In graded 100-mL flasks, prepare solutions of BSA containing 0, 100, 200, 300, and 400 $\mu g.mL^{-1}$ of BSA from the stock solution.
Dispense into test tubes 0.6 mL each dilution. A control tube contains only 0.6 mL of distilled water
Thoroughly mix each solution by inversion.
Add to each tube:
3 mL of reagent C
Close the tube with the plastic wrap and homogenise by inversion
Let the tubes stand for 10 minutes before adding 0.3 mL of Folin reagent.
Homogenize.
Wait 30 minutes in the dark and then measure the absorbance at 750 nm by adjusting the zero of the distilled water solution (concentration in BSA 0 $\mu g.\ mL^{-1}$).
Plot the curve OD = f (protein concentration).

6.2. Determination of proteins in the Yeast Protein Extract

In 3 test tubes add successively:

0.6 mL of extract diluted to $1/20^{th}$, $1/30^{th}$, and $1/40^{th}$ (30, 20 and 15 µl in 0.6 mL)
3 mL of reagent C; homogenize after sealing the tubes and let them stand for 10 minutes.
0.3 mL of Folin reagent, homogenise.

Wait 30 minutes in the dark

Measure the absorbance at 750 nm.

7. Calculations

Note: If the absorbance values are low, recommence the procedure using smaller dilutions of yeast protein extract ($1/10^{th}$, $1/5^{th}$, 1/4 i.e. 60, 120 and 150 µl in 0.6 mL).

Determine by comparing with the standard curve the protein concentration in the YPE in $\mu g.mL^{-1}$ then in $mg.mL^{-1}$ by direct reading or by using the regression line of the standard curve (specify on the standard curve the equation for the line and the correlation coefficient).

Appendix 2: Dinitrofluorobenzene method

1. Introduction

This method is used to quickly determine the amino nitrogen in a biological solution compared with a standard range produced with a solution of glycine.

2. Scope

Oenological products of plant or animal origin

3. Definition

Dinitrofluorobenzene (DNNFB) reacts with free NH_2 functions contained in the amino acids to give a bright yellow compound determined by colorimetry at 420 nm. The reaction takes place at pH> 9.3.

4. Reagents and Products

Reagents:

- Borax or sodium tetraborate
- 10 M Hydrochloric acid dinitrofluorobenzene
- Glycine

5. Equipment:

- haemolysis tubes
- micropipettes

- Visible spectrophotometer
- Water bath at 60°C

6. Sampling

Prepare a solution of 5% sodium tetraborate in pure water
Prepare a solution with DNFB: introduce 130 µl of DNFB in 10 mL of 95% pure ethanol.
Prepare a solution of hydrochloric acid 2M
Produce a standard range from a stock solution of glycine with 2 g/l (M = 75.07 g) e.g. 0.50 mg/l, 100 mg/l, 200 mg/l, 500 mg/l
Prepare a solution with 2 g/l of the product to be titrated

7. Procedure

In a test tube, insert:

- 380 µl of 5% Borax
- 20 µl of the sample to be titrated
- 20 µl of the DNFB solution perform in identical fashion with the glycine range

Stir and place in water bath at 60°C for 30 min
Add 3 mL of HCL 2M
Stir and read the specific absorbance at 420 nm for the sample
Produce a calibration curve with the Glycine range

8. Results

Plot the value of absorbance at 420 nm for the sample on the calibration curve
The results are expressed in g/l of Glycine.

Appendix 3: Protein separation by SDS-PAGE

1. Introduction:

SDS-PAGE (polyacrylamide gel electrophoresis) is a variant of electrophoresis commonly used to separate proteins by their molecular weight

2. Scope

Evaluation of the molecular weight of proteins of plant or animal origin. This method can be applied to all products of biological origin and winemaking products containing proteins.

3. Principle

The determination of the molecular weights of proteins is carried out by SDS-PAGE using the Laemmli method (1970). This technique enables the separation of proteins according to their molecular weight using Sodium Dodecyl Sulphate or SDS, a strongly negatively charged molecule, which standardizes their charges and make them lose their native three-dimensional structure. The migration rate of the whole SDS / denatured molecule depends only on the molecular weight of the proteins. Prior to protein denaturation with SDS, the disulphide bonds of the proteins must be reduced by 2-mercaptoethanol.

The migration medium consists of a polyacrylamide gel.

The gel is composed of two parts. A concentration gel which, as its name suggests, allows the proteins to concentrate before they migrate into the separating gel underneath.

The concentration gel contains 5% of acrylamide-bisacrylamide, while the separating gel contains 12%.

The migration takes place in the electrophoresis buffer chilled to 12°C and stirred for about 1 h 30 at a voltage of 80 V for the concentration gel and for nearly 3 hours at 170 V for the separating gel.

