Summary

To ensure consistency with the Food Standards Code it is requested to amend the table to clause 14 (Permitted processing aids with miscellaneous functions) of Standard 1.3.3 of the Food Standards Code to include chitin-glucan as a processing aid. In the revised food Standards Code it would fall under Schedule 18-9 (Processing Aids that perform various technological purposes). It is also requested that Standard 4.5.1 Wine Production Requirements (Australia only) be amended to include chitin-glucan in the table to clause 4.

The European Union requested the addition of chitosan and chitin-glucan of fungal origin to the Annex of the Wine Agreement in November 2010. Provisional approval was granted for the use of these products in European wine exported to Australia under the Wine Agreement. In 2004, FSANZ approved permission to use chitosan sourced from *A. niger* as a processing aid in the manufacture of various alcoholic beverages.

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-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.

Chitin-glucan is insoluble in alcoholic beverages. The precipitates it forms with unwanted components in alcoholic beverages during processing are removed via filtration or similar processes. Therefore, no analytical method is needed to check for chitosan residues. Standard 1.3.4 requires that substances added to food, including processing aids, comply with relevant specifications as detailed in the Code. Chitin-glucan meets the OIV specification which is one of the secondary references for specifications in Standard 1.3.4 (Identity and Purity). Therefore, no new specification is required for the Code.

Chitin has been assessed by EFSA and approved for use in the European Union. According to Bornet and Teissedre (2008) , in rats, there were no changes indicating obvious toxicity of chitins in clinical signs, body weight, food intake, hematology, serum, biochemistry or histopathological findings except a slight decrease in body weight gain. Chitosan derived from shrimp was recognized as GRAS substance (Substance Generally Recognized as Safe) through scientific procedures for use in foods in general, including meat and poultry, for multiple technique effects by the US Food and Drug Administration.

PART A: APPLICATION TO AMEND THE AUSTRALIA AND NEW ZEALAND FOOD STANDARD CODE FOR THE USE OF CHITIN GLUCAN AS A PROCESSING AID FOR WINE

Prepared for: Food Standards Australia New Zealand
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Prepared by: Winemakers Federation of Australia

31 January 2016

Table of Contents

Summary 3

3.1 GENERAL REQUIREMENTS 4
3.1.2 Applicant details 4
3.1.3 PURPOSE OF THE APPLICATION 5
3.1.4 JUSTIFICATION FOR THE APPLICATION 5
A. REGULATORY IMPACT INFORMATION 6
3.1.5 INFORMATION TO SUPPORT THE APPLICATION 7
A. Technical Information on the Processing Aid 7
1. Information on the type of processing aid 7
2. Information on the identity of the processing aid 8
3. Information on the chemical and physical properties of the processing aid 8
4. Manufacturing process 8
5. Specification for identity and purity 9
6. Analytical method of detection 9

B. Information Related to the safety of a chemical processing aid 9
1. General Information on the Industrial use of this chemical 9
2. General information on the use of the chemical as a food processing aid in other countries 10
5. Safety assessment reports prepared by international agencies or other national government agencies if available 10
F. Information Related to the Dietary Exposure to the Processing Aid. 12
3.1.6 Assessment Procedure 13
3.1.8 EXCLUSIVE CAPTURABLE BENEFIT. 13
3.1.9 INTERNATIONAL AND OTHER STANDARDS 13
3.1.10 STATUTORY DECLARATION 14
3.1.11 CHECKLIST 14

References 15
Attachments 15

Summary

To ensure consistency with the Food Standards Code it is requested to amend the table to clause 14 (Permitted processing aids with miscellaneous functions) of Standard 1.3.3 of the Food Standards Code to include chitin-glucan as a processing aid. In the revised food Standards Code it would fall under Schedule 18-9 (Processing Aids that perform various technological purposes). It is also requested that Standard 4.5.1 Wine Production Requirements (Australia only) be amended to include chitin-glucan in the table to clause 4.

The European Union requested the addition of chitosan and chitin-glucan of fungal origin to the Annex of the Wine Agreement in November 2010. Provisional approval was granted for the use of these products in European wine exported to Australia under the Wine Agreement. In 2004, FSANZ approved permission to use chitosan sourced from *A. niger* as a processing aid in the manufacture of various alcoholic beverages.

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-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.

Chitin-glucan is insoluble in alcoholic beverages. The precipitates it forms with unwanted components in alcoholic beverages during processing are removed via filtration or similar processes. Therefore, no analytical method is needed to check for chitosan residues. Standard 1.3.4 requires that substances added to food, including processing aids, comply with relevant specifications as detailed in the Code. Chitin-glucan meets the OIV specification which is one of the secondary references for specifications in Standard 1.3.4 (Identity and Purity). Therefore, no new specification is required for the Code.

3.1 GENERAL REQUIREMENTS

3.1.2 Applicant details

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3.1.3 PURPOSE OF THE APPLICATION

The intention of this application is to amend the table to clause 14 (Permitted processing aids with miscellaneous functions) of Standard 1.3.3 of the Food Standards Code to include Chitin glucan of fungal origin and to include chitin glucan in the table to clause 4 Standard 4.5.1 Wine Production Requirements (Australia only) to permit the use of chitin glucan as a processing aid.

In the revised food Standards Code it would fall under Schedule 18-9 (Processing Aids that perform various technological purposes).

Chitin-glucan is of fungal 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-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.

Chitin has been assessed by EFSA and approved for use in the European Union. According to Bornet and Teissedre (2008), in rats, there were no changes indicating obvious toxicity of chitins in clinical signs, body weight, food intake, hematology, serum, biochemistry or histopathological findings except a slight decrease in body weight gain. Chitosan derived from shrimp was recognized as GRAS substance (Substance Generally Recognized as Safe) through scientific procedures for use in foods in general, including meat and poultry, for multiple technique effects by the US Food and Drug Administration.

3.1.4 JUSTIFICATION FOR THE APPLICATION

a) Need for the Proposed Change.

The European Union requested the addition of chitosan and chitin-glucan of fungal origin to the Annex of the Wine Agreement in November 2010. Provisional approval was granted for the use of these products in European wine exported to Australia under the Wine Agreement.

To ensure consistency with the Food Standards Code it is requested to amend the table to clause 14 (Permitted processing aids with miscellaneous functions) of Standard 1.3.3 of the Food Standards Code

to include Chitin glucan of fungal origin and to include chitin glucan in the table to clause 4 of Standard 4.5.1 to permit the use of chitin glucan as a processing aid.

In 2004, FSANZ approved permission to use chitosan sourced from *A. niger* as a processing aid in the manufacture of various alcoholic beverages. FSANZ prepared a variation to Standard 1.3.3 to permit chitosan sourced from *A. niger* as a processing aid to be used in the manufacture of wine, beer, cider, spirits and food grade alcohol. FSANZ also prepared a variation to Standard 4.5.1 – Wine Production Requirements which is an Australian-only Standard for permission to use chitosan sourced from *A. niger* as a processing aid in the production of Australian produced wine. A separate permission is required to be incorporated into this Standard since it is a standalone Australian-only Standard that covers Australian-produced wine. Processing aid permissions for imported wine and New Zealand-produced wine are covered by Standard 1.3.3.

b) Advantages of the Proposed Change Over the Status Quo

Permitting chitin glucan as a processing aid for the manufacture of wine provides an overall benefit. There were no costs linked to permitting the processing aid while there were benefits to the wine sector to having an alternative processing aid to produce improved quality products at potentially lower costs of production. It will also enable to comply with its World Trade Organisation obligations and meet the requirements of the Australian-European Union Agreement on Trade in Wine (The Wine Agreement).

c) Status of Similar Application made in other Countries

No applications are being made by the applicant to other national jurisdictions.

A. REGULATORY IMPACT INFORMATION

1. Costs and benefits

a) Costs and benefits to the consumers

Consumers will have no change to their costs and benefits.

b) Costs and Benefits to Industry and Business in General.

c) Permitting chitin glucan as a processing aid for the manufacture of wine provides an overall benefit. There were no costs linked to permitting the processing aid while there were benefits to the wine sector to having an alternative processing aid to produce improved quality products at potentially lower costs of production.

d) Costs and Benefits to Government.

There will be no increased regulatory or enforcement costs for the government.

2. Impact on International Trade

It will provide other countries other than the European Union the ability to export wine to Australia that has been produced using chitin as a processing aid, consistent with our World Trade Organisation obligations and enable Australia to meet its obligations under the Australian-European Union Agreement on Trade in Wine (The Wine Agreement).

3.1.5 INFORMATION TO SUPPORT THE APPLICATION

1. General

(a) There are no negative public health implications. The application is consistent with Australia's World Trade Organisation obligations to provide equal treatment.

(b) Consumer Choice Issues

There are no consumer choice issues.

(c) Evidence of General Food Industry or Specific Company Support

Permitting chitin glucan as a processing aid for the manufacture of wine provides an overall benefit. There were no costs linked to permitting the processing aid while there were benefits to the wine sector to having an alternative processing aid to produce improved quality products at potentially lower costs of production.

A. Technical Information on the Processing Aid

1. Information on the type of processing aid

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-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. *Information on the identity of the processing aid*

CHITIN-GLUCAN

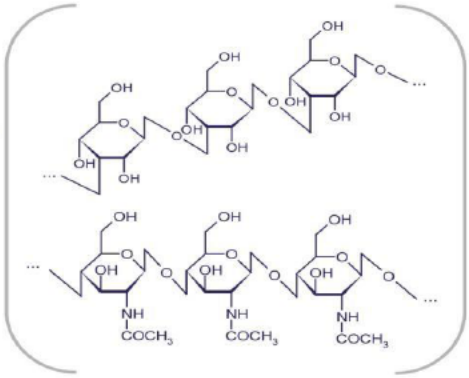
$[C_6H_{10}O_5]_m$ - $[C_8H_{13}NO_5]_n$

CAS number Chitin: 1398-61-4
CAS number c-glucan : 9041-22-9
(OIV-Oeno 367-2009
Synonyms: Poly(N-acetyl-D-glucosamine)-poly(D-glucose) and 1,3-ß-glucan

3. *Information on the chemical and physical properties of the processing aid*

Beta (1,3) glucan

'chitin



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

4. *Manufacturing process*

Chitin-glucan is a purified ingredient, presented in the form of a powder, which is composed largely of two polysaccharides: - chitin, composed of repeating units of N-acetyl-D-glucosamine (CAS number 1398-61- 4); - beta (1,3)-glucan, composed of repeating units of D-glucose (CAS number 9041-22-9). Chitin-glucan is the main component in the cell walls of the mycelium of a fungus from the Ascomycetes family: Aspergillus niger (A. niger). The two polymers are linked covalently and form a three-dimensional network. Chitin-glucan is obtained from the mycelium of non-genetically-modified strains of A. niger, a microorganism employed in the food and pharmaceutical industries for the production of citric acid.

According to Shahlaei and Pourhossein (2013) the alkali-insoluble cell-wall residue of the Aspergillus *Niger biomass* consist mainly of chitin and (1_3, 1_6)-b-D-glucan. Preparation of Chitin–glucan (CG) from the biomass of *A. Niger* was reported in 1979 by Muzzarell. The method was based on the alkali treatment of biomass with NaOH solution (2.5%) at ambient

temperature overnight and then aggressive alkali treatment with concentrated NaOH (40–45%) at 130°C for 4–6 hours. The procedure yielded a white powder containing 32% of the polyaminosaccharide and 15-20% glucan. Other methods for chitin-glucan extraction from the biomass of *A. Niger* involve the alkaline extraction to remove the proteins and alkali- soluble polysaccharides.

5. Specification for identity and purity

Specifications are given in the OIV INTERNATIONAL ŒNOLOGICAL CODEX Chitin-Glucan COEI-1-CHITGL: 2009 (attached). Chitin-glucan meets the OIV specification which is one of the secondary references for specifications in Standard 1.3.4 (Identity and Purity). Therefore, no new specification is required for the Code.

6. Analytical method of detection

Chitin-glucan is insoluble in alcoholic beverages. The precipitates it forms with unwanted components in alcoholic beverages during processing are removed via filtration or similar processes. Therefore, no analytical method is needed to check for chitosan residues. Tests are identified by the OIV in OIV INTERNATIONAL ŒNOLOGICAL CODEX Chitin-Glucan COEI-1-CHITGL: 2009 (attached).

B. Information Related to the safety of a chemical processing aid

In 2010, EFSA provided a scientific opinion on the safety of 'Chitin-glucan' as a Novel Food ingredient (EFSA Journal 2010; 8(7):1687). The Food ingredient called "KiOnutrime-CG™" has a content of more than 90 % chitin glucan, which is the main component in the cell walls of the mycelium of Aspergillus niger derived from a fermentation process. The compositional data and the manufacturing process do not give rise to concerns. The ingredient was intended to be marketed as a food supplement to increase the daily intake of fibre. The intended intake of chitin-glucan is 2 to 5 g/day. At the highest dose administered in a 13-week rat study, i.e. about 6.6 g/kg body weight (bw), no adverse effects were observed. This dose is approximately 80-fold higher than the maximum intended level of intake for humans on a g/kg bw basis. The Panel concluded that chitin-glucan was safe as a food ingredient at the proposed conditions of use and the proposed intake levels.

1. General Information on the Industrial use of this chemical

Chitin-glucan is also used as a food supplement (EFSA 2010), in the cosmetic and pharmaceutical industries and to remove contaminants from beverages.

2. General information on the use of the chemical as a food processing aid in other countries

Chitin glucan is a legally permitted processing aid in the European Union and for sale in countries it has wine agreements with: Chile, Australia, Canada, United States, Switzerland and South Africa.

3. Data on the toxicokinetics and metabolism of the chemical processing aid, and if necessary its metabolites

According to Bornet and Teissedre (2008) , in rats, fed of chitin powdered diet containing 5% concentration of this substance during 13 weeks, Nino et al. showed that there were no changes indicating obvious toxicity of chitins in clinical signs, body weight, food intake, hematology, serum, biochemistry or histopathological findings except a slight decrease in body weight gain. In a subacute toxicity study, Kim et al. found that no-observed adverse effect level for chitosan oligosaccharide was considered to be over 2,000 mg kg¡1 in rats. More recently, Qin et al. shown that the oral maximum tolerated dose of chitooligomers was more than 10 g kg¡1 body weight in mice. It had no mutagenicity judged by negative experimental results of AMES test (biological assay developed in 1974 by B.N. Ames for identifying possible carcinogens by studying their mutagenic effect on bacteria and widely used to detect possible chemical carcinogens. It is based on mutagenicity in Salmonella bacteria), mouse bone marrow cells micronucleus test and mouse sperm abnormality test. The results of the 30-day feeding study show that no abnormal symptoms and clinical signs or deaths were found in rats during the test. No significance difference in body weight, food consumption, hematology value, clinical chemistry value and organ/body weight ratio and abnormality of any organ during hispathological examination was found. It was concluded that short-term ingestion of chitooligomers was of non-toxic. Chitosan derived from shrimp was recognized as GRAS substance (Substance Generally Recognized as Safe) trough scientific procedures for use in foods in general, including meat and poultry, for multiple technique effects by the US Food and Drug Administration.

5. Safety assessment reports prepared by international agencies or other national government agencies if available

United States

The Food and Drug Administration (FDA) responded to a notice, dated November 17, 2011, submitted in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS); the GRAS proposal). FDA received the notice on December 15, 2011, filed it on January 5, 2012, and designated it as GRAS Notice No. GRN 000412.

The subject of the notice is chitin-glucan from *Aspergillus niger* (chitin-glucan). The notice informs FDA of the view of KitoZyme S.A. (KitoZyme) that chitin-glucan from *A. niger* is GRAS, through scientific procedures, for use in microbial stabilization, removal of contaminants, and/or clarification in alcoholic beverage production at levels between 10 and 500 grams per hectolitre (100 litres).

KitoZyme describes chitin-glucan, a mixture of chitin (poly-N-acetyl-D-glucosamine) and β-(1,3)-D-glucan, as an insoluble, non-digestible fibre derived from the post-fermentation biomass of non-viable *A. niger* used to manufacture food-grade citric acid. KitoZyme states that strains of *A. niger* used in the production of citric acid are non-pathogenic and nontoxigenic, and have a long history of safe use worldwide.

Hydrolysis of the *A. niger* material produces chitin and β-1(1,3)-glucan polysaccharides, which are dried, milled, and sieved to result in greater than 95 percent of the granulation particles sized less than 500 µm. All materials and processing aids used in the manufacture of chitin-glucan are food-grade. The notifier provides product specifications for chitin-glucan, including microbiological limits, heavy metals, and chemical characterization.

KitoZyme reported on a published 13-week sub chronic toxicity study in Wistar rats. Twenty rats per sex were fed chitin and β-glucan from *A. niger* in a 30:70 ratio. KitoZyme reported the No Observable Adverse Effect Level to be the highest dose tested, equivalent to 6,589 and 7,002 milligrams/kilogram body weight/day for the male and female rats, respectively.

When chitin-glucan is used in the production of alcoholic beverages, it is removed from the wine, must, beer, cider, or spirits at the end of the treatment using physical separation processes, such as racking, centrifugation, or filtering.

KitoZyme notes that chitin-glucan preparations are insoluble in both water and ethanol, and because the material is removed from solution, intake modelling is not considered necessary. In addition, high performance liquid chromatography of wine processed with chitin-glucan indicated that the final product was free from detectable chitin-glucan. In addition, KitoZyme notes that chitin-glucan is not digested by the human gastrointestinal tract, therefore, absorption and systemic exposure to chitin-glucan would not occur.

Based on the information provided by KitoZyme, as well as other information available to FDA, the agency has no questions at this time regarding KitoZyme's conclusion that chitin-glucan from *A. niger* is GRAS under the intended conditions of use.

EFSA

Following a request from the European Commission, the Panel on Dietetic Products, Nutrition and Allergies was asked to carry out the additional assessment for „Chitin-glucan" as a food ingredient in the context of Regulation (EC) No. 258/97. The Novel Food ingredient called "KiOnutrime-CG™" has a content of more than 90 % chitin-glucan, which is the main component in the cell walls of the mycelium of *Aspergillus niger* derived from a fermentation process. The compositional data and the manufacturing

process do not give rise to concerns. The ingredient is intended to be marketed as a food supplement to increase the daily intake of fibre. The intended intake of chitin-glucan is 2 to 5 g/day. At the highest dose administered in a 13-week rat study, i.e. about 6.6 g/kg body weight (bw), no adverse effects were observed. This dose is approximately 80-fold higher than the maximum intended level of intake for humans on a g/kg bw basis. The Panel concluded that Novel Food KiOnutrime-CG™ is safe as a food ingredient at the proposed conditions of use and the proposed intake levels (EFSA 2010).

F. Information Related to the Dietary Exposure to the Processing Aid.

Chitin-glucan is proposed for use as a processing-aid in the manufacture of wine.

Chitin-glucan is similar to chitosan derived from *A. niger*, and chemically and structurally equivalent to shellfish derived chitosan. Fungal chitosan is approved for the fining of wine.

Shellfish derived chitosan is widely available in the food supply through use in dietary supplement products, industrial, pharmaceutical, agricultural, and cosmetic applications, and background exposures to chitosan are therefore expected to exceed those occurring from the proposed food uses of fungal chitosan. Thus, based on the absence/trivial exposure to chitosan under the proposed food uses, calculation of estimated intakes was not deemed necessary in the assessment of the safety of the material under the proposed food uses of chitin -glucan in wine/ processing.

Published studies examining the metabolism and kinetics; acute, subchronic, and chronic toxicity; reproductive toxicity in animals; and safety in human of shellfish-derived chitosan or chitosan oligosaccharides are presented in the dossier.

2. The levels of residues of the processing aids or its metabolites for each food or food group

Regardless of the technological purpose, the sediments that contain the chitin-glucan are removed from the wine, must, or spirits at the end of the treatment by physical separation processes such as racking, centrifugation and/ or filtration. It is unlikely that any residual chitosan will remain in the treated products. Therefore, the estimated intake of chitin-glucan from all proposed technological uses can be considered as negligible.

3. Information on likely level of consumption

No information.

4. Percentage of food group to use processing aid

There is no information on the expected use of this processing aid in Australian wine or imported product currently being sold in Australia.

5. Information on residues in foods in other countries

There is no information on residues in wines where it is approved as a processing aid in other countries.

6. Where consumption has changed, information on likely consumption

Not applicable

3.1.6 Assessment Procedure

This application seeks the appropriate assessment procedure is **General Procedure Level 1.** The application seeks to permit the use of a processing aid in wine to give force to the EU-Australia Wine Agreement. There are no perceived health risks from its approval.

3.1.7 CONFIDENTIAL COMMERCIAL INFORMATION

No confidential or commercial information is incorporated in this application.

3.1.8 EXCLUSIVE CAPTURABLE BENEFIT.

There is no exclusive capturable benefit to the applicant.

3.1.9 INTERNATIONAL AND OTHER STANDARDS

A. JECFA

No JECFA standard.

B. Other National Standards

<u>European Union</u>

COMMISSION REGULATION (EC) No 606/2009 of 10 July 2009 laying down certain detailed rules for implementing Council Regulation (EC) No 479/2008 as regards the categories of grapevine products, oenological practices and the applicable restrictions (I.193/ Official Journal of the European Union, 24/07/2009)) outlines requirements for treatments of wine with chitin glucan.

Commission Regulation No 53/2011 laid down the following conditions:

Appendix 13 Requirements for the treatment of wines with chitosan of fungoid origin and for the treatment of wines with chitin-glucan of fungoid origin
Areas of application:

(a) reduction in the heavy metal content, particularly iron, lead, cadmium and copper;
(b) prevention of ferric casse and copper casse;
(c) reduction of possible contaminants, especially ochratoxin A;
(d) reduction in the populations of undesirable micro-organisms, in particular Brettanomyces, solely by means of treatment with chitosan.

Requirements: — The dose levels to be used are determined after a qualification test. The maximum dose level used may not exceed:
— 100 g/hl for applications (a) and (b),
— 500 g/hl for application (c), — 10 g/hl for application (d),
— sediments are removed using physical processes.

3.1.10 STATUTORY DECLARATION

Attached

3.1.11 CHECKLIST

Attached

References

1. Bornet, A. and Teissedre P. (2008) **Chitosan, chitin-glucan and chitin effects on minerals (iron, lead, cadmium) and organic (ochratoxin A) contaminants in wines**, Eur Food Res Technol. (2008) 226:681–689.
2. European Commission (2011), **Commission Regulation (EU) No 53/2011 of 21 January 2011 amending Regulation (EC) No 606/2009 laying down certain detailed rules for implementing Council Regulation (EC) No 479/2008 as regards the categories of grapevine products, oenological practices and the applicable restrictions**, Official Journal of the European Union, Volume 54, L 19/1-5.
3. EFSA (2010) **Scientific Opinion on the safety of 'Chitin-glucan' as a Novel Food ingredient,** EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), EFSA Journal 2010; 8(7):1687.
4. FDA (2012) Agency Response Letter GRAS Notice No. GRN 000412, CFSAN/Office of Food Safety, June 18, 2012.
5. Mohsen Shahlaei M. and Pourhossein S. (2013) **Biomass of *Aspergillus Niger*: Uses and Application,** Journal of Reports in Pharmaceutical Sciences, **2013, 2(1), 83-89.**
6. OIVa (2014) **Treatment of Wine and Musts using Chitin-Glucan** (various), International code of Oenological Practices, 2014 Issue.
7. OIVb (2009), **Chitin –glucan** (Monograph), International Oenological Codex, COEI-1-CHITGL: 2009.

Attachments

1. Statutory Declaration
2. Summary
3. Checklist

4. Bornet, A. and Teissedre P. (2008) **Chitosan, chitin-glucan and chitin effects on minerals (iron, lead, cadmium) and organic (ochratoxin A) contaminants in wines**, Eur Food Res Technol. (2008) 226:681–689.
5. European Commission (2011), **Commission Regulation (EU) No 53/2011 of 21 January 2011 amending Regulation (EC) No 606/2009 laying down certain detailed rules for implementing Council Regulation (EC) No 479/2008 as regards the categories of grapevine products, oenological practices and the applicable restrictions**, Official Journal of the European Union, Volume 54, L 19/1-5.
6. EFSA (2010) **Scientific Opinion on the safety of 'Chitin-glucan' as a Novel Food ingredient,** EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), EFSA Journal 2010; 8(7):1687.
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9. OIVa (2014) **Treatment of Wine and Musts using Chitin-Glucan** (various), International code of Oenological Practices, 2014 Issue.
10. OIVb (2009), **Chitin –glucan** (Monograph), International Oenological Codex, COEI-1-CHITGL: 2009.

11. Beran, M, Ademek, L., Molik, P. ISOLATION AND SOME APPLICATIONS OF FUNGAL CHITIN - GLUCAN COMPLEX AND CHITOSAn, Food Research Institute Prague, Radiová 7, 10231 Prague, Czech Republic.

Chitosan, chitin-glucan and chitin effects on minerals (iron, lead, cadmium) and organic (Ochratoxin A) contaminants in wines

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DOI 10.1007/s00217-007-0577-0

ORIGINAL PAPER

Chitosan, chitin-glucan and chitin effects on minerals (iron, lead, cadmium) and organic (ochratoxin A) contaminants in wines

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Abstract Chitosan (F7), chitin (F2), chitin-glucan (F1) and chitin glucan hydrolysate (F5) of fungal origin were tested for removal of mineral (Fe or Pb and Cd) and organic (ochratoxin A: OTA) contaminants in wines. Red, white and sweet wines are spiked with either Fe (20 mg L^{-1}), or Pb (500 μg L^{-1}) and Cd (20 μg L^{-1}) or OTA (5 μg L^{-1}). The wines were then treated with F1, F2, F5 or F7 at doses of 0.1 g, 0.5 and 2 g L^{-1}. After 2 days, the levels of iron, copper, lead and cadmium were measured using a flame and graphite furnace atomic absorption spectrophotometer. Depending on the treatment red wines showed reductions of iron by 73–90%, cadmium by 29–57% and lead by 33–74%. The same treatments with white wine gave reductions for iron of 32–91%, cadmium fell by 11–23%, lead by 50–65%. In the case of sweet wines iron was reduced by 51–90%, cadmium by 17–25%, and lead by 38–84%. For wine enriched in OTA, treatments with F1, F2, F5, F7 were carried out at doses of 2 and 5 g L^{-1}. After 2 days, the levels of OTA in wines were analyzed by HPLC with fluorimetric detection. OTA was reduced by 56.7–83.4% in red wine, by 53.4–64.5% in white wine and 26.1–43.5% in sweet wine. These findings indicate that chitosan, chitin, chitin-glucan, or chitin glucan hydrolysate from fungal origin may be useful ancillaries for wine limpidity prevention by reducing levels of Fe, heavy metals (Pb, Cd) and mycotoxins (OTA) and thereby improving wine safety.

Keywords Lead · Cadmium · Iron · Ochratoxin A · Chitin · Chitin-glucan · Chitosan · Technology auxiliary · Wine

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Introduction

During winemaking and storage of wines contaminants can cause stabilization and safety problems. For these reason contaminants such as iron, lead, cadmium and ochratoxin A have to be controlled. Unstable wines form different types of hazes. Excessive amounts of iron (10–20 mg L^{-1} or more) oxidized to the ferric form can cause a precipitation of pigmented materials (blue haze) or with orthophosphate ions (white haze). To produce a stable wine the level of iron level must be <5 mg L^{-1} prior to bottling.

Lead adversely affects multiple enzyme systems with the body, as any ligand with sulfhydryl groups is vulnerable. The best-known effect is that on the production of heme. Lead interferes with the critical phases of the dehydration of aminolevulinic acid and the incorporation of iron into the protoporphyrin molecule; the result is a decrease in heme production. Because heme is essential for cellular oxidation, deficiencies have far-reaching effects. Lead is renally excreted, but the

elimination rate varies, depending on the tissue that absorbed the lead.

The reduction of lead levels in foods and beverages is a necessity to improve food safety [1]. The OIV (Vine and Wine International Organization) established the maximum concentration of lead in wine to 150 μg L^{-1}. However, wine contains small amount of rhamnogalacturonan-II dimmer able to give strong complexes in vitro with lead and other selected cations [2]. This pectic polysaccharide should reduce intestinal absorption and tissue retention of Pb as reported in rats with a potential beneficial effect by minimising toxicity [3, 4].

The toxic effects of cadmium are due to the inactivation of enzymes containing sulphydryl groups and the uncoupling of oxidative phosphorylation in mitochondria [5]. Cadmium may also compete with other metals such as zinc and selenium for inclusion into metalloenzymes and it may compete with calcium for binding sites on regulatory proteins such as calmodulin [6]. The OIV established the maximum level of cadmium in wine as 10 μg L^{-1}.

Ochratoxin A (OTA) is a known nephrotoxin and carcinogen produced by several molds of the *Aspergillus flavus* and *Penicillium* genera, including *Aspergillus ochraceus*. It has been frequently detected in foods, especially cereal products, and beverages. In humans, exposure to OTA has been linked with Balken endemic nephropathy, a chronic kidney disease associated with tumors of the renal system. Wine and grape juice has been identified as a possible source of ochratoxin. The main molds responsible of OTA contaminations in grapes and wines are *Aspergillus carbonarius* and *Aspergillus nigri* [7–12]. The European Commission established, with regulation 123/2005, the maximum allowable concentration of OTA in wine, must and grape juice. It is now forbidden to market batches that contain >2 μg L^{-1} (ppb).

A variety of fining agents, including activated carbon, silica gel, potassium caseinate, egg albumin, and gelatin, were used in relation to their abilities to remove OTA in wines. Current clarification products (organic or inorganic fining agents) have variable levels of efficiency for reducing contents of OTA. Activated carbon and potassium caseinate were reported as interesting to remove OTA in wine. Potassium caseinate removed up to 82% of OTA with high doses (150 g hL^{-1}), whereas activated carbon showed the highest specific adsorption capacity due to a high surface area per mass [13] but could also modify phenolics composition in wine. Bentonite in white wine and yeast hulls in red wine have been described as the most effective non-carbonaceous fining agents for the removal of OTA [10, 11]. After various clarification products treatments to remove OTA, sensorial repercussions on wine quality as well as allergenic reactions for some sensitive wine consumers can't be totally excluded.

