RESOLUTION OIV/OENO 367/2009

MONOGRAPH ON CHITIN–GLUCANE

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,

CONSIDERING the works of the Specifications of Oenological Products experts group,

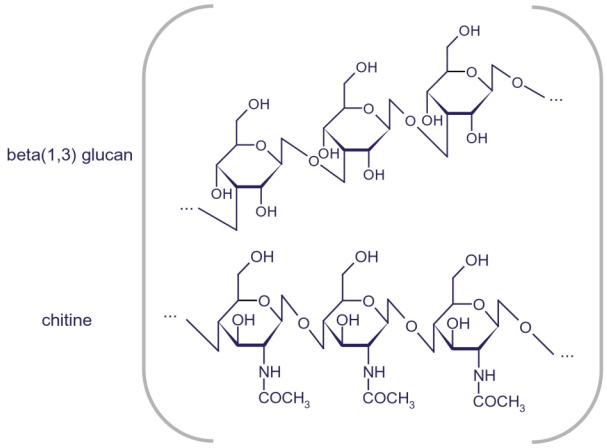
DECIDE to add in the International Oenological Codex the following monograph:

CHITIN-GLUCAN

$[C_6H_{10}O_5]_m$ - $[C_8H_{13}NO_5]_n$

CAS number Chitin: **□1398-61-4□**

CAS number □-glucan: **□9041-22-9□**



1. PURPOSE, ORIGIN AND SCOPE

Chitin-glucan is of fungus origin and is a natural polymer, the main component of the cellular walls of *Aspergillus niger*. It is initially extracted and purified from the mycelium of *Aspergillus niger*. This fungal resource is a by-product of the citric acid produced for the food and pharmaceutical markets.

Chitin-glucan is composed of polysaccharides chitin (repeat units N-acetyl-D-

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glucosamine) and 1,3-ß-glucan (repeat unit D-glucose). The two polymers are covalently connected and form a three-dimensional network. The chitin/glucan ratio ranges from 25:75 to 60:40 (m/m).

It is used as a fining agent of musts during racking in order to reduce the colloid content and cloudiness.

It is also used for stabilising wines prior to bottling after alcoholic fermentation. This polymer has a stabilising capacity with respect to ferric breakages. It also helps eliminate undesirable compounds such as heavy metals (lead, cadmium), mycotoxins, etc.

2. SYNONYMS

Poly(N-acetyl-D-glucosamine)-poly(D-glucose) and 1,3-ß-glucan

3. LABELLING

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

4. CHARACTERS

4.1. Aspect

Chitin-glucan 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. Identification and chitin-glucan ratio

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5.1.1. Determination of the chitin-glucan ratio

The chitin/glucan ratio is determined using the ^{13}C NMR spectrum in solid phase, by comparison with the spectrum of a pure chitin reference sample.

This method is detailed in appendix I.

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 chitin-glucan are as follows: HNO_3 (65 %) (Suprapur), HCl (37 %) (Suprapur), H_2O_2 (35 %). The 0.5 to 2 g sample of chitin-glucan 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 (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.

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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.

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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.

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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 V.

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 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.

6. OCHRATOXIN A TESTING

Prepare an aqueous solution (distilled water) of chitin-glucan 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.

7. STORAGE

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

Appendix I

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Determination of the chitin/glucan ratio

1. PRINCIPLE

This method consists in determining the chitin/glucan ratio using the ^{13}C RMN spectrum in solid phase.

2. REAGENTS AND MATERIALS

2.1. Chitin glucan sample
2.2. Osmosis purified water
2.3. Hydrochloric acid 1 M
2.4. Pure ethanol
2.5. Pure chloroform
2.6. Pure methanol
2.7. Acetone
2.8. Standard laboratory material, pipettes, cylindrical glass vases, porosity filters 30 µm...
2.9. Rotary shaker
2.10. Laboratory centifuge
2.11. Conductimeter
2.12. Nuclear Magnetic resonance apparatus

3. SAMPLE PREPARATION

Before the determination, samples are prepared according to a precise protocol as described below:

3.1. Washing with HCl 1 M (2.3)

This step consists in mixing 2 g of chitin-glucan (2.1) and 40 ml of HCl 1 M in a tube flask.

This mixture is agitated for 30 min at 320 rpm then centrifuged at 4000 rpm for 10 min. The supernatant is eliminated.

This step is repeated once.

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3.2. Washing with osmosis purified water

This step consists in mixing the sediment from the previous step with 40 ml of osmosis purified water (2.2).

This mixture is centrifuged for 10 min at 4000 rpm. The supernatant is eliminated.

This step is repeated until the supernatant conductivity is lower than 100 µS/cm.

3.3. Washing with ethanol

This step consists in mixing the sediment from the previous step with 40 ml of ethanol (2.4).

This mixture is centrifuged for 10 min at 4000 rpm. The supernatant is eliminated.

This step is repeated once.

3.4. Washing with chloroform/methanol

This step consists in mixing the sediment from the previous step with 40 ml of a of 50/50, v/v of chloroform (2.5) and methanol (2.6) mixture.

This mixture is agitated for 30 min at 320 rpm then centrifuged at 4000 rpm for 10 min. The supernatant is eliminated.

This step is repeated once.

3.5. Washing with acetone and drying

This step consists in mixing the sediment from the previous step with 40 ml of acetone (2.7).

This mixture is agitated for 30 min at 320 rpm then centrifuged at 4000 rpm for 10 min.

After centrifugation, pour the supernatant on a 30 µm filter, rinse the tube flask with acetone (2.7) and pour everything on the filter.

Place the material located on the filter in a crystallising dish and allow to dry.

After drying, the product is ready to be analysed by NMR.

4. PROCEDURE

The prepared samples are then analysed on the Brücker Avance DSX 400WB nuclear magnetic resonance instrument (or the equivalent).

The analysis conditions are as follows:

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- Magnetic field: 9.04 Tesla
- Larmor frequency: 83 kHz
- Time interval between 2 magnetic pulses: 5s
- Time period during which the magnetic pulse is applied: 5,5ms
- Number of magnetic pulse sequences: 3000

5. EXPRESSION OF THE RESULTS

5.1. The beta-glucan content is determined from the area of the four resonance bands.
5.2. The results are expressed in mol %.

Appendix II

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 chitin-glucan 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.

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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 the oenological products are expressed in mg/kg

Appendix III

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.

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Appendix IV

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.

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Appendix V

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 VI

Enumeration of the moulds by counting

YGC medium

Composition:

Yeast extract	5.0 g
D-Glucose	20 g

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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.

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