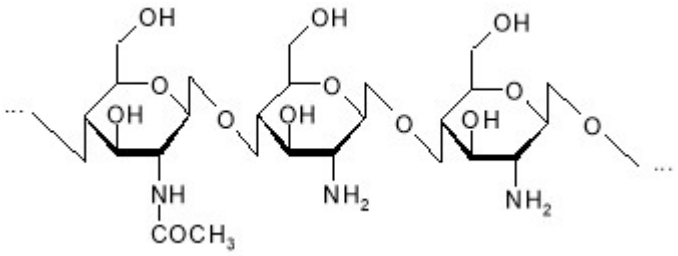
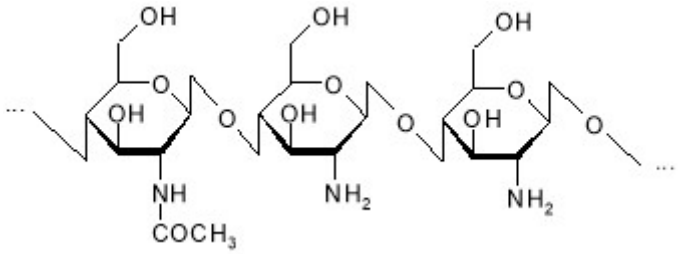
COEI-1-CHITOS Chitosan

[$C_6H_{11}NO_4$]n

CAS number Chitosan : [9012-76-4]



Chitosan



1. **Purpose, origin and applicability**

   Chitosan, a natural polysaccharide prepared of fungal origin, is initially extracted and purified from reliable and abundant food or biotechnological fungal sources such as *Agaricus bisporus* or *Aspergillus niger*.

   Chitosan is obtained by hydrolysis of a chitin-rich extract. Chitin is a polysaccharide composed of several N-acetyl-D-glucosamine units interconnected by □□ (1.4) type linkages.

   Chitosan is composed of glucosamine sugar units (deacetylated units) and N-acetyl-D-glucosamine units (acetylated units) interconnected by □□ (1.4) type linkages.

   It is used as a fining agent in the treatment of musts for flotation clarification to reduce cloudiness and the content of unstable colloids.

   It is also used for stabilising wines. This polymer actually helps eliminate undesirable micro-organisms such as *Brettanomyces.*

2. **Synonyms**

Poly(N-acetyl-D-glucosamine)-poly(D-glucose).

3. Labelling

The following information must be stated on the packaging label: exclusively fungal origin, product for oenological use, use and conservation conditions and use-by date.

4. Characters

4.1. Aspect and solubility

Chitosan comes in the form of a white, odourless and flavourless powder. Chitin-glucan is almost completely insoluble in aqueous or organic medium.

4.2. Purity and soluble residues

The purity of the product must be equal to or higher than 95 %.

Dissolve 5 g of chitin-glucan in 100 ml of bidistilled water and agitate for 2 minutes. Filter after cooling on a fine mesh filter or membrane.

Evaporate the filtrate and dry at 100-105 °C. The content of solubles should not be higher than 5 %.

5. Tests

5.1. Determination of the acetylation degree and chitosan origin

5.1.1. Determination of the acetylation degree

The acetylation degree is determined by potentiometric titration, using the method described in Appendix I.

5.1.2. Determination of the source

Chitosan, as a natural polymer, is extracted and purified from fungal sources; it is obtained by hydrolysis of a chitin-rich extract. This chitosan is considered identical to chitosan from shellfish in terms of structures and properties.

An identification of the origin of chitosan is made based on 3 characteristics: content of residual glucans (refer to method in annex II), viscosity of chitosan in solution 1 % and settled density (following settlement).

Only fungal origin chitosan has both contents of residual glucan > at 2 %, a settled density ≥ at 0,7 g/cm^3 and viscosity in solution 1 % in acetic acid 1 % < at 15 cPs

5.2. Loss during desiccation

In a glass cup, previously dried for 1 hour in an oven at 100-105 °C and cooled in a

desiccator, place 10 g of the analyte. Allow to desiccate in
the drying oven at 100-105 °C to constant mass. Weigh the dry residue amount after cooling in the desiccator.
The weight loss must be lower than 10 %.
Note: all the limits stated below are reported in dry weight except for the microbiological analyses