Chitin, chitosan and derivatives are non-toxic, biodegradable polymers that can remove metals and organic contaminants foodstuffs [8].

Chitosan was approved as food additive in some asian countries (e.g., Japan, Korea), and several studies shown that this compound is not toxic as reported by Hwang et al. [14]. Chitin and chitosan are listed as processing aids (clarifying, agents/filtration, aids/flocculating agents) for fruit juices and nectars (Codex Alimentarius Commission 2003) [15].

In rats, fed of chitin powdered diet containing 5% concentration of this substance during 13 weeks, Nino et al. [16] showed that there were no changes indicating obvious toxicity of chitins in clinical signs, body weight, food intake, hematology, serum, biochemistry or histopathological findings except a slight decrease in body weight gain. In a subacute toxicity study, Kim et al. [17] found that no-observed adverse effect level for chitosan oligosaccharide was considered to be over 2,000 mg kg^{-1} in rats. More recently, Qin et al. [18] shown that the oral maximum tolerated dose of chitooligomers was more than 10 g kg^{-1} body weight in mice. It had no mutagenicity judged by negative experimental results of AMES test (biological assay developed in 1974 by B.N. Ames for identifying possible carcinogens by studying their mutagenic effect on bacteria and widely used to detect possible chemical carcinogens. It is based on mutagenicity in Salmonella bacteria), mouse bone marrow cells micronucleus test and mouse sperm abnormality test. The results of the 30-day feeding study shows that no abnormal symptoms and clinical signs or deaths were found in rats during the test. No significance difference in body weight, food consumption, hematology value, clinical chemistry value and organ/body weight ratio and abnormality of any organ during hispathological examination was found. It was concluded that short-term ingestion of chitooligomers was of non-toxic. Chitosan derived from shrimp was recognized as GRAS substance (Substance Generally Recognized as Safe) trough scientific procedures for use in foods in general, including meat and poultry, for multiple technique effects by the US Food and Drug Administration [19].

Chitin exists widely in the cell walls of fungi, molds and yeast [20] and in the exoskeletons of invertebrates such as crustaceans, mollusks, crabs, shrimps, lobster, squid and insects [21]. Chitosan are found in only in a few species of fungi. Chitin and chitosan consist of 2-acetamido-2-deoxy-β-D-glucose and 2-amido-2-deoxy-β-D-glucose as repeating units, respectively. Chitin is

chemically identical to cellulose except that the secondary hydroxyl group on the alpha carbon atom of the cellulose molecule is substituted with acetoamide groups. Chitosan is the N-acetylated form of chitin and shows the deacetylation reaction of chitin. Numerous studies have demonstrated that chitosan and its derivatives have various biological activities such as antimicrobial activity [22–28]. Chitosan was also reported as a natural preservative for foods prone to fungal or bacterial spoilage in apple juice [29] and mayonnaise [30], mozzarella cheese [31].

This paper reports on the treatment of wine with chitin, chitin-glucan, chitin glucan hydrolysate and chitosan (Fig. 1) to lower the levels of lead, cadmium, iron and ochratoxin A, thereby reducing toxicity and improving safety in wines. An interesting innovation purposed by Kitozyme is the possibilty to obtain these polysaccharide molecules: chitin, chitin-glucan, chitin glucan hydrolysate and chitosan from fungal origin after some specific industrial hydrolysis process (Fig. 1, structures) [8]. Because of their high toxicity, minerals (lead and cadmium) or organic mycotoxin contaminants (ochratoxin A) need to be quantified and reduced as low as possible in beverages and in wine in particular to improve their safety. Possible haze formation in wine can be due to iron and is a well-known common problem. The level of this compound needs also to be reduced to protect wine from possible haze formation. In this work, we used polysaccharides (chitin, chitin-glucan, chitin glucan hydrolysate and chitosan from fungal origin, Kitozyme S.A.) in wines to remove significantly lead, cadmium, iron and ochratoxin A.

Materials and methods

Materials

The samples used in our tests were unfiltered, bottled wine (vintage 2003) from two cellars in southern France, namely: Chardonnay white wine (W), Merlot red wine (R) and Grenache-Macabeu, a natural sweet wine (S). The chemical characteristics of wines including: pH, alcohol level, total phenol content, total acidity, residual sugars and total sulfites are given in Table 1. The novel polysaccharide adsorbents were produced by Kitozyme S.A., Herstal, Belgium: chitin-glucan (F1) (46% chitin, 54% β-glucan); chitin (F2) (78% chitin and 22% β-glucan); chitin-glucan hydrolysate (F5) (25% chitin and 75% β-glucan), (chitosan F7) (9% chitin and 2% β-glucan). The chitin, chitosans and derivatives were in solid form with variable granulometry (particles between 75 and 125 pm).

Stabilization treatments and pH adjustments

Preliminary experiments indicated significant reductions in Fe, Pb, Cd and OTA at levels of 2 g L^{-1}. For each wine, the various adsorbents (chitins and chitosans) were added to the wines at 0.1, 0.5, 2 and 5 g L^{-1},

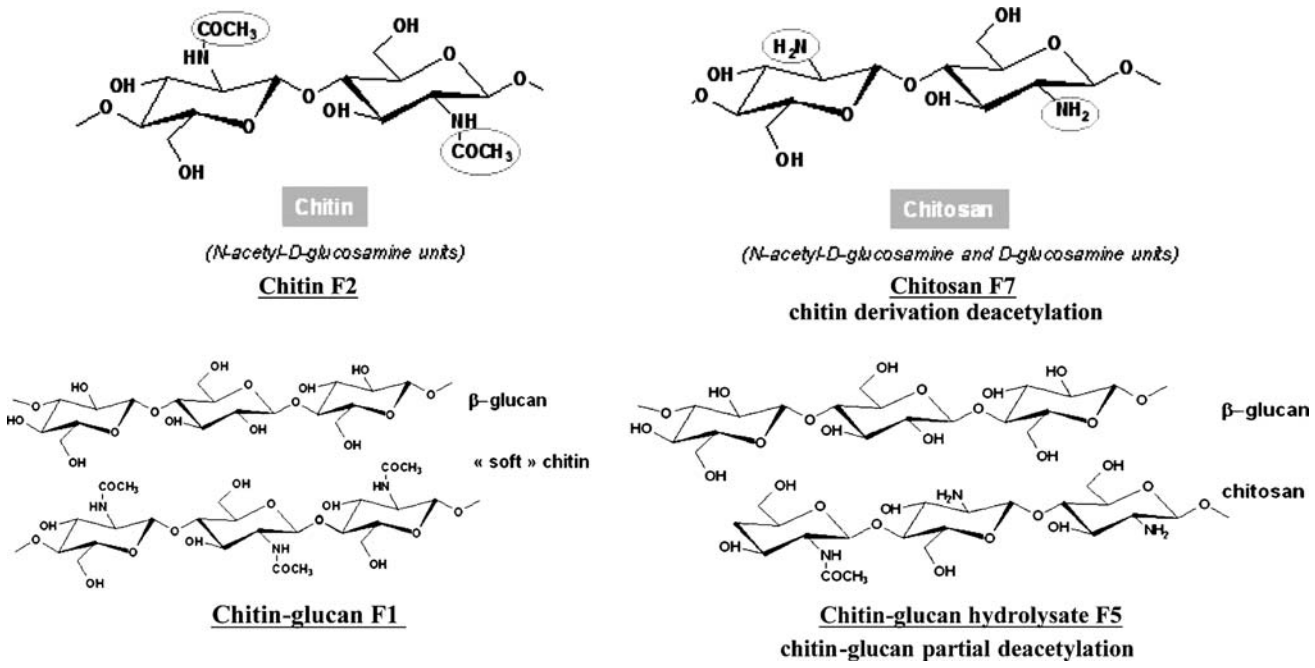

Fig. 1 Chemical structures of chitin, chitosan, chitin glucan, chitin glucan hydrolysate

Table 1 Chemical characteristic of wines and analytical characteristics for the determination of metals (Fe, Pb, Cd) and OTA in wines samples

Wine sample	CPT (mg GAE L^{-1})	AL (% vol)	TA (g H_2SO_4 L^{-1})	RS (g L^{-1})	SO_2 T (mg L^{-1})	pH
Red wine (R)	2,075	13.55	2.8	2	82	3.88
White wine (W)	273.3	12.85	3.01	2	121	3.55
Sweet wine (S)	370.8	15.95	2.63	120	127	3.86

Compound	LOD (pg)	Characteristic mass[a] (pg)	Calibration range[b]	*r*	Accuracy[c] recovery (%)	Precision[d] RSD (%)
Fe	480	16	0.0–5 mg L^{-1}	0.9991	99.1 ± 3.8	1.5–3.5
Cd	2	1.2	0.0–10 μg L^{-1}	0.9993	99.5 ± 2.5	1.3–3.3
Pb	22.5	19	0.0–30 μg L^{-1}	0.9994	99.0 ± 3.9	1.8–3.6
OTA	0.2	–	0.0–10 μg L^{-1}	0.9995	99.4 ± 2.7	1.8–3.8

LOD detection limit calculated according to IUPAC rules (25 μL), *CPT* total phenol content in mg of GAE (gallic acid equivalent) L^{-1}, *AL* alcohol level (% vol), *TA* total acidity in g H_2SO_4 L^{-1}, *RS* residual sugars in g L^{-1}, SO_2 *T* total sulfites in mg L^{-1}

[a] Characteristic mass in pg/0.0044A

[b] Calibration range in mg L^{-1} and coefficient of correlation (*r*) obtained for five points

[c] Mean value SD of determinations in two different samples

[d] Relative standard deviation RSD (%) of six determinations in five different samples

and the mixtures were stirred gently for up to 48 h at 20°C. The solutions were centrifuged at 3,000*g* for 30 min at room temperature (20°C) to sediment the absorbents and 50 mL aliquots of the supernatant were taken for analysis.

Preparation of samples for the effects of pH on removal of OTA and metals

Sodium hydroxide and sulfuric acid were added to the wines after centrifugation to obtain a pH of 0.85 units above and below the initial pH of the wine (3.55 for W, 3.88 for R, 3.86 for S).

Methods of analysis

All analyses were repeated at least three times, on the wines before and after absorbent treatment. Wines (W, R, S) were spiked with minerals (iron 20 mg L^{-1}, lead 500 μg L^{-1}, cadmium 20 μg L^{-1} or ochratoxin A 5 μg L^{-1}). Iron, lead and cadmium were brought on the same chemical forms (Fe^{3+}, Pb^{2+}, Cd^{2+}) than in wine by fluka standard solutions: $Fe(NO_3)_3$, $Pb(NO_3)_2$, $Cd(NO_3)_2$. The wines containing added metals were treated with F1, F2, F5 or F7 at doses of 0.1, 0.5 and 2 g L^{-1}. After 2 days of mild agitation, the levels of iron, copper, lead and cadmium were measured with flame and a graphite furnace atomic absorption spectrophotometer. Wine samples destined for iron measurement were evaporated in vacuo to remove ethanol, then diluted with distilled water to their original volumes. All measurements of metals an OTA were carried out according to OIV (International Wines and Vines Organization) validated methods using standard calibration curves having at least squares correlation coeficient of 0.99 or better. Wine containing added iron was measured directly by atomic absorption spectrophotometry at 248.3 nm using a standard measurements calibration curve [32]. Wines with added lead and cadmium were first diluted then measured with a graphite furnace atomic absorption spectrophotometry at 283.3 nm in comparison with standard measurements using a standard calibration curve [33]. Cadmium was measured directly with a graphite furnace atomic absorption spectrophotometry at 228.8 nm in comparison with standards measurements [34]. Wine samples with added ochratoxin A, were treated with F1, F2, F5 or F7 at 2 and 5 g L^{-1} then shaken gently at room temperature for 2 days. Aliquots of these wines were analyzed by a validated method [35] that measures OTA in red, white and rose and special wines within a range of 0–10 μg L^{-1}. Wines samples were diluted with a solution of g polyethylene glycol and sodium bicarbonate, then introduced to the immunoaffinity column. OTA was eluted with methanol and quantified by reverse phase HPLC using fluorimetric detection (excitation wavelength = 333 nm; emitting wavelength = 460 nm).

Analytical characteristics of metals (Fe, Pb, Cd) and OTA determination in wines samples are given in Table 1. The detection limits were calculated according to IUPAC rules [36] and the sensitivity of the analytical conditions also evaluated [37]. Accuracy was checked with recovery assays for four determinations in two different experimental wines samples, by adding

known amounts of analytes [38]. Precisions of the methods (as inter-day reproducibility) were also checked in six determinations of five different randomly chosen samples of wines. Detection limits and sensitivity were suitable for the range of Fe, Cd, Pb and OTA concentrations encountered and are compatible with estimates given by other authors. The techniques were accurate and reproducible.

Results and discussion

Influence of structures and doses of chitosans, chitins and derivatives

In our experiments (Table 2) at the highest treatment dose 2 g L^{-1}, for red wine (R), iron is reduced until 90% with F7, 85% with F5, 80% with F2, 73% with F1. In the case of white wine, iron is reduced by 91% with F7, 67% with F5, 34% with F2 and 32% with F1. For sweet wine, iron was reduced by 98% with F7, 4% with F5, 51% with F2 and 77% with F1.

Cadmium in red wine (R) was reduced by 29% with F7, 27% with F2, 35% with F5, 57% with F1. In the case of white wine (W), cadmium is decreased by 11% with F7, 24% with F5, 23% with F2 and 17% with F1. For sweet wine (S), cadmium is reduced by 23% when treated with F7, 17% with F2 and F5 and 25% with F1. An efficient dose treatment effect for lead removal with best results at 2 g L^{-1} was observed for F7, F1, F5 but not in the case of F2. We found for red wine that lead was decreased by 74% with F7 treatment, 51% with F2, 37% for F5, 33% with F1. In the case of white wine, lead was reduced by 78% with F5, 65% with F7, 50% with F2 and 58% with F1. For sweet wine, lead levels fell by 86% with F5, 84% with F7, 42% with F2 and 38% with F1. Generally in Table 2, we can observed that the higher the amount of polysaccharide added, the less the concentration of metal (Fe, Pb) remaining in wines, except for cadmium. In the case of sweet wine: the high content of sugars could reduce the efficiency of chitin (F2 at the doses of 0.5 and 2 g L^{-1}) to remove lead and on another hand the ability of lead adsorption by F2 could already be used for iron or other metals naturally present in wine (e.g., Cu, Zn, Mn, Ni, etc.).

Table 3, shows a dose dependance with the higher removal of OTA occurring at 5 g L^{-1} than 2 g L^{-1}. With the 2 g L^{-1} treatment, the reduction of OTA was 35% in red wine treated with F2, and 40% in sweet wine treated with either F5 or F7. For 5 g L^{-1} treatments the levels of OTA in red wine was reduced by 83.4% with F7, 73.3% by F5, 66.7% with F2 and 56.7% with F1. In the case of white wine (W), the levels of OTA are reduced until 42.2% for F5, 53.4% with F7 and F2, and 64.5% with F1. In the case of the sweet wine (S), the levels of OTA are reduced by 26.1% with F7, 34.8% with F2 and 43.5% with F1.

Influence of pH

Variation in pH can influence the removal of iron, lead and cadmium (Fig. 2). A lower pH close to 3.1 gave the best percent removal of iron for the different types of wines. An increase of 1 pH unit to around pH 4.1 reduced the efficiency of iron removal about two-fold for the white and sweet wines. It is known that citric acid (by the intermediary of it's three carboxylic groups) is a good iron–wine complexing agent. Citric acid is naturally present in wine around 0.5 g L^{-1} and could possibly complexes more iron with an increase of pH. This could explain the iron chelating differences of polysaccharides observed between red and white/sweet wines. The best removal of iron with F5 was 67–94%

Table 2 Levels of iron, lead, cadmium in wine for three doses of treatment by chitosan and chitin polysaccharides

	Iron (mg L^{-1})			Lead (μg L^{-1})			Cadmium (μg L^{-1})		
Wines	R	W	S	R	W	S	R	W	S
Initial levels	23	6	5	150	111	110	19	18	10
Treatments									
F1 2 g L^{-1}	6	4	1	101	47	68	8.8	15.2	7
F1 0.5 g L^{-1}	6	4	3	104	79	82	8.5	16	9
F1 0.1 g L^{-1}	7	5	4	118	100	75	8.2	14.8	8.8
F2 2 g L^{-1}	4	4	2	73	55	110	15.2	15	8.4
F2 0.5 g L^{-1}	6	5	4	89	62	94	14.1	13.9	8.4
F2 0.1 g L^{-1}	7	4	5	89	81	64	16.6	14.6	8
F5 2 g L^{-1}	3	2	0.5	95	24	15	12.5	14.7	8.4
F5 0.5 g L^{-1}	6	3	1	139	41	50	15.6	14.9	8.8
F5 0.1 g hL^{-1}	6	4	2	145	68	104	14.6	13.8	9
F7 2 g L^{-1}	2	0.5	0.1	39	38	18	14.4	16.6	8.4
F7 0.5 g L^{-1}	3	2	0.5	51	53	51	13.9	17.2	7.8
F7 0.1 g L^{-1}	5	5	2	94	63	58	14.3	16.1	9.6

RSD % 3.5 for Fe, 3.3 for Cd, 3.6 for Pb

R red wine, *W* white wine, *S* sweet wine

Table 3 Levels of ochratoxin A (OTA) in wine for a chitosan and chitin polysaccharides treatments

Wines	R	W	S	R	W	S
Treatment doses (g L^{-1})	2	2	2	5	5	5
OTA (μg L^{-1})						
Initial levels in wine	3.7	4.3	4.7	3.0	4.5	4.6
F1	2.6	3.3	3.6	1.3	1.6	2.6
F2	2.4	3.6	3.3	1	2.1	3
F5	2.8	3.4	2.8	0.8	2.6	4.4
F7	2.8	3.4	2.8	0.5	2.1	3.4

RSD 3.8%

R red wine, *W* white wine, *S* sweet wine

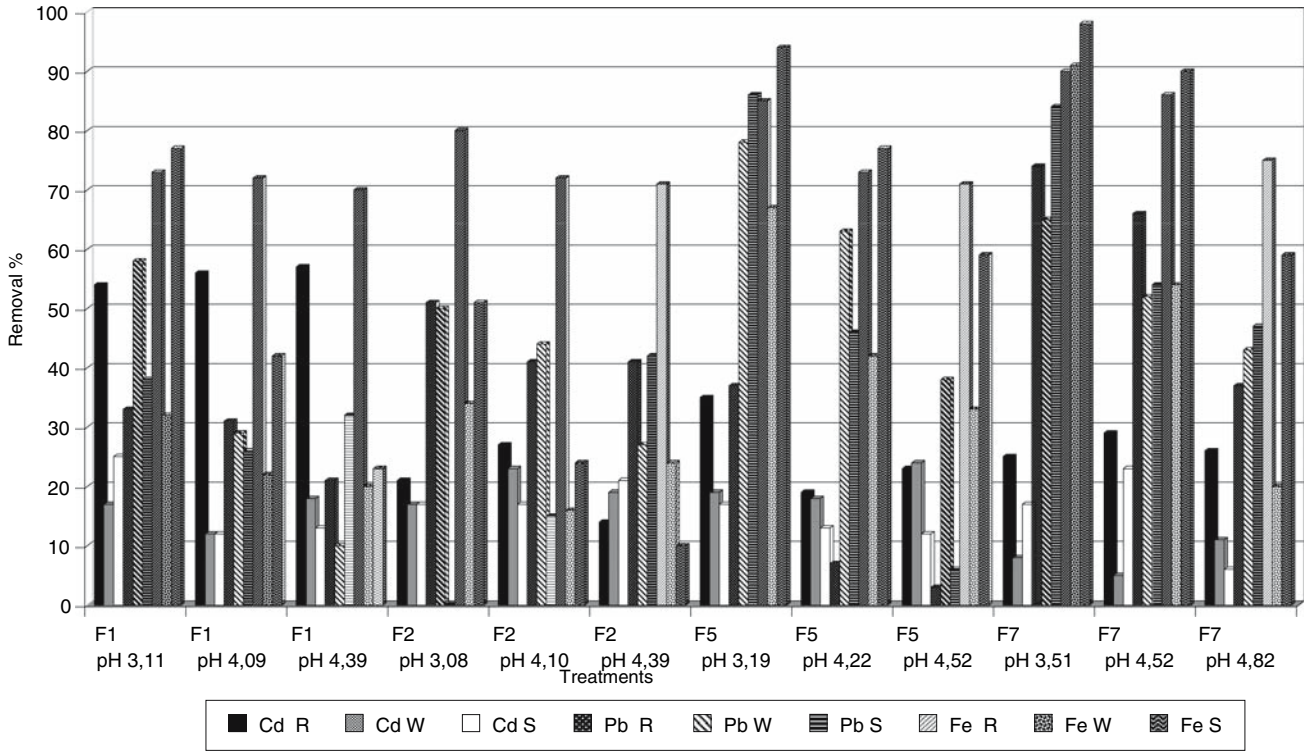

Fig. 2 Percentage of Fe, Cd, Pb removal in wines in function of chitosans treatments (2 g L^{-1}) with pH variation (initial levels of Fe: R = 23 mg L^{-1}, W = 6 mg L^{-1}, S = 5 mg L^{-1}; Cd: R = 19 μg L^{-1}, W = 18 μg L^{-1}, S = 10 μg L^{-1}; Pb: R = 150 μg L^{-1}, W = 111 μg L^{-1}, S = 110 μg L^{-1}). *W* Chardonnay white wine, *R* Merlot red wine, *S* Grenache-Macabeu natural sweet wine. *F1* chitin-glucan, *F2* chitin, *F5* chitin-glucan hydrolysate, *F7* chitosan

while with F7 it was 90–98% at the lower pH. The maximum removal of Cd (54–57%) is found for red wine with F1 treatment and pH appeared to have no major effect. Lead elimination was most effective with F5 (37–86%) and F7 (74–87%) at the lower pH. Except for sweet wines treated with F2, differences in Pb removal were associated with pH variation. In this case, the highest lead removal was observed at the higher pH. The reduction in cadmium is lower (57% maximum) than for lead (86% maximum) with of treatments and for all types of biopolymeric chitins and chitosans molecules used. It is likely a competition that lead and cadmium ions compete for amino groups and lead is fixed more because of its higher concentration.

The interactions of metals with chitosan are probably determined by a complex mixture of adsorption, ion-exchange and chelation factors [39]. Efficient iron removal may be due to the adsorption of Fe^{+3} ions on the surface of insoluble chitosan particles that takes place as the formation of a surface complex which involves nitrogen and oxygen atoms in the repeat unit of chitosan: $CHI–NH_2 + Fe^{3+} \Leftrightarrow [CHI–NH_2–Fe–]^{+3}$. The effects of time and degree of deacetylation on iron-adsorption capacities of chitosan increases with the degree of deacetylation of chitosan, indicating that free amino groups take part in binding the Fe^{+3} ions onto the chitosan surface. Hydroxyl groups and carbonyl groups may also take part in the process in cooperation with amino groups. Different mechanisms for the interaction of metal ions with chitosan solutions involving amine and hydroxyl groups have been also proposed [20, 40–42].

Chelation of heavy metal ions by chitosan has been studied, and this polysaccharide is known as a heavy metal-chelating agent [43]. Chitosan has free amines in some of its repeat units, which gets protonated in dilute acidic media. These protonated amines form the multiple bonding sites that are useful in chelating heavy metals like Cu^{2+} and Zn^{2+} [44]. Two other types of interactions may also explain the lead and cadmium removal: Pb^{2+} or Cd^{2+} chelation with hydroxyls groups located in position C-3 of chitosan or chitin, and the binding of hydroxyls groups located in position C-3 of β-glucan part. Possibly, an alternative to explain high adsorption abilities of chitosan particles for heavy metals ions could be attributed to the deposition of metal hydroxide aggregates in pores of chitosan particles rather than chelation to amine groups [45].

Removal of OTA by F7 from red wine was 66.7% at pH 3.5 and this increased at pH 4.5 (Fig. 3) to

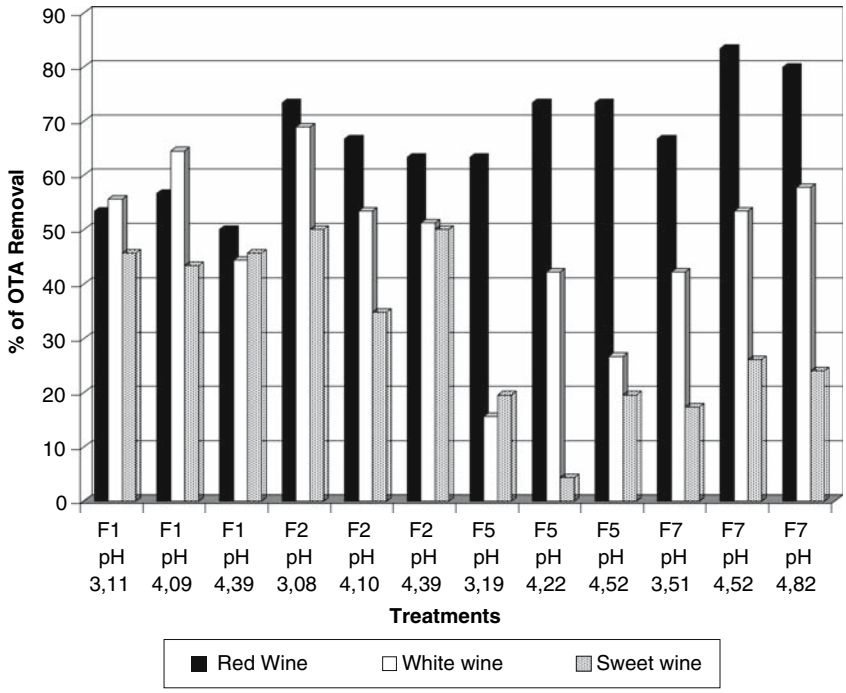

Fig. 3 Percentage of OTA removal in wines (initial levels R = 3 μg L^{-1}, W = 4.5 μg L^{-1}, S = 4.6 μg L^{-1}) in function of chitosans treatments (5 g L^{-1}) with pH variation

83.4% while with white wine removal was 57.4 and 26.1%. At pH 3.1, F2 treatment gave a maximal removal of OTA of 73.4% (R); 68.9% (W); 50% (S), and these values fell at increased pH. At pH values between 3.1 and 4.4, F1gave a maximum removal in OTA of 43.5% (S); 56.7% (R), and 64.5% (W). In function of pH, F5 use, gave lowest removal for OTA in the case of wines W and S, in comparison OTA was reduced between 63.4 and 73.4% in the same conditions. At wine pH, OTA molecule is fully protonated by the intermediary of its carboxylic group. With an acidic pH, both the amino groups of chitosan and chitin-glucan hydrolysate and the carboxylic groups of OTA are positively charged, and would have lower tendency to react. For these reasons, the acidic group of OTA could interact with amino groups of chitosan or chitin-glucan hydrolysate with highest pH. However, to explain adsorption abilities of chitin and chitin glucan particles for OTA, it could be also attributed to OTA aggregation with deposition in pores of chitin or chitin-glucan.

Conclusion

This study demonstrates that polysaccharides: chitosan, chitin, chitin-glucan and chitin glucan hydrolysate of fungal origin can reduce the levels of iron, heavy metals (Pb, Cd) and mycotoxins (ochratoxin A) and thereby to improve wine safety and quality. Flame and graphite furnace atomic absorption spectrophotometer and HPLC with fluorimetric detection are reliable methods with good limits of detection, precisions and accuracy for the determinations of Fe, Pb, Cd and OTA in wines. Treatments of wines with increasing doses of these polysaccharides (until 2 g L^{-1} for the metals and 5 g L^{-1} for OTA) gave reductions in Fe (32–91%), Cd (11–57%), Pb (33–84%), and OTA (26.1–83.4%). The degree of polymerization, deacetylation, distribution of acetyl and amino groups along the polymer chain as well as pH medium is of crucial importance for chitosan–contaminants interacting characteristics. Our results indicate that the biopolymers: chitosan, chitin-glucan, chitin-glucan hydrolysate and chitin are of real interest to reduce the levels in contaminants (iron, heavy metals, mycotoxins) and should become ancillaries especially for beverages (alcoholic or not) during production or storage to reduce the contribution of beverages to the dietary intake in contaminants. Toxicity of these polysaccharides is actually investigated in view to obtain their official use in wine industry.

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II

(Non-legislative acts)

REGULATIONS

COMMISSION REGULATION (EU) No 53/2011

of 21 January 2011

amending Regulation (EC) No 606/2009 laying down certain detailed rules for implementing Council Regulation (EC) No 479/2008 as regards the categories of grapevine products, oenological practices and the applicable restrictions

THE EUROPEAN COMMISSION,

Having regard to the Treaty on the Functioning of the European Union,

Having regard to Council Regulation (EC) No 1234/2007 of 22 October 2007 establishing a common organisation of agricultural markets and on specific provisions for certain agricultural products (Single CMO Regulation) [1], in particular the third and fourth paragraphs of Article 121 thereof,

Whereas:

(1) According to Article 3 of Commission Regulation (EC) No 606/2009 [2], the authorised oenological practices are laid down in Annex I to that Regulation. The International Organisation of Vine and Wine (OIV) has adopted new oenological practices. In order to meet the international standards in this field and to provide EU producers with the new possibilities available to third country producers, these new oenological practices should be authorised in the EU under the conditions of use defined by the OIV.

(2) Regulation (EC) No 606/2009 authorises clarification by means of pectolytic enzymes and enzymatic preparations of beta-glucanase. These enzymes and other enzymatic preparations are also used for maceration, clarification, stabilisation, filtration and for revealing the aromatic precursors of grapes present in the must and the wine. These oenological practices have been adopted by the OIV and they should be authorised under the conditions of use defined by the OIV.

