Application to amend the Australian New Zealand Food Standards Code

3.1.2 Applicant Details

[redacted]

b) Winemakers Federation of Australia
c) NFF house, 14-16 Brisbane Avenue, BARTON, ACT 2600

PO Box 3891, Manuka, ACT 2603 (Postal)

[redacted]

f) Peak industry body (Not-for-profit)
g) Lallemand Australia Pty Ltd (Technical input)

3.1.3 Purpose of the application

The purpose of the application is to amend Standard 1.3.3 and Standard 4.5.1 to permit the use of fungal chitosan as a processing aid (in particular as a fining agent and for microbial stabilisation) in wine , beer and cider.

3.1.4 Justification of the application

The technical application of fungal chitosan as a processing aid has been demonstrated in a number of wine producing countries around the world and it has been approved for use in some of Australia's major competitors. In addition, under the Australia – European wine agreement, wine from Europe made using fungal chitosan can be sold in Australia, but Australian producers cannot yet use it for domestic production.

A. Regulatory impact information

1. Costs and benefits

- Consumers will benefit from better techniques to stabilise wine
- Industry will benefit from increased availability of techniques to make better quality wine, increasing quality and lowering cost of production
- There are no regulatory impacts on government and this will enhance relations with Europe by implementing in regulation the ability to use fungal chitin for wine making.

2. Impact on international trade
   This will enhance the competitiveness of Australian wine domestically and in major export markets where fungal chitin is approved for wine making. It will make Australia consistant with the standards from other major producers.

3.1.5 Information to support the application

A. Data requirements

Attached as per the requirements in Section 3.3.2

3.1.6 Assessment procedure

This is an unpaid application and we would consider it as General Level 1 procedure.

3.1.7 Confidential commercial information

There is no confidential commercial information in this application.

3.1.8 Exclusive capturable commercial benefit

The applicant is a not-for-profit organisation and has no commercial or financial interest in the application. We are not seeking approval for a proprietary product and to the best of our knowledge there is no exclusive capturable commercial benefit.

3.1.9 International and other national standards

A. Other national standards or regulations

This application is for a processing aid and therefore the Codex General Standard for Food Additives is not applicable.

Resolutions permitting the use of fungal chitosan in winemaking as a fining agent and contaminant treatment have been granted by the International Organisation of Vine and Wine (OIV/OENO 336A/2009; 337A/2009; 338A/2009; 339A/2009) (OIV, 2011) (Attachments 1-4). A monograph for fungal chitosan has been added to the International Oenological Codex by decision if the OIV general assembly dated July 2009 considering the works of the group of experts "Specifications of Oenological Products" (OIV/OENO OIV/OENO 368/2009).

B. Other national standards or regulations

The corresponding approval for use of fungal chitosan in wine products marketed within the European Union has been issued by the European Commission (EU, 2011). Since 2011, chitosan from fungal origin is therefore approved as oenological practices for clarification according to ANNEX I of REGULATION (EU) No 53/2011 and for treatment of wines.

Fungal Chitosan (from *Aspergillus niger)* is GRAS self-affirmed for use as a processing-aid in the manufacture of alcoholic beverages. This GRAS self-affirmation was notified to the offices of the U.S. Food and Drug Administration (FDA), without objection from the Agency (FDA 2011, GRAS NOTICE No. 397).

The application of fungal chitosan in the winemaking process has been approved by the National Authority of Argentina.

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.

3.1.10 Statutory declaration

Attached

3.1.11 Checklist

Attached

(4)

STATUTORY DECLARATION – AUSTRALIA

The information provided in Parts 1 to 3 must be attested to by a statutory declaration in some suitable form along the following lines:

STATUTORY DECLARATION

Statutory Declarations Act 1959

[redacted]

I, [*Name, address and occupation of person making the declaration*]

make the following declaration under the *Statutory Declarations Act 1959*:

1. the information provided in this application fully sets out the matters required
2. the information provided in this application is true to the best of my knowledge and belief
3. no information has been withheld that might prejudice this application, to the best of my knowledge and belief

I understand that a person who intentionally makes a false statement in a statutory declaration is guilty of an offence under section 11 of the *Statutory Declarations Act 1959*, and I believe that the statements in this declaration are true in every particular.

[redacted]

[*Signature of person making the declaration*]

[redacted] ADELAIDE 18 of [~~*month*~~] [~~*year*~~] SEPTEMBER 2012

[redacted]

[~~*Signature of person before whom the declaration is made*~~]*

[*Full name, qualification and address of person before whom the declaration is made (in printed letters)*]

[redacted] Policy Officer and Commissioner for taking affidavits in the State of South Australia of 12, Queen St CROYDON SA 5008

* A statutory declaration must be made before a prescribed person under the *Statutory Declarations Act 1959*, available online at http://www.frli.gov.au/ComLaw/Legislation/ActCompilation1.nsf/current/bytitle/7E3AE20F8329B422CA256F71004DB642?OpenDocument&mostrecent=1.

CHECKLIST FOR GENERAL REQUIREMENTS

This Checklist will assist you in determining if you have met the information requirements as detailed in Section 3.1 – General Requirements. All applications must include this Checklist.

General Requirements (3.1)

3.1.1 Form of application
- ☑ *Executive Summary*
- ☑ *Relevant sections of Part 3 identified*
- ☑ *Pages sequentially numbered*
- ☐ *Electronic + 2 hard copies*
- ☑ *Electronic and hard copies identical*
- ☑ *Hard copies capable of being laid flat*
- ☑ *All references provided*

3.1.2 Applicant details

3.1.3 Purpose of the application

3.1.4 Justification for the application

3.2.5 Information to support the application

3.1.6 Assessment procedure
- ☑ *General*
- ☐ *Major*
- ☐ *Minor*

☑ 3.1.7 Confidential Commercial Information
- ☐ *Confidential material separated in both electronic and hard copy*
- ☐ *Justification provided*

☑ 3.1.8 Exclusive Capturable Commercial Benefit

☑ 3.1.9 International and Other National standards

☑ 3.1.10 Statutory Declaration

☑ 3.1.11 Checklist/s provided with Application
- ☑ *3.1 Checklist*
- ☐ *Any other relevant checklists for Sections 3.2-3.7*

(6)

CHECKLIST FOR STANDARDS RELATED TO SUBSTANCES ADDED TO FOOD

This Checklist is in addition to the Checklist for Section 3.1 and will assist you in determining if you have met the information requirements as specified in Sections 3.3.1-3.3.3.

Food Additives (3.3.1)

☐	A.1 Nature and technological function information	☐	B.1 Toxicokinetics and metabolism information
☐	A.2 Identification information	☐	B.2 Toxicity information
☐	A.3 Chemical and physical properties	☐	B.3 Safety assessments from international agencies
☐	A.4 Impurity profile	☐	C.1 List of foods likely to contain the food additive
☐	A.5 Manufacturing process	☐	C.2 Proposed levels in foods
☐	A.6 Specifications	☐	C.3 Likely level of consumption
☐	A.7 Food labelling	☐	C.4 Percentage of food group to contain the food additive
☐	A.8 Analytical detection method	☐	C.5 Use in other countries (if applicable)
☐	A.9 Additional functions	☐	C.6 Where consumption has changed, information on likely consumption

Processing Aids (3.3.2)

☑	A.1 Type of processing aid	☐	C.3. Allergenicity information of enzyme (enzyme only)
☑	A.2 Identification information	☑	C.4. Overseas safety Assessment Reports
☑	A.3 Chemical and physical properties	☐	D.1 Information on source organism (enzyme from microorganism only)
☑	A.4 Manufacturing process	☐	D.2 Pathogenicity and toxicity of source microorganism (enzyme from microorganism only)
☑	A.5 Specification information	☐	D.3 Genetic stability of source organism (enzyme from microorganism only)
☑	A.6 Analytical method for detection	☐	E.1 Nature of genetic modification of source organism (enzyme from GM source microorganism)
☑	B.1 Industrial use information (chemical only)	☑	F.1 List of foods likely to contain the processing aid
☑	B.2 Information on use in other countries (chemical only)	☑	F.2 Anticipated residue levels in foods

(7)

- ☑ B.3 Toxicokinetics and metabolism information (chemical only)
- ☑ B.4 Toxicity information (chemical only)
- ☑ B.5 Safety assessments from international agencies (chemical only)
- ☐ C.1 Information on enzyme use on other countries (enzyme only)
- ☐ C.2 Toxicity information of enzyme (enzyme only)
- ☑ F.3 Information on likely level of consumption
- ☑ F.4 Percentage of food group to use processing aid
- ☑ F.5 Information on residues in foods in other countries (if available)
- ☑ F.6 Where consumption has changed, information on likely consumption

Nutritive Substances (3.3.3)

- ☐ A.1 Identification information
- ☐ A.2 Chemical and physical properties
- ☐ A.3 Impurity profile information
- ☐ A.4 Manufacturing process
- ☐ A.5 Specification information
- ☐ A.6 Analytical detection method
- ☐ A.7 Proposed food label
- ☐ B.1 Toxicokinetics and metabolism information
- ☐ B.2 Animal or human toxicity studies
- ☐ B.3 Safety assessments from international agencies
- ☐ C.1 List of food groups or foods likely to contain the nutritive substance
- ☐ C.2 Proposed maximum levels in food groups or foods
- ☐ C.3 Likely level of consumption
- ☐ C.4 Percentage of food group to use nutritive substance
- ☐ C.5 Use in other countries (if available)
- ☐ C.6 Where consumption has changed, information on likely consumption
- ☐ D.1 Nutritional purpose
- ☐ E.1 Need for nutritive substance
- ☐ E.2 Demonstrated potential deficit or health benefit
- ☐ F.1 Consumer awareness and understanding
- ☐ F.2 Actual or potential behaviour of consumers
- ☐ F.3 Demonstration of no adverse effects on any population groups

(8)

Application for a NEW PROCESSING AID:

Fungal chitosan from *Aspergillus niger*

Prepared for: Food Standards Australia New Zealand
PO Box 786
CANBERRA BC ACT 2610

Prepared by: Winemakers Federation of Australia

August, 2012

Table of Contents

Summary

Chitosan is a naturally occurring carbohydrate polymer that is widely distributed in nature (crustacean shells, fungal cell walls). It is a non-allergenic, biodegradable polysaccharide of glucosamine and N-acetylglucosamine that is derived from chitin. Fungal chitosan is obtained by deacetylation of chitin present in the cell walls of non-genetically modified *A. niger* mycelium.

Fungal chitosan from *A. niger* is proposed for use as a processing-aid in the manufacture of wine, beer, cider and spirits, as well as grain and beet derived food grade ethanol. They are proposed for use in stabilizing through their antimicrobial effect on a number of economically important microbial contaminants such as *Brettanomyces* as well as for flotation, clarification to reduce cloudiness and the content of unstable colloids, for use as fining agent in the treatment of wine, for stabilization of the color, for riddling of sparkling wine, for reducing organic and mineral contaminants in wine and spirit, and for encapsulation of fermentation yeast, lactic bacteria and nutrients. Two fungal chitosans are proposed which differ slightly in their residual glucan contents as well as in their granulometry.

Fungal chitosan as a processing aid does not fall in any of the categories of processing aids listed in FSANZ standard 1.3.3.

Evidence that the form and the amount of the processing aid perform the intended function has been demonstrated through a thesis, laboratory and industrial experimentations. These assessments have been conducted in particular during two years consecutively experiments (year 2008-2009 and 2009-2010) under derogation of the DGCCRF (the French official body supervising regulations), in the frame of the Regulation EC 423/2008 (Art. 44) and Regulation EC 606/2009, respectively.

As fining agent in the treatment of wine, for stabilization of the color, for riddling of sparkling wine, clarification, and removal of mineral and organic contaminant, fungal chitosan is added at the end of the alcoholic fermentation.

For flotation clarification from must, chitosan can be added before or during the alcoholic fermentation. In spirits, it is used before filtration and bottling to remove mineral contaminants.

For microbiological stabilization in wine, cider, and beer, chitosan can be added during all process of winemaking.

Regardless of the technological purpose, the sediments that contain the chitosan 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. Since chitosan is insoluble at slightly acidic to neutral pH levels, as well as in aqueous and ethanol solutions, it is unlikely that any residual chitosan will remain in the treated

products. High-performance liquid chromatography (HPLC) analyses for residual chitosan in wine processed with chitosan indicate that the final product is free from chitosan carry-over products up to the limit of detection of the analysis method (10 mg/L).

Therefore, the estimated intake of chitosan from all proposed technological uses can be considered as negligible. Chitosan derived from *A. niger*, was shown to be chemically and structurally equivalent to shellfish derived chitosan. The principal difference between the two chitosan preparations is the presence of small quantities of *beta*-1,3-glucans in *A. niger* sources of chitosan that are not present in shellfish chitosan. Therefore, data establishing the safety of shellfish-derived chitosan are considered relevant to the safety evaluation of fungal chitosan for the proposed food uses described herein.