Once de-moulded, the gel undergoes staining to reveal the protein bands. The molecular weights of the bands can be measured using known size markers that have migrated with the samples. For example, using a marker sold by Sigma under the name of *Molecular Weight Standard Mixture* with the following sizes: 15, 25, 35, 50, 75, 100 and 150 Kda.

4. Reagents and Products

4.1. Denaturation buffer

- Buffer Tris Hcl 0.125 M pH 6.8
- distilled water;
- SDS to 4%
- 2-mercaptoethanol to 10%;
- bromophenol blue to 0.2%

- pure glycerol
- Complete with distillated water

4.2. Separating gel, preparation for 30 mL

- 7.50 mL of acrylamide/bis-acrylamide
- 11.25 mL Tris/Hcl buffer to 0,75 M pH 8.8
- 0.30 mL of SDS to 10%
- 10.95 mL of distilled water

30 microlitres of TEMED for polymerisation
after stirring, add 300 microlitres of ammonium persulfate to 20%

4.3. Concentration gel, preparation for 10 mL

- 1.25 mL of acrylamide/bis-acrylamide
- 1.25 mL Tris/Hcl buffer to 0,25 M pH 6.8
- 0.10 mL of SDS to 10%
- 7.4 mL of distilled water
- for the polymerisation, add 40 microlitres of TEMED
- after stirring, add 100 microlitres of ammonium persulfate to 20%

4.4. Migration buffer, preparation for 1 litre

- 12.5 mL Tris buffer 25mM pH 8,3
- 14.4 g of glycine
- 977.5 mL of distilled water
- 10 mL of SDS to 10%

5. Equipment

Electrophoresis equipment for

- the plates
- the clamps
- the seal

- the comb
- the spacers

6. Sampling

6.1. Denaturing the proteins of products

The samples are treated in the denaturing buffer, prepared just before the denaturing process.

50 microlitres of samples are mixed with 50 microlitres of denaturing buffer.

The mixture is then heated to boiling point for 4 minutes in order to promote protein denaturation.

7. Procedure

7.1. Preparation of plates

The electrophoresis plates are cleaned before use with water (and soap if necessary) and then with 70% alcohol.

Wipe the plates with a paper towel, leaving no fibres on the surfaces where the gel will be poured.

Place the seal on the round-edged plate.

Install the spacers and the second glass plate.

The assembly is then consolidated with clamps.

7.2. Pouring the separating gel

As soon as it has been prepared, the separating gel is poured between two plates using a pipette.

To avoid the presence of bubbles in the gel, the assembly is tilted during filling.

The gel is then covered with distilled water to obtain a perfectly horizontal surface.

7.3. Pouring the concentration gel

Remove the distilled water from the top of the separating gel

Holding the assembly tilted, fill it with the concentration gel to the upper level of the glass plates.

Put the comb in place to form wells in the concentration gel.

7.4. Sample deposits

Remove the comb

Place the plates and migration tanks in the electrophoresis tank.

Fill the tanks with the migration buffer starting with the upper and then the lower

part.

Add 50 microlitres of denatured protein sample in each well using a syringe and the submarine technique.

Also place a size marker in each of the wells located around the edges in order to frame the well containing the samples.

Once the deposits have been made, the migration is initiated relatively quickly to prevent the spread of the deposits

7.5. Starting and stopping the electrophoresis

The duration of the electrophoresis depends on several factors:

The generator used, the thickness of the gel, the amount of buffer used, its dilution, etc.

Close the lid of the tank

Check that the generator is switched off or disconnected

Connect the wires of the generator lid

maintain the temperature at 12°C

Connect the generator to the mains

Start the generator on the selected voltage: 80 volts for 1:30 for the concentration gel then 170 volts for about 3 hours for the migration in the separating gel

Stop the electrophoresis when the bromophenol blue reaches the bottom edge of the plates.

Switch off the generator and disconnect the power

Disconnect the wires from the lid

Open the electrophoresis tank

Remove the gel on its support

8. Results

The bands perpendicular to the path of migration for each molecule of protein can be revealed using several types of staining. The intensity and thickness of the bands depend on the protein concentration.

The size marker is used to directly assess the molecular weight of the proteins in each band.

8.1. Staining of electrophoresis gels

Several types of staining can be applied to characterise the proteins present as precisely as possible.

Coomassie blue staining

After migration, the electrophoresis gels are immersed in a Coomassie blue staining solution (0.025% Coomassie Blue R250, 40% methanol, 7% acetic acid) overnight.

The next day the electrophoresis gels are soaked for 30 minutes in an initial de-staining bath (40% methanol, 7% acetic acid) to remove the excess dye. They are then placed in a second bath (5% methanol, 7% acetic acid) which is changed regularly until a virtually colourless background is obtained. The staining and de-staining steps are carried out while stirring at room temperature.

The gels are stored in distilled water before being scanned for analysis.

8.2. Staining with silver nitrate

Staining with silver nitrate can detect smaller amounts of protein than Coomassie blue staining. The products used for this staining are provided by *Biorad* and form part of the *Silver Stain Plus* kit.