(3) Wines entitled to the protected designations of origin 'Malta' and 'Gozo' have a sugar content greater than 45 g/l and are produced in small quantities. Likewise, certain French white wines with a protected geographical indication may have a total alcoholic strength by volume greater than 15 % vol. and a sugar content greater than 45 g/l. In order to ensure the preservation of these wines, the Member States concerned, i.e., Malta and France, respectively, requested a derogation to the maximum sulphur dioxide contents given in Annex I B to Regulation (EC) No 606/2009. These wines should be mentioned in the list of wines having a maximum sulphur dioxide content of 300 milligrams per litre.

(4) Wines entitled to the traditional expression 'Késői szüretelésű bor' have a very high sugar content and are produced in small quantities. In order to ensure the preservation of these wines, Hungary requested a derogation to the maximum sulphur dioxide content. A maximum sulphur dioxide content of 350 milligrams per litre should be authorised for these wines.

(5) Wines entitled to the protected designation of origin 'Douro' followed by the statement 'colheita tardia' derogate from the maximum sulphur dioxide content. Wines entitled to the protected designation of origin 'Duriense' have the same characteristics as these wines. On the basis of this, Portugal requested a derogation from the maximum sulphur dioxide content. A maximum sulphur dioxide content of 400 milligrams per litre should be authorised for these wines.

(6) In order to render the names of vine varieties clearer, the names of the varieties should be given in the different languages of the countries where these varieties are used.

(7) Certain provisions concerning certain liqueur wines differ from the requirements laid down in the specifications for these wines. These provisions should be amended in accordance with the requirements in question.

[1] OJ L 299, 16.11.2007, p. 1.
[2] OJ L 193, 24.7.2009, p. 1.

(8) Regulation (EC) No 606/2009 should be amended accordingly.

(9) The making of wine from grapes harvested during the 2010 wine-growing year has already begun. In order not to distort competition between wine producers, the new oenological practices should be authorised for all these producers starting at the beginning of the 2010 wine-growing year. This regulation should apply retroactively from 1 August 2010, which marks the start of the 2010 wine-growing year.

(10) The measures provided for in this Regulation are in accordance with the opinion of the Regulatory Committee established by Article 195(3) of Regulation (EC) No 1234/2007,

HAS ADOPTED THIS REGULATION:

Article 1

Regulation (EC) No 606/2009 shall be amended as follows:

(a) Annex I A is amended in accordance with Annex I to this Regulation;

(b) Annex I B is amended in accordance with Annex II to this Regulation;

(c) Annex II is amended in accordance with Annex III to this Regulation;

(d) Annex III is amended in accordance with Annex IV to this Regulation.

Article 2

This Regulation shall enter into force on the day following its publication in the *Official Journal of the European Union*.

It shall apply from 1 August 2010.

This Regulation shall be binding in its entirety and directly applicable in all Member States.

Done at Brussels, 21 January 2011.

For the Commission
The President
José Manuel BARROSO

ANNEX I

Annex I A to Regulation (EC) No 606/2009 shall be amended as follows:

(1) The table shall be amended as follows:

(a) line 10 shall be replaced by the following:

'10	clarification by means of one or more of the following substances for oenological use: — edible gelatine, — plant proteins from wheat or peas, — isinglass, — casein and potassium caseinates, — egg albumin, — bentonite, — silicon dioxide as a gel or colloidal solution, — kaolin, — tannin, — chitosan of fungoid origin, — chitin-glucan of fungoid origin		The use of chitosan in the treatment of wines is limited to 100 g/hl. The use of chitin-glucan in the treatment of wines is limited to 100 g/hl'

(b) the following entries shall be added:

'44	Treatment using chitosan of fungoid origin	Under the conditions set out in Appendix 13	
45	Treatment using chitin-glucan of fungoid origin	Under the conditions set out in Appendix 13	
46	Acidification by means of electro-membranary treatment	Conditions and limits laid down in points C and D of Annex XVa to Regulation (EC) No 1234/2007 and Articles 11 and 13 of this Regulation Under the conditions set out in Appendix 14	
47	Use of enzymatic preparations for oneological purposes in maceration, clarification, stabilisation, filtration and to reveal the aromatic precursors of grapes present in the must and the wine	Without prejudice to the provisions of Article 9(2) of this Regulation, enzymatic preparations and the enzyme activities of these preparations (i.e., pectolyase, pectin methylesterase, polygalacturonase, hemicellulase, cellulase, betaglucanase and glycosidase) must comply with the corresponding purity and identification specifications of the International Oenological Codex published by the OIV'	

(2) Appendix 1 shall be deleted.

(3) The following Appendices 13 and 14 shall be added:

'Appendix 13

Requirements for the treatment of wines with chitosan of fungoid origin and for the treatment of wines with chitin-glucan of fungoid origin

Areas of application:

(a) reduction in the heavy metal content, particularly iron, lead, cadmium and copper;

(b) prevention of ferric casse and copper casse;

(c) reduction of possible contaminants, especially ochratoxin A;

(d) reduction in the populations of undesirable micro-organisms, in particular *Brettanomyces*, solely by means of treatment with chitosan.

Requirements:

— The dose levels to be used are determined after a qualification test. The maximum dose level used may not exceed:

  — 100 g/hl for applications (a) and (b),

  — 500 g/hl for application (c),

  — 10 g/hl for application (d),

— sediments are removed using physical processes.

Appendix 14

Requirements for acidification by means of electro-membranary treatment

— The cationic membranes must be constituted in such a way as to enable only the extraction of cations, in particular cation K^+.

— The bipolar membranes are impermeable to the anions and cations of must and wine.

— The treatment is to be carried out under the responsibility of an oenologist or qualified technician.

— The membranes used must comply with the requirements of Regulation (EC) No 1935/2004 and of Directive 2002/72/EC and with the national provisions adopted for the implementation of the Directive. The membranes must also comply with the requirements of the monograph "Electrodialysis Membranes" of the International Oenological Codex published by the OIV.'

ANNEX II

Part A, point 2, of Annex I B to Regulation (EC) No 606/2009 shall be amended as follows:

(1) point (c) shall be amended as follows:

(a) in the 13th indent, the following sub-indents shall be added:

'— Vin de pays de l'Agenais,

— Vin de pays des terroirs landais,

— Vin de pays des Landes,

— Vin de pays d'Allobrogie,

— Vin de pays du Var;'

(b) the following indent shall be added:

'— wines originating in Malta with a total alcoholic strength by volume greater than or equal to 13,5 % vol. and a sugar content greater than or equal to 45 g/l and entitled to the protected designation of origin "Malta" and "Gozo";'

(2) in point (d), the following indent shall be added:

'— wines entitled to the traditional expression "Késői szüretelésű bor".'

(3) in point (e), the ninth indent shall be replaced by the following:

'— white wines entitled to the protected designation of origin "Douro" or to the protected geographical indication "Duriense" followed by the statement "colheita tardia";'

ANNEX III

In Appendix 1 to Annex II to Regulation (EC) No 606/2009, the names of the following vine varieties shall be inserted in the list in the appropriate alphabetical order:

' "Albariño", "Macabeo B", "Toutes les Malvasías" and "Tous les Moscateles".'

ANNEX IV

Annex III to Regulation (EC) No 606/2009 shall be amended as follows:

(a) the second indent of Part A, point 4(a) shall be replaced by the following:

'— concentrated grape must, rectified concentrated grape must or must from raisined grapes to which neutral alcohol of vine origin has been added to prevent fermentation, for Spanish wine described by the traditional expression "vino generoso de licor" and provided that the increase in the total alcoholic strength by volume of the wine in question is not greater than 8 % vol.,';

(b) Part B shall be amended as follows:

(i) in point 3, the second paragraph shall be replaced by the following:

'However, as concerns liqueur wines with the protected designation of origin "Málaga" and "Jerez-Xérès-Sherry", the must of raisined grapes to which neutral alcohol of vine origin has been added to prevent fermentation, obtained from the Pedro Ximénez vine variety, may come from the "Montilla-Moriles" region.';

(ii) in point 10, the first indent shall be replaced by the following:

'— obtained from "vino generoso", as referred to in point 8, or from wine under flor capable of producing such a "vino generoso", to which has been added either must of raisined grapes to which neutral alcohol of vine origin has been added to prevent fermentation, or rectified concentrated grape must or "vino dulce natural",';

(c) Appendix 1 shall be amended as follows:

(i) in point A in the list for Spain, the following rows shall be inserted in the appropriate alphabetical order:

'Condado de Huelva	Pedro Ximénez Moscatel Mistela
Empordà	Mistela Moscatel'

(ii) in point B.5 in the list for Spain, the following row shall be inserted in the appropriate alphabetical order:

'Empordà	Garnacha/Garnatxa'

(d) Appendix 2 shall be amended as follows:

(i) in point A 2, liqueur wine with the protected designation of origin 'Trentino' shall be removed from the list for Italy;

(ii) in point A 3, the following list shall be added:

'**ITALY**

Trentino';

(e) in Appendix 3, the names of the following vine varieties shall be added:

'Moscateles — Garnacha'

I

(Acts adopted under the EC Treaty/Euratom Treaty whose publication is obligatory)

REGULATIONS

COMMISSION REGULATION (EC) No 606/2009

of 10 July 2009

laying down certain detailed rules for implementing Council Regulation (EC) No 479/2008 as regards the categories of grapevine products, oenological practices and the applicable restrictions

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Community,

Having regard to Council Regulation (EC) No 479/2008 of 29 April 2008 on the common organisation of the market in wine, amending Regulations (EC) No 1493/1999, (EC) No 1782/2003, (EC) No 1290/2005, (EC) No 3/2008 and repealing Regulations (EEC) No 2392/86 and (EC) No 1493/1999 [1], and in particular Articles 25(3) and 32 thereof,

Whereas:

(1) The definition of wine given in the first indent of point (c) of the second subparagraph of paragraph 1 of Annex IV to Regulation (EC) No 479/2008 listing the categories of grapevine products provides for a total alcoholic strength of not more than 15 % vol. However, that limit may be increased to 20 % vol. for wines produced without enrichment in certain wine-growing areas that should be defined.

(2) Chapter II of Title III of Regulation (EC) No 479/2008 and Annexes V and VI thereto lay down general rules on oenological practices and processes and refer for the rest to detailed implementing rules to be adopted by the Commission. The permitted oenological practices should be defined clearly and precisely, including the methods for sweetening wines, and limits on the use of certain substances and the conditions for using certain of those substances should be laid down.

(3) Annex IV to Council Regulation (EC) No 1493/1999 of 17 May 1999 on the common organisation of the market in wine [2] listed authorised oenological practices. That list of authorised oenological practices, described more clearly and more coherently and supplemented to take account of technical progress, should be kept in a single annex.

(4) Annex V A to Regulation (EC) No 1493/1999 lays down maximum levels of sulphites in wines produced in the Community that are higher than the limits laid down by the International Organisation of Vine and Wine (OIV). The limits should be aligned with those of the OIV, which are recognised internationally, and the derogations required for certain sweet wines produced in small quantities because of their higher sugar content and to ensure their good conservation should be kept. In the light of current scientific studies into the reduction and replacement of sulphites in wine and the sulphite intake from wine in the human diet, provision must be made for re-examining the maximum limits at a later date with a view to reducing them.

(5) The procedures by means of which the Member States may authorise certain oenological practices and processes not provided for by Community rules for a definite period and for experimental purposes should be laid down.

(6) The production of sparkling wines, quality sparkling wines and quality aromatic sparkling wines requires a number of specific practices in addition to the oenological practices permitted elsewhere. For reasons of clarity, those practices should be listed in a separate annex.

[1] OJ L 148, 6.6.2008, p. 1.

[2] OJ L 179, 14.7.1999, p. 1.

(7) The production of liqueur wines requires a number of specific practices in addition to the oenological practices permitted elsewhere and the production of liqueur wines with a protected designation of origin has certain particularities. For reasons of clarity, those practices and restrictions should be listed in a separate annex.

(8) Coupage is a widespread oenological practice and, in view of its possible consequences for the quality of wine, its use must be strictly defined and regulated in order to prevent abuse and to ensure high quality wines at the same time as promoting a more competitive sector. As far as rosé wine production is concerned, for the reasons mentioned above, this practice must be regulated more particularly for certain wines which are not subject to specifications.

(9) Purity and identification specifications of a large number of substances used in oenological practices are already laid down in the Community rules on foodstuffs and in the International Oenological Codex of the OIV. For the purposes of harmonisation and clarity, those specifications should be used in the first instance, while providing for additional rules specific to the situation in the Community.

(10) Wine products that do not comply with the provisions of Chapter II of Title III of Regulation (EC) No 479/2008 or those to be laid down in this Regulation may not be placed on the market. However, some of these products may be used for industrial purposes and the conditions for their use should be laid down so as to ensure adequate monitoring of their final use. In addition, to avoid financial losses for operators with stocks of certain products produced before the date of application of that Regulation, it should be laid down that products made in accordance with the rules in force before that date may be released for consumption.

(11) In accordance with paragraph 4 of point D of Annex V to Regulation (EC) No 479/2008, all enrichment, acidification and deacidification operations must be notified to the competent authorities. This also holds for quantities of sugar, concentrated grape must and rectified concentrated grape must held by the natural or legal persons undertaking such operations. The purpose of such notification is to allow the operations in question to be monitored. Notifications must therefore be addressed to the competent authority of the Member State on whose territory the operation is to take place and must be as accurate as possible. Where an increase in alcoholic strength is involved, the competent authority must be notified in sufficient time to permit it to carry out an effective check.

(12) In the case of acidification and deacidification, a check after the operation is sufficient. For that reason and to simplify administrative procedures, it must therefore be possible to make such notification, except for the first notification in the wine year, by updating records regularly verified by the competent authority. In certain Member States, the competent authorities carry out systematic analytical checks of all batches of products turned into wine. As long as this continues to be the case, declarations of intention to enrich wine are not absolutely necessary.

(13) Notwithstanding the general rule laid down in point D of Annex VI to Regulation (EC) No 479/2008, the pouring of wine [or grape must onto lees or grape marc or pressed 'aszú' or 'výber' pulp is an essential characteristic of the production of certain Hungarian and Slovak wines. The particular rules for that practice must be laid down in accordance with the national provisions in force in the Member States concerned on 1 May 2004.

(14) Article 31 of Regulation (EC) No 479/2008 lays down that the analysis methods for establishing the composition of the products covered by that Regulation and the rules for checking whether those products have been subjected to processes in violation of authorised oenological practice are those recommended and published by the OIV in the Compendium of International Methods of Analysis of Wines and Musts. Where specific analysis methods are necessary for certain Community wine products and they have not been established by the OIV, those Community methods should be described.

(15) So as to ensure greater transparency, a list of the analysis methods concerned and their description should be published at Community level.

(16) Consequently, Commission Regulations (EEC) No 2676/90 of 17 September 1990 determining Community methods for the analysis of wines [1] and (EC) No 423/2008 of 8 May 2008 laying down certain detailed rules for implementing Council Regulation (EC) No 1493/1999 and establishing a Community code of oenological practices and processes [2] should be repealed.

(17) The measures provided for in this Regulation are in accordance with the opinion of the Regulatory Committee established by Article 113(2) of Regulation (EC) No 479/2008,

HAS ADOPTED THIS REGULATION:

Article 1

Purpose

This Regulation lays down detailed rules for the application of Title III, Chapters I and II of Regulation (EC) No 479/2008.

[1] OJ L 272, 3.10.1990, p. 1.
[2] OJ L 127, 15.5.2008, p. 13.

Article 2

Wine-growing areas where wines may have a maximum total alcoholic strength of 20 % vol.

The wine-growing areas referred to in the first indent of point (c) of the second subparagraph of paragraph 1 of Annex IV to Regulation (EC) No 479/2008 shall be zones C I, C II and C III referred to in Annex IX to that Regulation and the areas of zone B in which white wines with the following protected geographical indications may be produced: 'Vin de pays de Franche-Comté' and 'Vin de pays du Val de Loire'.

Article 3

Authorised oenological practices and restrictions

1. The authorised oenological practices and restrictions applicable to the production and conservation of products covered by Regulation (EC) No 479/2008, referred to in Article 29(1) thereof, are laid down in Annex I hereto.

2. The authorised oenological practices and the conditions for and the limits on their use are set out in Annex I A.

3. The maximum sulphur dioxide contents of wines are given in Annex I B.

4. The maximum volatile acid contents are given in Annex I C.

5. The rules on sweetening are laid down in Annex I D.

Article 4

Experimental use of new oenological practices

1. For experimental purposes as referred to in Article 29(2) of Regulation (EC) No 479/2008, each Member State may authorise the use of certain oenological practices or processes not provided for in that Regulation or in this Regulation, for a maximum of three years, on condition that:

(a) the practices and processes concerned meet the requirements of Articles 27(2) and 30(b) to (e) of Regulation (EC) No 479/2008;

(b) such practices and processes are applied to quantities not exceeding 50 000 hectolitres per year for any one experiment;

(c) the Member State concerned informs the Commission and the other Member States at the beginning of the experiment of the terms of each authorisation;

(d) the processes shall be entered on the accompanying document referred to in Article 112(1) and in the register referred to in Article 112(2) of Regulation (EC) No 479/2008.

'Experiment' shall mean an operation or operations carried out in the context of a well-defined research project with a single experimental protocol.

2. The products obtained by the experimental use of such practices and processes may be placed on the market of a Member State other than the Member State concerned provided the Member State authorising the experiment gives prior notification to the competent authorities of the Member State of destination of the terms of the authorisation and the quantities involved.

3. During the three months following the end of the period referred to in paragraph 1, the Member State concerned shall forward to the Commission a report on the authorised experiment and the results thereof. The Commission shall notify the other Member States of those results.

4. Depending on these results, the Member State concerned may apply to the Commission for authorisation to continue the experiment, possibly with a larger quantity than in the original experiment, for a further maximum period of three years. The Member State shall submit an appropriate dossier in support of its application. The Commission, in accordance with the procedure referred to in Article 113(2) of Regulation (EC) No 479/2008, shall decide on the application to continue the experiment.

Article 5

Oenological practices applicable to categories of sparkling wines

The authorised oenological practices and restrictions, including enrichment, acidification and de-acidification, concerning sparkling wines, quality sparkling wines and quality aromatic sparkling wines, referred to in point (b) of the second paragraph of Article 32 of Regulation (EC) No 479/2008 are listed in Annex II hereto, without prejudice to the oenological practices and restrictions of general application laid down in Regulation (EC) No 479/2008 and in Annex I hereto.

Article 6

Oenological practices applicable to liqueur wines

The authorised oenological practices and restrictions concerning liqueur wines referred to in point (c) of the second paragraph of Article 32 of Regulation (EC) No 479/2008 are listed in Annex III hereto, without prejudice to the oenological practices and restrictions of general application laid down in Regulation (EC) No 479/2008 and in Annex I hereto.

Article 7

Definition of coupage

1. Within the meaning of point (d) of the second paragraph of Article 32 of Regulation (EC) No 479/2008, 'coupage' shall mean the mixing of wines or musts of different origins, different vine varieties, different harvest years or different categories of wine or of must.

2. The following shall be regarded as different categories of wine or must:

(a) red wine, white wine and the musts or wines suitable for yielding one of these categories of wine;

(b) wines without a protected designation of origin or geographical indication, wines with a protected designation of origin (PDO) and wines with a protected geographical indication (PGI) as well as musts or wines suitable for yielding one of these categories of wine.

For the purposes of this paragraph, rosé wine shall be regarded as red wine.

3. The following processes shall not be regarded as coupage:

(a) enrichment by the addition of concentrated grape must or rectified concentrated grape must;

(b) sweetening.

Article 8

General rules on blending and coupage

1. A wine may be obtained by blending or coupage only where the constituents of that blending or coupage possess the required characteristics for obtaining wine and comply with Regulation (EC) No 479/2008 and this Regulation.

Coupage of a non-PDO/PGI white wine with a non-PDO/PGI red wine cannot produce a rosé wine.

However, the second subparagraph does not exclude coupage of the type referred to therein where the final product is intended for the preparation of a cuvée as defined in Annex I to Regulation (EC) No 479/2008 or intended for the production of semi-sparkling wines.

2. Coupage of a grape must or a wine which has undergone the oenological practice referred to in paragraph 14 of Annex I A to this Regulation with a grape must or a wine which has not undergone that practice shall be prohibited.

Article 9

The purity and identification specifications of substances used in oenological practices

1. Where they are not laid down by Commission Directive 2008/84/EC [1], the purity and identification specifications of substances used in the oenological practices referred to in point (e) of the second paragraph of Article 32 of Regulation (EC) No 479/2008 shall be those laid down and published in the International Oenological Codex of the International Organisation of Vine and Wine.

Where necessary, those purity criteria shall be supplemented by the specific requirements provided for in Annex I A hereto.

2. The enzymes and enzymatic preparations used in the authorised oenological practices and processes listed in Annex I A shall meet the requirements of Regulation (EC) No 1332/2008 of the European Parliament and of the Council of 16 December 2008 on food enzymes [2].

Article 10

Conditions governing the holding, circulation and use of products not complying with Chapter II of Title III of Regulation (EC) No 479/2008 or this Regulation

1. Products not complying with Chapter II of Title III of Regulation (EC) No 479/2008 or this Regulation shall be destroyed. However, Member States may authorise the use of certain products, the characteristics of which they shall determine, by distilleries or vinegar factories or for industrial purposes.

2. Such products may not be held without legitimate cause by producers or traders and they may be moved only to distilleries, vinegar factories, or establishments using them for industrial purposes or products or elimination plants.

3. Member States may have denaturing agents or indicators added to wines as referred to in paragraph 1 in order to make them more easily identifiable. Where justified, they may also prohibit the uses provided for in paragraph 1 and have the products disposed of.

4. Wine produced before 1 August 2009 may be offered or supplied for direct human consumption provided that it complies with the Community or national rules in force prior to that date.

Article 11

General rules applicable to the enrichment, acidification and deacidification of products other than wine

The processes referred to in paragraph 1 of point D of Annex V to Regulation (EC) No 479/2008 must be carried out in a single operation. However, Member States may permit some of these processes to be carried out in more than one operation where this improves the vinification of the products concerned. In such cases, the limits laid down in Annex V to Regulation (EC) No 479/2008 shall apply to the whole operation concerned.

[1] OJ L 253, 20.9.2008, p. 1.

[2] OJ L 354, 31.12.2008, p. 7.

Article 12

Administrative rules applicable to enrichment

1. Notifications as referred to in paragraph 4 of point D of Annex V to Regulation (EC) No 479/2008 relating to operations to increase alcoholic strength shall be made by the natural or legal persons carrying out the operations concerned and in compliance with suitable time limits and control conditions set by the competent authority of the Member State on whose territory the operation takes place.

2. Notifications as referred to in paragraph 1 shall be made in writing and shall include the following information:

(a) the name and address of the person making the notification;

(b) the place where the operation is to be carried out;

(c) the date and time when the operation is to commence;

(d) the description of the product undergoing the operation;

(e) the process used for the operation, with details of the type of product to be used.

3. Member States may allow prior notifications covering several operations or a specified period to be sent to the competent authorities. Such notifications shall be accepted only if the person making the notification keeps a written record of each enrichment operation as provided for in paragraph 6 and of the information required by paragraph 2.

4. Where the person concerned is prevented by reasons of force majeure from carrying out the notified operation in due time, Member States shall specify the conditions under which that person is to submit a new notification to the competent authority so that the necessary checks can be carried out.

5. The notification referred to in paragraph 1 shall not be required in Member States in which the competent inspection authorities carry out systematic analytical checks of all batches of products turned into wine.

6. The particulars relating to operations to increase alcoholic strength shall be entered in the registers referred to in Article 112(2) of Regulation (EC) No 479/2008 immediately after the operation is completed.

In cases where prior notifications covering several operations do not indicate the date and time when the operations are to commence, an entry must also be made in those registers before each operation commences.

Article 13

Administrative rules applicable to acidification and deacidification

1. In the case of acidification and deacidification, operators shall make notifications as referred to in paragraph 4 of point D of Annex V to Regulation (EC) No 479/2008 not later than the second day following the first operation carried out in any wine year. Such notifications shall be valid for all operations in that wine year.

2. Notifications as referred to in paragraph 1 shall be made in writing and shall include the following information:

(a) the name and address of the person making the notification;

(b) the type of operation involved;

(c) the place where the operation took place.

3. The particulars relating to each acidification and deacidification operation shall be entered in the registers referred to in Article 112(2) of Regulation (EC) No 479/2008.

Article 14

Pouring of wine or grape must to lees or grape marc or pressed 'aszú'/'výber' pulp

The pouring of wine or grape must to lees or grape marc or pressed 'aszú'/'výber' pulp, provided for in paragraph 2 of point D of Annex VI to Regulation (EC) No 479/2008, shall be carried out as follows, in accordance with the national provisions in force on 1 May 2004:

(a) 'Tokaji fordítás' or 'Tokajský forditáš' shall be prepared by pouring must or wine on pressed 'aszú'/'výber' pulp;

(b) 'Tokaji máslás' or 'Tokajský mášláš' shall be prepared by pouring must or wine on the lees of 'szamorodni'/'samorodné' or 'aszú'/'výber'.

The products concerned must be from the same harvest year.

Article 15

Applicable Community analysis methods

1. The analysis methods referred to in the second paragraph of Article 31 of Regulation (EC) No 479/2008 applicable for the verification of certain wine products and certain limits laid down at Community level are set out in Annex IV hereto.

2. The Commission shall publish in the C Series of the *Official Journal of the European Union* the list and description of the analysis methods referred to the first paragraph of Article 31 of Regulation (EC) No 479/2008 and described in the Compendium of International Methods of Analysis of Wines and Musts of the International Organisation of Vine and Wine and applicable for verification of the limits and requirements laid down by Community rules for the production of wine products.

Article 16

Repeal

Regulations (EEC) No 2676/90 and (EC) No 423/2008 are repealed.

References to the repealed Regulations and to Regulation (EC) No 1493/1999 shall be construed as references to this Regulation and shall be read in accordance with the correlation table in Annex V.

Article 17

This Regulation shall enter into force on the seventh day following its publication in the *Official Journal of the European Union*.

It shall apply from 1 August 2009.

This Regulation shall be binding in its entirety and directly applicable in all Member States.

Done at Brussels, 10 July 2009.

For the Commission
Mariann FISCHER BOEL
Member of the Commission

ANNEX I A

AUTHORISED OENOLOGICAL PRACTICES AND PROCESSES.