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 in wine/alcoholic beverage processing for the GRAS determination.

A number of animal, human, and *in vitro* studies relevant to the safety of shellfish chitosan, which has a long history of safe use in the food supply, have been published. 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.

Shellfish derived chitosan has a long history of safe use in the food supply. It is currently approved for use as a natural food additive for general food use in Japan and Korea (Japan Food Chemical Research Foundation, 2011; KFDA, 2011), and has widespread use as a dietary supplement product in the United States, the European Union, and other regulatory jurisdictions throughout the world. Finally, fungal chitosan (derived from *Agaricus bisporus* and *Aspergillus niger* sources) has been granted Novel Food approval by the European Commission, for use in supplement products in the European Union based on its substantial equivalence to existing shellfish derived chitosan products that are currently in the market[1].

Resolutions permitting the use of fungal chitosan in winemaking as a fining agent and contaminant treatment have been granted by the International Organisation of Vine and Wine (OIV/OENO 336A/2009; 337A/2009; 338A/2009; 339A/2009) (OIV, 2011) (Attachments 1-4).

A monograph for fungal chitosan has been added to the International Oenological Codex by decision if the OIV general assembly dated July 2009 considering the works of the group of experts "Specifications of Oenological Products" (OIV/OENO OIV/OENO 368/2009).

The corresponding approval for use of fungal chitosan in wine products marketed within the European Union has been issued by the European Commission (EU, 2011). Since 2011, chitosan from fungal origin is therefore approved as oenological practices for clarification according to ANNEX I of REGULATION (EU) No 53/2011 and for treatment of wines.

Fungal Chitosan (from *Aspergillus niger)* is GRAS self-affirmed for use as a processing-aid in the manufacture of alcoholic beverages. This GRAS self-affirmation was notified to the offices of the U.S. Food and Drug Administration (FDA), without objection from the Agency (FDA 2011, GRAS NOTICE No. 397).

The application of fungal chitosan in the winemaking process has been approved by the National Authority of Argentina.

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.

A. Technical information on the processing aid

1. Information on the type of processing aid

1.1 Description

Chitosan is a linear polysaccharide of glucosamine and N-acetylglucosamine that is derived from chitin, a naturally occurring carbohydrate polymer that is widely distributed in nature (crustacean shells, fungal cell walls). Fungal chitosan is obtained by deacetylation of chitin present in the cell walls of non-genetically modified *A. niger* mycelium. Small quantities (10 to 15% wt/wt maximum) of *beta*-1,3-D-glucans also are present in the fungal chitosan as residuals from the manufacturing process. *beta*-1,3-D-Glucans are a major constituent of the cell walls of fungi, and also are present as structural components of many edible vegetables (Ko and Lin, 2004). *beta*-1,3-D-Glucans are composed of linear polysaccharide chains of varying molecular weight and can be linear (vegetable, and *A. niger* sources) or branched (Baker's yeast).

Fungal chitosan as a processing aid does not fall in any of the categories of processing aids listed in Standard 1.3.3. Evidence that the form and the amount of the processing aid perform the intended function has been demonstrated through laboratory and industrial experimentations. Report on industrial tests on chitosan from fungal source as a technological auxiliary on must and wine are enclosed in Appendix 1. These assessments have been conducted two years consecutively (year 2008-2009 and 2009-2010) under derogation of the DGCCRF (the French official body supervising such experiments) in France, in the frame of the Regulation EC 423/2008 (Art. 44) and Regulation EC 606/2009, respectively.

Fungal chitosan from *A. niger* is proposed for use as a processing-aid in the manufacture of wine, beer, cider, and spirits, as well as grain and beet derived food grade ethanol. Two fungal chitosans are proposed which differ slightly in their residual glucan contents as well as in their granulometry. There are proposed for use in stabilizing wine, cider and beer through their antimicrobial effect on a number of economically important microbial contaminants such as *Brettanomyces* as well as for flotation, clarification to reduce cloudiness and the content of unstable colloids, for use as fining agent in the treatment of wine, for stabilization of the color, for riddling of sparkling wine, for reducing organic and mineral contaminants in wine and spirit, and for encapsulation of fermentation yeast, lactic bacteria and nutrients. Proposed food uses and use-levels of chitosan are detailed below in Table 3.2-1. The amount (use-levels) proposed are maximum use levels for the technological function to be achieved

Table 1.2-1 Summary of the Individual Proposed Technological Food-Uses and Use-Levels for Chitosan*

Food Category	Proposed Food-Uses	Technological Use	Use-Level (%)
Beverages, Alcoholic	Wine	Microbiological stabilization	10 g/hl
	Wine	Removal of organic contaminants	500 g/hl
	Wine	Removal of mineral contaminants	100 g/hl
	Wine	Clarification wine or in bottle (riddling)	100 g/hl
	Wine	Encapsulation of fermentation yeast lactic bacteria and nutrients	5%[2]
	Spirit	Removal of mineral contaminants	100 g/hl
	Cider	Microbiological stabilization	10 g/hl
	Beer	Microbiological stabilization	10 g/hl*
	Beer	Clarification	100 g/hl*

*the use levels are given for information purpose but chitosan is removed after treatment by physical separation process

As fining agent in the treatment of wine, for stabilization of the color, for riddling of sparkling wine, clarification, and removal of mineral and organic contaminant, fungal chitosan is added at the end of the alcoholic fermentation.

For flotation clarification from must, chitosan can be added before or during the alcoholic fermentation. In spirits, it is used before filtration and bottling to remove mineral contaminants.

For microbiological stabilization in wine, cider, and beer, chitosan can be added during all process of winemaking. There is no analytical data available on residual chitosan after treatment of beer and cider.

[2] The 5% for encapsulation is based on data collected from the literature for food applications. The purpose is to use chitosan as an encapsule for sensitive molecules like lactic bacteria, yeast and yeast nutrients. This application has not been experimented yet and is provided as an example of other possible applications of chitosan in wine. The application is given for information only and there is not data on residual levels, although as chitosan is insoluble it will all be filtered out.

However, the product is expected to behave similarly in beer and cider than it behaves in wine. It is expected to be insoluble in beer and in cider as it is in wine and to be used at the same dose levels. The product is also removed after treatment by physical separation leaving behind negligible residual levels. Some tests have been performed on ciders showing the efficacy of the product to decrease the contamination of Brettanomyces. The product has been removed from cider by physical separation. No measurement of residual chitosan has been performed.

Regardless of the technological purpose, the sediments that contain the chitosan 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. Since chitosan is insoluble at slightly acidic to neutral pH levels, as well as in aqueous and ethanol solutions, it is unlikely that any residual chitosan will remain in the treated products. High-performance liquid chromatography (HPLC) analyses for residual chitosan in wine processed with chitosan indicate that the final product is free from chitosan carry-over products up to the limit of detection of the analysis method (10 mg/L).

Therefore, the estimated intake of chitosan from all proposed technological uses can be considered as negligible. Chitosan derived from *A. niger*, was shown to be chemically and structurally equivalent to shellfish derived chitosan. The principal difference between the two chitosan preparations is the presence of small quantities of *beta*-1,3-glucans in *A. niger* sources of chitosan that are not present in shellfish chitosan. Therefore, data establishing the safety of shellfish-derived chitosan are considered relevant to the safety evaluation of fungal chitosan for the proposed food uses described herein.

2. Information on the identity of the processing aid

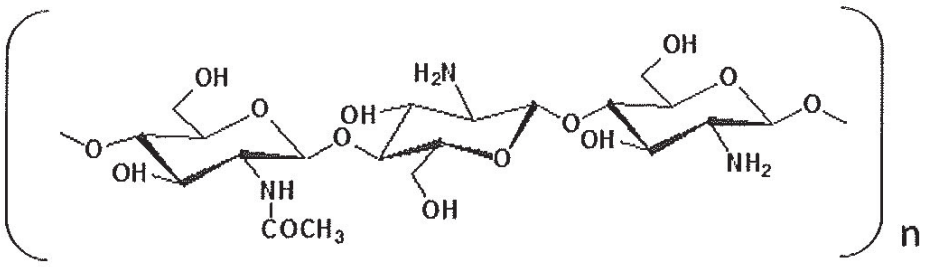
2.1 Chemical Abstracts name

2-Amino-2-deoxy-poly-D-glucosamine

2.2 International Union for Pure and Applied Chemistry name:

(2R, 3R, 4R, 5S, 6R) 3-amino-6-(hydroxymethyl) oxane-2, 4, 5-triol

2.3 Structural formula:



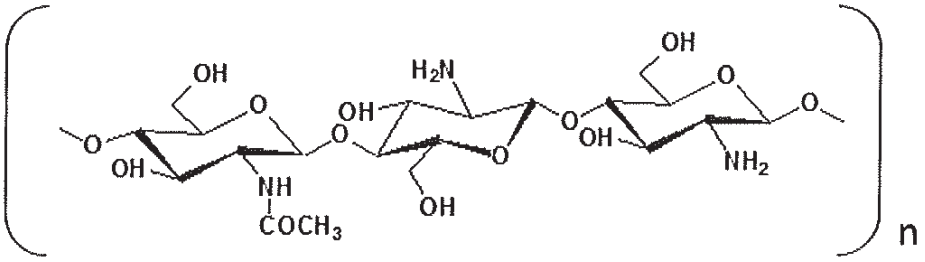
2.4 Common Name:
Fungal Chitosan

2.5 Manufacturer's code:
Tariff code: 3913900099

2.6 Marketing name:
At the moment fungal chitosan to be used in microbiological stabilization of wines is marketed under KiOfine-B, No Brett Inside but could be marketed also by third party distributors under their trade-names.
The fungal chitosan to be used in other wine applications (clarification, fining, removal of contaminants) is in final marketing development and trade-names are under final selection.

2.7 Chemical Abstract Service (CAS) Number
Chitosan: 9012-76-4

2.8 Chemical and Physical Characteristics

Molecular formula	Chitosan: $(C_6H_{11}NO_4)_n$
Chemical Structure	Chitosan 
Viscosity	Mv of 10-15K (Mv= viscosimetric molecular weight)
Appearance and Odor	Odorless, off white to slightly brownish fine free-flowing powder.

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

Fungal chitosan products are provided as an odorless, off white to slightly brownish fine free-flowing powder with a tapped density >0.7g/cm^3. The two products differ in terms of their granulometry, i.e. 90% less than 50 µm and 90% less than 500 µm, in relation with their intended use. Except for the

content of residual beta-glucans that slightly differ, the two products have an overall similar chemical composition and share the same specifications (see Table 5).

Chemical properties of chitosan are as follows: linear polyamine, presence of amino and hydroxyl groups,chelating properties (metal ions). The physicochemical properties of chitosan in solution are related to the degree of deacetylation and molecular weight.

As fining agent in the treatment of wine, for stabilization of the color, for riddling of sparkling wine, clarification, and removal of mineral and organic contaminant, fungal chitosan is added at the end of the alcoholic fermentation. For flotation clarification from must, chitosan can be added before or during the alcoholic fermentation. In spirits, it is used before filtration and bottling to remove mineral contaminants. For microbiological stabilization in wine, cider, and beer, chitosan can be added during all process of winemaking for fermentation yeast, lactic bacteria, and nutrients encapsulated within chitosan, it can be used during all process of winemaking

Regardless of the technological purpose, the sediments that contain the chitosan are removed from the wine, must, or spirits at the end of the treatment by physical separation processes such as racking, centrifugation, or filtration. Since chitosan is insoluble at slightly acidic to neutral pH levels, as well as in aqueous and ethanol solutions, it is unlikely that any residual chitosan will remain in the treated products. High-performance liquid chromatography (HPLC) analyses for residual chitosan in wine processed with chitosan indicate that the final product is free from chitosan carry-over products up to the limit of detection of the analysis method (10 mg/L) (see Appendix 2 for results).

Therefore, the estimated intake of chitosan from all proposed technological uses can be considered as negligible. For example, using a conservative estimate of chitosan carry-over into wine of 10 mg/L (detection limit of HPLC analysis), the consumption of two 750 mL bottles of wine per day would result in exposures chitosan exposures of 15 mg per person, equivalent to 214 µg/kg bodyweight for a 70 kg individual. This intake estimate represents a gross overestimate of exposure to chitosan under the proposed food uses. Moreover, shellfish derived chitosan is widely available in the food supply through use in dietary supplement products, and industrial, pharmaceutical (excipient), agricultural (pesticide), 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 described in Table 1.2-1, calculation of estimated intakes was not deemed necessary in the assessment of the safety of the material under the proposed food uses in wine/alcoholic beverage processing for the GRAS determination.