5.3. Ashes
Incinerate without exceeding 600 °C the residue left from the determination of the loss during desiccation as described in 5.2. Allow to calcine for 6 hours. Allow the crucible to cool in a desiccator and weigh.
The total ash content should not be higher than 3 %.
5.4. Preparation of the test solution
Before determining the metals, the sample is dissolved by acid digestion (HNO_3, H_2O_2 and HCl). Mineralisation is performed in a closed microwave system. The sample undergoes neither crushing nor drying before mineralisation.
The reagents used for the mineralisation of chitosan are as follows: HNO_3 (65 %) (Suprapur), HCl (37 %) (Suprapur), H_2O_2 (35 %). The 0.5 to 2 g sample of chitosan is placed in a flask to which are added 25 ml of HNO_3, 2 ml of HCl and 3 ml of H_2O_2. This is submitted to microwave digestion with a maximum power of 1200 watts; Power of 60 % for 1 min, 30 % for 10 min, 15 % for 3 min, and 40 % for 15 min). The solution is diluted in a volumetric flask with bidistilled water to a final volume of 25.0 ml.
The metal contents can then be determined.

5.5. Lead
Lead is determined by atomic absorption spectrophotometry, using the method described in appendix II.
The lead content must be lower than 1 mg/kg.
It is also possible to achieve lead determination by atomic absorption, using the method described in chapter II of the International Oenological Codex.

5.6. Mercury
Mercury is determined by atomic absorption spectrophotometry, using the method described in appendix II.

The mercury content must be lower than 0.1 mg/kg.

It is also possible to achieve mercury determination by atomic absorption, using the method described in chapter II of the International Oenological Codex.

5.7. Arsenic

Arsenic is determined by atomic absorption spectrophotometry, using the method described in appendix II.

The arsenic content must be lower than 1 mg/kg.

It is also possible to achieve arsenic determination by atomic absorption, using the method described in chapter II of the International Oenological Codex.

5.8. Cadmium

Cadmium is determined by atomic absorption spectrophotometry, using the method described in appendix II.

The cadmium content must be lower than 1 mg/kg.

It is also possible to achieve cadmium determination by atomic absorption, using the method described in chapter II of the International Oenological Codex.

5.9. Chromium

Chromium is determined by atomic absorption spectrophotometry, using the method described in appendix II.

The chromium content must be lower than 10 mg/kg.

It is also possible to achieve chromium determination by atomic absorption, using the method described in chapter II of the International Oenological Codex.

5.10. Zinc

Zinc is determined by atomic absorption spectrophotometry, using the method described in appendix II.

The zinc content must be lower than 50 mg/kg.

It is also possible to achieve zinc determination by atomic absorption, using the method described in chapter II of the International Oenological Codex.

5.11. Iron

Iron is determined by atomic absorption spectrophotometry, using the method described in appendix II.

The iron content must be lower than 100 mg/kg.

It is also possible to achieve iron determination by atomic absorption, using the method described in chapter II of the International Oenological Codex.

5.12. Copper

Copper is determined by atomic absorption spectrophotometry, using the method described in appendix II.

The copper content must be lower than 30 mg/kg.

It is also possible to achieve copper determination by atomic absorption, using the method described in chapter II of the International Oenological Codex.

5.13. Microbiological control

5.13.1. Total bacteria count

The total bacteria count is performed according to the horizontal method by means of the colony count technique at 30 °C on the PCA medium described in appendix III.

Less than 1000 CFU/g of preparation.

It is also possible to carry out the enumeration as described in chapter II of the International Oenological Codex.

5.13.2. Enterobacteria

The enumeration of *Enterobacteria* is carried out according to the horizontal method by means of the colony count technique at 30 °C described in appendix IV.

Less than 10 CFU/g of preparation.

5.13.3. Salmonella

Carry out the enumeration as described in chapter II of the International Oenological Codex.

Absence checked on a 25 g sample.

5.13.4. Coliform bacteria

Carry out the enumeration as described in chapter II of the International Oenological Codex.

Less than 100 CFU/g of preparation.

5.13.5. Yeasts

The enumeration of yeasts is carried out according to the horizontal method by means of the colony count technique at 25 °C on the YGC medium described in appendix VI.

Less than 100 CFU/g of preparation.

It is also possible to carry out the enumeration as described in chapter II of the International Oenological Codex.

5.13.6. Moulds

The enumeration of moulds is carried out according to the horizontal method by means of the colony count technique at 25 °C on the YGC medium described in appendix VII.

Less than 100 CFU/g of preparation.

It is also possible to carry out the enumeration as described in chapter II of the International Oenological Codex.

6. Ochratoxin A testing

Prepare an aqueous solution (distilled water) of chitosan at 1 % and agitate for 1 hour, then carry out determination using the method described in the Compendium of International Methods of Analysis of Wine and Musts.

Less than 5 µg/kg.

6.1. Storage

Keep container closed and store in a cool and dry place.

Appendix I: Determination of the acetylation degree

1. Principle

This method consists in determining the acetylation degree of chitosan by titration of the amino groups. The acetylation degree is the ratio of the number of N-acetyl-glucosamine units to the number of total monomers.