	1	2	3
	Oenological practice	Conditions of use [1]	Limits on use Applications
1	Aeration or oxygenation using gaseous oxygen		
2	Heat treatments		
3	Centrifuging and filtration with or without an inert filtering agent		Use of an agent must not leave undesirable residues in the treated product
4	Use of carbon dioxide, argon or nitrogen, either alone or combined, in order to create an inert atmosphere and to handle the product shielded from the air		
5	Use of yeasts for wine production, whether dry or in wine suspension	Only with fresh grapes, grape must, partially fermented grape must, partially fermented grape must obtained from raisined grapes, concentrated grape must and new wine still in fermentation and for the second alcoholic fermentation of all categories of sparkling wine.	
6	The use, to encourage yeast development, of one or more of the following substances, with the possible addition of microcrystalline cellulose as an excipient:		
	— addition of diammonium phosphate or ammonium sulphate	Only with fresh grapes, grape must, partially fermented grape must, partially fermented grape must obtained from raisined grapes, concentrated grape must and new wine still in fermentation and for the second alcoholic fermentation of all categories of sparkling wine.	No more than 1 g/l (expressed in salts) [2] or 0,3 g/l for the second fermentation of sparkling wines.
	— addition of ammonium bisulphite	Only with fresh grapes, grape must, partially fermented grape must, partially fermented grape must obtained from raisined grapes, concentrated grape must and new wine still in fermentation	No more than 0,2 g/l (expressed in salts) [2] and up to the limits set in point 7.
	— addition of thiamin hydrochloride	Only with fresh grapes, grape must, partially fermented grape must, partially fermented grape must obtained from raisined grapes, concentrated grape must and new wine still in fermentation and for the second alcoholic fermentation of all categories of sparkling wine.	No more than 0,6 mg/l (expressed in thiamin) for each treatment
7	Use of sulphur dioxide, potassium bisulphite or potassium metabisulphite, also called potassium disulphite or potassium pyrosulphite		Limits (i.e. maximum quantity in the product placed on the market) as laid down in Annex I B
8	Elimination of sulphur dioxide by physical processes	Only with fresh grapes, grape must, partially fermented grape must, partially fermented grape must obtained from raisined grapes, concentrated grape must, rectified concentrated grape must and new wine still in fermentation	

1		2	3
	Oenological practice	Conditions of use [1]	Limits on use Applications
9	Treatment with charcoal for oenological use	Only for musts and new wines still in fermentation, rectified concentrated grape must and white wines	No more than 100 g of dry product per hl
10	Clarification by means of one or more of the following substances for oenological use: — edible gelatine, — plant proteins from wheat or peas, — isinglass, — casein and potassium caseinates, — egg albumin, — bentonite, — silicon dioxide as a gel or colloidal solution, — kaolin, — tannin, — pectolytic enzymes, — enzymatic preparations of beta-glucanase	Conditions for using beta-glucanase laid down in Appendix 1	
11	Use of sorbic acid in potassium sorbate form		Maximum sorbic acid content in the product so treated and placed on the market: 200 mg/l
12	Use of tartaric L(+) acid, malic L acid, DL malic acid, or lactic acid for acidification purposes	Conditions and limits laid down in points C and D of Annex V to Regulation (EC) No 479/2008 and Articles 11 and 13 of this Regulation. Specifications for L(+) tartaric acid laid down in paragraph 2 of Appendix 2	
13	Use of one or more of the following substances for deacidification purposes: — neutral potassium tartrate, — potassium bicarbonate, — calcium carbonate, which may contain small quantities of the double calcium salt of L(+) tartaric and L(-) malic acids, — calcium tartrate, — L(+) tartaric acid — a homogeneous preparation of tartaric acid and calcium carbonate in equivalent proportions and finely pulverised	Conditions and limits laid down in points C and D of Annex V to Regulation (EC) No 479/2008 and Articles 11 and 13 of this Regulation. Specifications for L(+) tartaric acid laid down in Appendix 2	
14	Addition of Aleppo pine resin	Under the conditions set out in Appendix 3	

1		2	3
Oenological practice		Conditions of use (1)	Limits on use Applications
15	Use of preparations from yeast cell walls		No more than 40 g/hl
16	Use of polyvinylpolypyrrolidone		No more than 80 g/hl
17	Use of lactic bacteria		
18	Addition of lysozyme		No more than 500 mg/l (where added to both the must and the wine, the total overall quantity must not exceed 500 mg/l)
19	Addition of L ascorbic acid		Maximum content in wine thus treated and placed on the market: 250 mg/l (3)
20	Use of ion exchange resins	Only with grape must intended for the manufacture of rectified concentrated grape must under the conditions set out in Appendix 4	
21	Use in dry wines of fresh lees which are sound and undiluted and contain yeasts resulting from the recent vinification of dry wine	For the products defined in paragraphs 1, 3, 4, 5, 6, 7, 8, 9, 15 and 16 of Annex IV to Regulation (EC) No 479/2008	Quantities not exceeding 5 % of the volume of product treated
22	Bubbling using argon or nitrogen		
23	Addition of carbon dioxide	For partially fermented must for direct human consumption as such and the products defined in paragraphs 1, 7 and 9 of Annex IV to Regulation (EC) No 479/2008	In the case of still wines the maximum carbon dioxide content in the wine so treated and placed on the market is 3 g/l, while the excess pressure caused by the carbon dioxide must be less than 1 bar at a temperature of 20 °C
24	Addition of citric acid for wine stabilisation purposes	For partially fermented must for direct human consumption as such and the products defined in paragraphs 1, 3, 4, 5, 6, 7, 8, 9, 15 and 16 of Annex IV to Regulation (EC) No 479/2008	Maximum content in wine thus treated and placed on the market: 1g/l
25	Addition of tannins	For partially fermented must for direct human consumption as such and the products defined in paragraphs 1, 3, 4, 5, 6, 7, 8, 9, 15 and 16 of Annex IV to Council Regulation (EC) No 479/2008	
26	The treatment: — of white and rosé wines with potassium ferrocyanide, — of red wines with potassium ferrocyanide or with calcium phytate	For partially fermented must for direct human consumption as such and the products defined in paragraphs 1, 3, 4, 5, 6, 7, 8, 9, 15 and 16 of Annex IV to Regulation (EC) No 479/2008 under the conditions set out in Appendix 5	In the case of calcium phytate, no more than 8 g/hl
27	Addition of metatartaric acid	For partially fermented must for direct human consumption as such and the products defined in paragraphs 1, 3, 4, 5, 6, 7, 8, 9, 15 and 16 of Annex IV to Regulation (EC) No 479/2008	No more than 100 mg/l

1		2	3
Oenological practice		Conditions of use (1)	Limits on use Applications
28	Use of acacia	For partially fermented must for direct human consumption as such and the products defined in paragraphs 1, 3, 4, 5, 6, 7, 8, 9, 15 and 16 of Annex IV to Regulation (EC) No 479/2008	
29	Use of DL tartaric acid, also called racemic acid, or of its neutral salt of potassium, for precipitating excess calcium	For partially fermented must for direct human consumption as such and the products defined in paragraphs 1, 3, 4, 5, 6, 7, 8, 9, 15 and 16 of Annex IV to Regulation (EC) No 479/2008 and under the conditions laid down in Appendix 5	
30	To assist the precipitation of tartaric salts, use of: — potassium bitartrate or potassium hydrogen tartrate, — calcium tartrate	For partially fermented must for direct human consumption as such and the products defined in paragraphs 1, 3, 4, 5, 6, 7, 8, 9, 15 and 16 of Annex IV to Regulation (EC) No 479/2008	In the case of calcium tartrate, no more than 200 g/hl
31	Use of copper sulphate or cupric citrate to eliminate defects of taste or smell in the wine	For partially fermented must for direct human consumption as such and the products defined in paragraphs 1, 3, 4, 5, 6, 7, 8, 9, 15 and 16 of Annex IV to Regulation (EC) No 479/2008	No more than 1 g/hl, provided that the copper content of the product so treated does not exceed 1 mg/l
32	Addition of caramel within the meaning of Directive 94/36/EC of the European Parliament and of the Council of 30 June 1994 on colours for use in foodstuffs (4), to reinforce the colour	Only with liqueur wines	
33	Use of discs of pure paraffin impregnated with allyl isothiocyanate to create a sterile atmosphere	Only for partially fermented must for direct human consumption as such, and wine. Permitted solely in Italy as long as it is not prohibited under that country's legislation and only in containers holding more than 20 litres	No trace of allyl isothiocyanate must be present in the wine
34	Addition of dimethyldicarbonate (DMDC) to wine for microbiological stabilisation	For partially fermented must for direct human consumption as such and the products defined in paragraphs 1, 3, 4, 5, 6, 7, 8, 9, 15 and 16 of Annex IV to Regulation (EC) No 479/2008 and under the conditions laid down in Appendix 6	No more than 200 mg/l with no detectable residues in the wine placed on the market
35	Addition of yeast mannoproteins to ensure the tartaric and protein stabilisation of wines	For partially fermented must for direct human consumption as such and the products defined in paragraphs 1, 3, 4, 5, 6, 7, 8, 9, 15 and 16 of Annex IV to Regulation (EC) No 479/2008	

1		2	3
Oenological practice		Conditions of use (1)	Limits on use Applications
36	Electrodialysis treatment to ensure the tartaric stabilisation of the wine	For partially fermented must for direct human consumption as such and the products defined in paragraphs 1, 3, 4, 5, 6, 7, 8, 9, 15 and 16 of Annex IV to Regulation (EC) No 479/2008 and under the conditions laid down in Appendix 7	
37	Use of urease to reduce the level of urea in the wine	For partially fermented must for direct human consumption as such and the products defined in paragraphs 1, 3, 4, 5, 6, 7, 8, 9, 15 and 16 of Annex IV to Regulation (EC) No 479/2008 and under the conditions laid down in Appendix 8	
38	Use of oak chips in winemaking and ageing, including in the fermentation of fresh grapes and grape must	Under the conditions laid down in Appendix 9	
39	Use: — of calcium alginate, or, — of potassium alginate,	Only for the manufacture of all categories of sparkling and semi-sparkling wines obtained by fermentation in bottle and with the lees separated by disgorging	
40	Partial dealcoholisation of wine	Only with wine and under the conditions laid down in Appendix 10	
41	Use of polyvinylimidazole/polyvinylpyrrolidone (PVI/PVP) copolymers in order to reduce the copper, iron and heavy metal content	Under the conditions laid down in Appendix 11	No more than 500 mg/l (where added to both the must and the wine, the total overall quantity must not exceed 500 mg/l)
42	Addition of carboxymethylcellulose (cellulose gums) to ensure tartaric stabilisation	Only with wine and all categories of sparkling and semi-sparkling wine	No more than 100 mg/l
43	Treatment with cation exchangers to ensure the tartaric stabilisation of the wine	For partially fermented must for direct human consumption as such and the products defined in paragraphs 1, 3, 4, 5, 6, 7, 8, 9, 15 and 16 of Annex IV to Regulation (EC) No 479/2008 and under the conditions laid down in Appendix 12	

(1) Unless otherwise stated, the practice or process described may be used for fresh grapes, grape must, partially fermented grape must, partially fermented grape must from raisined grapes, concentrated grape must, new wine still in fermentation, partially fermented grape must for direct human consumption, wine, all categories of sparkling wine, semi-sparkling wine, aerated semi-sparkling wine, liqueur wines, wines made from raisined grapes and wines made from over-ripened grapes.

(2) These ammonium salts may also be used in combination, up to an overall limit of 1g/l, without prejudice to the specific limits of 0,3 g/l or 0,2 g/l set above.

(3) The use limit is 250 mg/l for each treatment.

(4) OJ L 237, 10.9.1994, p. 13.

Appendix 1

Requirements for beta-glucanase

1. International code for beta-glucanase: E.C. 3-2-1-58

2. Beta-glucan hydrolase (breaking down the glucan in *Botrytis cinerea*)

3. Origin: *Trichoderma harzianum*

4. Area of application: breaking down the beta-glucans present in wines, in particular those produced from botrytised grapes

5. Maximum dose: 3 g of the enzymatic preparation containing 25 % total organic solids (TOS) per hectolitre

6. Chemical and microbiological purity specifications:

Loss on drying	Less than 10 %
Heavy metals	Less than 30 ppm
Pb	Less than 10 ppm
As	Less than 3 ppm
Total coliforms	Absent
Escherichia coli	Absent in 25 g sample
Salmonella spp	Absent in 25 g sample
Aerobic count:	Less than 5×10^4 cells/g

Appendix 2

L(+) tartaric acid

1. Tartaric acid, the use of which for deacidification purposes is provided for in paragraph 13 of Annex I A, may be used only for products that:

   are from the Elbling and Riesling vine varieties; and

   are obtained from grapes harvested in the following wine-growing regions in the northern part of wine-growing zone A:

   — Ahr,

   — Rheingau,

   — Mittelrhein,

   — Mosel,

   — Nahe,

   — Rheinhessen,

   — Pfalz,

   — Moselle luxembourgeoise.

2. Tartaric acid, the use of which is provided for in paragraphs 12 and 13 of this Annex, also called L(+) tartaric acid, must be of agricultural origin and extracted specifically from wine products. It must also comply with the purity criteria laid down in Directive 2008/84/EC.

Appendix 3

Aleppo pine resin

1. Aleppo pine resin, the use of which is provided for in paragraph 14 of Annex I A, may be used only to produce 'retsina' wine. This oenological practice may be carried out only:

   (a) in the geographical territory of Greece;

   (b) using grape must from grape varieties, areas of production and wine-making areas as specified in the Greek provisions in force at 31 December 1980;

   (c) by adding 1 000 grams or less of resin per hectolitre of the product used, before fermentation or, where the actual alcoholic strength by volume does not exceed one third of the overall alcoholic strength by volume, during fermentation.

2. Greece shall notify the Commission in advance if it intends to amend the provisions referred to in paragraph 1(b). If the Commission does not respond within two months of such notification, Greece may implement the planned amendments.

Appendix 4

Ion exchange resins

The ion exchange resins which may be used accordance with paragraph 20 of Annex I A are styrene and divinylbenzene copolymers containing sulphonic acid or ammonium groups. They must comply with the requirements laid down in Regulation (EC) No 1935/2004 of the European Parliament and of the Council [1] and Community and national provisions adopted in implementation thereof. In addition, when tested by the analysis method laid down in paragraph 2, they must not lose more than 1 mg/l of organic matter into any of the solvents listed. They must be regenerated with substances permitted for use in the preparation of foodstuffs.

These resins may be used only under the supervision of an oenologist or technician and in installations approved by the authorities of the Member States on whose territory they are used. Such authorities shall lay down the duties and responsibility incumbent on approved oenologists and technicians.

Analysis method for determining the loss of organic matter from ion exchange resins:

1. SCOPE AND AREA OF APPLICATION

The method determines the loss of organic matter from ion exchange resins.

2. DEFINITION

The loss of organic matter from ion exchange resins. The loss of organic matter is determined by the method specified.

3. PRINCIPLE

Extracting solvents are passed through prepared resins and the weight of organic matter extracted is determined gravimetrically.

4. REAGENTS

All reagents shall be of analytical quality.

Extracting solvents.

4.1. Distilled water or deionised water of equivalent purity.

4.2. Ethanol, 15 % v/v. Prepare by mixing 15 parts of absolute ethanol with 85 parts of water (paragraph 4.1).

4.3. Acetic acid, 5 % m/m. Prepare by mixing 5 parts of glacial acetic acid with 95 parts of water (paragraph 4.1).

5. APPARATUS

5.1. Ion exchange chromatography columns.

5.2. Measuring cylinders, capacity 2 l.

5.3. Evaporating dishes capable of withstanding a muffle furnace at 850 °C.

5.4. Drying oven, thermostatically controlled at 105 ± 2 °C.

5.5. Muffle furnace, thermostatically controlled at 850 ± 25 °C.

5.6. Analytical balance, accurate to 0.1 mg.

5.7. Evaporator, hot plate or infra-red evaporator.

[1] OJ L 338, 13.11.2004, p. 4.

6. PROCEDURE

6.1. Add to each of three separate ion exchange chromatography columns (paragraph 5.1) 50 ml of the ion exchange resin to be tested, washed and treated in accordance with the manufacturer's directions for preparing resins for use with food.

6.2. For the anionic resins, pass the three extracting solvents (paragraphs 4.1, 4.2 and 4.3) separately through the prepared columns (paragraph 6.1) at a flow rate of 350 to 450 ml/h. Discard the first litre of eluate in each case and collect the next two litres in measuring cylinders (paragraph 5.2). For the cationic resins, pass only solvents referred to in paragraphs 4.1 and 4.2 through the columns prepared for this purpose.

6.3. Evaporate the three eluates over a hotplate or with an infrared evaporator (paragraph 5.7) in separate evaporating dishes (paragraph 5.3) which have been previously cleaned and weighed (m0). Place the dishes in an oven (paragraph 5.4) and dry to constant weight (m1).

6.4. After recording the constant weight (paragraph 6.3), place the evaporating dish in the muffle furnace (paragraph 5.5) and ash to constant weight (m2).

6.5. Calculate the organic matter extracted (paragraph 7.1). If the result is greater than 1 mg/l, carry out a blank test on the reagents and recalculate the weight of organic matter extracted.

The blank test shall be carried out by repeating the operations referred to in paragraphs 6.3 and 6.4 but using two litres of the extracting solvent, to give weights m3 and m4 in paragraphs 6.3 and 6.4 respectively.

7. EXPRESSION OF THE RESULTS

7.1. Formula and calculation of results

The organic matter extracted from ion exchange resins, in mg/l, is given by:

$$500\ (m1 - m2)$$

where m1 and m2 are expressed in grams.

The corrected weight (mg/l) of the organic matter extracted from ion exchange resins is given by:

$$500\ (m1 - m2 - m3 + m4)$$

where m1, m2, m3 and m4 are expressed in grams.

7.2. The difference in the results between two parallel determinations carried out on the same sample must not exceed 0,2 mg/l.

Appendix 5

Potassium ferrocyanide

Calcium phytate

DL tartaric acid

Potassium ferrocyanide or calcium phytate, the use of which is provided for in paragraph 26 of Annex I A, or DL tartaric acid, the use of which is provided for in paragraph 29 of Annex I A, may be used only under the supervision of an oenologist or technician officially approved by the authorities of the Member State in whose territory the process is carried out, the extent of whose responsibility shall be fixed, if necessary, by the Member State concerned.

After treatment with potassium ferrocyanide or calcium phytate, the wine must contain traces of iron.

Supervision of the use of the product referred to in the first paragraph shall be governed by the provisions adopted by the Member States.

Appendix 6

Requirements for dimethyldicarbonate

AREA OF APPLICATION

Dimethyldicarbonate may be added to wine for the following purpose: microbiological stabilisation of bottled wine containing fermentable sugar.

REQUIREMENTS

— the addition must be carried out only a short time prior to bottling, defined as putting the product concerned up for commercial purposes in containers of a capacity not exceeding 60 litres,

— the treatment may only be applied to wine with a sugar content of not less than 5 g/l,

— the product used must comply with the purity criteria laid down in Directive 2008/84/EC,

— this treatment is to be recorded in the register referred to in Article 112(2) of Regulation (EC) No 479/2008.

Appendix 7

Requirements for electrodialysis treatment

The purpose is to obtain tartaric stability of the wine with regard to potassium hydrogen tartrate and calcium tartrate (and other calcium salts) by extraction of ions in supersaturation in the wine under the action of an electrical field and using membranes that are either anion-permeable or cation-permeable.

1. MEMBRANE REQUIREMENTS

1.1. The membranes are to be arranged alternately in a 'filter-press' type system or any other appropriate system separating the treatment (wine) and concentration (waste water) compartments.

1.2. The cation-permeable membranes must be designed to extract cations only, in particular K^+, Ca^{++}.

1.3. The anion-permeable membranes must be designed to extract anions only, in particular tartrate anions.

1.4. The membranes must not excessively modify the physico-chemical composition and sensory characteristics of the wine. They must meet the following requirements:

— they must be manufactured according to good manufacturing practice from substances authorised for the manufacture of plastic materials intended to come into contact with foodstuffs as listed in Annex II to Commission Directive 2002/72/EC [1],

— the user of the electrodialysis equipment must show that the membranes used meet the above requirements and that any replacements have been carried out by specialised personnel,

— they must not release any substance in quantities endangering human health or affecting the taste or smell of foodstuffs and must meet the criteria laid down in Directive 2002/72/EC,

— their use must not trigger interactions between their constituents and the wine liable to result in the formation of new compounds that may be toxic in the treated product.

The stability of fresh electrodialysis membranes is to be determined using a simulant reproducing the physico-chemical composition of the wine for investigation of possible migration of certain substances from them.

The experimental method recommended is as follows:

The simulant is a water-alcohol solution buffered to the pH and conductivity of the wine. Its composition is as follows:

— absolute ethanol: 11 l,

— potassium hydrogen tartrate: 380 g,

— potassium chloride: 60 g,

— concentrated sulphuric acid: 5 ml,

— distilled water: to make up 100 litres,

This solution is used for closed circuit migration tests on an electrodialysis stack under tension (1 volt/cell), on the basis of 50 l/m2 of anionic and cationic membranes, until 50 % demineralisation of the solution. The effluent circuit is initiated by a 5 g/l potassium chloride solution. Migrating substances are tested for in both the simulant and the effluent.

[1] OJ L 220, 15.8.2002, p. 18.

Organic molecules entering into the membrane composition that are liable to migrate into the treated solution will be determined. A specific determination will be carried out for each of these constituents by an approved laboratory. The content in the simulant of all the determined compounds must be less than 50 g/l.

The general rules on controls of materials in contact with foodstuffs must be applied to these membranes.

2. MEMBRANE UTILISATION REQUIREMENTS

The membrane pair is formulated so that the following conditions are met:

— the pH reduction of the wine is to be no more than 0,3 pH units,

— the volatile acidity reduction is to be less than 0,12 g/l (2 meq expressed as acetic acid),

— treatment must not affect the non-ionic constituents of the wine, in particular polyphenols and polysaccharides,

— diffusion of small molecules such as ethanol is to be reduced and must not cause a reduction in alcoholic strength of more than 0,1 % vol.,

— the membranes must be conserved and cleaned by approved methods with substances authorised for use in the preparation of foodstuffs,

— the membranes are marked so that alternation in the stack can be checked,

— the equipment is to be run using a command and control mechanism that will take account of the particular instability of each wine so as to eliminate only the supersaturation of potassium hydrogen tartrate and calcium salts,

— the treatment is to be carried out under the responsibility of an oenologist or qualified technician.

The treatment is to be recorded in the register referred to in Article 112(2) of Regulation (EC) No 479/2008.

Appendix 8

Requirements for urease

1. International code for urease: EC 3-5-1-5, CAS No: 9002-13-5.

2. Activity: urease activity (active at acidic pH), to break down urea into ammonia and carbon dioxide. The stated activity is not less than 5 units/mg, one unit being defined as the amount that produces one µmol of ammonia per minute at 37 °C from 5 g/l urea at pH 4.

3. Origin: *Lactobacillus fermentum*.

4. Area of application: breaking down urea present in wine intended for prolonged ageing, where its initial urea concentration is higher than 1 mg/l.

5. Maximum dose: 75 mg of enzyme preparation per litre of wine treated, not exceeding 375 units of urease per litre of wine. After treatment, all residual enzyme activity must be eliminated by filtering the wine (pore size < 1 µm).

6. Chemical and microbiological purity specifications:

Loss on drying	Less than 10 %
Heavy metals	Less than 30 ppm
Pb	Less than 10 ppm
As	Less than 2 ppm
Total coliforms	Absent
Salmonella spp	Absent in 25 g sample
Aerobic count	Less than 5×10^4 cells/g

Urease used in the treatment of wine must be prepared under similar conditions to those for urease as covered by the opinion of the Scientific Committee for Food of 10 December 1998.

Appendix 9

Requirements for pieces of oak wood

PURPOSE, ORIGIN AND AREA OF APPLICATION

Pieces of oak wood are used in winemaking and ageing, including in the fermentation of fresh grapes and grape must, to pass on certain characteristics of oak wood to wine.

The pieces of oak wood must come exclusively from the *Quercus* genus.

They may be left in their natural state, or heated to a low, medium or high temperature, but they may not have undergone combustion, including surface combustion, nor be carbonaceous or friable to the touch. They may not have undergone any chemical, enzymatic or physical processes other than heating. No product may be added for the purpose of increasing their natural flavour or the amount of their extractible phenolic compounds.

LABELLING

The label must mention the origin of the botanical species of oak and the intensity of any heating, the storage conditions and safety precautions.

DIMENSIONS

The dimensions of the particles of wood must be such that at least 95 % in weight are retained by a 2 mm mesh filter (9 *mesh*).

PURITY

The pieces of oak wood may not release any substances in concentrations which may be harmful to health.

This treatment is to be recorded in the register referred to in Article 112(2) of Regulation (EC) No 479/2008.

Appendix 10

Requirements for the partial dealcoholisation of wine

The aim of this treatment is to produce a partially dealcoholised wine, by eliminating some of the alcohol (ethanol) in it using physical separation techniques.

Requirements

— The wines treated must have no organoleptic faults and must be suitable for direct human consumption,

— Elimination of alcohol from the wine cannot be carried out if one of the enrichment operations laid down in Annex V to Regulation (EC) No 479/2008 was applied to one of the wine products used in the preparation of the wine in question,

— Reduction of the actual alcoholic strength by volume may not be more than 2 % vol. and the actual alcoholic strength by volume of the final product must comply with that defined in point (a) of the second subparagraph of paragraph 1 of Annex IV to Regulation (EC) No 479/2008.

— The treatment is to be carried out under the responsibility of an oenologist or qualified technician,

— This treatment is to be recorded in the register referred to in Article 112(2) of Regulation (EC) No 479/2008,

— The Member States may require this treatment to be notified to the competent authorities.

Appendix 11

Requirements for treatment with PVI/PVP copolymers

The purpose of this treatment is to reduce excessively high concentrations of metals and to prevent defects caused by this excessively high content, such as ferric casse, through the addition of copolymers that adsorb these metals.

Requirements

— The added copolymers must be eliminated by filtering within two days at most of their addition to the wine, taking account of the precautionary principle.

— In the case of musts, the copolymers must be added no earlier than two days before filtering.

— The treatment is to be carried out under the responsibility of an oenologist or qualified technician.

— The adsorbant copolymers used must comply with the requirements of the International Oenological Codex published by the International Organisation of Vine and Wine, especially as regards the maximum monomer content ([1]).

(1) Treatment with PVI/PVP copolymers can only occur after the purity and identification specifications for authorised copolymers have been laid down and published in the OIV's International Oenological Codex.

Appendix 12

Requirements for treatment with cation exchangers to ensure the tartaric stabilisation of the wine

The purpose is to obtain tartaric stability of the wine with regard to potassium hydrogen tartrate and calcium tartrate (and other calcium salts).

Requirements

1. The treatment must be limited to the elimination of excess cations.

   — The wine must first of all be cooled.

   — Only the minimum fraction of wine necessary to obtain stability must be treated with cation exchangers.

2. The treatment is to be carried out on acid-regenerated cation-exchanger resins.

3. All the operations are to be carried out under the responsibility of an oenologist or qualified technician. The treatment must be recorded in the register referred to in Article 112(2) of Regulation (EC) No 479/2008.

4. The cationic resins used must comply with the requirements of Regulation (EC) No 1935/2004 of the European Parliament and of the Council [1], the Community and national provisions adopted thereunder and the analytical requirements laid down in Appendix 4 to this Regulation. Their use must not excessively modify the physico-chemical composition or the organoleptic characteristics of the wine and must comply with the limits set out in point 3 of the International Oenological Codex monograph 'Cation-exchange resins' published by the OIV.

[1] OJ L 338, 13.11.2004, p. 4.

ANNEX I B

THE MAXIMUM SULPHUR DIOXIDE CONTENT OF WINES

A. THE SULPHUR DIOXIDE CONTENT OF WINES

1. The total sulphur dioxide content of wines, other than sparkling wines and liqueur wines, on their release to the market for direct human consumption, may not exceed:

   (a) 150 milligrams per litre for red wines;

   (b) 200 milligrams per litre for white and rosé wines.

2. Notwithstanding paragraph 1(a) and (b), the maximum sulphur dioxide content shall be raised, as regards wines with a sugar content, expressed as the sum of glucose and fructose, of not less than five grams per litre, to:

   (a) 200 milligrams per litre for red wines;

   (b) 250 milligrams per litre for white and rosé wines;

   (c) 300 milligrams per litre for:

   — wines entitled to the description 'Spätlese' in accordance with Community provisions,

   — white wines entitled to one of the following protected designations of origin: Bordeaux supérieur, Graves de Vayres, Côtes de Bordeaux-Saint-Macaire, Premières Côtes de Bordeaux, Côtes de Bergerac, Haut Montravel, Côtes de Montravel, Gaillac, Rosette and Savennières;

   — white wines entitled to the protected designations of origin Allela, Navarra, Penedès, Tarragona and Valencia and wines entitled to a protected designation of origin from the Comunidad Autónoma del País Vasco and described as 'vendimia tardia',

   — the sweet wines entitled to the protected designation of origin 'Binissalem-Mallorca',

   — wines originating in the United Kingdom produced in accordance with UK legislation where the sugar content is more than 45 g/l,

   — wines from Hungary with the protected designation of origin 'Tokaji' and described in accordance with Hungarian provisions as 'Tokaji édes szamorodni' or 'Tokaji szàraz szamorodni',

   — wines entitled to one of the following protected designations of origin: Loazzolo, Alto Adige and Trentino described by the terms or one of the terms: 'passito' or 'vendemmia tardiva',

   — wines entitled to the protected designation of origin: 'Colli orientali del Friuli' accompanied by the term 'Picolit',

   — wines entitled to the protected designations of origin 'Moscato di Pantelleria naturale' and 'Moscato di Pantelleria',

   — wines from the Czech Republic entitled to the description 'pozdní sběr',

   — wines from Slovakia entitled to a protected designation of origin and described by the term 'neskorý zber' and Slovak 'Tokaj' wines entitled to the protected designation of origin 'Tokajské samorodné suché' or 'Tokajské samorodné sladké',

   — wines from Slovenia entitled to a protected designation of origin and described by the term 'vrhunsko vino ZGP — pozna trgatev',

   — white wines with the following protected geographical indications, with a total alcoholic strength by volume of more than 15 % vol. and a sugar content of more than 45 g/l:

   — Vin de pays de Franche-Comté,

   — Vin de pays des coteaux de l'Auxois,

   — Vin de pays de Saône-et-Loire,

— Vin de pays des coteaux de l'Ardèche,

— Vin de pays des collines rhodaniennes,

— Vin de pays du comté Tolosan,

— Vin de pays des côtes de Gascogne,

— Vin de pays du Gers,

— Vin de pays du Lot,

— Vin de pays des côtes du Tarn,

— Vin de pays de la Corrèze,

— Vin de pays de l'Ile de Beauté,

— Vin de pays d'Oc,

— Vin de pays des côtes de Thau,

— Vin de pays des coteaux de Murviel,

— Vin de pays du Val de Loire,

— Vin de pays de Méditerranée,

— Vin de pays des comtés rhodaniens,

— Vin de pays des côtes de Thongue,

— Vin de pays de la Côte Vermeille,

— sweet wines originating in Greece with an actual alcoholic strength by volume equal to or more than 15 % vol. and a sugar content equal to or more than 45 g/l and entitled to one of the following protected geographical indications:

— Τοπικός Οίνος Τυρνάβου (Regional wine of Tyrnavos),

— Αχαϊκός Τοπικός Οίνος (Regional wine of Ahaia),

— Λακωνικός Τοπικός Οίνος (Regional wine of Lakonia),

— Τοπικός Οίνος Φλώρινας (Regional wine of Florina),

— Τοπικός Οίνος Κυκλάδων (Regional wine of Cyclades),

— Τοπικός Οίνος Αργολίδας (Regional wine of Argolida),

— Τοπικός Οίνος Πιερίας (Regional wine of Pieria),

— Αγιορείτικος Τοπικός Οίνος (Regional wine of Mount Athos- Regional wine of Holy Mountain),

— sweet wines originating in Cyprus with an actual alcoholic strength by volume equal to or less than 15 % vol. and a sugar content equal to or more than 45 g/l and entitled to the protected designation of origin Κουμανδαρία (Commandaria),

— sweet wines originating in Cyprus produced from overripe grapes or from raisined grapes with a total alcoholic strength by volume equal to or more than 15 % vol. and a sugar content equal to or more than 45 g/l and entitled to one of the following protected geographical indications:

— Τοπικός Οίνος Λεμεσός (Regional wine of Lemesos),

— Τοπικός Οίνος Πάφος (Regional wine of Pafos),

— Τοπικός Οίνος Λάρνακα (Regional wine of Larnaka),

— Τοπικός Οίνος Λευκωσία (Regional wine of Lefkosia);

(d) 350 milligrams per litre for:

— wines entitled to the description 'Auslese' in accordance with Community provisions,

— Romanian white wines entitled to one of the following protected designations of origin: Murfatlar, Cotnari, Târnave, Pietroasa, Valea Călugărească,

— wines from the Czech Republic entitled to the description 'výběr z hroznů',

— wines from Slovakia entitled to a protected designation of origin and described by the term 'výber z hrozna' and Slovak 'Tokaj' wines entitled to the protected designation of origin 'Tokajský máslášˇ' or 'Tokajský forditášˇ',

— wines from Slovenia entitled to a protected designation of origin and described by the term 'vrhunsko vino ZGP — izbor';

(e) 400 milligrams per litre for:

— wines entitled to the descriptions 'Beerenauslese', 'Ausbruch', 'Ausbruchwein', 'Trockenbeerenauslese', 'Strohwein', 'Schilfwein' and 'Eiswein' in accordance with Community provisions,

— white wines entitled to one of the following protected designations of origin: Sauternes, Barsac, Cadillac, Cérons, Loupiac, Sainte-Croix-du-Mont, Monbazillac, Bonnezeaux, Quarts de Chaume, Coteaux du Layon, Coteaux de l'Aubance, Graves Supérieures, Sainte-Foy Bordeaux, Saussignac, Jurançon except where followed by the term 'sec', Anjou-Coteaux de la Loire, Coteaux du Layon followed by the name of the commune of origin, Chaume, Coteaux de Saumur, Pacherenc du Vic Bilh except where followed by the term 'sec', Alsace et Alsace grand cru followed by the term 'vendanges tardives' or 'sélection de grains nobles',

— sweet wines originating in Greece produced from overripe grapes and from raisined grapes with a residual sugar content, expressed as sugar, equal to or more than 45 g/l and entitled to one of the following protected designations of origin: Σάμος (Samos), Ρόδος (Rhodes), Πατρα (Patras), Ρίο Πατρών (Rio Patron), Κεφαλονία (Kefallonia), Λήμνος (Limnos), Σητεία (Sitia), Σαντορίνη (Santorini), Νεμέα (Nemea), Δαφνές (Daphnes) and sweet wines produced from overripe grapes and from raisined grapes entitled to one of the following protected geographical indications: Σιάτιστας (Siatista), Καστοριάς (Kastoria), Κυκλάδων (Cyclades), Μονεμβάσιος (Monemvasia), Αγιορείτικος (Mount Athos — Holy Mountain),

— wines from the Czech Republic entitled to the descriptions 'výběr z bobulí', 'výběr z cibéb', 'ledové víno' or 'slámové víno',

— wines from Slovakia entitled to a protected designation of origin and described by the terms 'bobuľový výber', 'hrozienkový výber', 'cibébový výber', 'ľadové víno' or 'slamové víno' and Slovak 'Tokaj' wines entitled to the protected designation of origin 'Tokajský výber', 'Tokajská esencia' or 'Tokajská výberová esencia',

— wines from Hungary entitled to a protected designation of origin and described in accordance with Hungarian provisions as 'Tokaji máslás', 'Tokaji fordítás', 'Tokaji aszúeszencia', 'Tokaji eszencia', 'Tokaji aszú' or 'Töppedt szőlőből készült bor',

— wines entitled to the protected designation of origin 'Albana di Romagna' and described by the term 'passito',

— Luxemburg wines entitled to a protected designation of origin and described by the terms 'vendanges tardives', 'vin de glace' or 'vin de paille',

— white wines entitled to the protected designation of origin 'Douro' followed by the term 'colheita tardia',

— wines from Slovenia entitled to a protected designation of origin and described by the terms 'vrhunsko vino ZGP — jagodni izbor', 'vrhunsko vino ZGP — ledeno vino' or 'vrhunsko vino ZGP — suhi jagodni izbor',

— white wines originating in Canada entitled to the description 'Icewine'.