4. Manufacturing process

4.1 Raw Material

Fungal chitosan is manufactured from the non-viable post-fermentation microbial biomass of non-genetically modified *A. niger,* which is sourced from manufacturers of food grade citric acid and is permitted for use in feed applications in the European Union. Commercial sources of *A. niger* used for production of chitosan are subject to strict quality control for compliance with specifications as described in Table 4.1.1. Sources of *A. niger* used for manufacture of chitosan are authorized for sale for

use in animal feed and conform to the European legislation (EC Directives 2002/32/EC, 2003/57/EC, and 2003/100/EC) regarding contaminants (heavy metals and aflatoxins), and pesticide residues (EC, 2002, 2003a,b). All raw materials and processing aids used in the manufacture of chitosan are suitable food-grade materials and are used in accordance with applicable U.S. federal regulations, and/or are permitted for use in food as described in Table 4.1.2 below.

Table 4.1.1 Quality Control Specifications for *A. niger* Raw Material Used for Production of Chitosan

Analysis Parameter	Specification
Water Content (%)	≤10%
Ash (% dw)	≤2%
Protein (%)	≤10%
Lipids (%)	≤1%
Density (kg/m^3)	≤670
Metals (ppm) As Hg Pb Cu Cd Zn	 ND ND ≤0.3 ≤ 0.3 - 4.5 ≤0.2 ≤17 – 20
Bacteria cfu/g	≤1000
Yeasts and Molds cfu/g	≤10

dw = dry weight; ND = not detected

Table 4.1.2 Raw Materials and Processing Aids Used in the Manufacture of Chitosan

Material	Use	Regulatory Status
Aspergillus niger	Source of chitin	Permitted for use in the production of citric acid for use in food in the United States (21 CFR §173.280 – U.S. FDA, 2011)
Processing-Aids		
Water	Solvent	N/A
Sodium Hydroxide	Processing-aid	In accordance with 21 CFR §184.1763, sodium hydroxide is permitted for use in food as a processing aid with no limitations other than cGMP (U.S. FDA, 2011).
Acetic acid	Processing-aid	In accordance with 21 CFR §184.1005, acetic acid is a direct food substance affirmed as generally recognized as safe (U.S. FDA, 2011).

N/A = Not applicable

4.2 Method of Manufacture

Fungal chitosans are obtained through the deacetylation (*i.e.,* hydrolysis of acetyl groups) of chitin present within the cell wall of *A. niger* mycelium. For the manufacture of chitosan, the manufacturer follows the Belgian legislation relative to auto-control in the food industry[3] and 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 Hazard Analysis and Critical Control Points (HACCP) method[4]. A schematic of the manufacturing process is described below (Figure 4.2-1). Briefly, *A. niger* raw material, meeting quality control standards, is introduced to a stainless steel vessel, and the material is subject to thermal alkali hydrolysis (sodium hydroxide) conditions which result in the partial deacetylation of chitin as well as the hydrolysis of native proteins and nucleic acids, and saponification of cellular lipids. Following several wash steps, the chitosan is solubilized using acetic acid, the acid insoluble constituents are removed through filtration and washing, and the chitosan is then precipitated using sodium hydroxide. The chitosan is then washed, concentrated, and dried. The dried material is held in quarantine for quality control testing prior to packaging and storage. Two chitosan are produced. During manufacturing, one is subject to an additional filtration step which results in a reduction in the amount of insoluble *beta*-(1,3)-D-glucans in the material relative, rendering the ingredient more suitable for use in microbial growth control applications; otherwise the materials are compositionally indistinguishable.

[3] Arrêté Royal 27/04/2007; Arrêté Royal 24/10/2005; Arrêté Royal 22/01/2004; Arrêté Royal 14/11/2003 (AFSCA, 2011)

[4] http://www.naredi.be/engels/home.htm (Naredi, 2011)

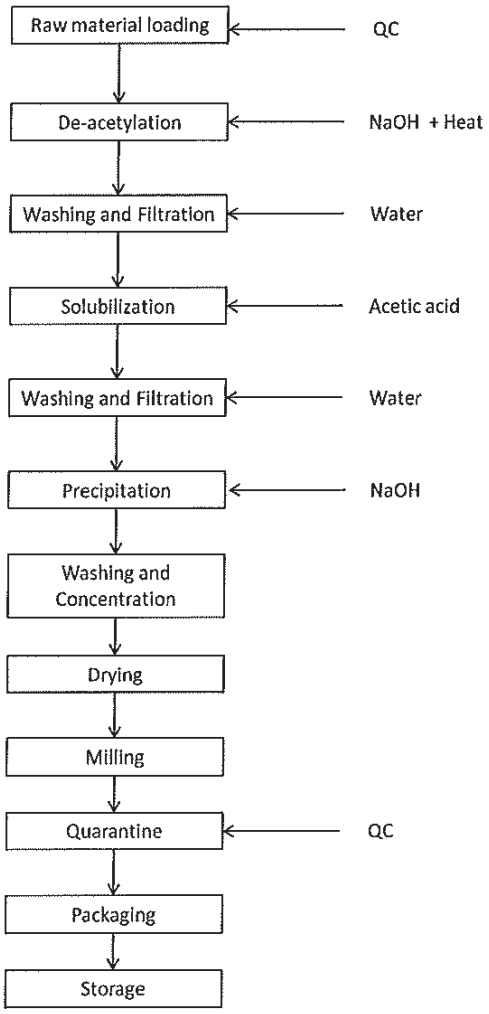


Figure 4.2.-1 Schematic Overview of the Manufacturing Process for Chitosan

The processing aid has been tested for the presence of the food allergens including sulphites, milk (including lactose, casein), eggs, peanuts, nuts, almonds, pistachio, gluten, soy, celery, mustard, sesame seeds, lupin, mollusks, crustaceans, fish. Results are negative (or at concentration below the detection limit) which confirms that the manufacturing process does not carry-over any allergens.

5. Specification for identity and purity

5.1. Product specifications

The detailed product specifications for fungal chitosan are provided in Table 5 below. Analytical methods are also described below. Some methods are internal analytical methods developed and validated by the manufacturer.

Table 5 Product Specifications for fungal chitosan

Test Parameters/Test	Specification	
Identification-Composition		
Degree of acetylation (mol%)	0 – 30	
Residual glucans (%, w/w)	≥2	
Viscosity 1% in HAc 1% (mPa.s)	1 – 15	
Taped density (g/cm^3)	≥0.7	
Loss on drying	≤10	
Ash (%, w/w)	≤3	
Soluble residues (%, w/w)	≤5	
Heavy Metals		
Mercury (mg/kg)	≤0.1	
Lead (mg/kg)	≤1	
Arsenic (mg/kg)	≤1	
Cadmium (mg/kg)	≤1	
Chromium (mg/kg)	≤10	
Zinc (mg/kg)	≤50	
Iron (mg/kg)	≤100	
Copper (mg/kg)	≤30	
Microbial		
Aerobic microbial count (cfu/g)	<1000	
Yeasts and moulds (cfu/g)	<100	
Coliforms (cfu/g)	<100	
Enterobacteriaceae (cfu/g)	<10	
Salmonella	Absence/25 g	

In addition to these specifications, information on purity and particle size can also be provided. The purity, refereed here is the chitosan content which is calculated on the basis of the sample dried weight by removing the values of glucans, proteins and ashes from the sample dried weight.

The specification on the particle size is ≥ 90% less than 50 µm for the first product and ≥90% less than 500 µm for the other one.

5.2 Analytical methods

Test methods are briefly described below.

5.2.1 Degree of acetylation

The degree of acetylation of is determined by potentiometric titration, using. The method relies on the titration of hydrochloric acid in excess to amino groups of chitosan using sodium hydroxide. Briefly, chitosan is dissolved in an excess of dilute hydrochloric acid prepared from hydrochloric R solution. The solution is then titrated with dilute sodium hydroxide prepared from sodium hydroxide R solution, using an automatic titrator (KEM, automatic potentiometric titrator, AT-500N), and the pH is measured. The titration curve shows two inflection points. The difference between these two inflection points among the abscissa corresponds to the amount of acid required to protonate the amino groups of chitosan.

5.2.2 Residual glucans

The determination of residual beta-glucan is performed by UV spectrophotometry (Thermospectronic Biomate 3. Briefly, beta-glucan is thermally decomposed by addition of hot sulphuric acid R, leading to

hydroxymethylfurfural moieties that are able to react with phenol and form a yellowish to brown colored product which absorbs at a wavelength of 420 nm. The absorption intensity of the solution is then compared to an external calibration curve established with a reference oat beta-glucan (medium viscosity, lot #60501, Megazyme) using the same method.

5.2.3 Viscosity

The apparent viscosity of the chitosan solution is measured using a calibrated rotational viscometer (Brookfield digital viscosimeter model DV-II+pro) at controlled temperature, using an appropriate spindle, spindle rotation speed and a temperature-controlled bath.

5.2.4 Loss on drying

The loss on drying of is determined thermogravimetrically. Briefly, a known quantity of sample is heated at 105°C, and the sample weight loss is continuously measured using a calibrated moisture analyser (Ohaus MB 45) until reaching a value less than 1mg per 90s. When this value is reached, the weight of dry matter is calculated by removing the loss on drying value from the total weight.

5.2.5. Ash

The total ash content is determined. The porcelain crucible is weighed. A known quantity of chitosan is placed in the porcelain crucible and heated for 10hrs at 600°C in a calibrated muffle oven (Carbolite, 201). After combustion, porcelain crucible containing the chitosan sample is weighed.

5.2.6. Soluble residues

A known quantity of chitosan is washed with water and filtered. The residual matter is then filtered and weighed.

5.2.7. Mercury, Lead, Arsenic, Cadmium, Chromium, Zinc, Iron Copper

The individual content of metals is determined by ICP-MS derived from the ISO 11885 standard.

5.2.8. Total viable aerobic microbial count

The total viable aerobic microbial count is determined according to the ISO 4833:2003 standard, an horizontal method for the enumeration of microorganisms, by counting the colonies growing in a solid medium after aerobic incubation at 30°C.

5.2.9. Total yeasts and molds count

The total yeasts and molds count is determined according to the ISO 7954 standard, a horizontal method for the enumeration of yeasts and molds, by counting the colonies growing in a solid medium after aerobic incubation.

5.2.10. Coliformes

The amount of *Escherichia coli* is determined according to the ISO7251 standard, a horizontal method for the enumeration of beta-glucuronidase-positive *Escherichia coli* (Colony-count technique at 44°C using 5-bromo-4-chloro-3-indolyl beta-D-glucuronide)

5.2.11. Enterobacteriaceae

The amount of *Enterobacteriaceae* is determined according to the ISO 215-28-2 standard, a horizontal method for the detection and enumeration of *Enterobacteriaceae* (Colony-count method).

5.2.12. Salmonella

The amount of *Salmonella* is determined according to the ISO 6579 standard, a horizontal method for the detection of *Salmonella*, including *Salmonella typhi* and *Salmonella paratyphi*

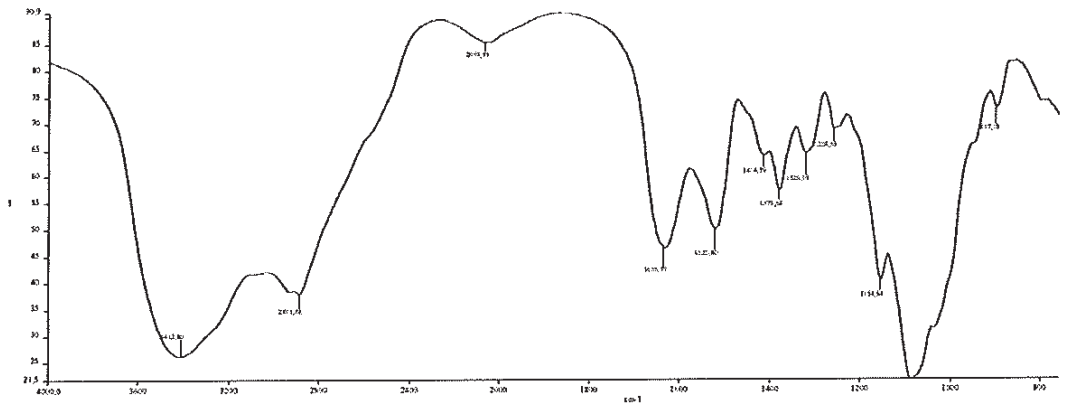
5.3 Analytical method for identification

The methods for identification of chitosan are reported below.