Once electrophoresis has been completed, the gel is placed in a bath of 400 mL of fixing solution (50% methanol, 10% acetic acid, 10% fixative enhancer concentrate (kit), 30% distilled water) for 20 min while stirring at room temperature.

The gel is then rinsed twice with 400 mL of distilled water for 10 min to remove any acetic acid which may impair the staining step.

Staining is performed with 100 mL of a solution prepared as indicated below:

In a large beaker, place:

- 35 mL of distilled water
- 5 mL of "Silver Complex" solution
- 5 mL of "Reduction Moderator" solution
- 5 mL of "Image development" reagent

Once these products have been well mixed and just before use, 50 mL of the "Developer Accelerator" solution at room temperature are added to the beaker. The preparation is then poured over the gel in the staining dish. After a period of 20 to 60 minutes, depending on the sample and its concentration, brownish bands appear.

The reaction is then stopped with a solution of 5% acetic acid for at least 15 min, then the gel is placed for about 5 min in ultrapure water. The gels are then ready to be scanned to determine the molecular weights of the protein bands.

8.3. Staining of glycoproteins

This method is used to express the presence of glycoproteins in yeast-type products.

It is performed using the "GelCode® Glycoprotein Staining" kit marketed by Pierce Biotechnology.

After electrophoresis, the gel is fixed by immersion in a bath of 300 mL of 50% methanol for 30 min while being stirred.

Wash the gel in a bath containing 300 mL of 3% acetic acid for 10 min. Repeat this step once. (The staining can be stopped after this step by placing the gel in distilled water overnight).

The gel is then covered with 25 mL of "Oxidizing Solution" while being stirred for 15 min.

The gel is then washed 3 times with 300 mL of 3% acetic acid for 5 min.

A solution of 25 mL of GelCode® Glycoprotein Staining is placed on the gel for 15 min while being stirred.

Add 25 mL of "Reducing Solution" while gently stirring for 5 min.

Wash the gel thoroughly with a solution of 3% acetic acid. The glycoproteins appear as magenta bands. The gel can be stored in a solution of 3% acetic acid before being scanned.

Appendix 4: Gel permeation chromatography

1. Introduction

The proposed method is a molecule separation method. This type of chromatography is also called gel filtration or exclusion chromatography.

2. Scope

The polymer profiles are studied in the biological products using gel permeation chromatography on a column optimised for protein analysis. A double detection at 280 nm/214 nm is used to monitor the elution of molecules containing amino acids with aromatic rings and peptide bonds.

3. Definition

Gel permeation chromatography allows the separation of molecules depending on their size and shape using a column containing porous granular gel. Large molecules (with a diameter greater than that of the pores) are excluded and eluted first at the dead volume (Vm or V0). Small and medium sized molecules are eluted later, their migration being impeded by their inclusion in the gel. The solutes are therefore eluted in reverse order of molecular weight. There is a linear relationship between the elution volume and the molecular weight logarithm.

4. Reagents and products

$NaH_2PO_4,2H_2O$

$Na_2HPO_4,2H_2O$

NaCl

NaOH 10 M

Compound	Molecular weight in kDa
Bovine albumin	66
Egg albumin	45
Glyceraldehyde 3 phosphate dehydrogenase	36
Bovine carbonic anhydrase	29
Bovine trypsinogen pancreas	24
Soy trypsin inhibitor	20
Lactalbumin	14.2

5. Equipment

GE Healthcare chromatography column: Superdex 200 (diameter 10 mm x length 300 mm)
Cellulose-ester filters with porosity: 0.22 µm

- Cup
- 2L beaker
- 1L volumetric flask
- 0.45 µm membranes for aqueous solution

6. Procedure

6.1. Chromatography buffer and conditions
In a cup, weigh:

- NaH_2PO_4, 2 H_2O = 1.56 g
- Na_2HPO_4, 2 H_2O = 1.58 g
- NaCl = 14.63g

Decant into a beaker containing 0.9 litres of ultrapure water. The pH of this solution should be approximately 6.5. Bring it to pH = 7.2 using NaOH 10 M.

Decant into a 1L volumetric flask and make up to 1L with ultrapure water. Filter through 0.45 µm membrane for an aqueous solution.

The flow is set at 0.6 mL/min.

6.2. Preparing the samples

To analyse a product in a powder form for example, dilute 1 g in 100 mL of ultrapure water. Filter the sample through a 0.22 µm cellulose-ester filter

6.3. Column calibration

To calibrate the column, plot a curve from the log of the molecular weight of the molecular weight solutions according to the retention time.

Inject the sample solution according to this.

7. Results

Inject the molecular weight solution mixture as indicated in point 7.3. Plot the curve of the log of the solutions' molecular weight according to the retention time. Determine the molecular weights of the product's peaks by referring to this curve.