3. The lists of wines bearing a protected designation of origin or a protected geographical indication given in subparagraphs (c), (d) and (e) of paragraph 2 may be amended where the production conditions of the wines concerned are amended or the designation of origin or geographical indication is changed. The Member States shall provide the Commission, in advance, with all the necessary technical information for the wines concerned, including their product specifications and the annual quantities produced.

4. Where climate conditions make this necessary, the Commission may decide in accordance with the procedure referred to in Article 113(2) of Regulation (EC) No 479/2008 that in certain wine-growing areas of the Community the Member States concerned may authorise an increase of a maximum of 50 milligrams per litre in the maximum total sulphur dioxide levels of less than 300 milligrams per litre referred to in this point for wines produced within their territory. The list of cases in which the Member States may permit such an increase is given in Appendix 1.

5. Member States may apply more restrictive provisions to wines produced within their territory.

B. THE SULPHUR DIOXIDE CONTENT OF LIQUEUR WINES

The total sulphur dioxide content of liqueur wines, on their release to the market for direct human consumption, may not exceed:

150 mg/l where the sugar content is less than 5 g/l;

200 mg/l where the sugar content is not less than 5 g/l.

C. THE SULPHUR DIOXIDE CONTENT OF SPARKLING WINES

1. The total sulphur dioxide content of sparkling wines, on their release to the market for direct human consumption, may not exceed:

   (a) 185 mg/l for all categories of quality sparkling wine; and

   (b) 235 mg/l for other sparkling wines.

2. Where climate conditions make this necessary in certain wine-growing areas of the Community, the Member States concerned may authorise an increase of up to 40 mg/l in the maximum total sulphur dioxide content for the sparkling wines referred to in paragraph 1(a) and (b) produced in their territory, provided that the wines covered by this authorisation are not sent outside the Member State in question.

Appendix 1

Increase in the maximum total sulphur dioxide content where the climate conditions make this necessary

(Annex I B to this Regulation)

	Year	Member State	Wine-growing areas(s)	Wines concerned
1.	2000	Germany	All wine-growing areas of Germany.	All wines obtained from grapes harvested in 2000.
2.	2006	Germany	The wine-growing areas in the regions of Baden-Württemberg, Bavaria, Hessen and Rhineland Palatinate.	All wines obtained from grapes harvested in 2006.
3.	2006	France	The wine-growing areas in the departments of Bas-Rhin and Haut-Rhin.	All wines obtained from grapes harvested in 2006.

ANNEX I C

THE MAXIMUM VOLATILE ACID CONTENT OF WINES

1. The volatile acid content may not exceed:

   (a) 18 milliequivalents per litre for partially fermented grape must;

   (b) 18 milliequivalents per litre for white and rosé wines; or

   (c) 20 milliequivalents per litre for red wines.

2. The levels referred to in paragraph 1 shall apply:

   (a) to products from grapes harvested within the Community, at the production stage and at all stages of marketing;

   (b) to partially fermented grape must and wines originating in third countries, at all stages following their entry into the geographical territory of the Community.

3. Derogations from paragraph 1 may be granted:

   (a) for certain wines with a protected designation of origin or a protected geographical indication:

      — where they have been aged for a period of at least two years, or

      — where they have been produced according to particular methods;

   (b) wines with a total alcoholic strength by volume of at least 13 % vol.

   The Member States must notify these derogations to the Commission, which must then inform the other Member States.

ANNEX I D

LIMITS AND CONDITIONS FOR THE SWEETENING OF WINES

1. The sweetening of wine may be authorised only if carried out using one or more of the following products:

   (a) grape must;

   (b) concentrated grape must;

   (c) rectified concentrated grape must.

   The total alcoholic strength by volume of the wine in question may not be increased by more than 4 % vol.

2. The sweetening of imported wines intended for direct human consumption and bearing a geographical indication is forbidden within the territory of the Community. The sweetening of other imported wines shall be subject to the same conditions as wines produced in the Community.

3. The sweetening of a wine with a protected designation of origin may be authorised by a Member State only if it is carried out:

   (a) in accordance with the conditions and limits laid down in this Annex;

   (b) within the region in which the wine was produced or within an area in immediate proximity.

   The grape must and concentrated grape must referred to in paragraph 1 must originate in the same region as the wine for the sweetening of which it is used.

4. The sweetening of wines shall be authorised only at the production and wholesale stages.

5. The sweetening of wines must be carried out in accordance with the following specific administrative rules:

   (a) Any natural or legal person intending to carry out a sweetening operation shall notify the competent authority of the Member State on whose territory the operation is to take place.

   (b) Notice shall be given in writing. It shall reach the competent authority at least forty-eight hours before the day on which the sweetening operation is to take place.

   (c) However, where an undertaking frequently or continuously carries out sweetening operations, Member States may allow a notification covering several operations or a specified period to be sent to the competent authorities. Such notification shall be accepted only on condition that the undertaking keeps a written record of each sweetening operation and records the information required by point (d).

   (d) Notifications shall include the following information:

      — the quantity and the total and actual alcoholic strengths of the wine to be sweetened,

      — the quantity and the total and actual alcoholic strengths of the grape must or the quantity and density of the concentrated grape must or rectified concentrated grape must to be added, as the case may be,

      — the total and actual alcoholic strengths of the wine after sweetening.

   The persons referred to in point (a) shall keep goods inwards and outwards registers showing the quantities of grape must, concentrated grape must or rectified concentrated grape must which they are holding for sweetening operations.

ANNEX II

AUTHORISED OENOLOGICAL PRACTICES AND RESTRICTIONS APPLICABLE TO SPARKLING WINES, QUALITY SPARKLING WINES AND QUALITY AROMATIC SPARKLING WINES

A. **Sparkling wine**

1. For the purposes of this point and points B and C of this Annex:

   (a) 'tirage liqueur' means;

   the product added to the cuvée to provoke secondary fermentation;

   (b) 'expedition liqueur' means;

   the product added to sparkling wines to give them special taste qualities.

2. The expedition liqueur may contain only:

   — sucrose,

   — grape must,

   — grape must in fermentation,

   — concentrated grape must,

   — rectified concentrated grape must;

   — wine, or

   — a mixture thereof,

   with the possible addition of wine distillate.

3. Without prejudice to enrichment authorised pursuant to Regulation (EC) No 479/2008 for the constituents of a cuvée, any enrichment of the cuvée shall be prohibited.

4. However, each Member State may, in respect of regions and varieties for which it is technically justified, authorise the enrichment of the cuvée at the place of preparation of the sparkling wines provided that:

   (a) none of the constituents of the cuvée has previously undergone enrichment;

   (b) the said constituents are derived solely from grapes harvested in its territory;

   (c) the enrichment is carried out in a single operation;

   (d) the following limits are not exceeded:

      (i) 3 % vol. for a cuvée comprising constituents from wine-growing zone A;

      (ii) 2 % vol. for a cuvée comprising constituents from wine-growing zone B;

      (iii) 1,5 % vol. for a cuvée comprising constituents from wine-growing zone C;

   (e) the method used is the addition of sucrose, concentrated grape must or rectified concentrated grape must.

5. The addition of tirage liqueur and expedition liqueur shall be considered neither as enrichment nor as sweetening. The addition of tirage liqueur may not cause an increase in the total alcoholic strength by volume of the cuvée of more than 1,5 % vol. This increase shall be measured by calculating the difference between the total alcoholic strength by volume of the cuvée and the total alcoholic strength by volume of the sparkling wine before any expedition liqueur is added.

6. The addition of expedition liqueur shall be carried out in such a way as not to increase the actual alcoholic strength by volume of the sparkling wine by more than 0,5 % vol.

7. Sweetening of the cuvée and its constituents shall be prohibited.

8. In addition to any acidification or deacidification of the constituents of the cuvée in accordance with Regulation (EC) No 479/2008, the cuvée may be subject to acidification or deacidification. Acidification and deacidification of the cuvée shall be mutually exclusive. Acidification may be carried out only up to a maximum of 1,5 grams per litre, expressed as tartaric acid, i.e. 20 milliequivalents per litre.

9. In years of exceptional climate conditions, the maximum limit of 1,5 grams per litre or 20 milliequivalents per litre may be raised to 2.5 grams per litre or 34 milliequivalents per litre, provided that the natural acidity of the products is not less than 3 g/l, expressed as tartaric acid, or 40 milliequivalents per litre.

10. The carbon dioxide contained in the sparkling wines may be produced only as a result of the alcoholic fermentation of the cuvée from which such wine is prepared.

    Such fermentation, unless it is intended for processing grapes, grape must or partially fermented grape must directly into sparkling wine, may result only from the addition of tirage liqueur. It may take place only in bottles or in closed tanks.

    The use of carbon dioxide in the case of the process of transfer by counter-pressure is authorised under supervision and on condition that the pressure of the carbon dioxide contained in the sparkling wine is not thereby increased.

11. In the case of sparkling wines other than sparkling wines with a protected designation of origin:

    (a) the tirage liqueur intended for their preparation may contain only:

        — grape must,

        — grape must in fermentation,

        — concentrated grape must,

        — rectified concentrated grape must, or

        — sucrose and wine;

    (b) the actual alcoholic strength by volume, including the alcohol contained in any expedition liqueur added, shall be not less than 9,5 % vol.

B. **Quality sparkling wine**

1. The tirage liqueur intended for the production of a quality sparkling wine may contain only:

    (a) sucrose,

    (b) concentrated grape must,

    (c) rectified concentrated grape must,

    (d) grape must or partially fermented grape must, or

    (e) wine.

2. Producer Member States may define any supplementary or more stringent characteristics or conditions of production and circulation for the quality sparkling wines covered by this Title and produced in their territory.

3. The manufacture of quality sparkling wines is also covered by the rules referred to in:

    — paragraphs 1 to 10 of point A,

    — paragraph 3 of point C for the actual alcoholic strength, paragraph 5 of point C for the minimum excess pressure and paragraphs 6 and 7 of point C for the minimum length of the production process, without prejudice to paragraph 4(d) of this point,

4. As regards quality aromatic sparkling wines:

   (a) except by way of derogation, these may be obtained only by making exclusive use, when constituting the cuvée, of grape must or partially fermented grape must derived from wine varieties contained on the list given in Appendix 1. However, quality aromatic sparkling wine may be produced in the traditional way by using as constituents of the cuvée wines obtained from grapes of the 'Prosecco' variety harvested in the regions of Trentino-Alto Adige, Veneto and Friuli-Venezia Giulia;

   (b) control of the fermentation process before and after the cuvée has been constituted, in order to render the cuvée sparkling, may be effected only by refrigeration or other physical processes;

   (c) the addition of expedition liqueur shall be prohibited;

   (d) the length of the production process for quality aromatic sparkling wines may not be less than one month.

C. **Sparkling wines and quality sparkling wines with a protected designation of origin**

1. The total alcoholic strength by volume of the cuvées intended for the preparation of quality sparkling wines with a protected designation of origin shall be not less than:

   — 9,5 % vol. in wine-growing zones C III,

   — 9 % vol. in other wine-growing zones.

2. However, the cuvées intended for the preparation of quality sparkling wines with the protected designations of origin 'Prosecco di Conegliano Valdobbiadene' and 'Montello e Colli Asolani' and prepared from a single vine variety may have a total alcoholic strength by volume of not less than 8,5 % vol.

3. The actual alcoholic strength by volume of quality sparkling wines with a protected designation of origin, including the alcohol contained in any expedition liqueur added, shall be not less than 10 % vol.

4. The tirage liqueur for sparkling wines and quality sparkling wines with a protected designation of origin may contain only:

   (a) sucrose,

   (b) concentrated grape must,

   (c) rectified concentrated grape must;

   and:

   (a) grape must,

   (b) partially fermented grape must,

   (c) wine;

   suitable for yielding the same sparkling wine or quality sparkling wine with a protected designation of origin as that to which the tirage liqueur is added.

5. Notwithstanding paragraph 5(c) of Annex IV to Regulation (EC) No 479/2008, when kept at a temperature of 20 °C in closed containers of a capacity of less than 25 cl., quality sparkling wines with a protected designation of origin must have an excess pressure of not less than 3 bar.

6. The duration of the process of making quality sparkling wines with a protected designation of origin, including ageing in the undertaking where they are made and reckoned from the start of the fermentation process designed to make the wines sparkling, may not be less than:

   (a) six months where the fermentation process designed to make the wines sparkling takes place in closed tanks;

   (b) nine months where the fermentation process designed to make the wines sparkling takes place in the bottles.

7. The duration of the fermentation process designed to make the cuvée sparkling and the duration of the presence of the cuvée on the lees shall not be less than:

   — 90 days,

   — 30 days if the fermentation takes place in containers with stirrers.

8. The rules laid down in paragraphs 1-10 of point A and paragraph 2 of point B shall also apply to sparkling wines and quality sparkling wines with a protected designation of origin.

9. As regards quality aromatic sparkling wines with a protected designation of origin:

   (a) these wines may be obtained solely by using, for constituting the cuvée, grape must or partially fermented grape must of vine varieties on the list given in Appendix 1, provided that these varieties are recognised as suitable for the production of quality sparkling wines with a protected designation of origin in the region whose name the quality sparkling wines with a protected designation of origin bear. By derogation, a quality aromatic sparkling wine with a protected designation of origin may be produced by using as constituents of the cuvée wines obtained from grapes of the 'Prosecco' vine variety harvested in the regions of the designations of origin 'Conegliano-Valdobbiadene' and 'Montello e Colli Asolani';

   (b) control of the fermentation process before and after the cuvée has been constituted, in order to render the cuvée sparkling, may be effected only by refrigeration or other physical processes;

   (c) the addition of expedition liqueur shall be prohibited;

   (d) the actual alcoholic strength by volume of quality aromatic sparkling wines with a protected designation of origin may not be less than 6 % vol.;

   (e) the total alcoholic strength by volume of quality aromatic sparkling wines with a protected designation of origin may not be less than 10 % vol.;

   (f) when kept at a temperature of 20 °C in closed containers, quality aromatic sparkling wines with a protected designation of origin must have an excess pressure of not less than 3 bar;

   (g) notwithstanding paragraph 6 of point C, the duration of the process of producing quality aromatic sparkling wines with a protected designation of origin must not be less than one month.

Appendix 1

List of vine varieties grapes of which may be used to constitute the cuvée for preparing quality aromatic sparkling wines and quality sparkling wines with a protected designation of origin

Airén

Aleatico N

Alvarinho

Ασύρτικο (Assyrtiko)

Bourboulenc B

Brachetto N.

Busuioacă de Bohotin

Clairette B

Colombard B

Csaba gyöngye B

Cserszegi fűszeres B

Devín

Fernão Pires

Freisa N

Gamay N

Gewürztraminer Rs

Girò N

Γλυκερύθρα (Glykerythra)

Huxelrebe

Irsai Olivér B

Macabeu B

All the Malvoisies

Mauzac blanc and rosé

Monica N

Μοσχοφίλερο (Moschofilero)

Müller-Thurgau B

All the Muscatels

Manzoni moscato

Nektár

Pálava B

Parellada B

Perle B

Piquepoul B

Poulsard

Prosecco

Ροδίτης (Roditis)

Scheurebe

Tămâioasă românească

Torbato

Touriga Nacional

Verdejo

Zefír B

ANNEX III

AUTHORISED OENOLOGICAL PRACTICES AND RESTRICTIONS APPLICABLE TO LIQUEUR WINES AND LIQUEUR WINES WITH A PROTECTED DESIGNATION OF ORIGIN OR PROTECTED GEOGRAPHICAL INDICATION

A. **Liqueur wines**

1. The products referred to in paragraph 3(c) of Annex IV to Regulation (EC) No 479/2008 and used for preparing liqueur wines and liqueur wines with a protected designation of origin or a protected geographical indication may have undergone, where appropriate, only the oenological practices and processes referred to in Regulation (EC) No 479/2008 or this Regulation.

2. However,

   (a) the increase in natural alcoholic strength by volume may be due only to the use of the products referred to in paragraph 3(e) and (f) of Annex IV to Regulation (EC) No 479/2008; and

   (b) by derogation, Spain is authorised to permit the use of calcium sulphate for Spanish wines described by the traditional terms 'vino generoso' or 'vino generoso de licor' where this practice is traditional and provided that the sulphate content of the product so treated is not more than 2,5 g/l, expressed as potassium sulphate. These products may undergo additional acidification up to a maximum limit of 1,5 g/l.

3. Without prejudice to any provisions of a more restrictive nature which the Member States may adopt for liqueur wines and liqueur wines with a protected designation of origin or a protected geographical indication prepared within their territory, the oenological practices referred to in Regulation (EC) No 479/2008 and in this Regulation shall be authorised for those products.

4. The following are also authorised:

   (a) sweetening, subject to a declaration and registration requirement, where the products used have not been enriched with concentrated grape must, by means of:

      — concentrated grape must or rectified concentrated grape must, provided that the increase in the total alcoholic strength by volume of the wine in question is not more than 3 % vol.,

      — concentrated grape must, rectified concentrated grape must or partially fermented grape must obtained from raisined grapes for Spanish wines described by the traditional term 'vino generoso de licor' and provided that the increase in the total alcoholic strength by volume of the wine in question is not more than 8 % vol.,

      — concentrated grape must or rectified concentrated grape must for liqueur wines with the protected designation of origin 'Madeira' and provided that the increase in the total alcoholic strength by volume of the wine in question is not more than 8 % vol.;

   (b) the addition of alcohol, distillate or spirits, as referred to in paragraphs 3(e) and (f) of Annex IV to Regulation (EC) No 479/2008, in order to compensate for losses due to evaporation during ageing;

   (c) ageing in vessels at a temperature not exceeding 50 °C, for liqueur wines with the protected designation of origin 'Madeira'.

5. The vine varieties from which the products referred to in paragraph 3(c) of Annex IV to Regulation (EC) No 479/2008 used for the preparation of liqueur wines and liqueur wines with a protected designation of origin or a protected geographical indication are produced shall be selected from those referred to in Article 24(1) of Regulation (EC) No 479/2008.

6. The natural alcoholic strength by volume of the products referred to in paragraph 3(c) of Annex IV to Regulation (EC) No 479/2008 used for the preparation of a liqueur wine other than a liqueur wine with a protected designation of origin or a protected geographical indication may not be less 12 % vol.

B. **Liqueur wines with a protected designation of origin (provisions other than those laid down in point A of this Annex and concerning specifically liqueur wines with a protected designation of origin)**

1. The list of liqueur wines with a protected designation of origin whose production involves the use of grape must or the mixture of grape must with wine, referred to in the fourth indent of paragraph 3(c) of Annex IV to Regulation (EC) No 479/2008, is given in Appendix 1 A to this Annex.

2. The list of liqueur wines with a protected designation of origin to which the products referred to in paragraph 3(f) of Annex IV to Regulation (EC) No 479/2008 may be added is given in Appendix 1 B to this Annex.

3. The products referred to in paragraph 3(c) of Annex IV to Regulation (EC) No 479/2008 and concentrated grape must and partially fermented grape must obtained from raisined grapes referred to in paragraph 3(f)(iii) of that Annex IV used for the preparation of liqueur wine with a protected designation of origin must come from the region whose name the liqueur wine with a protected designation of origin in question bears.

However, for liqueur wines with the protected designations of origin 'Málaga' and 'Jerez-Xérès-Sherry', the grape must, concentrated grape must or, pursuant to paragraph 4 of point B of Annex VI to Regulation (EC) No 479/2008, the partially fermented grape must obtained from raisined grapes referred to in paragraph 3(f)(iii) of Annex IV to Regulation (EC) No 479/2008 obtained from the 'Pedro Ximénez' vine variety may come from the Montilla-Moriles region.

4. The operations referred to in paragraphs 1 to 4 of point A of this Annex for the preparation of a liqueur wine with a protected designation of origin may be performed only within the region referred to in paragraph 3.

However, as regards the liqueur wine with a protected designation of origin for which the designation 'Porto' is reserved for the product prepared from grapes obtained from the region delimited as the 'Douro', the additional manufacturing and ageing processes may take place either in the aforementioned region or in Vila Nova de Gaia — Porto.

5. Without prejudice to any provisions of a more restrictive nature which the Member States may adopt for liqueur wines with a protected designation of origin prepared within their territory:

(a) the natural alcoholic strength by volume of the products referred to in paragraph 3(c) of Annex IV to Regulation (EC) No 479/2008 used for the preparation of a liqueur wine with a protected designation of origin may not be less than 12 % vol. However, some liqueur wines with a protected designation of origin on one of the lists given in Appendix 2 A to this Annex may be obtained from:

(i) grape must with a natural alcoholic strength by volume of not less than 10 % vol. in the case of liqueur wines with a protected designation of origin obtained by the addition of spirit obtained from wine or grape marc with a designation of origin, possibly from the same holding; or

(ii) partially fermented grape must or, in the case of the second indent below, from wine with an initial natural alcoholic strength by volume of not less than:

— 11 % vol. in the case of liqueur wines with a protected designation of origin obtained by the addition of neutral alcohol, or of a distillate of wine with an actual alcoholic strength by volume of not less than 70 % vol., or of spirit of vinous origin,

— 10,5 % vol. for wines prepared from white grape must referred to in list 3 given in Appendix 2 A,

— 9 % vol. in the case of a Portuguese liqueur wine with the protected designation of origin 'Madeira', the production of which is traditional and customary in accordance with the national legislation, which makes express provision for such a wine;

(b) the list of liqueur wines with a protected designation of origin with, notwithstanding paragraph 3(b) of Annex IV to Regulation (EC) No 479/2008, a total alcoholic strength by volume of less than 17,5 % vol. but not less than 15 % vol., where national legislation applicable thereto before 1 January 1985 expressly so provides, is given in Appendix 2 B.

6. The specific, traditional terms 'οίνος γλυκύς φυσικός', 'vino dulce natural', 'vino dolce naturale' and 'vinho doce natural' shall be used only for liqueur wines with a protected designation of origin:

— obtained from harvests at least 85 % of which are of the vine varieties listed in Appendix 3,

— derived from musts with an initial natural sugar content of at least 212 grams per litre,

— obtained by adding alcohol, distillate or spirits, as referred to in paragraph 3(e) and (f) of Annex IV to Regulation (EC) No 479/2008 to the exclusion of any other enrichment.

7. Insofar as is necessary to conform to traditional production practices, Member States may, for liqueur wines with a protected designation of origin produced within their territory, stipulate that the specific traditional name 'vin doux naturel' is used only for liqueur wines with a protected designation of origin which are:

   — made directly by producers harvesting the grapes and exclusively from their harvests of Muscatel, Grenache, Maccabeo or Malvoisie grapes; however, harvests may be included which have been obtained from vineyards that are also planted with vine varieties other than the four indicated above provided these do not constitute more than 10 % of the total stock,

   — obtained within the limit of a yield per hectare of 40 hl of grape must referred to in the first and fourth indents of paragraph 3(c) of Annex IV to Regulation (EC) No 479/2008, any greater yield resulting in the entire harvest ceasing to be eligible for the description 'vin doux naturel',

   — derived from a grape must as referred to above with an initial natural sugar content of at least 252 grams per litre,

   — obtained, to the exclusion of any other enrichment, by the addition of alcohol of vinous origin amounting in pure alcohol to a minimum of 5 % of the volume of the grape must as referred to above used and a maximum represented by the lower of the following two proportions:

      — either 10 % of the volume of the abovementioned grape must used, or,

      — 40 % of the total alcoholic strength by volume of the finished product represented by the sum of the actual alcoholic strength by volume and the equivalent of the potential alcoholic strength by volume calculated on the basis of 1 % vol. of pure alcohol for 17,5 grams of residual sugar per litre.

8. The specific traditional name 'vino generoso' shall be used only for dry liqueur wines with a protected designation of origin developed totally or partly under flor and:

   — obtained only from white grapes obtained from the Palomino de Jerez, Palomino fino, Pedro Ximénez, Verdejo, Zalema and Garrido Fino vine varieties,

   — released to the market after it has been matured for an average of two years in oak barrels.

   Development under flor as referred to in the first subparagraph means the biological process which, occurring when a film of typical yeasts develops spontaneously at the free surface of the wine after total alcoholic fermentation of the must, gives the product specific analytic and organoleptic characteristics.

9. The specific traditional name 'vinho generoso' shall be used only for liqueur wines with the protected designations of origin 'Porto', 'Madeira', 'Moscatel de Setubal' and 'Carcavelos' in association with the respective designation of origin.

10. The specific traditional name 'vino generoso de licor' shall be used only for liqueur wines with a protected designation of origin:

   — obtained from 'vino generoso', as referred to in paragraph 8, or from wine under flor capable of producing such a 'vino generoso', to which either partially fermented grape must obtained from raisined grapes or concentrated grape must has been added,

   — released to the market after it has been matured for an average of two years in oak barrels,

Appendix 1

The list of liqueur wines with a protected designation of origin whose production involves special rules

A. LIST OF LIQUEUR WINES WITH A PROTECTED DESIGNATION OF ORIGIN WHOSE PRODUCTION INVOLVES THE USE OF GRAPE MUST OR A MIXTURE THEREOF WITH WINE

(Paragraph B 1 of this Annex)

GREECE

Σάμος (Samos), Μοσχάτος Πατρών (Patras Muscatel), Μοσχάτος Ρίου Πατρών (Rio Patron Muscatel), Μοσχάτος Κεφαλληνίας (Kefallonia Muscatel), Μοσχάτος Ρόδου (Rhodes Muscatel), Μοσχάτος Λήμνου (Lemnos Muscatel), Σητεία (Sitia), Νεμέα (Nemea), Σαντορίνη (Santorini), Δαφνές (Dafnes), Μαυροδάφνη Κεφαλληνίας (Mavrodafne of Kefallonia), Μαυροδάφνη Πατρών (Mavrodafne of Patras)

SPAIN

Liqueur wines with a protected designation of origin	Description of product as established by Community rules or national legislation
Alicante	Moscatel de Alicante
	Vino dulce
Cariñena	Vino dulce
Jerez-Xérès-Sherry	Pedro Ximénez
	Moscatel
Malaga	Vino dulce
Montilla-Moriles	Pedro Ximénez
	Moscatel
Priorato	Vino dulce
Tarragona	Vino dulce
Valencia	Moscatel de Valencia
	Vino dulce

ITALY

Cannonau di Sardegna, Giró di Cagliari, Malvasia di Bosa, Malvasia di Cagliari, Marsala, Monica di Cagliari, Moscato di Cagliari, Moscato di Sorso-Sennori, Moscato di Trani, Masco di Cagliari, Oltrepó Pavese Moscato, San Martino della Battaglia, Trentino, Vesuvio Lacrima Christi.

B. LIST OF LIQUEUR WINES WITH A PROTECTED DESIGNATION OF ORIGIN WHOSE PRODUCTION INVOLVES THE ADDITION OF THE PRODUCTS REFERRED TO IN PARAGRAPH 3(f) OF ANNEX IV TO REGULATION (EC) No 479/2008

(Paragraph 2 of point B of this Annex)

1. **List of liqueur wines with a protected designation of origin whose production involves the addition of wine alcohol or dried-grape alcohol with an actual alcoholic strength of not less than 95 % vol. and not more than 96 % vol.**

(First indent of paragraph 3(f)(ii) of Annex IV to Regulation (EC) No 479/2008)

GREECE

Σάμος (Samos), Μοσχάτος Πατρών (Patras Muscatel), Μοσχάτος Ρίου Πατρών (Rio Patron Muscatel), Μοσχάτος Κεφαλληνίας (Kefallonia Muscatel), Μοσχάτος Ρόδου (Rhodes Muscatel), Μοσχάτος Λήμνου (Lemnos Muscatel), Σητεία (Sitia), Σαντορίνη (Santorini), Δαφνές (Dafnes), Μαυροδάφνη Πατρών (Mavrodafne of Patras), Μαυροδάφνη Κεφαλληνίας (Mavrodafne of Kefallonia).