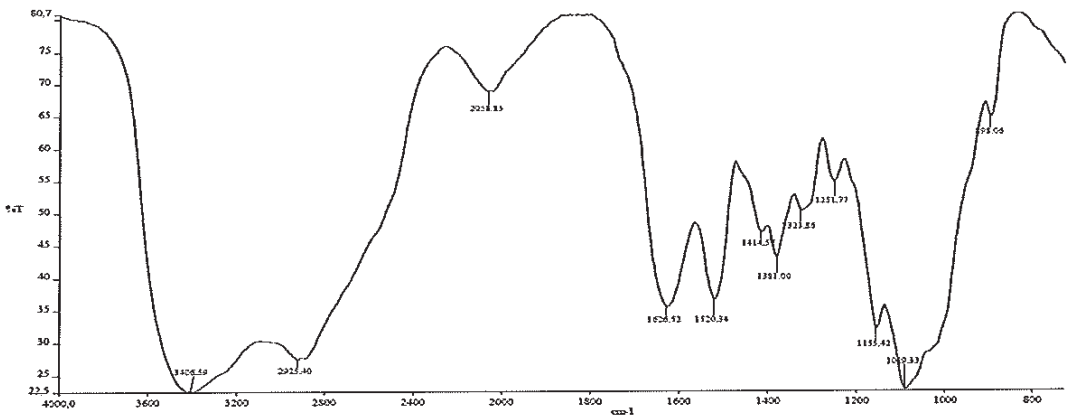
5.3.1. Infrared Spectroscopy Analysis

Infrared (IR) spectroscopy is one of the most common methods used for chemical characterization of chitosan (Kumirska *et al.*, 2010). Using Fourier Infrared Transmission (FTIR) spectroscopy chemical analysis, shellfish chitosan sources were compared to *A. niger* derived chitosan. Two shellfish sources of chitosan were used for the comparison: a commercial food grade source of chitosan currently on the market for use in food, and supplement products throughout the world; and chitosan chlorhydrate, a qualified European Pharmacopoeia chitosan standard (Ph. Eur., 2004). As shown in Figure 2.2.8-1 below, all three sources of chitosan produced comparable FTIR absorption spectral profiles, supporting the chemical equivalence of *A. niger* derived chitosan sources to shellfish sources.

A – European Pharmacopoeia Standard Chitosan Chlorhydrate



B – *A. niger* Derived Chitosan (fungal chitosan)



C – Shellfish Derived Chitosan (Food grade)

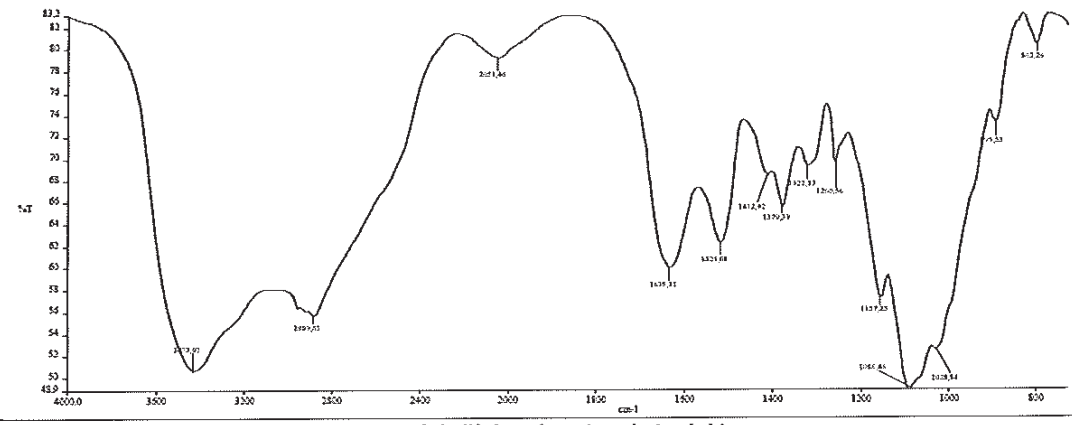


Figure 2.2.8-1 FTIR spectral comparison of shellfish and *A. niger* derived chitosan

Each peak within the absorption spectrum corresponds to the vibration frequency of a component of the molecule, which allows for quantitative differentiation of bond types within chemically similar materials. **A)** European Pharmacopoeia chitosan chlorohydrate standard (shellfish derived); **B)** commercial food grade chitosan (shellfish derived); **C)** *A. niger* derived chitosan Analysis conducted in accordance with the Ph.Eur. monograph of chitosan chlorhydrate 1774.

5.3.2 NMR Spectroscopy

A. niger derived chitosan was further characterized using Nuclear Magnetic Resonance (NMR) spectroscopy of carbon 13 in the solid state (13C-NMR), a technique that is well suited for use with polysaccharides such as chitosan (Kumirska *et al.*, 2010). As depicted in Figure 2.2.8-2, the NMR spectrum generated for *A. niger* derived chitosan was equivalent to that of shellfish derived chitosan. The two additional peaks in the NMR spectrum *A. niger* derived chitosan were identified to be a carboxyl group (173 ppm) and methyl group (23 ppm) of the N-acetylglucosamine units.

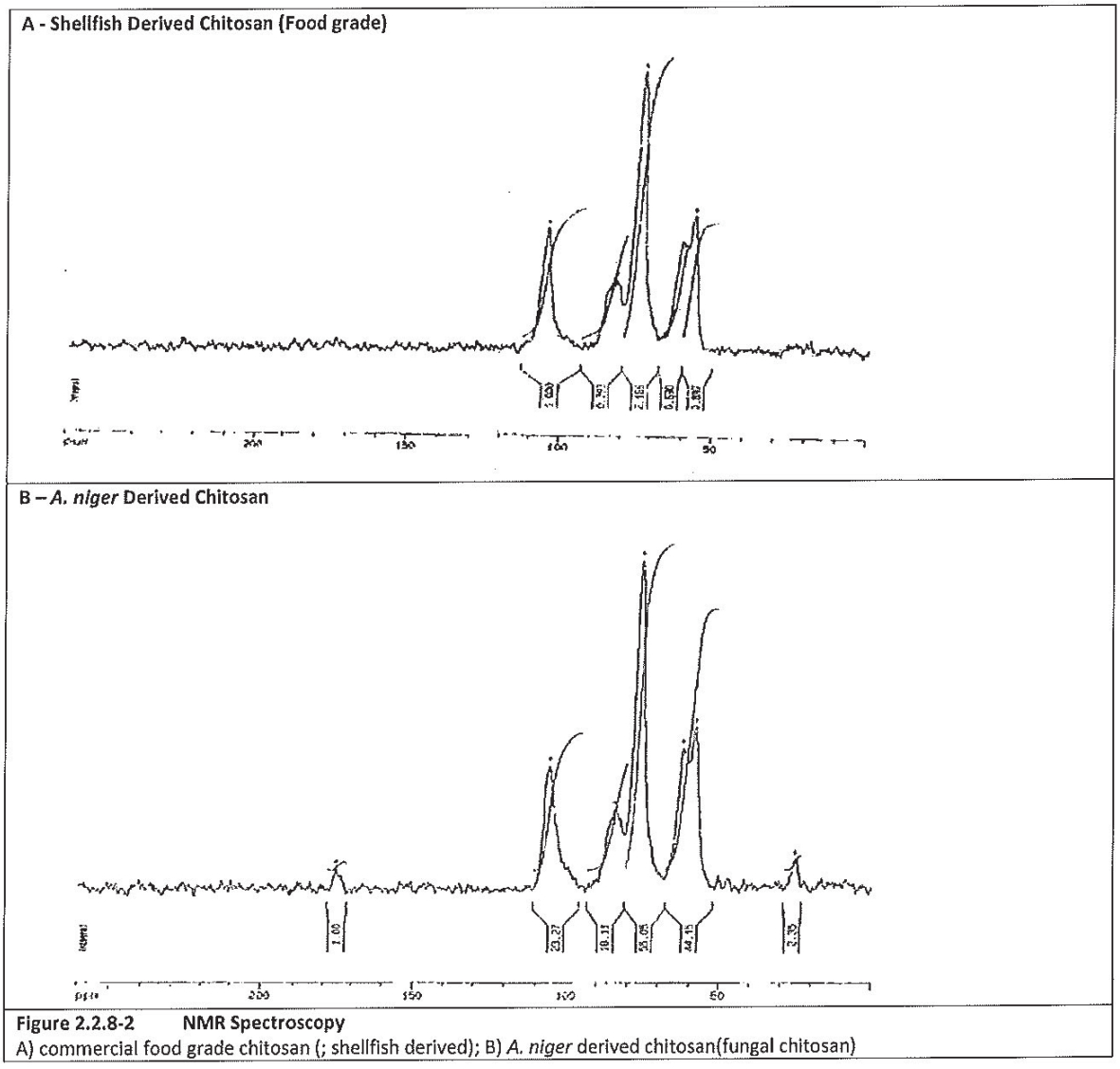


Figure 2.2.8-2 NMR Spectroscopy
A) commercial food grade chitosan (; shellfish derived); B) *A. niger* derived chitosan(fungal chitosan)

6. Analytical method for detection

Since no residues are likely to be present in the wine after treatment, there is no need to provide analytical method for detection and quantification of the amount of processing aid remaining in the wine. It should however be pointed out the HPLC (High Performance Liquid Chromatography) may be suitable method for detection of chitosan, although the limit of detection is quite high in regard with the use levels.

B/ Information related to the safety of a chemical processing aid

B1. General information on the non-food industrial use of the chemical

Due to its physical and chemical properties, chitosan is being used in a wide variety of product and applications going from pharmceutical, medical and cosmetics products to water treatment and plant protection. In pharmaceutical applications, chitosan is used as an excipient for drug delivery systems. In medical devices, it is uses as for its haemostatic, wound healing and antibacterial properties in different forms (sponges, granules, bandages, fibers, sutures...). In cosmetics, chitosan is used for hair care (in shampoos...), skin care (in body washes, creams, lotions...) and oral care (in toothpastes, mouthwashes, chew-gum...) to prevent formation of plaque. Other industrial applications of chitosan include water engineering (flocculating agent, chelating agent and heavy metal trappers), textile and paper industry; and agriculture. Considerable amount of chitosan are used in waste water treatment. For all these applications, shellfish derived chitosan has been used so far since fungal chitosan has been developed recently.

A number of animal, human, and *in vitro* studies relevant to the safety of shellfish chitosan, which has a long history of safe use in the food supply, have been published. Chitosan derived from *A. niger*, was shown to be chemically and structurally equivalent to shellfish derived chitosan (section 5.3). The principal difference between the two chitosan preparations is the presence of small quantities of *beta*-1,3-glucans in *A. niger* sources of chitosan that are not present in shellfish chitosan. Therefore, data establishing the safety of shellfish-derived chitosan are considered relevant to the safety evaluation of fungal chitosan for the proposed food uses described herein.

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 summarized below. Generally available studies conducted in adult subjects have evaluated the safety and tolerability of repeated consumption of chitosan, and multiple studies investigating the effects of consuming shellfish-derived chitosan on various biological parameters (*e.g.*, plasma lipid levels, mineral and vitamin absorption, weight gain, sugar metabolism) have been reported.

Shellfish derived chitosan has a long history of safe use in the food supply. It is currently approved for use as a natural food additive for general food use in Japan and Korea (Japan Food Chemical Research Foundation, 2011; KFDA, 2011), and has widespread use as a dietary supplement product in the United States, the European Union, and other regulatory jurisdictions throughout the world. Supplement products typically promote consumption of 1 to 2 g/person/ day for use in weight control, and/or maintenance of cardiovascular health. Several chitosan hydrolysate formulations also are approved for use as a plant growth enhancer, and as a substance that boosts the ability of plants to defend against fungal infections. Exemption from the requirement of a tolerance requirement for these applications has been granted by the United States Environmental Protection Agency (Proposed final rule; U.S. EPA, 2008).

Finally, fungal chitosan (derived from *Agaricus bisporus* and *Aspergillus niger* sources) has been granted Novel Food approval by the European Commission, for use in supplement products in the European Union based on its substantial equivalence to existing shellfish derived chitosan products that are currently in the market[5].

Below are general information relevant to safety of chitosan as reported in the literature for shellfishes-derived chitosan.

1.1 Metabolic Fate

A limited number of studies have examined the metabolic fate of chitosan. Similar to dietary fibers, chitosan is poorly absorbed, is not subject to digestive processes within the gastrointestinal tract, and therefore travels intact throughout the small intestine to the colon where it is then subject to microbial fermentation and excretion in the feces. These findings have been summarized in brief below.

1.2.1 Absorption and Distribution

Chitosan is poorly absorbed due its highly insoluble physico-chemical properties (reviewed in Kean and Thanou, 2010). Upon ingestion, chitosan is solubilized by hydrochloric acid in the stomach and converted into a viscous liquid that emulsifies dietary fat droplets. As this viscous chitosan gel enters the duodenum, it starts to precipitate due to the gradual increase in pH and is excreted in the feces (described in Furda 2000). *In vitro* cellular models have also provided additional evidence that chitosan is poorly absorbed. Chitosan with molecular weight of 30 kDa or higher was not taken up by intestinal epithelial Caco-2 cells (Schipper *et al.*, 1997). A subsequent study also demonstrated water-soluble chitosans of 230 kDa did not penetrate through the Caco-2 cell layer (Chae *et al.*, 2005).