This method is based on the method described by Rinaudo et al., (1999).

The titration of a chitosan solution by means of NaOH at 0.1 M must be performed in order to identify two pH jumps from 0 to 14.

Chitosan is dissolved in 0.1M HCl, the amino groups (on the deacetylated glucosamine units (G)) are positively charged (HCl in excess)).

The chitosan solution (of known quantity) is titrated by NaOH of known concentration. In the first part of the reaction, the excess quantity of HCl is determined:

1.1. $HCl\ (excess) + NaOH + NH_3^+Cl^- \rightarrow NaCl + H_2O + NH_3^+Cl^-$

After the first pH jump, the quantity of charged amino groups is determined:

1.2. $HCl + H_2O + NH_3^+Cl^- + NaOH \dashrightarrow NH_2 + 2H_2O + 2NaCl$

1.3. After the second pH jump, the excess quantity of NaOH is measured.

The determination of the NaOH volume between the two jumps makes it possible to identify the quantity of charged amines.

2. Reagents and materials

2.1. Commercial preparation of chitosan

2.2. Distilled or deionised water

2.3. Chlorhydric acid 0,3 M

2.4. Sodium Hydroxide 0,1M

2.5. Glass cylindrical flasks, pipettes, burettes…

2.6. Magnetic mixer and stir bar

2.7. pH-meter with temperature sensor.

3. Samples preparation

Before determination, the samples are prepared according to the protocol described hereafter:

100 mg of chitosan are placed into a cylindrical flask to which 3 ml of 0.3 M HCl and 40 ml of water are added. Agitate for 12 hours.

4. Procedure

First introduce the pH electrode of the pH-meter as well as the temperature sensor into the cylindrical flask. Check that the pH value is lower than 3.

To bring to pH = 1, add a V1 volume (ml) of HCl 0.3 M and agitate.

Then to bring to pH = 7 with a V2 volume (ml) of 0.1 M NaOH

These operations can be carried out using an automatic titrator.

5. Expression of results

The acetylation degree of chitosan is expressed in %. This formula is the ratio of the mass of acetylated glucosamine (aG) units in g actually present in the sample, to the mass in g that would be present if all the groups were acetylated, where:

$$Q = (V_{NaOH} \times 0.1)/(1000 \times M_{cs}) = \text{specific concentration in amino groups}$$

- Mcs: dry weight of chitosan in g

$$V_{NaOH} = V2 - V1 = \text{Volume of 0.1 M NaOH between 2 pH jumps in ml}$$

For a 1 g sample
With G = Glucosamine part; a = acetylated part
aG weight actually present (in g) =

$$1g - (\text{Number of moles of G groups/g}) \times \text{G molecular weight} = 1g - Q \times 162$$

aG weight if all the deacetylated groups were acetylated (in g) =

$$1g + (\text{Number of moles of G groups/g}) \times \text{molecular weight a} = 1g + Q \times 42$$

The acetylation degree will be equal to DA, where:

$$\mathbf{DA} = (1 - 162 \times Q)/(1 + 42 \times Q)$$

Bibliography

- Rinaudo, M., G. Pavlov and J. Desbrieres. 1999. Influenced of acetic acid concentration on the solubilization of chitosan. *Polym.* 40, 7029-7032.

Appendix II: Determination of the residual glucan content

1. Principle

This method consists in determining the content of residual glucans in chitosan by means of spectrophotometry.
This method is based on a colorimetric reaction with a response depending on the degradation of the starch hydrolysates by hot concentrated sulphuric acid.
This degradation gives a brown yellow compound with a colour intensity proportional to the content of residual glucans.

2. Reagents and materials

2.1. Glucan 97% (Société Mégazyme)
2.2. Commercial preparation of chitosan
2.3. Distilled or deionised water

2.4. Ethanol
2.5. Acetic acid 1%
2.6. Solution of phenol 5%
2.7. Glacial acetic acid 100%
2.8. Glass cylindrical flasks, pipettes, volumetric flasks,...
2.9. Magnetic mixer and stir bar
2.10. Chronometer

3. Preparation of the standard range

A stock solution of glucan (glucan with a purity of 97 % is provided by the company Megazyme) is prepared according to the precise protocol described hereafter:

500 mg of glucan are introduced into a volumetric flask of 100 ml into which 6 ml of ethanol and 80 ml of distilled water are added.

Agitate and boil out to allow glucan dissolution

Allow to cool, adjust to the filling mark with water

Agitate for 30 minutes.

Pour 1 ml of this solution into a 50 ml volumetric flask and adjust to the filling mark with 1 % acetic acid.