SPAIN

Condado de Huelva, Jerez-Xérès-Sherry, Manzanilla-Sanlúcar de Barrameda, Málaga, Montilla-Moriles, Rueda, Terra Alta.

CYPRUS

Κουμανδαρία (Commandaria).

2. **List of liqueur wines with a protected designation of origin whose production involves the addition of spirits distilled from wine or grape marc with an actual alcoholic strength of not less than 52 % vol. and not more than 86 % vol.**

(Second indent of paragraph 3(f)(ii) of Annex IV to Regulation (EC) No 479/2008)

GREECE

Μαυροδάφνη Πατρών (Mavrodafne of Patras), Μαυροδάφνη Κεφαλληνίας (Mavrodafne of Kefallonia), Σητεία (Sitia), Σαντορίνη (Santorini), Δαφνές (Dafnes), Νεμέα (Nemea).

FRANCE

Pineau des Charentes or Pineau charentais, Floc de Gascogne, Macvin du Jura.

CYPRUS

Κουμανδαρία (Commandaria).

3. **List of liqueur wines with a protected designation of origin whose production involves the addition of spirits distilled from dried grapes with an alcoholic strength of not less than 52 % vol. but less than 94,5 % vol.**

(Third indent of paragraph 3(f)(ii) of Annex IV to Regulation (EC) No 479/2008)

GREECE

Μαυροδάφνη Πατρών (Mavrodafne of Patras), Μαυροδάφνη Κεφαλληνίας (Mavrodafne of Kefallonia).

4. **List of liqueur wines with a protected designation of origin whose production involves the addition of partially fermented grape must obtained from raisined grapes**

(First indent of paragraph 3(f)(iii) of Annex IV to Regulation (EC) No 479/2008)

SPAIN

Liqueur wines with a protected designation of origin	Description of product as established by Community rules or national legislation
Jerez-Xérès-Sherry	Vino generoso de licor
Málaga	Vino dulce
Montilla-Moriles	Vino generoso de licor

ITALY

Aleatico di Gradoli, Giró di Cagliari, Malvasia delle Lipari, Malvasia di Cagliari, Moscato passito di Pantelleria

CYPRUS

Κουμανδαρία (Commandaria).

5. **List of liqueur wines with a protected designation of origin whose production involves the addition of concentrated grape must obtained by the action of direct heat, complying, with the exception of this operation, with the definition of concentrated grape must.**

(Second indent of paragraph 3(f)(iii) of Annex IV to Regulation (EC) No 479/2008)

SPAIN

Liqueur wines with a protected designation of origin	Description of product as established by Community rules or national legislation
Alicante	
Condado de Huelva	Vino generoso de licor
Jerez-Xérès-Sherry	Vino generoso de licor
Málaga	Vino dulce
Montilla-Moriles	Vino generoso de licor
Navarra	Moscatel

ITALY

Marsala

6. **List of liqueur wines with a protected designation of origin whose production involves the addition of concentrated grape must**

(Third indent of paragraph 3(f)(iii) of Annex IV to Regulation (EC) No 479/2008)

SPAIN

Liqueur wines with a protected designation of origin	Description of product as established by Community rules or national legislation
Málaga	Vino dulce
Montilla-Moriles	Vino dulce
Tarragona	Vino dulce

ITALY

Oltrepó Pavese Moscato, Marsala, Moscato di Trani.

Appendix 2

A. **Lists referred to in paragraph 5(a) of Annex III B**

1. **List of liqueur wines with a protected designation of origin produced from grape must with a natural alcoholic strength by volume of not less than 10 % vol. obtained by the addition of spirit obtained from wine or grape marc with a registered designation of origin, possibly from the same holding.**

FRANCE

Pineau des Charentes or Pineau charentais, Floc de Gascogne, Macvin du Jura.

2. **List of liqueur wines with a protected designation of origin produced from fermenting grape must with an initial natural alcoholic strength by volume of not less than 11 % vol. obtained by the addition of neutral alcohol or of a distillate of wine with a an actual alcoholic strength by volume of not less than 70 % vol., or of spirit of vinous origin.**

PORTUGAL

Porto — Port

Moscatel de Setúbal, Setúbal

Carcavelos

Moscatel do Douro.

ITALY

Moscato di Noto

Trentino

3. **List of liqueur wines with a protected designation of origin produced from wine with an initial natural alcoholic strength by volume of not less than 10,5 % vol.**

SPAIN

Jerez-Xérès-Sherry

Manzanilla-Sanlúcar de Barrameda

Condado de Huelva

Rueda

4. **List of liqueur wines with a protected designation of origin obtained from fermenting grape must with an initial natural alcoholic strength by volume of not less than 9 % vol.**

PORTUGAL

Madeira.

B. **List referred to in paragraph 5(b) of Annex III B**

List of liqueur wines with a protected designation of origin with a total alcoholic strength by volume of less than 17,5 % vol. but not less than 15 % vol., where national laws applicable thereto before 1 January 1985 expressly so provided

(Paragraph 3(b) of Annex IV to Regulation (EC) No 479/2008)

SPAIN

Liqueur wines with a protected designation of origin	Description of product as established by Community rules or national legislation
Condado de Huelva	Vino generoso
Jerez-Xérès-Sherry	Vino generoso
Manzanilla-Sanlúcar de Barrameda	Vino generoso
Málaga	Seco
Montilla-Moriles	Vino generoso
Priorato	Rancio seco
Rueda	Vino generoso
Tarragona	Rancio seco

ITALY

Trentino

PORTUGAL

Liqueur wines with a protected designation of origin	Description of product as established by Community rules or national legislation
Porto — Port	Branco leve seco

Appendix 3

List of varieties that may be used to produce liqueur wines with a protected designation of origin that bear the specific, traditional terms 'vino dulce natural', 'vino dolce naturale', 'vinho doce natural' and 'οινος γλυκυς φυσικος'

Muscats — Grenache — Garnacha Blanca — Garnacha Peluda — Listán Blanco — Listán Negro-Negramoll — Maccabéo — Malvoisies — Mavrodaphne — Assirtiko — Liatiko — Garnacha tintorera — Monastrell — Palomino — Pedro Ximénez — Albarola — Aleatico — Bosco — Cannonau — Corinto nero — Giró — Monica — Nasco — Primitivo — Vermentino — Zibibbo.

ANNEX IV

SPECIAL COMMUNITY ANALYSIS METHODS

A. ALLYL ISOTHIOCYANATE

1. **Principle of the method**

Any allyl isothiocyanate present in the wine is collected by distillation and identified by gas chromatography.

2. **Reagents**

2.1. Ethanol, absolute.

2.2. *Standard* solution: solution of allyl isothiocyanate in absolute alcohol containing 15 mg of allyl isothiocyanate per litre.

2.3. Freezing mixture consisting of ethanol and dry ice (temperature – 60 °C).

3. **Apparatus**

3.1. Distillation apparatus as shown in the figure. A stream of nitrogen is passed continuously through the apparatus.

3.2. Heating mantle, thermostatically controlled.

3.3. Flowmeter.

3.4. Gas chromatograph fitted with a flame spectrophotometer detector equipped with a selective filter for sulphur compounds (wavelength = 394 nm) or any other suitable detector.

3.5. Stainless steel chromatograph column of internal diameter 3 mm and length 3 m filled with Carbowax 20M at 10 % on Chromosorb WHP, 80 to 100 mesh.

3.6. Microsyringe, 10µl.

4. **Procedure**

Put two litres of wine into the distillation flask, introduce a few millilitres of ethanol (paragraph 2.1) into the two collecting tubes so that the porous parts of the gas dispersion rods are completely immersed. Cool the two tubes externally with the freezing mixture. Connect the flask to the collecting tubes and begin to flush the apparatus with nitrogen at a rate of three litres per hour. Heat the wine to 80 °C with the heating mantle, distil and collect 45 to 50 ml of the distillate.

Stabilize the chromatograph. It is recommended that the following conditions are used:

— injector temperature: 200 °C,

— column temperature: 130 °C,

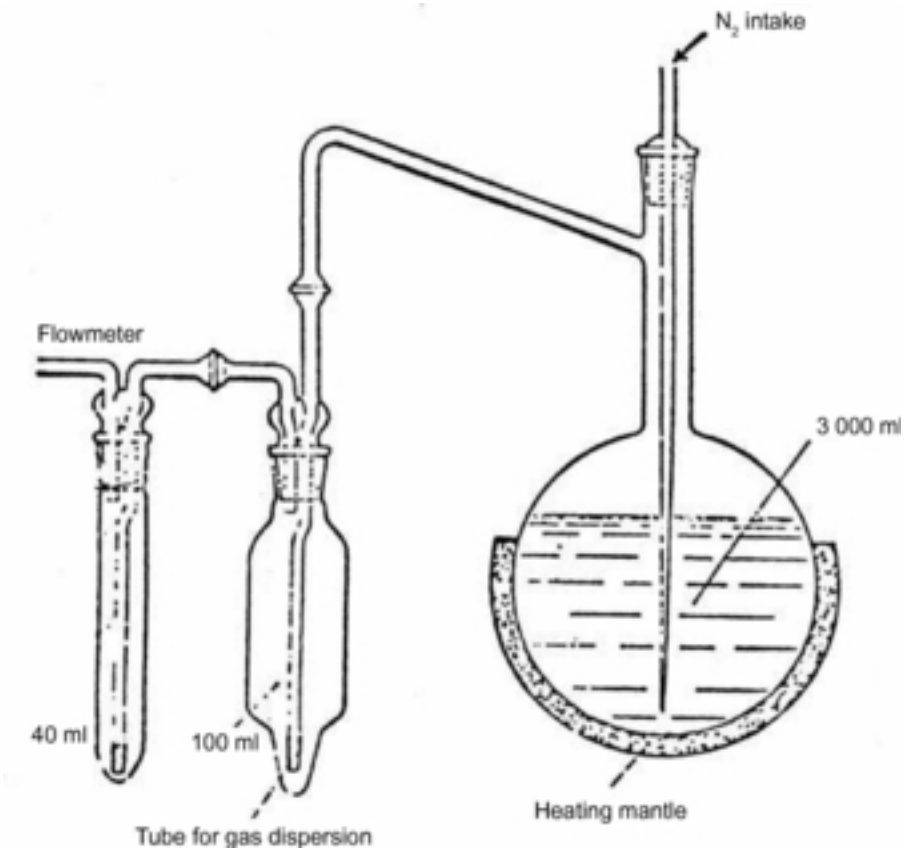
— helium carrier gas flow rate: 20 ml per minute.

With the microsyringe, introduce a volume of the *standard* solution such that the peak corresponding to the allyl isothiocyanate can easily be identified on the gas chromatogram.

Similarly introduce an aliquot of the distillate into the chromatograph. Check that the retention time of the peak obtained corresponds with that of the peak of allyl isothiocyanate.

Under the conditions described above, compounds naturally present in the wine will not produce interfering peaks on the chromatogram of the sample solution.

Apparatus for distillation under a current of nitrogen



B. SPECIAL ANALYSIS METHODS FOR RECTIFIED CONCENTRATED GRAPE MUST

(a) ***Total cations***

1. **Principle**

The test sample is treated by a strongly acid cation exchanger. The cations are exchanged with H^+. Total cations are expressed by the difference between the total acidity of the effluent and that of the test sample.

2. **Apparatus**

2.1. Glass column of internal diameter 10 to 11 mm and length approximately 300 mm, fitted with a drain tap.

2.2. pH meter with a scale graduated at least in 0,1 pH units.

2.3. Electrodes:

— glass electrode, kept in distilled water,

— calomel/saturated potassium chloride reference electrode, kept in a saturated solution of potassium chloride, or

— a combined electrode, kept in distilled water,

3. **Reagents**

3.1. Strongly acid cation exchange resin in H + form pre-swollen by soaking in water overnight.

3.2. Sodium hydroxide solution, 0,1 M.

3.3. Paper pH indicator.

4. **Procedure**

4.1. *Preparation of sample*

Use the solution obtained by diluting the rectified concentrated must to 40 % (m/v): introduce 200 g of accurately weighed rectified concentrated must into a 500 ml volumetric flask. Make up to the mark with water and homogenise.

4.2. *Preparation of the ion exchange column*

Introduce into the column approximately 10 ml pre-swollen ion exchanger in H + form. Rinse the column with distilled water until all acidity has been removed, using the paper indicator to monitor this.

4.3. *Ion exchange*

Pass 100 ml of the rectified concentrated must solution prepared as in paragraph 4.1 through the column at the rate of one drop every second. Collect the effluent in a beaker. Rinse the column with 50 ml of distilled water. Titrate the acidity in the effluent (including the rinse water) with the 0,1 M sodium hydroxide solution until the pH is 7 at 20 °C. The alkaline solution should be added slowly and the solution continuously shaken. Let n ml be the volume of 0,1 M sodium hydroxide solution used.

5. **Expression of the results**

The total cations are expressed in milliequivalents per kilogram of total sugar to one decimal place.

5.1. *Calculations*

— Acidity of the effluent expressed in milliequivalents per kilogram of rectified concentrated must:

Where E = The free sulphur dioxide in milligrams per litre is 2,5 n.

— Total acidity of the rectified concentrated must in milliequivalents per kilogram: a.

— Total cations in milliequivalents per kilogram of total sugars:

((2,5n-a)/(P)) × 100

P = percentage concentration (m/m) of total sugars.

(b) ***Conductivity***

1. **Principle**

The electrical conductivity of a column of liquid defined by two parallel platinum electrodes at its ends is measured by incorporating it in one arm of a Wheatstone bridge.

The conductivity varies with temperature and it is therefore expressed at 20 °C.

2. **Apparatus**

2.1. Conductivity meter enabling measurements of conductivity to be made over a range from 1 to 1 000 microsiemens per cm (μS cm^{-1}).

2.2. Waterbath for bringing the temperature of samples to be analysed to approximately 20 °C (20 ± 2 °C).

3. **Reagents**

3.1. Demineralised water with specific conductivity below 2 μS cm^{-1} at 20 °C.

3.2. *Reference solution of potassium chloride*

Dissolve 0,581 g of potassium chloride, KCl, previously dried to constant mass at a temperature of 105 °C, in demineralised water (paragraph 3.1). Make up to one litre with demineralised water (paragraph 3.1). This solution has a conductivity of 1 000 μS cm^{-1} at 20 °C. It should not be kept for more than three months.

4. **Procedure**

4.1. *Preparation of the sample to be analysed*

Use the solution with a total sugar concentration of 25 % (m/m) (25° Brix): weigh a mass equal to 2 500/P and make up to 100 g with water (paragraph 3.1), where P = percentage (m/m) concentration of total sugars in the rectified concentrated must.

4.2. *Determination of conductivity*

Bring the sample to be analysed to 20 °C by immersion in a waterbath. Maintain the temperature to within ± 0,1 °C.

Rinse the conductivity cell twice with the solution to be examined.

Measure the conductivity and express the result in μS cm^{-1}.

5. **Expression of the results**

The result is expressed in microsiemens per cm (μScm^{-1}) at 20 °C to the nearest whole number for the 25 % (m/m) (25° Brix) solution of rectified concentrated must.

5.1. *Calculations*

If the apparatus does not have temperature compensation, correct the measured conductivity using Table I. If the temperature is below 20 °C, add the correction; if the temperature is above 20 °C, subtract the correction.

Table I

Corrections to be made to the conductivity for temperatures different from 20 °C (μS cm^{-1})

Conductivity	Temperature (°C)									
	20,2 19,8	20,4 19,6	20,6 19,4	20,8 19,2	21,0 19,0	21,2 18,8	21,4 18,6	21,6 18,4	21,8 18,2	22,0 (1) 18,0 (2)
0	0	0	0	0	0	0	0	0	0	0
50	0	0	1	1	1	1	1	2	2	2
100	0	1	1	2	2	3	3	3	4	4
150	1	1	2	3	3	4	5	5	6	7
200	1	2	3	3	4	5	6	7	8	9
250	1	2	3	4	6	7	8	9	10	11
300	1	3	4	5	7	8	9	11	12	13
350	1	3	5	6	8	9	11	12	14	15
400	2	3	5	7	9	11	12	14	16	18
450	2	3	6	8	10	12	14	16	18	20
500	2	4	7	9	11	13	15	18	20	22
550	2	5	7	10	12	14	17	19	22	24
600	3	5	8	11	13	16	18	21	24	26

(1) Subtract the correction.
(2) Add the correction.

(c) ***Hydroxymethylfurfural (HMF)***

1. **Principle of the methods**

1.1. *Colorimetric method*

Aldehydes derived from furan, the main one being hydroxymethylfurfural, react with barbituric acid and paratoluidine to give a red compound which is determined by colorimetry at 550 nm.

1.2. *High-performance liquid chromatography (HPLC)*

Separation through a column by reversed-phase chromatography and determination at 280 nm.

2. **Colorimetric method**

2.1. *Apparatus*

2.1.1. Spectrophotometer for making measurements between 300 and 700 nm.

2.1.2. Glass cells with optical paths of 1 cm.

2.2. *Reagents*

2.2.1. Barbituric acid, 0,5 % solution (m/v).

Dissolve 500 mg of barbituric acid, $C_4O_3N_2H_4$, in distilled water and heat slightly over a waterbath at 100 °C. Make up to 100 ml with distilled water. The solution keeps for about a week.

2.2.2. Paratoluidine solution, 10 % (m/v).

Place 10 g of paratoluidine, $C_6H_4(CH_3)NH_2$, in a 100 ml volumetric flask; add 50 ml of isopropanol, $CH_3CH(OH)CH_3$, and 10 ml of glacial acetic acid, CH_3COOH (ρ_{20} = 1,05 g/ml). Make up to 100 ml with isopropanol. This solution should be renewed daily.

2.2.3. Ethanal (acetaldehyde), CH_3CHO, 1 % (m/v) aqueous solution.

Prepare just before use.

2.2.4. Hydroxymethylfurfural, $C_6O_3H_6$, 1 g/l aqueous solution.

Prepare successive dilutions containing 5, 10, 20, 30 and 40 mg/l. The 1 g/l and the diluted solutions must be freshly prepared.

2.3. *Procedure*

2.3.1. Preparation of sample

Use the solution obtained by diluting the rectified concentrated must to 40 % (m/v): introduce 200 g of accurately weighed rectified concentrated must into a 500 ml volumetric flask. Make up to the mark with water and homogenise. Carry out the determination on 2 ml of this solution.

2.3.2. Colorimetric determination

Into each of two 25 ml flasks *a* and *b* fitted with ground glass stoppers place 2 ml of the sample prepared as in paragraph 2.3.1. Place in each flask 5 ml of paratoluidine solution (paragraph 2.2.2); mix. Add 1 ml of distilled water to flask *b* (control) and 1 ml barbituric acid solution (paragraph 2.2.1) to flask *a*. Shake to homogenize. Transfer the contents of the flasks into spectrophotometer cells with optical paths of 1 cm. Zero the absorbence scale using the contents of flask *b* for a wavelength of 550 nm. Follow the variation in the absorbence of the contents of flask *a*; record the maximum value A, which is reached after two to five minutes.

Samples with hydroxymethylfurfural concentrations above 30 mg/l must be diluted before the analysis.

2.3.3. Preparation of the calibration curve

Place 2 ml of each of the hydroxymethylfurfural solutions with 5, 10, 20, 30 and 40 mg/l (paragraph 2.2.4) into two sets of 25 ml flasks *a* and *b* and treat them as described in paragraph 2.3.2.

The graph representing the variation of absorbence with the hydroxymethylfurfural concentration in mg/l is a straight line passing through the origin.

2.4. *Expression of results*

The hydroxymethylfurfural concentration in rectified concentrated musts is expressed in milligrams per kilogram of total sugars.

2.4.1. Method of calculation

The hydroxymethylfurfural concentration C mg/l in the sample to be analysed is that concentration on the calibration curve corresponding to the absorbence A measured on the sample.

The hydroxymethylfurfural concentration in milligrams per kilogram of total sugars is given by:

$250 \times ((C)/(P))$

P = percentage (m/m) concentration of total sugars in the rectified concentrated must.

3. **High-performance liquid chromatography**

3.1. *Apparatus*

3.1.1. High-performance liquid chromatograph equipped with:

— a loop injector, 5 or 10 µl,

— spectrophotometer detector for making measurements at 280 nm,

— column of octadecyl-bonded silica (e.g.: Bondapak C_{18} — Corasil, Waters Ass.),

— a recorder and, if required, an integrator,

Flow rate of mobile phase: 1,5 ml/minute.

3.1.2. Membrane filtration apparatus, pore diameter 0,45 µm.

3.2. *Reagents*

3.2.1. Doubly distilled water.

3.2.2. Methanol, CH_3OH, distilled or HPLC quality.

3.2.3. Acetic acid, CH_3COOH, (ρ_{20} = 1,05 g/ml).

3.2.4. Mobile phase: water-methanol (paragraph 3.2.2)-acetic acid (paragraph 3.2.3) previously filtered through a membrane filter (0,45 µm), (40:9:1 v/v).

This mobile phase must be prepared daily and outgassed before use.

3.2.5. Reference solution of hydroxymethylfurfural, 25 mg/l (v/v).

Into a 100 ml volumetric flask, place 25 mg of hydroxymethylfurfural, $C_6H_3O_6$, accurately weighed, and make up to the mark with methanol (paragraph 3.2.2). Dilute this solution $1/10^e$ with methanol (paragraph 3.2.2) and filter through a membrane filter (0,45 µm).

If kept in a hermetically sealed brown glass bottle in a refrigerator, this solution will keep for two to three months.

3.3. *Procedure*

3.3.1. Preparation of sample

Use the solution obtained by diluting the rectified concentrated must to 40 % (m/v) (introduce 200 g of accurately weighed rectified concentrated must into a 500 ml volumetric flask. Make up to the mark with water and homogenise) and filter it through a membrane filter (0,45 µm).

3.3.2. Chromatographic determination

Inject 5 (or 10) µl of the sample prepared as described in paragraph 3.3.1. and 5 (or 10) µl of the reference hydroxymethylfurfural solution (paragraph 3.2.5) into the chromatograph. Record the chromatogram.

The retention time of hydroxymethylfurfural is approximately six to seven minutes.

3.4. *Expression of results*

The hydroxymethylfurfural concentration in rectified concentrated musts is expressed in milligrams per kilogram of total sugars.

3.4.1. Method of calculation

Let the hydroxymethylfurfural concentration in the 40 % (m/v) solution of the rectified concentrated must be C mg/l.

The hydroxymethylfurfural concentration in milligrams per kilogram of total sugars is given by:

$250 \times ((C)/(P))$

P = percentage (m/m) concentration of total sugars in the rectified concentrated must.

(d) ***Heavy metals***

1. **Principle**

I. *Rapid method for evaluation of heavy metals*

Heavy metals are revealed in the suitably diluted rectified concentrated must by the coloration produced by the formation of sulphides. They are assessed by comparison with a standard lead solution corresponding to the maximum admissible concentration.

II. *Determination of lead content by atomic absorption spectrophotometry*

The chelate given by lead with ammonium pyrrolidinedithiocarbamate is extracted with methylisobutylketone and the absorbence measured at 283,3 nm. The lead content is determined by using known additional amounts of lead in a set of reference solutions.

2. **Rapid method for evaluation of heavy metals**

2.1. *Reagents*

2.1.1. Dilute hydrochloric acid, 70 % (m/v).

Take 70 g of hydrochloric acid, HCl (ρ_{20} = 1,16 to 1,19 g/ml), and make up to 100 ml with water.

2.1.2. Dilute hydrochloric acid, 20 % (m/v).

Take 20 g of hydrochloric acid, HCl (ρ_{20} = 1,16 to 1,19 g/ml), and make up to 100 ml with water.

2.1.3. Dilute ammonia.

Take 14 g of ammonia, NH_3 (ρ_{20} = 0,931 to 0,934 g/ml) and make up to 100 ml with water.

2.1.4. pH 3,5 buffer solution.

Dissolve 25 g of ammonium acetate (CH_3COONH_4), in 25 ml of water and add 38 ml of dilute hydrochloric acid (paragraph 2.1.1). Adjust the pH if necessary with the dilute hydrochloric acid (paragraph 2.1.2) or the dilute ammonia (paragraph 2.1.3) and make up to 100 ml with water.

2.1.5. Thioacetamide solution, (C_2H_5NS), 4 % (m/v).

2.1.6. Glycerol solution, ($C_3H_8O_3$, 85 % (m/v)

($n_D^{20\,°C}$ = 1,449 to 1,455).

2.1.7. Thioacetamide reagent.

To 0,2 ml of thioacetamide solution (paragraph 2.1.5) add 1 ml of a mixture of 5 ml of water, 15 ml of 1 M sodium hydroxide solution and 20 ml of glycerol (paragraph 2.1.6). Heat over a waterbath at 100 °C for 20 seconds. Prepare just before use.

2.1.8. Solution containing 0,002 g/l of lead.

Prepare a 1 g/l lead solution by dissolving 0,400 g of lead nitrate, Pb $(NO_3)_2$, in water and making up to 250 ml with water. At the time of use, dilute this solution with water to two parts in 1 000 (v/v) in order to obtain a 0,002 g/l solution.

2.2. *Procedure*

Dissolve a test sample of 10 g of the rectified concentrated must in 10 ml of water. Add 2 ml of the pH 3,5 buffer solution (paragraph 2.1.4); mix. Add 1,2 ml of the thioacetamide reagent (paragraph 2.1.7). Mix at once. Prepare the control under the same conditions by using 10 ml of the 0,002 g/l lead solution (paragraph 2.1.8).

After two minutes, any brown coloration of the rectified concentrated must solution should not be more intense than that of the control.

2.3. *Calculations*

Under the conditions of the above procedure, the control sample corresponds to a maximum admissible heavy metal concentration expressed as lead of 2 mg/kg of rectified concentrated must.

3. **Determination of lead content by atomic absorption spectrophotometry**

3.1. *Apparatus*

3.1.1. Atomic absorption spectrophotometer equipped with an air-acetylene burner.

3.1.2. Lead hollow cathode lamp.

3.2. *Reagents*

3.2.1. Dilute acetic acid.

Take 12 g of glacial acetic acid (ρ_{20} = 1,05 g/ml) and make up to 100 ml with water.

3.2.2. Solution of ammonium pyrrolidinedithiocarbamate, $C_5H_{12}N_2S_2$, 1 % (m/v).

3.2.3. Methylisobutylketone, $(CH_3)_2\ CHCH_2COCH_3$.

3.2.4. Solution containing 0,010 g/l of lead.

Dilute the 1 g/l lead solution (paragraph 2.1.8) to 1 % (v/v).

3.3. *Procedure*

3.3.1. Preparation of solution to be examined

Dissolve 10 g of rectified concentrated must in a mixture of equal volumes of dilute acetic acid (paragraph 3.2.1) and water, and make up to 100 ml with this mixture.

Add 2 ml of ammonium pyrrolidinedithiocarbamate solution (paragraph 3.2.2) and 10 ml of methylisobutylketone (paragraph 3.2.3). Shake for 30 seconds while protected from bright light. Leave the two layers to separate. Use the methylisobutylketone layer.

3.3.2. Preparation of reference solutions

Prepare three reference solutions containing, in addition to 10 g of rectified concentrated must, 1, 2 and 3 ml respectively of the solution containing 0,010 g/l of lead (paragraph 3.2.4). Treat these in the same way as the solution to be examined.

3.3.3. Control

Prepare a control by proceeding under the same conditions as in paragraph 3.3.1, but without the addition of the rectified concentrated must.

3.3.4. Determination

Set the wavelength to 283,3 nm.

Atomise the methylisobutylketone from the control sample in the flame and zero the absorbence scale.

By operating with their respective solvent extracts, determine the absorbences of the solution to be examined and the reference solutions.

3.4. *Expression of results*

Express the lead content in milligrams per kilogram of rectified concentrated must to one decimal place.

3.4.1. Calculations

Plot the curve giving the variation in absorbence as a function of the lead concentration added to the reference solutions, zero concentration corresponding to the solution to be examined.

Extrapolate the straight line joining the points until it cuts the negative part of the concentration axis. The distance of the point of intersection from the origin gives the lead concentration in the solution to be examined.

(e) ***Chemical determination of ethanol***

This method is used for the determination of the alcoholic strength of low-alcohol liquids such as musts, concentrated musts and rectified concentrated musts.

1. **Principle**

Simple distillation of the liquid. Oxidation of the ethanol in the distillate by potassium dichromate. Titration of the excess dichromate with an iron (II) solution.

2. **Apparatus**

2.1. Distillation apparatus used to measure the alcoholic strength

3. **Reagents**

3.1. *Potassium dichromate solution.*

Dissolve 33,600 g of potassium dichromate, ($K_2Cr_2O_7$), in sufficient quantity of water to make one litre of solution at 20 °C.

One millilitre of this solution oxidizes 7,8924 mg of alcohol.

3.2. *Iron (II) ammonium sulphate solution.*

Dissolve 135 g of iron (II) ammonium sulphate, Fe SO_4, $(NH_4)_2SO_4$, 6 H_2O in sufficient quantity of water to make one litre of solution and add 20 ml of concentrated sulphuric acid, (H_2SO_4), (ρ_{20} = 1,84 g/ml). This solution more or less corresponds to half its volume of dichromate solution when just prepared. Subsequently, it oxidizes slowly.

3.3. *Potassium permanganate solution.*

Dissolve 1,088 g of potassium permanganate, $KMnO_4$, in a sufficient quantity of water to make one litre of solution.

3.4. *Sulphuric acid, diluted 1:2 (v/v).*

A little at a time and stirring continuously, add 500 ml of sulphuric acid, (H_2SO_4) (ρ_20 = 1,84 g/ml) to 500 ml of water.