Zeng *et al.* (2008a) labeled chitosan with FITC (fluorescein isothiocyanate) and administered it to female Kunming mice *via* oral gavage. Following a dose of 500 mg/kg of FITC-chitosan, blood samples were collected at 30, 60, 120, and 240 minutes. Four preparations of chitosans were used: chitosan oligomer (MW=0.99 kDa); middle molecular weight chitosan (M-chitosan, MW=32.7 kDa), water-soluble chitosan (MW=39.1 kDa); and high molecular weight chitosan (H-chitosan, MW=760 kDa). The authors reported that the extent of chitosan absorption was inversely related to its molecular weight, and occurred in the following rank order: H-chitosan < M-chitosan < chitosan oligosaccharide. Water-soluble chitosan had the greatest amount absorbed compared to all of the compounds tested despite its moderate molecular weight, which may be attributed to its greater water solubility. In another study, FITC-labeled chitosan was orally administered to Sprague-Dawley rats at a dose of 20 mg/kg (Chae *et al.*, 2005). Chitosans, ranging from 3.8 to 230 kDa, were detected in the plasma with levels peaking at 30 minutes after oral administration. However, high molecular weight chitosan (230 kDa) was not absorbed and negligible amounts were detected in the plasma. Furthermore, the area-under-the-curve (AUC) values for the 3.8 kDa chitosan was nearly 12 times higher than that for 22 kDa chitosan.

[5] Notification #108 Pursuant to Article 5 of Regulation (EC) No 258/97 of the European Parliament and of the Council (April 2007) (EC, 1997, 2007)

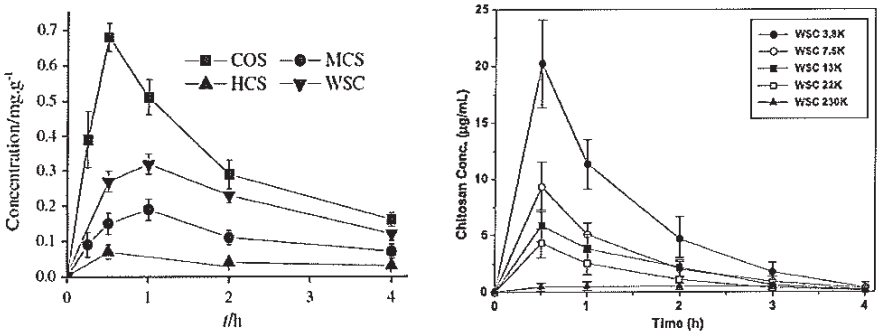


Figure 1.2.1-1 Plasma concentration following oral administration of chitosan.
Data were taken from Zeng *et al.*, 2008a (left) and Chae *et al.*, 2005 (right). COS = chitosan oligosaccharide; MCS = middle molecular weight chitosan; HCS = high molecular weight chitosan; WSC = water-soluble chitosan. WSC 3.8K to WSC 2430K refers to WSC ranging in size from 3.8 to 230 kDa.

Interpretation of these findings is complicated by several limitations in study design. For example, FITC labeling of chitosan is known to be highly variable, with coupling efficiency ranging between 24 to 91% (Ma *et al.*, 2008). This variability is due to the fact that isothiocyanate reacts optimally with neutral amine groups at pH 9.0, and most chitosan are insoluble when amine groups are neutral at pH ≥6.5, preventing homogenous labeling. Also, no control groups were used in the studies by Chae *et al.* (2005) or Zeng *et al.* (2008a), and non-specific binding of the FITC label may have occurred. Thus, the significance of the authors' findings must await confirmation using other sensitive assays.

1.2.2 Metabolism

The human digestive tract can efficiently hydrolyze glucose polymers linked by *alpha*-glycosidic linkages, such as those found in starch and glycogen, and the *beta*-glycosidic bond in lactose can be hydrolyzed by *beta*-galactosidase (Wisker *et al.*, 1985). The *beta*-glycosidic bonds in chitosan are resistant to hydrolyzation by hydrochloric acid present in the stomach; however, these bonds can be hydrolyzed by chitosanases to produce a mixture of chitosan oligomers ranging between 2 and 8 degrees of polymerization, which can be subsequently degraded further to glucosamine (reviewed in Shaikh and Deshpande, 1993; Muzzarelli *et al.*, 1997).

Chitin can also be hydrolyzed by chitinases (reviewed in Shaikh and Deshpande, 1993; Muzzarelli *et al.*, 1997). Although traditionally thought to be chitin-specific, non-specific chitinases have been shown to hydrolyze chitosan (reviewed in Aam *et al.*, 2010). At least 2 functional chitinases have been identified in humans; chitotriosidase is unlikely to be involved in the digestion of ingested chitosan as it is expressed primarily in the lymph nodes, bone marrow, and lungs. Acidic mammalian chitinase (AMCase) have been isolated in the human stomach, tears, sinus mucosa, and lungs, though its activity on chitosan has yet to be characterized.

Recent *in vitro* studies have reported that other digestive enzymes, including pepsin, amylase, and lipase, can hydrolyze chitosan at rates comparable to chitosanase (reviewed in Xia *et al.*, 2008). However, it is unknown whether these enzymes can effectively hydrolyze chitosan in the human gastrointestinal tract. Lysozyme, in addition to hydrolyzing the glycosidic linkages of bacterial cell wall peptidoglycans, can also

hydrolyze chitosan and chitin (Aam *et al.*, 2010). Varum *et al.* (1997) incubated three different chitosan with different degrees of deacetylation (42, 51, and 60%) in human serum, and measured degradation rates by changes in viscosity as a function of time. The degradation rate increased proportionally with the degree of deacetylation. Addition of lysozyme increased degradation rates, while addition of allosamidin, a chitinase inhibitor, had no effect. The authors concluded that the degradation of chitosan in human serum is mediated primarily by lysozyme and not other enzymes or depolymerizing mechanisms. In another study, chitosan with approximately 70% deacetylation was found to have the greatest susceptibility to lysozyme degradation (Sashiwa *et al.*, 1990).

1.2.3 Fermentation by Intestinal Microflora

Bacteria are known to express chitosanases (Gooday, 1989), and it is plausible that the intestinal microflora may also possess the ability to hydrolyze chitosan. Although it is unknown whether the intestinal microflora in humans expresses chitosanases or other enzymes that can degrade chitosan, rat bacterial enzymes isolated from the colon were able to degrade chitosan *in vitro* (Zhang and Neau, 2002). In another study examining the utility of chitosan as an excipient for the delivery of peptide drugs, pills containing 5(6)-carboxyfluorescein (CF) that were encapsulated by chitosan were not degraded when incubated with artificial gastric or intestinal juice, but its contents were released when incubated with rat cecal contents (Tozaki *et al.*, 1997). Moreover, oral administration of chitosan encapsulated pills containing insulin resulted in the systemic absorption of insulin, with the hypoglycemic effects observed as the capsules entered the colon.

Although it is unknown whether bacterial strain with chitosanase activity exists in the human intestines, *Clostridium paraputrificum* was isolated from human feces containing endochitinase and *beta*-N-acetylglucosaminidase activity. *In vitro* cultivation of the bacteria with colloidal chitin resulted in the production of hydrogen, carbon dioxide, acetate, and lactate, as well as minute quantities of propionate and butyrate (Simunek *et al.*, 2002).

1.2.4 Elimination

Onishi *et al.* (1999) administered FITC-labeled chitosan (50% deacetylation) intraperitoneally (29 mg/kg body weight) to 3 male ddy mice. Urine was collected at 1, 14, and 24 hours following chitosan administration; approximately 25% of the dose was excreted in the urine within 1 hour, and nearly the entire chitosan dose was accounted for in the urine by 14 hours. In addition, Richardson *et al.* (1999) administered [^{125}I]-labeled chitosan of various molecular weights (<5 kDa, between 5 to 10 kDa, and >10 kDa) intravenously to male Wistar rats. Chitosan with molecular weights >5 kDa was rapidly cleared from the plasma. At 60 minutes following injection, <10% of the administered dose recovered in the plasma while more than 50% of the administered dose was recovered in the liver. In contrast, approximately 30% of chitosan with smaller molecular weight (<5 kDa) was recovered in the plasma, and approximately 30% was found in the liver, at 60 minutes post-injection.

As a conclusion, neither chitosan nor *beta*-1,3-D-glucans are absorbed from the gastrointestinal tract, thus systemic exposure does not occur. Some evidence that small molecular weight chitosan preparations (*i.e.*, chitosan oligomers, ~1kDa) may be absorbed intact in mice was reported. However, these studies were confounded by poor study design and the use of non-qualitative analytical methods

for detection of chitosan oligosaccharides/metabolites in the plasma, limiting the reliability and usefulness of the data. Moreover, monomeric constituents of chitosan (*i.e.* glucosamine and N-acetylglucosamine) are known to be poorly absorbed in most animals species tested (Simon *et al.*, 2011), which contradicts literature reports that chitosan oligomers may be bioavailable.

Although there is no evidence presented in the literature to suggest that chitosan would be digested/hydrolyzed during gastrointestinal transit, putative hydrolysis products generated during transit would consist of compounds (chitosan oligomers, glucosamine, N-acetylglucosamine and glucose) that are known to be poorly bioavailable, and non-toxic even when consumed at high dietary concentrations in animals and humans (Lee *et al.*, 2004; Anderson *et al.*, 2005; Takahashi *et al.*, 2009). Therefore it can be concluded that neither chitosan nor *beta*-1,3-D-glucans would be chemically altered (metabolized) in the human gastrointestinal tract; limited colonic metabolism of chitosan by endogenous microflora could potentially occur. Microbial fermentation of chitosan and *beta*-1,3-glucans would produce innocuous metabolites of fermentation such as short-chain fatty acids, and H_2, CO_2, and CH_4 gases. Exposure to these metabolites occurs daily from the consumption of non-digestible dietary fiber.

B2. General information on the use of the chemical as a food processing aid in other countries:

2.1. OIV Resolution and Monograph

Resolutions permitting the use of fungal chitosan in winemaking as a fining agent and contaminant treatment have been granted by the International Organisation of Vine and Wine (OIV/OENO 336A/2009; 337A/2009; 338A/2009; 339A/2009) (OIV, 2011) (Attachments 1-4)

A monograph for fungal chitosan has been added to the International Oenological Codex by decision if the OIV general assembly dated July 2009 considering the works of the group of experts "Specifications of Oenological Products" (OIV/OENO OIV/OENO 368/2009) (Attachment 5).

In appendix the following documents are attached:

- Appendix 3 Resolution OIV oeno 336A-2009

- Appendix 4 Resolution OIV oeno 337A-2009

- Appendix 5 Resolution OIV oeno 338A-2009

- Appendix 6 Resolution OIV oeno 339A-2009

- Appendix 7 Resolution OIV oeno 368-2009

2.2. Approval in European Union

The corresponding approval for use of fungal chitosan in wine products marketed within the European Union has been issued by the European Commission (EU, 2011).

Since 2011, chitosan from fungal origin is therefore approved as oenological practices for clarification according to ANNEX I of REGULATION (EU) No 53/2011 and for treatment of wines under the conditions set up in Appendix 13. Commission Regulation CE 53/2011 (ENG) is provided in Appendix 8.

2.3 Approval in US

Fungal Chitosan (from *Aspergillus niger)* is GRAS self-affirmed for use as a processing-aid in the manufacture of alcoholic beverages. This GRAS self-affirmation was notified to the offices of the U.S. Food and Drug Administration (FDA), without objection from the Agency (FDA 2011, GRAS NOTICE No. 397, Appendix 9)

2.4 Approval in Argentina:

The application of fungal chitosan has been approved by the National Authority (Appendix 10).

3. Information on the toxicity of the chemical processing aid and if necessary its majors metabolites

Unless specified, information reported below has been collected from the literature on shellfish chitosan, or chitosan oligomers.

3.1 Acute Toxicity Studies

The acute oral toxicity of fungal chitosan, (KiOmedine-CsU[6]), was examined in female Sprague-Dawley rats (6/group). A single dose of 2,000 mg/kg body weight or vehicle control was administered by gavage and animals were followed for 14 days after administration. No clinical signs were presented and no changes were observed by macroscopic examination. The LD_{50} was determined to be greater than 2,000 mg/kg body weight in this study (KitoZyme 2008a).

The acute systemic toxicity of fungal chitosan was also examined in male Swiss mice (5/group). The polar extract of KiOmedine-CsU Chitosan was administered intravenously at a dose of 50 mL/kg body weight, and no clinical signs were observed up to 3 days after administration. As such, the LD_{50} of KiOmedine-CsU Chitosan was determined to be greater than 50 mL/kg body weight in this study (KitoZyme 2008b).

An additional study was identified in the literature that examined the acute toxicity of chitosan oligosaccharide in Kunming mice (Qin *et al.*, 2006). Mice of both sexes were administered a single dose of chitosan oligosaccharide (molecular weight of 1.86 kDa) *via* oral gavage at doses of 0, 1,000, 2,150, 4,640, or 10,000 mg/kg body weight and monitored for 7 days after treatment. Mice administered chitosan oligosaccharide did not exhibit any clinical signs of toxicity and no mortalities were recorded. The authors concluded that the oral maximum tolerant dose was greater than 10,000 mg/kg in mice.