The solution is ready to use to produce the standard range according to the protocol hereafter.

Stock solution V (ml)	Water V (ml)	Glucan M (µg)
0	1	0
0.1	0.9	10
0.3	0.7	30
0.5	0.5	50
0.7	0.3	70

4. Samples preparation

Before determination, the samples are prepared according to the protocol described hereafter:

100 mg of chitosan are placed into a 50 ml volumetric flask to which 25 ml of 1 % acetic acid are added.

Agitate for 12 hours then adjust to the filling mark.

5. Procedure

In a test tube, add 1 ml of the analyte solution, 1 ml of phenol at 5 % and 5 ml of concentrated sulphuric acid.

Agitate this mixture using a vortex for 10 s, then allow to cool for 1 hour.

The absorbance A is measured at 490 nm.

6. Expression of the results

Determine the glucan content in µg/g from the calibration curve (0-70 µg). This content is expressed in µg/g of chitosan.

Appendix III: Metal determination by atomic emission spectroscopy

1. Principle

This method consists in measuring atomic emission by an optical spectroscopy technique.

2. Sample preparation

Before the determination of metals, the sample is dissolved by acid digestion (HNO_3, H_2O_2 and HCl). Mineralisation takes place in closed microwave system. The sample undergoes neither crushing nor drying before mineralisation.

The reagents used for the mineralisation of chitosan are as follows: HNO_3 (65 %) (Suprapur), HCl (37 %) (Suprapur), H_2O_2 (35 %). The 0.5 to 2 g sample of chitosan is placed in a flask to which are added 25 ml of HNO_3, 2 ml of HCl and 3 ml of H_2O_2. The whole is then submitted to microwave digestion (Power of 60 % for 1 min, 30 % for 10 min, 15 % for 3 min, and 40 % for 15 min). The solution is then diluted in a volumetric flask with bidistilled water to a final volume of 25.0 ml.

The metal contents can then be determined.

3. Procedure

The dissolved samples are nebulised and the resulting aerosol is transported in a plasma torch induced by a high frequency electric field. The emission spectra are dispersed by a grating spectrometer and the line intensity is evaluated by a detector (photomultiplier). The detector signals are processed and controlled by a computer system. A background noise correction is applied to compensate for the background noise variations.

4. Expression of the results

The metal concentrations in chitosan are expressed in mg/kg.

Appendix IV: Total bacteria count by counting the colonies obtained at 30 °C

PCA medium

Composition:

Peptone	5.0 g
Yeast extract	2.5 g
Glucose	1.0 g
Agar-agar	15 g
Adjusted to	pH 7.0
Water	complete to 1000 ml

The medium is sterilised before use in an autoclave at 120 °C for 20 min.

The Petri dishes are inoculated by pour plate method and spiral plating method.

After inoculation, they are incubated at 30 °C in aerobiosis for 48 to 72 hours.

Count the CFU number.

Appendix V: Enumeration of *Enterobacteria* is carried out according to the horizontal method by means of the colony count technique at 30 °C

VRBG medium

Composition:

Peptone	7 g
Yeast extract	3 g
Glucose	10 g
Sodium Chloride	5 g
Crystal Violet	0.002 g
Neutral Red	0.03 g
Agar-agar	13 g
Bile salts	1.5 g
Adjusted to	pH 7.4
Water	complete to 1000 ml

The medium is sterilised before use in an autoclave at 120 °C for 20 min.
The Petri dishes are inoculated by pour plate method and spiral plating method.
After inoculation, they are incubated at 30 °C in aerobiosis for 18 to 24 hours.
Count the CFU number.

Appendix VI : Enumeration of yeasts by counting

YGC medium

Composition:

Yeast extract	5.0 g
D-glucose	20 g
Agar-agar	14.9 g
Choramphenicol	0.1 g
Adjusted to	pH 6.6
Water	complete to 1000 ml

The medium is sterilised before use in an autoclave at 120 °C for 20 min.

The Petri dishes are inoculated by pour plate method and spiral plating method.

After inoculation, they are incubated at 25 °C in aerobiosis for 3 to 5 days without being turned over.

Count the number of yeasts.

Appendix VII : Enumeration of the moulds by counting

YGC medium

Composition:

Yeast extract	5.0 g
D-glucose	20 g
Agar-agar	14.9 g
Choramphenicol	0.1 g
Adjusted to	pH 6.6
Water	complete to 1000 ml

The medium is sterilised before use in an autoclave at 120 °C for 20 min.

The Petri dishes are inoculated by pour plate method and spiral plating method.

After inoculation, they are incubated at 25 °C in aerobiosis for 3 to 5 days without being turned over.

Count the number of moulds.