3.5. *Ferrous orthophenanthroline reagent.*

Dissolve 0,695 g of ferrous sulphate, $FeSO_4$, 7 H_2O, in 100 ml of water, and add 1,485 g of orthophenanthroline monohydrate, $C_{12}H_8N_2$, H_2O. Heat to help the dissolution. This bright red solution keeps well.

4. **Procedure**

4.1. *Distillation*

Place 100 g of rectified concentrated must and 100 ml of water in the distillation flask. Collect the distillate in a 100 ml volumetric flask and make up to the mark with water.

4.2. *Oxidation*

Take a 300 ml flask with a ground glass stopper and with a widened neck enabling the neck to be rinsed without loss. In the flask, place 20 ml of the titrant potassium dichromate solution (paragraph 3.1) and 20 ml of the 1:2 (v/v) dilute sulphuric acid (paragraph 3.4) and shake. Add 20 ml of the distillate. Stopper the flask, shake, and wait at least 30 minutes, shaking occasionally. (This is the 'measurement' flask.)

Carry out the titration of the iron (II) ammonium sulphate solution (paragraph 3.2) with respect to the potassium dichromate solution by placing in an identical flask the same quantities of reagents but replacing the 20 ml of distillate by 20 ml of distilled water. (This is the 'control' flask.)

4.3. *Titration*

Add four drops of the orthophenanthroline reagent (paragraph 3.5) to the contents of the 'measurement' flask. Titrate the excess dichromate by adding to it the iron (II) ammonium sulphate solution (paragraph 3.2). Stop adding the ferrous solution when the mixture changes from green-blue to brown.

To judge the end-point more precisely, change the colour of the mixture back from brown to green-blue with the potassium permanganate solution (paragraph 3.3). Subtract a tenth of the volume of this solution used from the volume of the iron (II) solution added. Let the difference be n ml.

Proceed in the same way with the 'control' flask. Let n′ ml be the difference here.

5. **Expression of the results**

The ethanol is expressed in grams per kilogram of total sugars and is quoted to one decimal place.

5.1. *Method of calculation*

n′ ml of ferrous solution reduces 20 ml of dichromate solution which oxidizes 157,85 mg of pure ethanol.

One millilitre of iron (II) solution has the same reducing power as:

((157,85)/(n)) mg of ethanol

n-n′ ml of iron (II) solution have the same reducing power as:

157,85 × ((n′ — n)/(n)) mg of ethanol.

Ethanol concentration in g/kg of rectified concentrated must is given by:

7,892 × ((n′ — n)/(n))

Ethanol concentration in g/kg of total sugars is given by:

789,2 × ((n′ — n)/(n′ × P))

P = percentage (m/m) concentration of total sugars in the rectified concentrated must.

(f) ***Meso-inositol, scyllo-inositol and sucrose***

1. **Principle**

Gas chromatography of silylated derivatives.

2. **Reagents**

2.1. Internal standard: xylitol (aqueous solution of about 10 g/l to which a spatula tip of sodium azide is added)

2.2. Bis(trimethylsilyl)trifluoroacetamide — BSTFA — ($C_8H_{18}F_3NOSi_2$)

2.3. Trimethylchlorosilane (C_3H_9ClSi)

2.4. Pyridine p.A. (C_5H_5N)

2.5. Meso-inositol ($C_6H_{12}O_6$)

3. **Apparatus**

3.1. Gas chromatograph equipped with:

3.2. Capillary column (e.g. in fused silica, coated with OV 1, film thickness of 0,15 µ, length 25 m and internal diameter of 0,3 mm).

Operating conditions: carrier gas: hydrogen or helium

— carrier gas flow rate: about 2 ml/minute,

— injector and detector temperature: 300 °C,

— programming of temperature: 1 minute at 160 °C, 4 °C per minute to 260 °C, constant temperature of 260 °C for 15 minutes,

— splitter ratio: about 1:20.

3.3. Integrator.

3.4. Microsyringe, 10 µl.

3.5. Micropipettes, 50, 100 and 200 µl.

3.6. 2 ml flasks with Teflon stopper.

3.7. Oven.

4. **Procedure**

An accurately weighed sample of about 5 g of rectified concentrated must is placed in a 50 ml flask. 1 ml of standard solution of xylitol (paragraph 2.1) is added and water added to capacity. After mixing, 100 µl of solution is taken and placed in a flask (point 3.6) where it is dried under a gentle stream of air. 100 µl of absolute ethyl alcohol may be added if necessary to facilitate evaporation.

The residue is carefully dissolved in 100 µl of pyridine (paragraph 2.4) and 100 µl of bis(trimethylsilyl)trifluoroacetamide (paragraph 2.2) and 10 µl of trimethylchlorosilane (paragraph 2.3) are added. The flask is closed with the Teflon stopper and heated at 60 °C for one hour.

Draw off 0,5 µl of clear fluid and inject using a heated hollow needle in accordance with the stated splitter ratio.

5. **Calculation of results**

5.1. A solution is prepared containing:

60 g/l of glucose, 60 g/l of fructose, 1 g/l of meso-inositol and 1 g/l of sucrose.

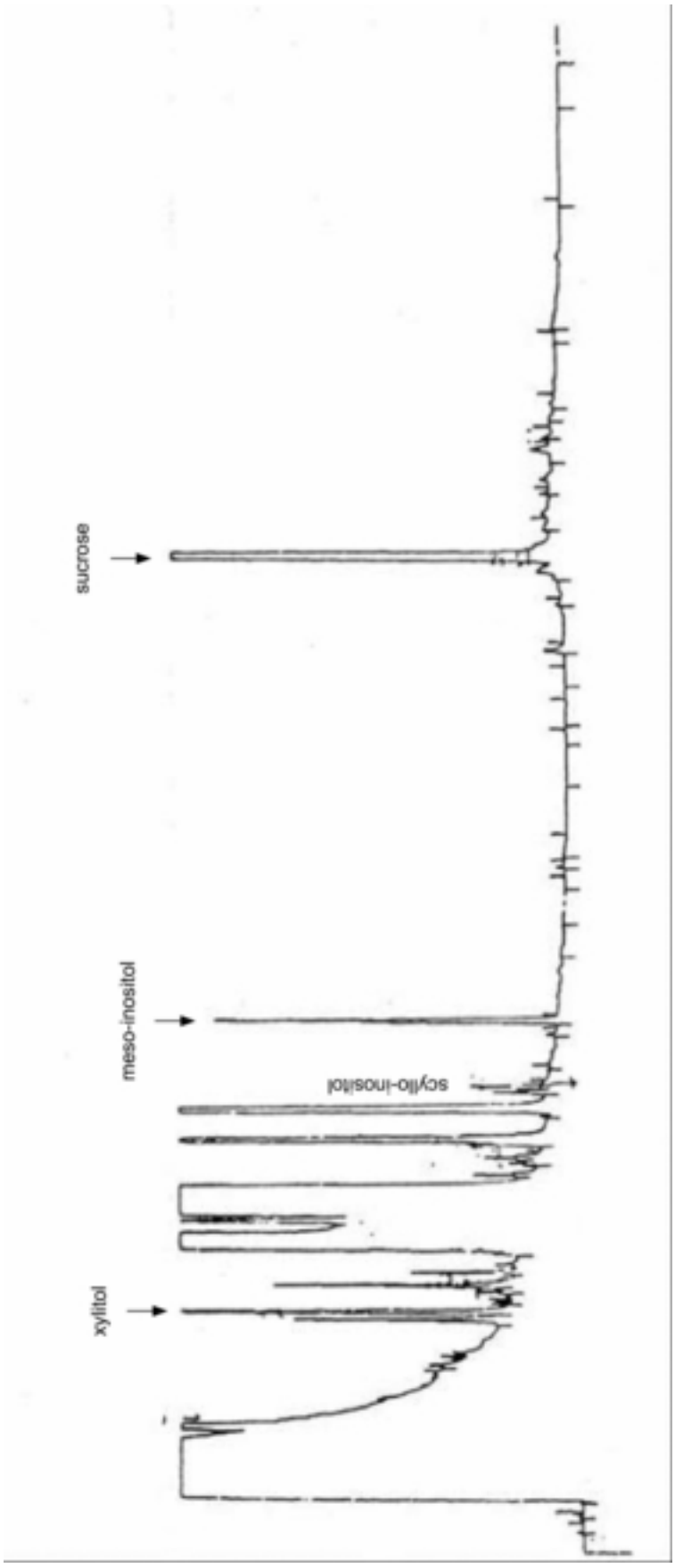
5 g of the solution is weighed and the procedure at paragraph 4 followed. The results for meso-inositol and sucrose with respect to xylitol are calculated from the chromatogram.

In the case of scyllo-inositol, which is not commercially available and has a retention time lying between the last peak of the anomeric form of glucose and the peak for meso-inositol (see diagram), the same result as for meso-inositol is taken.

6. **Expression of the results**

6.1. Meso-inositol and scyllo-inositol are expressed in milligrams per kilogram of total sugars.

Sucrose is expressed in grams per kilogram of must.


sucrose
meso-inositol
scyllo-inositol
xylitol

ANNEX V

CORRELATION TABLE REFERRED TO IN THE SECOND PARAGRAPH OF ARTICLE 16

Regulation (EC) No 1493/1999	Regulation (EC) No 2676/90	Regulation (EC) No 423/2008	This Regulation
—	—	Article 1	Article 1
—	—	—	Article 2
Article 43(1)	—	Article 5	Article 3(1)
Article 43)(2), first indent	—	Article 23	Article 3(2)
Article 43)(2), first indent	—	Article 24	Article 3(3)
Article 43)(2), first indent	—	Articles 34, 35 and 36	Article 3(4)
—	—	Article 44	Article 4
Article 43)(2), second indent	—	—	Article 5
Article 43)(2), third indent	—	—	Article 6
—	—	Article 38	Article 7
Article 42(6)	—	Article 39	Article 8
—	—	Article 6	Article 9
—	—	Article 46	Article 10(1)
—	—	Article 45	Article 10(2)
—	—	Article 32	Article 11
—	—	Article 29	Article 12
—	—	Article 30	Article 13
—	—	Article 21	Article 14
—	Article 1(1)	Article 47	Article 15
—	—	Article 48	Article 16
Annex IV	—	Articles 7 and 12	Annex I A
—	—	Article 10	Annex I A, Appendix 1
—	—	Article 8	Annex I A, Appendix 2
—	—	Article 9	Annex I A, Appendix 3
—	—	Article 13	Annex I A, Appendix 4
—	—	Articles 14, 15 and 16	Annex I A, Appendix 5
—	—	Article 17	Annex I A, Appendix 6
—	—	Article 18	Annex I A, Appendix 7
—	—	Article 19	Annex I A, Appendix 8
—	—	Article 22	Annex I A, Appendix 9
Annex V A	—	—	Annex I B
Annex V B	—	—	Annex I C
Annex V F	—	—	Annex I D
Annex V H	—	Article 28	Annex II A
Annex V I	—	Article 4	Annex II B
Annex VI K	—	—	Annex II C
Annex V J	—	Articles 25 and 37	Annex III A
—	—	Article 43	Annex III A
Annex VI L	—	Articles 40 and 41	Annex III B
—	Annex, paragraph 39	—	Annex IV-A
—	Annex, paragraph 42	—	Annex IV-B

European Food Safety Authority

EFSA Journal 2010; 8(7):1687

SCIENTIFIC OPINION

Scientific Opinion on the safety of 'Chitin-glucan' as a Novel Food ingredient[1]

EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA)[2], [3]

European Food Safety Authority (EFSA), Parma, Italy

ABSTRACT

Following a request from the European Commission, the Panel on Dietetic Products, Nutrition and Allergies was asked to carry out the additional assessment for 'Chitin-glucan' as a food ingredient in the context of Regulation (EC) No. 258/97. The Novel Food ingredient called "KiOnutrime-CG™" has a content of more than 90 % chitin-glucan, which is the main component in the cell walls of the mycelium of *Aspergillus niger* derived from a fermentation process. The compositional data and the manufacturing process do not give rise to concerns. The ingredient is intended to be marketed as a food supplement to increase the daily intake of fibre. The intended intake of chitin-glucan is 2 to 5 g/day. At the highest dose administered in a 13-week rat study, i.e. about 6.6 g/kg body weight (bw), no adverse effects were observed. This dose is approximately 80-fold higher than the maximum intended level of intake for humans on a g/kg bw basis. The Panel concludes that Novel Food KiOnutrime-CG™ is safe as a food ingredient at the proposed conditions of use and the proposed intake levels.

KEY WORDS

Chitin-glucan, novel food ingredient, Kitozyme

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[1] On request from the European Commission, Question EFSA-Q-2009-00762. Adopted on 9 July 2010.

[2] Panel members: Carlo Virginio Agostoni, Jean-Louis Bresson, Susan Fairweather-Tait, Albert Flynn, Ines Golly, Hannu Korhonen, Pagona Lagiou, Martinus Løvik, Rosangela Marchelli, Ambroise Martin, Bevan Moseley, Monika Neuhäuser-Berthold, Hildegard Przyrembel, Seppo Salminen, Yolanda Sanz, Sean (J.J.) Strain, Stephan Strobel, Inge Tetens, Daniel Tomé, Hendrik van Loveren and Hans Verhagen. Correspondence: nda@efsa.europa.eu

[3] Acknowledgement: The Panel wishes to thank for the preparatory work on this scientific opinion: The members of the Working Group on Novel Foods: Karl-Heinz Engel, Ines Golly, Marina Heinonen, Pagona Lagiou, Rosangela Marchelli, Bevan Moseley, Monika Neuhäuser-Berthold, Annette Pöting, Seppo Salminen, Hendrik Van Loveren and Hans Verhagen, and EFSA's staff member Wolfgang Gelbmann for the support provided to this scientific opinion.

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SUMMARY

Following a request from the European Commission, the EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) was asked to deliver a scientific opinion on the safety of 'Chitin-glucan' as a novel food ingredient in the context of Regulation (EC) No. 258/97 taking account of the comments/objections of a scientific nature raised by the Member States.

The Novel Food ingredient "KiOnutrime-CG™" has a content of more than 90 % chitin-glucan, which is the main component in the cell walls of the mycelium of *Aspergillus niger* derived from a fermentation process. The *A. niger* strain is non-toxic and non-pathogenic and has a history of safe use in production of food ingredients. The compositional data and the manufacturing process do not give rise to concerns.

The ingredient is intended to be marketed as a food supplement in the form of a powder in different formats such as gelatine capsules or tablets. According to the applicant it is designed to increase the daily intake of fibres. The intended intake of chitin-glucan is 2 to 5 g/day.

At the highest dose administered in the 13-week rat study, i.e. about 6.6 g/kg bodyweight (bw), no adverse effects were observed. This dose is approximately 80-fold higher than the maximum intended level of intake for humans on a g/kg bw basis. On basis of the data provided and taking into account the nature of the novel food ingredient, the Panel considers that there are no safety concerns under the proposed conditions of use.

The Panel concludes that Novel Food KiOnutrime-CG™ is safe as a food ingredient at the proposed conditions of use and the proposed intake levels.

TABLE OF CONTENTS

Abstract 1
Summary 2
Table of contents 3
Background as provided by the European Commission 4
Terms of reference as provided by the European Commission 5
Assessment 6
1. Specification of the Novel Food (NF) 6
2. Effect of the production process applied to the NF 9
3. History of the organism used as the source of the NF 10
4. Anticipated intake/extent of the use of the NF 11
5. Information from previous exposure to the NF or its source 11
6. Nutritional information on the NF 11
7. Microbiological information on the NF 12
8. Toxicological information on the NF 12
8.1. Genotoxicity 12
8.2. Animal studies 13
8.2.1. Acute toxicity studies 13
8.2.2. Sub-acute toxicity (OECD 407) 13
8.2.3. Sub-chronic toxicity (OECD 408) 13
8.2.4. Allergenicity 14
8.3. Human studies 14
8.4. Effect on the bioavailability of micronutrients 15
8.5. Other information 15
Discussion 16
Documentation provided to EFSA 16
References 16

BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

On 15 January 2008, KitoZyme submitted a request under Article 4 of the Novel Food Regulation (EC) N° 258/97 to place on the market 'Chitin-glucan' as a novel food ingredient.

On 23 January 2009, the competent authorities of Belgium forwarded to the Commission their initial assessment report, which came to the conclusion that an additional assessment was required.

On 12 March 2009, the Commission forwarded the initial assessment report to the other Member States. Several of the Member States submitted additional comments.

In consequence, a Community Decision was required under Article 7, paragraph 1 of Regulation (EC) No 258/97.

The concerns of a scientific nature raised by the Member States can be summarized as follows:

- No data given on the composition of the product's lipid and protein fraction.
- Unclear data on specification and composition, thus it remains unclear whether the composition complies with the specification.
- Only a single production batch was examined in the manufacturer's test facility.
- No indication of GMP conditions or a HACCP plan.
- Insufficient information on mycotoxin and total heavy metal and lead testing (not in accordance with European Pharmacopoeia, no information whether laboratory was accredited, insufficient information on the applied methods).
- Insufficient data on the product's stability. No results from analyses showing the stability of the product under realistic storage conditions.
- Information on the 'levels of exposure' or 'anticipated intakes' of other age groups such as children, the elderly, pregnant and lactating women was also not evident.
- The applicant should consider the effect of chitin-glucan on metabolism of lipids, fat-soluble vitamins and minerals.
- The provided human study with 20 male adults is insufficient to demonstrate safety. No information on whether chitin-glucan might influence the bioavailability and serum levels of other nutrients such as minerals and fat-soluble vitamins.
- Uncertainty of the intestinal fate of chitin-glucan, vague information to what extent chitin-glucan is fermented in the large bowel.
- Lack of characterisation of the up to 6 % protein fraction, concerns regarding potential allergenicity and cross-reactivity in relation to IgE sensitisation to *Aspergillus fumigatus*, a major respiratory and skin allergen.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

In accordance with Article 29 (1) (a) of Regulation (EC) No 178/2002, the European Food Safety Authority is asked to carry out the additional assessment for 'Chitin-glucan' as food ingredient in the context of Regulation (EC) N° 258/97.

EFSA is asked to carry out the additional assessment and to consider the elements of scientific nature in the comments raised by the other Member States.

ASSESSMENT

In accordance with the Commission Recommendation 97/618/EC chitin-glucan from *Aspergillus niger* is allocated to Class 2.1 'a complex (non-GM derived) novel food ingredient, the source of the novel food having a history of food use in the community'. The assessment of the safety of this novel food ingredient is based on data supplied in the original application, the initial assessment by the competent authority of Belgium, the concerns and objections of the other Member States and the responses of the applicant to these questions and those of Belgium. The data are required to comply with the information required for novel foods of Class 2.1 i.e. structured schemes I, II, III, IX, X, XI, XII and XIII. It is noted that the novel ingredient is intended by the applicant to be marketed for consumption as a food supplement in the form of a powder in different prescription formats (gelatine capsules, tablets and possibly other). This assessment concerns only risk that might be associated with consumption and is not an assessment of the efficacy of chitin-glucan with regard to any claimed benefits.

1. Specification of the Novel Food (NF)

Chitin-glucan is a purified ingredient, presented in the form of a powder, which is composed largely of two polysaccharides:

- chitin, composed of repeating units of N-acetyl-D-glucosamine (CAS number 1398-61- 4);
- beta(1,3)-glucan, composed of repeating units of D-glucose (CAS number 9041-22-9).

Chitin-glucan is the main component in the cell walls of the mycelium of a fungus from the Ascomycetes family: *Aspergillus niger* (*A. niger*). The two polymers are linked covalently and form a three-dimensional network.

Chitin-glucan is obtained from the mycelium of non-genetically-modified strains of *A. niger*, a microorganism employed in the food and pharmaceutical industries for the production of citric acid. The applicant uses two sources for its novel food ingredient.

The Novel Food ingredient (KiOnutrime-CG™, KiOnutrime-CG®, KiOnutrime-CG) is a white odourless powder with a yellowish tinge which has a dry matter content $\geq$ 90 %. It is insoluble in aqueous and organic media. It is intended to be marketed in different food supplement formats.

The applicant proposed the following specifications for the novel food ingredient (see **Table 1**).

Table 1: Specification for KiOnutrime-CG™

Parameter	Specification	Methods
Loss on drying (%)	≤ 10	Gravimetric method
Chitin-glucan content (%)	≥ 90	Internal Method: total weight minus ash, minus protein
Ratio of chitin-glucan	30:70 to 60:40	Internal Method based on 13C NMR
Ash (%)	≤ 3	Gravimetric method
Lipids (%)	≤ 1	Gravimetric method
Proteins (%)	≤ 6	Colorimetric method
Total heavy metals (ppm)	≤ 20	ICP-MS
Mercury (ppm)	≤ 0.2	ICP-MS
Lead (ppm)	≤ 1	ICP-MS
Arsenic (ppm)	≤ 1	ICP-MS
Cadmium (ppm)	≤ 0.5	ICP-MS

Aerobic count (cfu/g)	< 1000	ISO 4833
E. coli (cfu/g)	< 10	ISO 16649
Yeast and mould count (cfu/g)	< 1000	ISO 7954

In response to Member State comments, the applicant provided certificates from its *A. niger*-mycelium sources and information on the laboratory methods and accreditation of laboratories that conducted the analyses.

As concerns batch variation, five non-consecutive batches were analysed to demonstrate the ability of KitoZyme to produce within these specifications. Analytical results are presented in **Table 2** and indicate that the specifications were met.

The analytical methods used for the analyses followed ISO (International Standard Organization) norms. Analyses were run mostly in external laboratories that were accredited under ISO 17025 (amino acid profile, fatty acid profile, carbohydrate profile, mycotoxins, sterols, DNA, heavy metals, microbiology, water activity); the few internal methods were done according to existing in-house protocols. For parameters for which no standard tests are available, KitoZyme in-house QC laboratory has developed internal validated methods.

Table 2 : Batch testing

Parameter	**Batch N° L09093CG**	**Batch N° L09068CG**	**Batch N° L09070CG**	**Batch N° L09071CG**	**Batch N° L09072CG**	**Mean**	**Max.**	**Min.**
Loss on drying (%)	5.0	8.0	7.0	7.0	10.0	7.4	10.0	5.0
Chitin-glucan content (%)	94.0	94.0	95.0	94.0	94.0	94.0	95.0	94.0
Ratio of chitin-glucan	30:70	35:65	30:70	30:70	32:68	NA	NA	NA
Ash (%)	2.5	3.0	2.0	3.0	3.0	2.7	3.0	2.0
Lipids (%)	0.6	0.7	0.7	0.7	0.7	0.6	0.7	0.7
Proteins (%)	3.0	3.5	3.0	3.4	3.5	3.3	3.5	3.0
Total heavy metals (ppm)	2.3	1.9	2.1	2.0	2.0	2.0	2.3	1.9
Mercury (ppm)	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Lead (ppm)	< 0.25	< 0.25	< 0.25	< 0.25	< 0.25	< 0.25	< 0.25	< 0.25
Arsenic (ppm)	< 0.25	< 0.25	< 0.25	< 0.25	< 0.25	< 0.25	< 0.25	< 0.25
Cadmium (ppm)	< 0.25	< 0.25	< 0.25	< 0.25	< 0.25	< 0.25	< 0.25	< 0.25
Aerobic count (cfu/g)	< 10	30	<10	10	<10	20	30	< 10
Yeast and mould count (cfu/g)	< 10	<10	<10	<10	<10	< 10	< 10	< 10

Protein fraction

The protein content is ≤ 6%. The method for protein determination was based on colorimetry after reaction with ninhydrin and UV-absorption at 564 nm. Conventional methods for determination of nitrogen/protein content such as the Bradford protein assay, the Lowry method, the Kjeldahl method, and the BCA (bicinchoninic acid) method could not be used because of the presence of the amid group of chitin which interferes with these assays as well as the use of sodium hydroxide for several

hours for the extraction of chitin-glucan from the mycelium which is expected to denature or partly hydrolyse any protein component from the source.

As concerns amino acid composition, ten batches of chitin-glucan extracted from two different sources of *A. niger* have been analyzed, i.e. three batches of chitin-glucan from a first source and 7 batches of chitin-glucan from a second source. The eight most abundant amino acids are Leucine, Phenylalanine, Glutamic acid, Aspartic acid, Isoleucine, Valine, Alanine, and Tyrosine; the standard deviations between assays were small. The total amino acid content represents 2.9 % of chitin-glucan (2.9 g per 100 g of chitin-glucan; average values from 10 batches). This value is very close to the average value of 3.3 % reported by the applicant and which is the average protein content as determined by the KitoZyme method on the same batches.

Lipid fraction

The lipid content in chitin-glucan of KiOnutrime-CG™ in the specification is ≤ 1% as assessed by gravimetry after solvent extraction.

A literature review on lipids of *A. niger* mycelium from different strains shows that all strains contain phospholipids, glycolipids, and neutral lipids (triglyceride, diglyceride, monoglyceride, sterols and pigments) (Chattopadhyay et al., 1985; 1987). According to the applicant, the main difficulty in the determination of these fractions is related to the overall low lipid content in chitin-glucan, therefore requiring multiple solvent extractions to obtain a lipid fraction compatible with quantitative analysis. In addition, determination of these compounds was not possible using normalized methods (except for sterols). The applicant provided a review article showing that *A. niger* contains C_{16} to C_{18} saturated and unsaturated fatty acids. Small amounts of long chain (C_{20} to C_{24}) and short chain (C_{10} to C_{14}) saturated and unsaturated acids are also present. Linoleic acid, oleic acid, and palmitic acid are the major acids, while stearic acid and linolenic acid are the minor ones (Chattopadhyay et al., 1987).

For the determination of the lipid fraction, the following analyses have been performed:

- Total hydrolysis followed by fatty acid extraction according to the method described in Regulation EC N° 152/20092.

- Determination of the lipid content extracted by the Folch method (Folch, 1957); preparation and analysis by gaseous phase chromatography of the methyl esters of fatty acids according to NF EN ISO 5509 and NF ISO 5508; determination of sterol content (individual and total sterols) according to NF EN ISO 12228. The results on the fatty acid profile indicate that the 5 most abundant fatty acids are oleic acid (55.1 %), linoleic acid (19.9 %), palmitic acid (12.7%), stearic acid (5.1 %) and lignoceric acid (3.3%), albeit that some batches had too low a lipid content to allow analyses; other fatty acids are present at < 1% each. Three major fatty acids represent ~ 90 % of the total fatty acids: oleic acid, linoleic acid and palmitic acid. The ratio of unsaturated fatty acids/saturated fatty acids is 77/23. The sequence of the first 5 fatty acids is the same as the one reported in literature for *A. niger*. The results on the determination of the phytosterol content show that the phytosterols represent a low fraction of the extracted lipid fraction (73.9 mg/100 g of chitin-glucan), ergosterol being the main constituent of the phytosterols (74 %).

In conclusion and according to the applicant, the lipid fraction of chitin-glucan is similar to the lipid profile of *A. niger* reported in the literature (Chattopadhyay, 1985; 1987).

Carbohydrate

The applicant determined the total carbohydrate value by adding the content of glucose, saccharose, lactose, galactose, maltose and fructose measured by ionic chromatography. The determination of the carbohydrate content in chitin-glucan according to this method has been performed on ten batches of

chitin-glucan extracted from the two different sources of *A. niger*. The total carbohydrate value is less than 0.2 % for all batches.

The Panel considers that the protein, lipid and carbohydrate fractions do not indicate a safety concern as the ingredients found are regular constituents of the diet.

Heavy metals

The applicant reported that specifications for heavy metals and microbial contamination are in compliance with regulation EC N°629/2008 (Table 2).

Total heavy metal content as well as each specific metal listed in the certificate of analysis are determined by ICP-MS. The limits of detection were for Hg (0.1 ppm) and for Pb, As and Cd (0.25 ppm).

Mycotoxins

The mycotoxin analyses have been selected based on publication pointing out that certain strains of *A. niger* produce both ochratoxin and fumonisins (Frisvad, 2007). The results are presented in Table 3 for *A. niger* and chitin-glucan samples. The results for mycotoxins such as aflatoxin (B1, B2, G1, G2), fumonisin B1 and B2 and ochratoxin are below detection limit. The number of samples tested was not provided; the detection limit is assumed to be the value indicated in the Table after "<".

Table 3 : Mycotoxin analysis in samples of A. niger and chitin-glucan

Sample analysed (µg/kg)	A. niger	Chitin-glucan
Aflatoxin B1	< 0.1	< 0.1
Aflatoxin B2	< 0.1	<0.1
Aflatoxin G1	< 0.1	<0.1
Aflatoxin G2	< 1	<1
Ochratoxin	< 1	<1
Fumonisin B1	< 100	<100
Fumonisin B2	< 100	<100

The Panel considers that the contaminants measured (heavy metals, mycotoxins, micro-organisms) do not indicate a safety concern.

2. Effect of the production process applied to the NF

The source of the novel food ingredient [mycelium of *A. niger*] is obtained by means of fermentation, in accordance with different processes depending on the producers concerned, followed by stages involving extraction from citric acid and washing. At the end of this production process, the source is dried and packaged.

Chitin-glucan is obtained by a process of digestion by hydrolysis of the source (the mycelium of *A.niger)*, purification in an aqueous medium and drying. The process is described by patent No WO/2003/068824 filed by KitoZyme, albeit that this patent is very broad with respect to process conditions such as temperature, time of treatment and percentage of alkali. Upon request more specific production process data have been obtained from the applicant; these were marked by the applicant as "confidential" and caused the Panel no concern.

After drying, the resulting powder has a dry matter content of more than 90 %. The chitin-glucan powder is then packed in double-layer polyethylene bags, which are sealed and stored at ambient temperature. A sample is taken to check compliance with the specifications.

The applicant follows the auto-control guide established by the Belgian Federation for Food Supplements, Dietary and Organic Products, NAREDI, for the traceability, mandatory notification and risk analysis based on the HACCP method.

As concerns stability of chitin-glucan, the applicant claims that a shelf life of two years has been established for chitin-glucan by the applicant based on 3 stability studies and on the very low water activity of chitin-glucan:

Stability Study N°1 started July 2007: two batches were stored at room temperature. Follow up for microbiological content every 6 months. Both batches are within the specification during two years.