3.2 Subacute Toxicity Study

Chitosan Oligosaccharides

Kim *et al.* (2001) evaluated the subacute toxicity of chitosan oligosaccharide in rats. The study was not conducted using GLP, and study methodologies were not reported to be consistent with recognized U.S., or International guidelines for toxicity testing of chemicals. Five-week-old male and female SPF Sprague-Dawley rats (n=9/sex/group) were administered 0, 500, 1,000, or 2,000 mg chitosan oligosaccharide/kg body weight/day by gavage for 28 consecutive days. Body weight and feed consumption were monitored weekly during the administration period. After the administration period, rats were killed by exsanguination under phenobarbitol anesthesia. No significant between-group differences at any time point were observed with respect to feed intake, body weight, clinical signs, or mortality. All urinalysis parameters measured at necropsy (*i.e.,* color, pH, and concentrations of glucose, ketone bodies, nitrites, protein, occult blood, urobilinogen, and bilirubin) were within normal ranges with the exception of increased mean leukocyte concentrations in the urine of male rats administered 500 mg chitosan oligosaccharide/kg body weight/day. However, this finding was not considered to be toxicologically relevant as it was observed only in males, and its occurrence was not dose-related.

Hematological analysis revealed significantly increased mean leukocyte concentration in male rats administered 2,000 mg chitosan oligosaccharide/kg body weight/day (compared to controls) and significantly decreased percentage of granulocytes in female rats administered 1,000 mg chitosan oligosaccharide/kg body weight/day. These changes were within normal ranges and were therefore not considered to be toxicologically relevant. A significant increase in mean platelet volume in male rats administered 1,000 mg chitosan oligosaccharide/kg body weight/day was reported, but its toxicological significance was not addressed by the study authors. However, given that increased mean platelet volume was observed in males only, and not in a dose-dependent manner, this finding is unlikely to be toxicologically significant. No significant differences between the treated and control groups were observed for the remaining hematological parameters assessed (*i.e.,* red blood cell count, hemoglobin, hematocrit, mean corpuscular volume, platelet count, lymphocytes, and mid-range population).

Significantly decreased albumin and total blood protein concentrations were observed in male rats administered 500 mg chitosan oligosaccharide/kg body weight/day (compared to controls), although this difference was not considered to be toxicologically relevant due to a lack of a dose-response relationship. No significant between-group differences were observed for any other biochemical parameter assessed (*i.e.,* alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, blood urea nitrogen, lactate dehydrogenase, glucose, and triglycerides). In addition, no significant between-group differences attributable to treatment with chitosan oligosaccharide were reported with respect to organ weights or histopathological findings. The authors determined the no-observed-adverse-effect level (NOAEL) to be 2,000 mg/kg body weight/day in rats, the highest dose tested in this study.

3.3 Subchronic Toxicity Studies

Chitosan Oligosaccharides

One 90-day subchronic toxicity study was conducted in four week old female Kunming mice (10/group), weighing 20 to 26 g (Zeng *et al.*, 2008b). Four different types of chitosan with different molecular weights were prepared, including high molecular weight chitosan (H-chitosan; MW=760 kDa), middle molecular weight chitosan (M-chitosan; MW=32.7 kDa), water-soluble chitosan (MW=39.1 kDa) and chitosan oligomer (MW=0.99 kDa). Animals were fed a control diet or diet supplemented with chitosan at 1.05% provided *ad libitum* for 90 days, equivalent to intakes of approximately 500 mg/kg bw/day (U.S. FDA, 1993). Food intake was monitored daily, and animals were weighed and checked for signs of toxicity weekly. Upon termination of the study, the vital organs were removed for gross and histopathological examination. The levels of trace elements were also measured in the organs. No mortality and no significant clinical signs (*i.e.* food intake, abnormal behaviors and state of feces or fur) were observed in all treatment groups. No differences in body weight were observed among the chitosan treatment groups when compared to controls. Relative organ weights of the heart, liver, spleen, thymus, and kidney were not altered in the H-chitosan, M-chitosan, and chitosan oligomer treatment groups. However, relative thymus weight was significantly increased by 46% for animals in the water-soluble chitosan group compared to controls (0.22 *vs.* 0.15 g/100 g body weight). Gross examination of the organs did not reveal any treatment-related abnormalities, and histopathological findings were normal in all treatment groups. Trace levels of iron, zinc, and copper in the liver, heart, spleen and kidneys of animals in the H-chitosan, water-soluble chitosan, and chitosan oligomer groups did not differ from controls. However, compared to controls, animals in the M-chitosan group exhibited significantly higher iron levels in the liver (168 *vs.* 146 µg/g; $p < 0.05$) and spleen (958 *vs.* 843 µg/g; $p < 0.05$); significantly higher zinc levels in the liver (55 *vs.*32 µg/g; $p < 0.05$), spleen (45 *vs.*32 µg/g; $p < 0.05$), and heart (66 *vs.* 38 µg/g; $p < 0.05$); and copper levels in the liver (5.4 *vs.* 4.9 µg/g; $p < 0.05$). The authors attributed this finding to the greater accumulation of M-chitosan in the liver and other organs where they exert metal-chelating properties. Although oral administration of chitosan for 90-days did not produce explicit adverse effects, the use of a single dose of each test article and failure to adhere to GLP and OECD guidelines for toxicity testing present limitations to interpretation of the data.

Naito *et al.* (2007) conducted a 90-day toxicity study to determine the safety profile of oligoglucosamine (OG)[7], in rats. Six-week-old male and female F344 rats (10/sex/group) were given *ad libitum* access to food containing 0, 0.04, 0.2, or 1% OG for 90 days. The study was carried out in accordance with the Guidelines for Designation of Food Additives and for Revision of Standards of Use of Food Additives of the Japanese Ministry of Health, Labour and Welfare (Notification Eika No.29, March 22nd, 1996). No mortality was observed during the study period, and no abnormal clinical signs were observed in the control or 0.2% groups. Animals administered the 1% dose of OG developed clinical signs including swelling of the snout, auricals and forelimbs, alopecia of the forelimbs, and emaciation. All adverse clinical signs disappeared by the end of the study period, with the exception of piloerection. The observed erythema and loss of fur was considered by the study authors to be due to topical inflammation due to OG adhering to the skin and fur rather than systemic effects of OG ingestion. The study authors also proposed that OG may inhibit the absorption of vitamin E in the small intestine, potentially exacerbating any existing skin irritation.

[7] Chitosan oligosaccharide with 100% deacetylation

From Study Day 22 until the end of the study period, mean body weights of males and females in the 1% group were significantly lower than those of the control group. The study authors attributed this effect to feeding difficulties caused by the observed concomitant dermatitis. In males in the 1% group, feed consumption was significantly reduced by compared to control starting from Study Week 3 until the end of the study period ($p < 0.01$). In females in the 1% group, feed consumption was significantly reduced compared to control only on Study Days 15, 22, and 29 ($p < 0.01$).

Significantly more males in the 1% group were positive for proteinuria, ketone bodies, and bilirubin compared to the control group. Urinary volume also was significantly increased in males in the 1% group, and sodium, potassium, and chloride excretion were significantly decreased compared to controls. Males in the 0.2% group had significantly decreased urinary sodium and potassium excretion compared to controls, although concentrations remained within historical ranges. Females in the 1% group had significantly decreased levels of urinary sodium excretion, and significantly more females in the 1% group showed traces of urinary protein and ketone bodies compared to controls. No abnormalities were detected upon histological examination, and the increases in urinary protein are not likely to be indicative of renal dysfunction. In addition, the study authors suggested that the observed increases in urinary ketone bodies may have been due to increased use of body fat to compensate for reduced feed intake. No other significant differences with respect to urinary parameters (*i.e.,* color, pH, occult blood, red blood cells, crystals, casts, white blood cells, and epithelial cells) were reported.

In males in the 1% group, significant decreases in hemoglobin, and counts of red blood cells, lymphocytes, and platelets, and increases in mean corpuscular volume, mean corpuscular hemoglobin, and neutrophil count, compared to controls, were reported. In males in the 0.2% group, a significant decrease in red blood cell count (which remained within background control range), and significant increases in mean corpuscular volume and mean corpuscular hemoglobin, were reported. The only significant differences compared to controls in females were an increase in neutrophil count and a decrease in lymphocyte count. Changes in mean corpuscular volume and hemoglobin were within background control ranges, and were not considered to be toxicologically significant. The authors noted that changes in leukocyte and platelet counts may have been caused by the dermal inflammation. No other significant differences with respect to hematological parameters (*i.e.,* hematocrit, mean corpuscular hemoglobin concentration, eosinophils, basophils, and monocytes) were reported.

In males in the 1% group, significant decreases in blood levels of total protein, albumin, albumin:globulin ratio, creatinine, glucose, total cholesterol, triglycerides, calcium, and sodium compared to controls were reported. Albumin:globulin ratios were significantly reduced in dose-dependent manner in males in the 0.2 and 0.04% groups. However, these changes were within background control ranges and not accompanied by changes in albumin levels, and were therefore not considered to be toxicologically relevant. In females, significant decreases in blood levels of albumin, albumin:globulin ratio, calcium, and sodium were observed in the 1% group only. The study authors noted that the observed changes in blood biochemical parameters may have been attributable to reduced feed intake in the 1% group, and that reduced cholesterol levels may have been due to chitosan-induced increases in fecal cholesterol excretion. The toxicological relevance of the observed changes in creatinine concentration is unknown due to the absence of pathological kidney or liver abnormalities.

In males in the 1% group, significant decreases in most absolute organ weights (*i.e.,* thymus, heart, lungs, liver, spleen, kidneys, testes, prostate, and seminal vesicles), and significant increases in most organ weights relative to body weight (*i.e.,* brain, heart, lungs, kidneys, testes, and submaxillary, pituitary, thyroid, and adrenal glands) were observed. In addition, males in the 0.2% group had significantly increased absolute and relative pituitary gland weight. In females in the 1% group, significant reductions in several absolute organ weights (*i.e.,* thymus, liver, spleen, uterus, ovaries, and pituitary gland), and significant increases in several relative organ weights (*i.e.,* brain, heart, lungs, kidneys, and adrenal glands) were observed. Relative liver weights were significantly decreased in 0.04% group females. In females in the 0.2 and 1% groups, increased absolute and relative submaxillary gland weights were observed (compared to controls), although the relevance of this finding was not elaborated. The study authors attributed the decreased absolute and increased relative organ weights to malnutrition-induced suppression of body weight gain.

Significant microscopic abnormalities of the spleen, with significantly fewer male animals in the 1% group having extramedullary hematopoesis of the spleen compared to controls. Abnormal histopathology was also seen in the thymus, with animals developing degeneration or necrosis of the lymphocytes in the cortex of the thymus. No significant microscopic findings were reported for female animals. The study authors suggested that the observed effects on the spleen and thymus in the 1% group may have been secondary to malnutrition and/or decreased blood lymphocyte counts. In males, significantly more animals in the 1% group showed evidence of testicular toxicity (*i.e.,* sertoli cell vacuolization, unilaterally decreased germ cell production, and luminar cell debris). The study authors suggested that the observed testicular effects may have been due to decreased absorption of zinc and vitamins A and E, deficiencies of which have been reported to induce testicular atrophy and inhibit spermatogenesis.

Unilateral corneal opacities were observed in 1 male in each of the 0.04, 0.2, and 1% groups. Failure of mydriasis with synechia, increased light reflection by the retina, and distension of the eyeball also were observed in the animal in the 1% group that displayed corneal opacities. In addition, unilateral increases in light reflection by the retina were observed in 1 male in the 0.04% group, and 1 female in each of the control and 0.2% groups, and lens opacity was observed in 1 female in the 0.04% group. In the 0.04% group, enlargement of the left eye in 1 male, and opacity in the right eye in 1 female were observed, although these findings were considered to be incidental. Although the study authors noted that no significant differences in macroscopic eye examination were observed, the toxicological significance of the ophthalmological findings were not mentioned.

The authors concluded that "from these results, oligoglucosamine gave rise to no adverse effects in rats up to the dose level of 0.2 (w/w)%. Thus, the no-observed-adverse-effect level was determined to be 0.2(w/w) % for rats of either sex (124.0 mg/kg/day in males, 142.0 mg/kg/day in females)"

3.4 Developmental and Reproductive Toxicity Studies

Chitosan

The developmental and reproductive effects of chitosan have not been fully explored. A study was conducted where B6C3F1 female mice (15/group) that were induced to ovulate were orally administered

water-soluble chitosan (approximately 300 kDa; >90% deacetylation), at daily doses of 480 mg/kg body weight/day for 4 days (Choi *et al.*, 2002). Chitosan treatment did not have any effects on the oocyte and fertilization rates in animals fed a standard control diet. In contrast, chitosan treatment increased the number of ovulated oocytes and normal oocytes, as well as the *in vivo* and *in vitro* fertilization rates, compared to controls in animals fed a high-fat diet. The authors suggested that chitosan "might improve the functions of the ovary and the oviduct in obese mice".

3.5 Chitosan Oligosaccharide

In a study by Yoon et al., (2005), 4 generations of ICR mice ingested approximately 10 mg/kg bw/day of chitosan oligosaccharide *via* drinking water for up to 180 days. Though developmental and reproductive toxicity endpoints were not specifically examined in the study, no adverse effects were reported in all generations. Male and female ICR mice of the parental generation were provided with drinking water containing 0.1% chitosan oligosaccharide (equivalent to approximately 1 µg chitosan oligosaccharide/kg body weight/day) for 30 days. It was not indicated whether a control group was included in the parental generation. Subsequent generations (referred to as F1, F2, and F3 generations) were provided drinking water containing 0, 0.01, 0.1, or 1% chitosan oligosaccharide (equivalent to approximately 0, 0.1, 1, or 10 µg chitosan oligosaccharide/kg body weight/day) for 7, 60, or 180 days. Timing and conditions of mating and killing of animals were not specified (age of parental generation at mating was not specified, although animals were purchased at 8 to 10 weeks of age). Following the experimental periods, bone marrow was taken from the femur of each mouse and used to assess the formation of chromosomal aberrations. The authors noted that no significant differences with respect to chromosomal aberrations were observed between any of the treated groups compared to the control group. Other adverse effects or safety parameters were not assessed.

3.6 Chronic Toxicity Studies

The chronic toxicity of chitosan was evaluated by the National Toxicology Program in US (NTP, 2009). Shellfish chitosan was administered to Sprague-Dawley rats (10/sex/group) in the feed at dietary concentrations of 0, 1, 3, or 9% chitosan, equivalent to calculated intakes of 0, 500, 1,500, or 4,500 mg/kg body weight/day (U.S. FDA, 1993). Although the final study report by NTP has not been published, tabulated data/findings are publically available. The NTP reported that total body weight was reduced by approximately 10% in both male and female animals fed the highest dose of chitosan (no statistical testing described); no notable differences or trends towards chitosan related effects on body weight were observed for the other doses. In the absence of between group differences in feed consumption the observation of reduced weight gain is likely attributed to caloric dilution occurring from the addition of large dietary concentrations of non-caloric fiber to the diet. Statistically significant differences in the incidences of various non-neoplastic lesions were reported between select treatment groups and controls. The incidence of hematopoietic cell proliferation in the liver was significantly higher in males - but not females - treated with 9% chitosan compared to control animals (60% *vs.* 20% respectively). The incidence of kidney nephropathy was significantly lower in females treated with 9% chitosan compared to controls (0% *vs.* 50%), though similar incidence was observed in animals treated with 1% and 3% as the controls, and no difference were reported in males. Females treated with 9% chitosan also had significantly lower incidence of fatty changes in the periportal liver compared to controls (0% *vs.* 70%), and animals in the 1% and 3% treatment group also trended to lower incidences (40%). However, such effects were not observed in males. The differences in the incidence of these non-neoplastic lesions are unlikely to be biologically relevant given the lack of dose-response and inconsistencies between sexes.

No neoplastic lesions attributed to chitosan were reported. A NOAEL determination has not been reported by the NTP at this time.

3.7 Genotoxicity/Mutagenicity Studies

(i) In vitro: Ames

The genotoxic/mutagenic potential and the anti-genotoxic/anti-mutagenic potential of chitosan were examined in both *in vitro* and *in vivo* assays. These studies are summarized in Table IV.E-1. The Ames reverse mutation test was conducted using fungal chitosan (KiOmedine-CsU), in accordance with OECD Guideline 471 for the Testing of Chemicals (OECD, 1997). Chitosan (at doses up to 1,000 µg/plate) did not increase the number of revertent colonies in 4 strains of *Salmonella* Typhimurium (TA98, TA100, TA1535, TA1537) and in 1 *Escherichia coli* WP2 strain (pKM101), in the presence and absence of metabolic activation by S9 fractions (KitoZyme, 2008c). In another study, chitosan oligomers (0.5, 5, 50, 500 and 5000 µg/plate) tested negative in the Ames reverse mutation test conducted in 4 *Salmonella* Typhimurium strains (TA97, TA98, TA100 and TA102), in presence and absence of metabolic activation by S9 fractions (Qin *et al.*, 2006).

(ii) In vivo: Micronucleus

It was also reported that chitosan oligosaccharide tested negative for the mouse bone marrow micronucleus test in Kunming male and female mice (5/group/sex), as well as the mouse sperm abnormality test in Kunming male mice (5/group), following oral gavage of a single dose of up to 5,000 mg/kg body weight (Qin *et al.*, 2006). These studies were conducted in accordance with standard protocols set by the Ministry of Health of the People's Republic of China (2003). Yoon *et al.* (2005) examined the incidence of micronuclei formation and chromosomal aberrations in ICR male mice following exposure to chitosan oligosaccharide (0, 0.01, 0.1, and 1% w/v) through drinking water for up to 180 days. No differences in micronuclei frequency and chromosomal aberrations were observed between treated and control mice. The authors estimated the 1% (w/v) dose of chitosan oligosaccharide is equivalent to exposure of 10 mg/kg body weight/day. Furthermore, no differences in chromosomal aberrations were observed between treated and control mice in the F1, F2, and F3 generations also exposed to chitosan oligomers for up to 180 days under the same experimental paradigm. The authors did not mention following any guidelines or whether the studies were GLP-compliant.

Together, these *in vitro* and *in vivo* studies provide evidence that chitosan does not have mutagenic or genotoxic effects.

Table 3.7-1 Summary of Genotoxicity Studies for Chitosan

Model	Assay	Test article	Dose and route of administration	Findings	Reference
In vitro					
Salmonella typhimurium	• Ames test	Chitosan derived from fungal source	0, 10, 33,100, 333, 1000 µg/plate	• Ames test revealed no significant differences in revertant colonies	KitoZyme internal data (unpublished)-Kitozyme2008c
Salmonella typhimurium	• Ames test	Chitosan oligomer Source: shrimp DAC: 85% MW: 1.86 kDa	0, 0.5, 5, 50, 500, 5000 µg/plate	• Ames test revealed no significant differences in revertant colonies	Qin *et al.*, 2006
A. cepa Human lymphocyte cell cultures	• *A. cepa* assay for chromosome damage • Cytogenetic assay	Chitosan polymerized with poly(methacrylic acid) nanoparticles Source: NR DAC: 94% MW: 71.3 kDa	1.8, 19, 180 mg/L	• No differences in mean mitotic index values in *A. cepa* test • No numerical or structural changes in chromosomes	De Lima *et al.*, 2010
Euglena gracilis	• *E. gracilis* mutagenicity assay	N-carboxyethyl derivatives of chitosan Source: NR DAC: NR MW: 150 kDa	10, 50, 100, 200 µg/mL	• N-carboxyethyl chitosan did not cause formation of mutant colonies at any concentration tested • No change in cell viability observed • Co-treatment of carboxyethyl chitosan protected against acridine orange genotoxicity	Kogan *et al.*, 2004
In vivo					
Male ICR mice (20/group)	• Bone marrow micronuclei test • Chromosome aberration test (4 generations)	Chitosan oligomer Source: NR DAC: 90% MW: <10 kDa	0, 0.01%, 0.1%, 1% dietary chitosan oligosaccharide administered for 7, 60 and 180 days	• No differences in formation of micronuclei in bone marrow cells • No differences in chromosome aberrations in parents and F1-3	Yoon *et al.*, 2005

Table 3.7-1 Summary of Genotoxicity Studies for Chitosan					
Model	**Assay**	**Test article**	**Dose and route of administration**	**Findings**	**Reference**
Kunming mice (5/sex/group)	• Micronucleus test	Chitosan oligomer (single dose) Source: shrimp DAC: 85% MW: 1.86 kDa	• 1.2, 2.5, 5 g/kg bw by oral gavage	• No differences in frequencies of micronucleus in mice	Qin *et al.*, 2006
Kunming male mice /group)	• Mouse sperm abnormality test	Chitosan oligomer (single dose) Source: shrimp DAC: 85% MW: 1.86 kDa	• 1.2, 2.5, 5 g/kg bw by oral gavage	• No differences in frequency of mouse sperm abnormalities	Qin *et al.*, 2006
Anti-genotoxic properties					
Chinese hamster lung cells (CHL)	• Sister chromatid exchange	Chitin and chitosan	20 mg/mL	• Chitin and chitosan was anti-genotoxic when co-treated with 4-nitroquinoline N-oxide, dinitropyrene, mitomycin C, or adriamycin	Ohe, 1996
Female CF1 mice (12 to 13 per group)	• Determination of aberrant crypts and proliferative indices in colon	Low molecular weight chitosan (LMWC) Source: NR DAC: 80% MW: 20 kDA High molecular weight chitosan (HMWC) Source: NR DAC: 80% MW: 20 kDA	Pretreatment with azoxymethane (known colon-specific carcinogen) for 2 weeks (i.p.) Diets supplemented with 2% LMWC or HMWC for 6 weeks	• 2% HMWC significantly decreased number of aberrant crypt foci, and decreased crypt height and circumference, in mice exposed to azoxymethane • 2% LMWC decreased (not significant) number of aberrant crypt foci in mice exposed to azoxymethane • 2% LMWC and HMWC significantly decreased number of mitotic figures per crypt in azoxymethane treated mice	Torzsas *et al.*, 1996

bw = body weight; DAC = Degree of deacetylation; HMWC = High molecular weight chitosan; i.p. = intraperitoneal; LMWC = Low molecular weight chitosan; MW = molecular weight; NR = not reported

3.8 Other Relevant Animal Studies

Studies on selective biological endpoints of orally administered chitosan or chitosan oligomers to animals are summarized Table IV.F-1. Although these studies were not designed as safety studies, the endpoints evaluated are relevant to the safety assessment of chitosan under the proposed food uses. Chitosan treatment did not alter body or organ weights, and no changes in urinalysis, blood biochemistry and hematological parameters were observed. Deuchi *et al.* (1995) reported significant reduction in serum levels of minerals (Ca, Fe, and Mg) and lipidsoluble vitamins (A, D, E, and K) in Sprague-Dawley rats fed diets supplemented with 5% chitosan. However, these findings have not been replicated in other animal studies (Gordon and Beach-Williford, 1984; Kimura *et al.*, 2004; Jung *et al.*, 2006). In several randomized, double-blind, placebo-controlled human clinical trials, supplementation of the diet with grams of chitosan did not affect the absorption of fat-soluble vitamins or minerals (Pittler *et al.*, 1999; Mhurchu *et al.*, 2004; Tapola *et al.*, 2008). Overall, no observations were identified in these studies which raise concerns over the safety of the proposed uses of chitosan in alcoholic beverage production.