Stability Study N°2: one batch of chitin-glucan stored at two temperature conditions, i.e. (1) at room temperature and (2) at 40°C in closed polyethylene container. The duration of this study was extending over 13 months (RT) and over 6 months (40°C). Parameters followed during the stability study were loss on drying which allows characterisation of the water tightness of the packaging and the hygroscopic characteristics of the chitin-glucan; total microbiological content and yeasts/moulds. Results for these three parameters are within the specifications showing that chitin-glucan is stable for 13 months at room temperature and 6 months at 40°C.

Stability Study N°3: Three batches (a sample size of 20 g) according to ICH Q1A (stability testing of new drug substances and products) in recommended storage conditions, i.e. in the final closure container at 25 +/- 2°C. The testing program, start and end dates are reported in Table 4. A first long-term study at 25 +/- 2°C is set up for a duration of 36 months. A second study at 40 +/- 2°C (and 75 +/- 5% Relative Humidity) is set up under accelerated conditions for a duration of 6 months. The samples are stored in qualified ICH climatic chambers. At pre-determined time intervals, samples are collected and analysed for loss on drying, water activity and microbiological parameters (aerobic microbial count, total yeasts and moulds count, *Escherichia coli*, *Listeria monocytogenes*, Enterobacteriaceae, *Salmonella*) according to specified ISO methods.

Table 4 : Testing program for the stability study of KiOnutrime-CG®

Storage conditions	Temperature	Relative Humidity	Test intervals (T in month)	Start date	End date
Accelerated conditions	40 ± 2°C	75 ± 5 %	T0, T3, and T6	June 09	December 2009
Recommended storage conditions	25 ± 2°C	60 ± 5 %	T0, T3, T6, T9, T12, T18, T24, T30 and T36	June 09	June 2012

3. History of the organism used as the source of the NF

The *A. niger* strain used as the raw material for manufacture of KiOnutrime-CG™ is non-genetically modified, non-pathogenic and non-toxic for humans and animals. It does not produce ochratoxin A. This species has been commonly used in food production since the 1920s. The strain used in this process is a "privately developed strain of a proprietary nature" that was specifically selected for citric acid production. The citric acid has been sold in the US, EU and other countries since 1993.

Also, the mycelium of *A. niger* is currently used as a feed alternative to supplement the diet of ruminants with protein and nutritional fibre.

A. niger has a long history of use in the food industry as a source of enzymes. Several enzymes derived from the organism are authorized for use in the manufacture of food ingredients in Europe and the United States. FAO/WHO has repeatedly reviewed and accepted enzyme preparations from *A. niger*. The FDA in the United States has accepted numerous enzymes for food use, recognizing that α-amylase, cellulase, amyloglucosidase, catalase, glucose oxidase, lipase and pectinase from *A. niger* can be 'generally regarded as safe' (GRAS) under the condition that non-pathogenic and non-toxigenic strains and current good manufacturing practices be used in production (Schuster et al., 2002).

4. Anticipated intake/extent of the use of the NF

According to the applicant, chitin-glucan is intended for consumption as a food supplement in the form of a powder in different prescription formats (gelatine capsules, tablets and possible other) and is designed to increase the daily intake of fibre. It is supposed to be consumed either in a short-term manner (intestinal comfort) or in a prolonged manner as a fibre nutritional supplement. The intended intake of chitin-glucan is 2 to 5 g/day, split into two or three doses, taken preferably with food and with some liquid to help it swell.

The applicant indicates that the recommended dietary intake for fibre in European countries is between 25 - 35 g/day (for adults), In addition, the applicant provides data that the dietary fibre intakes vary from 10 - 20 g/day in young children (< 10 - 12 years), from 15 - 30 g/day in adolescents, and from 16 - 29 g/day in adults (EFSA, 2010).

Although not specifically indicated by the applicant the Panel assumes that the target group is the general population.

5. Information from previous exposure to the NF or its source

Chitin-glucan is obtained from a defined source: the mycelium of *Aspergillus niger*, the microorganism used to produce citric acid which is produced by several industrial companies. A number of producers of citric acid are commercializing the mycelium of *A. niger* as a feed ingredient.

6. Nutritional information on the NF

Food supplements containing KiOnutrime-CG™ are intended by the applicant to increase the dietary fibre intake. The caloric value is low given that chitin-glucan is an insoluble fibre which contains indigestible carbohydrates. Dietary fibre has a low caloric value (EFSA, 2010). The intended intake of chitin-glucan is 2 to 5 g/day.

Chitin-glucan being insoluble, it is not expected to be digested by human enzymes to any significant extent. Digestion of insoluble fibres does not occur in the small intestine; therefore, the majority of the material is expected to travel intact through the gastrointestinal tract to the colon to be subject to fermentation by the resident microbiota.

The fermentation of beta-glucan is well described in literature, and the metabolic products of fermentation are expected to be innocuous compounds (H_2, CO_2, CH_4, and volatile fatty acids). Chitin is fairly resistant to microbial fermentation and it is therefore expected to be excreted as such in faeces.

Proof that fermentation of chitin-glucan takes place in the colon is provided by results from the subacute and subchronic *in vivo* studies on Wistar rats (see below) showing the increase of caecal content. Such effects have also been confirmed by a 4 weeks *in vivo* study in the high-fat mouse model. Chitin-glucan is a fibre according the definition given into Directive EC N°2008/1004, therefore the energy content (or metabolisable energy) of chitin-glucan can be estimated to be around 2 kcal/g of product coming solely from fermentation in the colon.

On the basis of this information, it is concluded that the novel food product is not nutritionally disadvantageous.

7. Microbiological information on the NF

The applicant provided methods (*Annex 4 of the original application*) and results on an unknown number of batches as presented in **Table 5**.

Table 5 : Microbiological methods and testing results from one batch

Microbiology	Methods	Result
Total mesophilic bacteria (cfu/g)	NF-V-05-051	≤ 1000
Yeasts and moulds (cfu/g)	NF-V-05-059	≤ 1000
Pathogens		
Enterobacteriaceae	NF-V08-054	≤ 10
Total coliforms at 30°C (cfu/g)		≤ 1000
E.coli (cfu/g)	ISO 16649-2 NF-V-08-053	≤ 10
Listeria monocytogenes	Derived from ISO-13720	None / 25g
Salmonella	NMKL 71	None / 25g

The Panel notes that the value ≤ 10 cfu/g for E. coli does not comply with the European Pharmacopeia.

8. Toxicological information on the NF

8.1. Genotoxicity

The applicant provided a study report on an AMES test of a product called "chitin-glucan KiOfine" (a test substance which was reportedly similar to KiOnutrime-CG) carried out by the company Vivotechnia (ES) under GLP and according to OECD guideline 471 and method B13/B14 of Directive EC N° 2000/327. Up to 2.5 mg in DMSO per plate was tested in both the absence and presence of a metabolic activation system. Chitin-glucan was not mutagenic under these conditions.

In addition the applicant provided a literature review commissioned by the applicant on test substances related to chitin-glucan, albeit not similar. These were non-genotoxic: beta-glucan from barley in a mouse bone marrow micronucleus test (Delaney et al., 2004), 6-O-carboxymethylchitin-glucan in a mouse bone marrow micronucleus test (Chorvatovicova et al., 1998), and chitooligomers in an Ames test, a mouse micronucleus test, and in a mouse sperm abnormality test (Qin et al., 2006).

It is concluded that these results do suggest non-genotoxicity of the novel food ingredient.

8.2. Animal studies

8.2.1. Acute toxicity studies

Acute oral toxicity in rats and acute intravenous toxicity in mice were studied with a test product called "Chitin-Glucan KiOfine-26" in 2005 by the company Phycher BioDéveloppement (FR).

Table 6 : Acute toxicity testing

Protocol	Administration - species	Dose	Result
OECD 425	Oral - rats (n=6)	990-5000 mg/kg-bw of chitin-glucan	LD_{50} > 5000 mg/kg-bw
ISO10993-11	Intravenous – mice (n= 10)	50 ml/kg-bw of an aqueous extract of chitin-glucan[4]	LD_{50} > 50 ml/kg-bw

In addition, the applicant conducted a HET-CAM test with chitin-glucan on eye irritation in which it came out as non-irritant to the eyes.

8.2.2. Sub-acute toxicity (OECD 407)

A 28-day oral toxicity study in rats (in accordance with OECD protocol 407 and under GLP) was conducted with chitin-glucan. In this trial, the rats were given repeated doses of 0% (control group), 1 %, 5 % and 10 % of chitin-glucan in the feed. These percentages correspond to doses of 0.8, 4 and 8 g/kg body weight per day (TNO, 2009a).

No significant difference was found in terms of body weight, daily food intake, the weight of the organs and the biochemical parameters of blood and plasma. Statistically significant caecum enlargements were noted in the highest dose group (males) and two highest dose groups (females); caecal enlargement is not uncommon with large doses of fibres/poorly digestible carbohydrates, viz is considered a physiological rather than toxic response. There also was no histological anomaly observed in the organs. It was concluded from the test that chitin-glucan was not toxic, even at the highest dose (8 g/kg body weight/day).

The applicant also reported a 4-week study in which rats on a high fructose diet were administered KiOnutrime-CG at 10 % in the diet. No adverse effects were reported for KiOnutrime-CG.

8.2.3. Sub-chronic toxicity (OECD 408)

A 13-week oral toxicity study with chitin-glucan (KiOnutrime-CG™) in rats (in accordance with OECD protocol 408 and under GLP) was provided (TNO, 2009b).

Chitin-glucan was fed at constant dietary levels of 0 % (control), 1 % w/w (low-dose), 5 % w/w (mid-dose) and 10 % w/w (high-dose) to groups of 20 male and 20 female Wistar rats. These dietary levels were equal to overall mean intake levels of 0.63, 3.2 and 6.6 g chitin-glucan/kg body weight per day

[4] The extract of chitin-glucan is obtained by dispersing the chitin-glucan powder (3 g/15 mL) in a physiological buffer for 72 hours at 73°C, then harvesting the supernatant after centrifugation, in accordance with Annex A of Standard NF EN ISO-10993 part 10, page 23.

in males, and 0.68, 3.4 and 7.0 g chitin-glucan/kg body weight per day in females of the low-, mid- and high-dose group, respectively.

None of the rats died during the study and there were no treatment-related clinical signs. Body weight was not affected by the test substance. There were no treatment-related toxicological effects. As with the 28-day study, the full and empty weight of the caecum were increased in mid- and high-dose males, dose dependently, and in high-dose females, which caecal enlargement was considered a physiological response to the feeding of a high amount of poorly digestible carbohydrate. It is concluded that at an intake of 10 % of chitin-glucan in the diet, the highest dose tested, no adverse effects were observed. This dietary level was equivalent to an overall intake of 6.6 and 7.0 g chitin-glucan/kg body weight/day in males and females, respectively.

It is concluded from the animal studies (acute toxicity, subacute and subchronic studies) that these do not indicate a safety concern.

8.2.4. Allergenicity

A. niger is not currently known to be used as a direct food ingredient, but it is commonly detectible in fruits, certain vegetables, green coffee beans, onions, mango, corn and other cereals, peanuts, dried fruit products, and other food stuffs (Frisvad et al., 2007). The consumption of the organism is therefore expected to occur in the diet of most individuals.

There are some known allergens of *A. niger*: Asp n 14, Beta-Xylosidase, which enzyme is an additive used in the food industry and which presents an allergenic activity when it is inhaled (Horner et al., 1995; Kurup et al., 2000) whereas no allergenicity during the oral ingestion was listed in the scientific literature for this enzyme. Other allergens are Asp n 18, vacuolar serin protease, Pectinase; Glucoamylase, MG 66 000; Xylanase; Phytase; Cellulase; Flaviastase renamed (Doekes et al., 1999). The 3-phytase B is authorized as a feed additive and produced by *A. niger*.

An 8-year follow-up study on clinical reactions to *A. niger* was conducted in a biotechnology plant producing citric acid by fermentation of molasses with *A. niger* and belonging to Tate & Lyle. The authors concluded that *A. niger* was a weak antigen and that simple hygiene measures protect the workforce (Seaton and Wales, 1994). *A. niger* and *A. fumagitus* share some antigens and therefore cross-reactivity is possible.

For glucosamine hydrochloride, a novel food ingredient derived from a fermentation process with *A. niger*, the Panel noted that despite the fact that there was no evidence for the presence of protein in the assessed novel food ingredient, the possibility of allergenicity could not be fully excluded (EFSA, 2009). For the concerned Novel Foods ingredient "Chitin-Glucan" the specification indicate ≤ 6 % protein.

The Panel concludes that an allergenic risk cannot be ruled out, but is expected not to be higher than the consumption of other *A. niger* derived products.

8.3. Human studies

Under the supervision of the Diabetology, Nutrition and Metabolic Diseases Department, the Clinical Pharmacology Unit at Liège University Teaching Hospital has conducted a 4-week trial in 30 healthy male volunteers. Twenty volunteers received 4.5 g chitin-glucan (KiOnutrime-CG) per day and 10 were controls. There were no data on randomization or blinding of subjects. The study investigated markers for cardiovascular risk and metabolic syndrome. The study report indicated "After analysis of the study results, no toxicity of the compound was observed. All hepatic (γGT, TGO, TGP) and renal (creatine, urea) parameters were normal. Some side effects were however mentioned by some volunteers: among the 20 participants receiving KiOnutrime-CG™, seven subjects felt side effects

associated with ingestion of the compound. These gastrointestinal side effects mentioned by the volunteers were mild, transient and are common after ingestion of a certain quantity of fibers. It is noted that no side effects were reported in the control group. However, the study report did not specify what product or substance was used for the placebo. This human study was poorly reported as a small 3-page report with little detail; therefore this study is considered of very limited value to demonstrate the safety in humans.

According to the applicant, chitin-glucan has been classified as 'non-irritant to the skin' on the basis of a '24-hour occlusive single patch test' carried out on 10 volunteers, which corresponds to the lowest level of irritation, and chitin-glucan has been classified as 'non-irritant' to the skin and as 'non-sensitising' to the skin in accordance with the Marzulli-Maibach method in a 6 weeks trial on 50 volunteers (Study Report, Kitozyme, 2004).

8.4. Effect on the bioavailability of micronutrients

Some Member States had questions related to the bioavailability of micronutrients (lipids, fat-soluble vitamins and minerals).

In order to provide more information on the potential reduction of the bioavailability of nutrients, a literature review was performed by the applicant. Since no specific literature exists on chitin-glucan, the review was focused on beta-glucans of similar structure like those from plants, yeasts and mushrooms, as well as on carboxymethyl-chitin-glucan and chitin. Studies suggested that various pectins, gums, lactulose, oligofructose and indigestible sugars improve mineral bioavailability (Greger et al, 1999). However it is noted that this review addressed soluble fibre, and that chitin-glucan is an insoluble fibre.

The consumption of dietary fibre (like beta-glucans) has been reported to reduce the bioavailability of various minerals, an effect which is predominantly a function of the phytate content of the food containing beta-glucans. Therefore, the applicant has performed a titration of phytic acid in chitin-glucan in order to ensure the absence of phytate. KitoZyme shows that the mycelium of *A. niger* used for the production of chitin-glucan does not contain phytate, the concentration is below 0.05 % (m/m) which corresponds to the detection limit. *A. niger* is also reported to be a source of phytase (Pandey et al., 2001), an enzyme active against phytic acid.

Based on the scientific literature and on data provided by the applicant, particularly compositional data and the proposed intake levels, the Panel considers that the Novel Ingredient "chitin-glucan" under the proposed conditions of use has no relevant impact on bioavailability of nutrients.

8.5. Other information

In addition to the data provided on the novel food ingredient, the applicant provided literature data on compounds related to the novel food ingredient (beta-glucan, glucosamine, N-acetyl-glucosamine, chitin). Chitin-glucan is a copolymer composed of metabolites of D-glucosamine, N-acetyl-D-glucosamine and glucose. The polysaccharides related to chitin-glucan and on which toxicological data are available are chitin (of crustacean origin), chitosan (derived from chitin, of crustacean origin), beta-glucans (of vegetable and fungal origin) and oligomers of chitosan. No safety concerns arise from these data.

DISCUSSION

The applicant intends to market the Novel Food "KiOnutrime-CG™" with a content of more than 90 % chitin-glucan as a food supplement in different formats. According to the applicant it is designed to increase the daily intake of fibre. The intended intake of chitin-glucan is 2 to 5 g/day. The compositional data and the manufacturing process do not give rise to concerns.

At the highest dose administered in the 13-week study in rats, i.e. about 6.6 g/kg bw, no adverse effects were observed. This dose is approximately 80-fold higher than the max intended level of intake for humans on a g/kg bw basis. On basis of the data provided and taking into account the nature of the novel food ingredient, the Panel considers that there are no safety concerns under the proposed conditions of use.

There are no specific safety data for children but there are also no suggestions that they may be particularly susceptible to adverse effects.

CONCLUSIONS

The Panel concludes that Novel Food KiOnutrime-CG™ is safe as a food ingredient at the proposed conditions of use and the proposed intake levels.

DOCUMENTATION PROVIDED TO EFSA

1. Dossier on 'Chitin-glucan' (KiOnutrime-CG™) received on 10th September 2009. Submitted by KitoZyme. Additional data were provided on 22 of December 2009 and on 16 June 2010.
2. Letter from the European Commission to the European Food Safety Authority with the request for an opinion on the safety of 'Chitin-glucan'. SANCO E4/AK/mm (2009) D/540563, dated 27 August 2009.
3. Initial assessment report carried out by Belgium: Advisory Report of the Superior Health Council on a marketing authorization application for chitin-glucan as a novel food ingredient under Regulation EC No 258/97
4. Member States' comments and objections
5. Response by the applicant to the initial assessment report and the Member States' comments and objections

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3. WINES

3.2 CLARIFICATION OF WINE

3.2.1 FINING (OENO 7/99), (OENO 6/04), (OENO 9/04) (OIV-OENO 339A-2009), (OIV-OENO 339B-2009, OENO 417-2011)

Definition :

Clarification of wine by addition of substances that precipitate particles in suspension :
- Whether by promoting the natural sedimentation of the former, or
- By coagulating around the particles to be eliminated and by entraining them in sediments.

Objectives :

a) To complete spontaneous clarification on those occasions when it is unsatisfactory.
b) To soften red wines by removing from them some of the tannins and polyphenols.
c) To clarify wines with haze problems, stirred up lees, insoluble coloured matter, etc.

Prescriptions :

a) For clarifying agents promoting simply the sedimentation of particles, refer to Treatment with bentonites[11]
b) For the coagulating clarifying agents, only the following products are admissible: gelatin, albumin and white of egg, isinglass, skim milk, casein, alginates, colloidal solution of silicon dioxide, kaolin, potassium caseinate, proteins of plant origin, chitosan, chitin-glucan, yeast protein extracts.
c) The substances used shall to comply with the prescriptions of the International Oenological Codex.

Recommendation of the OIV :

[11] This definition applies solely to wines stricto sensu such as defined in the Part I, chap.3 "Wines" of the present Code.

Accepted.

3. WINES

3.2.2 FILTRATION (2/89)

Definition :

Physical process consisting of passing the wine through appropriate filters that retain particles in suspension.

Objectives :

a) Clarifying the wine, if necessary by successive stages (clarifying filtration) .
b) Obtaining biological stability of the wine by elimination of microorganisms (sterilising filtration).

Prescriptions :

Filtration can be undertaken :

a) By continuous deposition, using appropriate additives such as diatomaceous earth, perlite, cellulose…,
b) On pads of cellulose or other appropriate materials,
c) On mineral or organic membranes of a porosity greater than or equal to 0.2 μm (microfiltration).

The filter materials used shall comply with the prescriptions of the *International Oenological Codex .*

Recommendation of OIV:

Refer to the practices and treatments mentioned hereafter.

3. WINES

3.2.2.1 FILTRATION BY CONTINUOUS DEPOSITION (1/90)

Definition :

Filtration of the wine, after formation of a filter bed, which is then fed constantly by a continuous addition of filter material into the wine to be clarified.

Objective :

To obtain a suitable level of clarity, to a given technological stage, by the elimination of substances in suspension in the wine.

Prescriptions :

a) The nature of the filter material (such as diatomaceous earth, perlite and cellulose) and the necessary dose are determined by the turbidity of the wine and the clarification sought.
b) The filter materials used shall comply with the prescriptions of the *International Oenological Codex* .

Recommendation of OIV:

Accepted.

3. WINES

3.2.2.2 STERILISING FILTRATION (1/90)

Definition :

Filtration of wines on materials allowing the elimination of microorganisms.

Objective :

To obtain biologically stable wines for bottling.

Prescriptions :

a) The objective can be achieved by the use of the following, having regard to conditions of pressure, flow and recommended duration:
- Special prefabricated pads of cellulose or other materials ;
- Membranes having a maximum average pore diameter of 0.65 µm.

b) The filtration equipment must be sterilised beforehand by passing hot water or steam through them.
c) The materials used shall comply with the prescriptions of the *International Oenological Codex* .

Recommendation of OIV :

Accepted.

3. WINES

3.2.3 RACKING (16/70), (OENO 6/02)

Definition :

Operation involving the transfer of wine from one wine container to another by allowing the separation of solid deposits from the liquid.

Objectives:

a) Separate wine from the lees, and/or the deposits resulting from the addition of clarifying agents, deposited at the bottom of the container

b) Separate the wine from the micro-organisms at the end of the alcoholic and/or malolactic fermentation, or the bacterial or yeast sediment

c) Enable the carrying out of all wine making operations, treatment or transportation of wines.

d) Enable the tartric stabilization by cooling and the separation of tartrate crystals (potassium bitartrate and calcium tartrate).

Prescriptions :

Racking can occur:

a) Either in the absence of air to avoid oxidation,

b) Or with aeration to eliminate hydrogen sulfide or to reduce carbon dioxide or to create controlled oxidation,

c) Or at room temperature, or after cooling to avoid possible carbonic gas loss,

d) Or using the law of interconnected vessels, either with pumps or hand containers

e) In the case of racking in the absence of air, the container to be filled must be rendered inert with carbon dioxide, nitrogen or with argon. These gases must comply with International Oenological Codex prescriptions

Recommendation of OIV :

Accepted.

3. WINES

3.2.4 TREATMENT WITH SILICON DIOXIDE (1/91)

Definition :

Addition to wine of a colloidal solution (gel) of silicon dioxide coupled with the addition of a gelatin solution or, possibly, with other proteinaceous finings.

Objective :

To achieve the flocculation of the gelatin and possibly other proteinaceous finings, with a view to clarification.

Prescriptions :

a) The product is added to young white wines and rosés and occasionally to red wines.
b) Preliminary tests are necessary to determine optimal doses of the colloidal solutions of silicon dioxide and gelatin or possibly, of other proteinaceous finings.
c) The products shall comply with the prescriptions of the *International Oenological Codex .*

Recommendation of OIV:

Accepted

3. WINES

3.2.5 DECANTING (RACKING) (5/88), (OENO 1/04)

Definition :

Operation consisting of transferring from one wine tank to another:
- A still wine at normal pressure,

Objectives :

a) To separate wines from their lees, deposited on the bottom of the container.
b) To prepare for mixing or blending operations.
c) To prepare for physical clarification by filtration, centrifugation, etc.
d) To achieve bulk transportation of the wine.

Prescriptions :

Decanting can be performed:

a) Either under protection from air so as to avoid all oxidation, or
b) With aeration, to eliminate hydrogen sulphide or to reduce the carbon dioxide or to create a managed oxidation.

Recommendation of OIV:

Accepted.

3. WINES

3.2.6 TANNIN ADDITION (16/70)

Definition :

Addition of tannin to wine.

Objectives :

a) To facilitate the clarification of new wines by partial precipitation of excess proteinaceous matter.
b) To facilitate fining.

Prescription :

The tannins used shall comply with the prescriptions of the *International Oenological Codex .*

Recommendation of OIV:

Accepted.

3. WINES

3.2.7 FINING USING PROTEINS OF PLANT ORIGIN (OENO 8/04)

Objectives :

Use of protein matter of plant origin for the fining of wines in order to improve their clarity, stability and gustatory properties.

Prescriptions:

1. The doses to be used are determined after a preliminary test trial. The maximum usage dose should be less than 50 g/hl. After racking, the wines are analysed (turbidity, colour, absorbance at 280nm) and tasted. The dose retained corresponds to the sample which clarifies the wine without excess and gives a better result for tasting.

2. Proteins of plant origin can be used with other admitted products such as tannins, bentonite, silica gel

3. Proteins of plant origin must comply with the prescriptions of the *International Oenological Codex* .

Recommendation of OIV:

Accepted.

3. WINES

3.2.8 USE OF ENZYMES FOR IMPROVING FILTERABILITY (OENO 15/04)

Definition:

The addition to wine of enzymatic preparations with in particular, polygalacturonase, pectin lyase, pectinmethylesterase and/or glucanase activities that catalyse the degradation of pectic polysaccharides and/or fungal β-glucans.

Objective:

To improve the filterability of wines by specific hydrolysis of colloids.

Prescription:

The enzymes used must comply with the prescriptions of the *International Oenological Codex*.

Recommendation of OIV:

Accepted.

3. WINES

3.2.9 USE OF ENZYMES FOR THE RELEASE OF FLAVOURING SUBSTANCES (OENO 17/04)

Definition:

The addition to wine of enzymatic preparations with, in particular, glycosidase activities.

Objective:

To enhance the aromatic potential in wine, from glycosylated precursors from grapes

Prescription:

The enzymes used must comply with the prescriptions of the *International Oenological Codex*.

Recommendation of OIV:

Accepted.

3. WINES

3.2.10 USE OF ENZYMES FOR IMPROVING THE SOLUBILISATION OF YEAST COMPOUNDS (OENO 18/04)

Definition:

The addition to wine during winemaking on lees of enzymatic preparations notably with β-glucanase activities that catalyse the degradation of yeast cell walls.

Objectives:

a) To facilitate the release of yeast-soluble constituents into wine.
b) To improve the colloidal stability of wines.

Prescription:

The enzymes used must comply with the prescriptions of the *International Oenological Codex*.

Recommendation of OIV:

Accepted.

3. WINES

3.2.11 USE OF ENZYMES FOR THE CLARIFICATION OF WINES (OENO 12/04)

Definition:

The addition to wine of enzymatic preparations with in particular, polygalacturonase, pectin lyase, pectinmethylesterase and/or β-glucanase activities that catalyse the degradation of pectic polysaccharides and/or fungal β-glucans.

Objective:

To facilitate the clarification of wines.

Prescription:

The enzymes used must comply with the prescriptions of the *International Oenological Codex*.

Recommendation of the OIV

Admitted

3. WINES

3.2.12 FINING USING CHITOSAN (OIV-OENO 337A-2009)

Definition:

Addition of chitosan of fungal origin for the purpose of fining wines

Objectives:

a) To reduce turbidity by precipitating particles in suspension.
b) To carry out a treatment to prevent protein haze by the partial precipitation of excess proteinaceous matter.

Prescriptions:

a) The doses to be used are determined after preliminary testing. The maximum dose used must not exceed 100 g/hl.
b) Sediments are eliminated by physical procedures.
c) Chitosan of fungal origin may be used alone or together with other admitted products.
d) Chitosan must comply with the requirements of the International Oenological Codex.

Recommendation of the OIV

Admitted

3. WINES

3.2.13 FINING USING CHITIN-GLUCAN (OIV-OENO 337B-2009)

Definition:

Addition of chitin-glucan of fungal origin for the purpose of fining wines

Objectives:

a) To reduce turbidity by precipitating particles in suspension
b) To carry out a treatment to prevent protein haze by the partial precipitation of excess proteinaceous matter.

Prescriptions:

a) The doses to be used are determined after preliminary testing. The maximum dose used must not exceed 100 g/hl.
b) Sediments are eliminated by physical procedures.
c) Chitin-glucan of fungal origin may be used alone or together with other admitted products.
d) Chitin-glucan must comply with the requirements of the International Oenological Codex.

Recommendation of the OIV

Admitted

3. WINES

3.2.14 FINING USING YEAST PROTEIN EXTRACTS (OENO 417-2011)

Definition:

Addition of yeast protein extracts for fining wines

Objectives:

a) Reduce turbidity of wines by precipitating suspended particles
b) Preserve chromatic characteristics of wines
c) Eliminate excess tannin
d) Improve wine filterability

Prescriptions:

a) The doses used are determined beforehand by laboratory trials (fining point)
b) Maximum dose to be used as determined by an efficiency test conducted in laboratory must not exceed 60 g/hl for red wine and 30 g/hl for white and rosé wine
c) The yeast protein extracts can be used alone or associated with other authorised fining products
d) The deposits from the fining are to be eliminated from wine by physical procedures
e) The yeast protein extracts must comply with the prescriptions of the International Oenological Codex

Recommendation of the OIV:

Admitted.

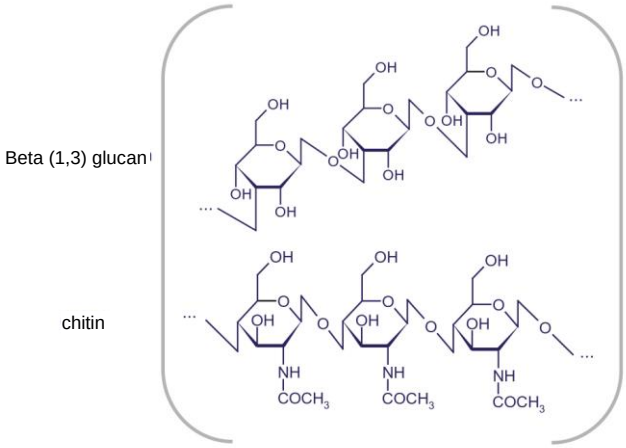
CHITIN-GLUCAN

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

CAS number Chitin: [**1398-61-4**]

CAS number β-glucan: [**9041-22-9**]

(OIV-Oeno 367-2009)



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-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

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.
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 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
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.

- 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:

- 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.

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.

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.

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
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.