Table 3.8-1 Summary of Other Relevant Animal Studies for Chitosan

Species (Strain), Sex, and Number of Animals	Route of Admin. and Study Duration	Test Article	Dose (mg/kg bw/d)	Parameters Measured Related to Safety	Reported Effects[a, b]	Reference
Rats						
Rats (Sprague-Dawley), male, 7/group	Dietary 28 days	Chitosan Source: shrimp shell DAC: 83% Size: 625 kDa	Group 1: 0 (control) Group 2: 5% (5,000[1])	• Body weight • Liver weight • Liver metabolizing enzymes	• Significantly lower final body weight in chitosan group • Significantly lower absolute and relative liver weight • Lower levels of CYP 3A, 1A1 in chitosan group, decrease in glutathione S-transferase	Yao *et al.*, 2010
Rats (Long Evans), female, 5/group	56 days	Chitosan, dietary Source: shrimp shells DAC: 85 to 98% Size: 350 kDa	Group 1: 0 Group 2: 2% (2,000[1])	Body weight and food consumption	NSD in weight and food consumption	Hossain *et al.*, 2007
				Plasma cholesterol	Plasma total cholesterol decreased by 16%	
				Liver lipids	NSD in liver lipids	
				Plasma fatty acid profile	NSD in plasma palmitic and steric acid levels, increases in oleic, linoleic, and docosapentaenoic acid; decreased arachidonic acid	
Rats (Sprague-Dawley) 8 males/group	Dietary 28 days	Chitosan Source: crab shell DAC: NR Size: NR	Group 1: 0 (control) Group 2: 2% (2,000[1]) Group 3: 5% (5,000[1])	• Food intake • Body weight gain • Plasma lipids • Microsomal CYP7A1 activity	• NSD body weight gain, food intake, food efficiency ratio • Chitosan-treated rats had significantly lower plasma total cholesterol and LDL-cholesterol concentration • Consumption of chitosan resulted in elevated activity of CYP7A1 by 123% in group 2, and 165% in group 3	Moon *et al.*, 2007

Table 3.8-1 Summary of Other Relevant Animal Studies for Chitosan

Species (Strain), Sex, and Number of Animals	Route of Admin. and Study Duration	Test Article	Dose (mg/kg bw/d)	Parameters Measured Related to Safety	Reported Effects[a, b]	Reference
Rats (Sprague-Dawley), Female, ovarectomized, 8/group	Dietary 42 days	Chito-oligosaccharides,	Group 1: 0 (control) Group 2: 2% (2,000[1])	Body weight, food consumption	NSD in weight gain, food intake, total calcium intake	Jung *et al.*, 2006
				Urinary and fecal calcium	Rate of calcium loss into feces significantly lower in ovariectomized rats in chitooligosaccharide group (retain calcium better)	
				Serum Calcium	NSD in treatment group	
				Bone mineral density	Chitooligosaccharide increased the bone marrow density in distal region of femur.	
Rats (Sprague-Dawley) 10/sex/group	Dietary 30 days	Chitosan oligomer Source: shrimp DAC: NR Size: 1.86 kDa	Group 1: 0% (control) Group 2: 0.75% (750[1]) Group 3: 1.5% (1,500[1]) Group 4: 3.0% (3,000[1])	• Daily food intake • Weekly body weight • Hematology test • Clinical chemistry tests • Organ weights • Histopathological examination	• NSD food intake, feces, hair, behavior, body weight • NSD in absolute or relative body weights • NSD in hematology and clinical chemistry parameters	Qin *et al.*, 2006
Rat (Sprague-Dawley) 9/sex/group	Oral (gavage) 28 days	Chitosan oligosaccharide Source: NR DAC: NR Size: <1 kDa	Group 1: 0 (control) Group 2: 500 Group 3: 1,000 Group 4: 2,000	• Clinical signs • Body weight • Hematological and biochemical parameters • Histopathological examinations	• NSD in behavior or external appearance • Normal body weight, food consumption • Normal urinalysis, hematology, blood chemistry, relative organ weights • Normal histopathological findings • NOAEL >2,000 mg/kg bw/day	Kim *et al.*, 2001

Table 3.8-1 Summary of Other Relevant Animal Studies for Chitosan

Species (Strain), Sex, and Number of Animals	Route of Admin. and Study Duration	Test Article	Dose (mg/kg bw/d)	Parameters Measured Related to Safety	Reported Effects[a, b]	Reference
Rats (Sprague-Dawley) 6/group	Dietary 28 days	Chitosan (high viscosity) Chitosan (low viscosity) Source: shrimp shell DAC: 90% Size: 480 kDa (high viscosity) 340 kDa (low viscosity)	Group 1: 0 (control) Group 2: 5% high viscosity chitosan (5,000[1]) Group 3: 5% low viscosity chitosan (5,000[1])	• Liver weight • Plasma lipid, transaminase, lactic acid, frutosamine, *beta*-hydroxybutyric acid, free fatty acid levels • Plasma and liver lipid peroxides • Liver and fecal lipids • Liver glucose-6-phosphate dehydrogenase	• NSD in body weight • Decreased relative liver weight • Higher liver lipid peroxide in chitosan (high viscosity) group • NSD plasma lipid peroxide values • NSD found in other tissue weights • Chitosan decreased plasma total cholesterol, VLDL-cholesterol, LDL-cholesterol, HDL-cholesterol • Decreased liver total lipids, but no significant difference in liver triacylglycerol content	Chiang *et al.*, 2000
Rats (Sprague-Dawley), male, 8-9/group	Dietary 18 days	Chitosan Source: NR DAC: NR Size: NR	Group 1: 0 (control) Group 2: Week 1: 10% (10,000[1]) Week 2+: 7.5% (7,500[1])	• Body weight, food intake • Liver lipids • Fecal fat • Cholesterol absorption	• Chitosan group had a slower rate of growth • Reduced food intake with 10% and 7.5% supplementation • Lower liver cholesterol contents in chitosan group • Higher fat excretion • No changes in intestinal contents supernantant viscosity	Gallaher *et al.*, 2000
Rats (Sprague-Dawley) 10/group	Dietary 14 days	Chitosan Source: NR DAC: 90% Size: NR	Group 1: 0 (cellulose control) Group 2: 5% (5,000[1])	• Body weight • Food efficiency • Apparent fat digestibility • Vitamin and mineral status	• Body weight gain reduced in chitosan group • Food efficiency ratio decreased in chitosan group • Apparent fat digestibility decreased in chitosan group • Chitosan group had lower Ca, Mg, Fe absorption, and lower bone mineral content • Liver retinol and retinyl palmitate lower in chitosan groups • Lower serum and liver vitamin E	Deuchi *et al.*, 1995

47

Table 3.8-1 Summary of Other Relevant Animal Studies for Chitosan

Species (Strain), Sex, and Number of Animals	Route of Admin. and Study Duration	Test Article	Dose (mg/kg bw/d)	Parameters Measured Related to Safety	Reported Effects[a, b]	Reference
					observed in chitosan group • Lower serum triglyceride • higher plasma vitamin K concentration	
Rats (Wistar), Male	Dietary 21 days	Chitosan Source: NR DAC: 94% Size: 250 kDa	Group 1: 0 (control) Group 2: 2% (2,000[1]) Group 3: 5% (5,000[1])	• Body weight • Food intake • Liver weight • Fecal weight • Serum cholesterol • Fecal neutral sterol excretion • Fecal bile acid excretion	• NSD in growth, food intake, liver weight, dried fecal weight • NSD in fecal excretion of neutral sterols and bile acids • Composition of bile acids and neutral sterols in cecum was statistically different in 5% chitosan group; chitosan expanded the neutral sterol pool and cholesterol, and decreased coprostanol • Statistically significant decrease in serum cholesterol in 5% chitosan group	Fukada *et al.*, 1991
Rats (Sprague-Dawley) • 6-7/group • 6/group	Dietary • 22 days • 28 days	Chitosan Source: crab shell DAC:81-99% Size: NR	Group 1: 0 (control) Group 2: 2% (2,000[1]) Group 3: 5% (5,000[1])	• Food intake • Growth • Organ weights • Serum cholesterol levels • Serum and liver lipids	• NSD in body weight, food intake • Relative liver weight was lower in chitosan groups • Chitosan prevented the rise of serum cholesterol due to feeding cholesterol • Liver cholesterol concentrations decreased in chitosan groups	Sugano *et al.*, 1988
Rats (Sprague-Dawley), Male 10/group	Dietary, 58 days	Chitosan Source: NR DAC: NR Size: NR	Group 1: 0 (control) Group 2: 1% (1,000[1])	Body weight	Weight gain reductions occurred in groups 5 and 6	Landes and Bough, 1976
				Food intake	Efficiency of food utilization was decreased in groups 5 and 6	

45

Table 3.8-1 Summary of Other Relevant Animal Studies for Chitosan

Species (Strain), Sex, and Number of Animals	Route of Admin. and Study Duration	Test Article	Dose (mg/kg bw/d)	Parameters Measured Related to Safety	Reported Effects[a, b]	Reference
			Group 3: 2.5% (2,500[1]) Group 4: 5% (5,000[1]) Group 5: 10% (10,000[1]) Group 6: 15% (15,000[1])	Hematology	Hemoglobin and packed cell volume decreased in groups 5 and 6; total serum protein decreased in group 6	
				Absolute and relative organ weights	Relative liver and kidney weights were reduced in group 6.	
Mice						
Mice (C57bl6/J), male 8/group	10 weeks	Fungal Chitosan from kitoZyme Source: *A. bisporus* DAC: NR Size: NR	Group 1: 0 (high-fat diet) Group 2: 5% (7,500; in high-fat diet)	Body weight gain, feed efficiency, fat mass development	Decreased body weight gain compared to non-supplemented high-fat diet; feed efficiency was significantly lower compared to control	Neyrinck *et al.*, 2009
				Liver weight, epididymal, visceral, and subcutaneous white adipose tissue weight	NSD in liver weight; white adipose tissue weight was systematically lower compared to controls	
				Oral glucose tolerance test	NSD in glucose tolerance	
				Plasma insulin, glucose, triglycerides, cholesterol, non-esterified fatty acids, and β-hydroxybuterate	NSD in insulin resistance index; decreased serum triglycerides, cholesterol; NSD in serum non-esterified fatty acids.	
				Lipid analysis in cecal	Fat staining of the tissue demonstrate	

Table 3.8-1 Summary of Other Relevant Animal Studies for Chitosan

Species (Strain), Sex, and Number of Animals	Route of Admin. and Study Duration	Test Article	Dose (mg/kg bw/d)	Parameters Measured Related to Safety	Reported Effects[a, b]	Reference
				content, liver and muscle	that lipid accumulation was reduced in liver and muscle compared to controls	
Mice (Kunming), female 10/group	90 days	Chitosan, high-molecular weight (HCS) Source: NR DAC: 85.5% Size: 760 kDa Chitosan, middle molecular weight (MCS); Source: NR DAC: 85.2% Size: 32.7kDa Chito-oligomer (COS); Source: NR DAC: 85.7%	Group 1: 0 (control) Group 2: 1.05% HCS (1575[1]) Group 3: 1.05% MCS (1575[1]) Group 4: 1.05% COS (1575[1]) Group 5: 1.05% WSC (1575[1])	General condition	NSD in appearance and behavior	Zeng *et al.*, 2008b
				Body weight	NSD in chitosan groups compared to control	
				Food intake	NSD	
				Absolute and relative organ weights	In WSC group: statistically significant increase in relative thymus weight. Other groups: NSD in relative heart, liver, spleen, thymus, kidney, and lung weights.	
				Histopathology	NSD in chitosan groups compared to control	
				Trace iron	Iron levels in liver, heart, spleen, kidney not different in groups 2, 4, 5 when compared to control; iron level in liver and spleen elevated in MCS group.	
				Trace zinc	Zinc levels in liver, heart, spleen, kidney not different in groups 2, 4, 5 when compared to control; zinc level	

Table 3.8-1 Summary of Other Relevant Animal Studies for Chitosan						
Species (Strain), Sex, and Number of Animals	**Route of Admin. and Study Duration**	**Test Article**	**Dose (mg/kg bw/d)**	**Parameters Measured Related to Safety**	**Reported Effects[a, b]**	**Reference**
		Size: 0.99 kDa Chitosan, water-soluble (WSC) Source: NR DAC: 52.6% Size: 39.1kDa			in liver, spleen, heart significant elevated in MCS group.	
				Trace copper	Copper levels in liver, heart, spleen, kidney not different in groups 2, 4, 5 when compared to control; copper level in liver, spleen significant elevated in MCS group	
Mice (C57Bl/6J), Males, 4/group	Oral (gavage) 140 days (20 weeks)	Water-soluble chitosan Source: NR DAC: NR Size: 46 kDa	Group 1: 0 (control) Group 2: 200 Group 3: 600	Body weight and food consumption	NSD in weight gain until week 17: group 3 had reduced body weight gain when fed high-fat diet.	Sumiyoshiand Kimura, 2006
				Plasma triglycerides, total cholesterol	NSD in plasma triglycerides; group 3 inhibited the increase of total cholesterol when fed a high-fat diet	
				Liver weight and lipids	Group 3 had significantly lower liver weight and hepatic triglyceride and total cholesterol	
				Liver and kidney damage markers	NSD in glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, and blood nitrogen urea	

Table 3.8-1 Summary of Other Relevant Animal Studies for Chitosan						
Species (Strain), Sex, and Number of Animals	**Route of Admin. and Study Duration**	**Test Article**	**Dose (mg/kg bw/d)**	**Parameters Measured Related to Safety**	**Reported Effects[a, b]**	**Reference**
Mice (Swiss Webster), male and female, 29-30/group	Dietary, 70 days	Chitosan Source: NR DAC: NR Size: NR	Group 1: 0 (control) Group 2: 10% (15,000[1])	Body weight	Chitosan group had reduction in weight gain at 10 weeks	Kimura *et al.*, 2004
				Small intestine length	Increased in chitosan group	
				Liver weight	Absolute and relative liver mass increased in chitosan group	
				Retinol concentration	NSD in whole-blood, tissue accumulation, and fecal and urinary excretion during 2-week retinol exposure period	
Mice (ICR) Female 13/group	Dietary, 63 days	Chitin-chitosan (80% chitosan) Source: NR DAC: NR Size: NR	Group 1: 0 (control) Group 2: 3% (4,500[1]) Group 3: 7% (10,500[1]) Group 4: 15% (22,500[1])	Body weight	Groups 2, 3, 4 significantly reduced the increase in body weight following high-fat diet	Han *et al.*, 1999
				Liver weight	Reduced in groups 3, 4 following a high-fat diet	
				Serum lipids, cholesterol	Serum triacylglycerol significantly reduced in groups 2, 3, 4	

Table 3.8-1 Summary of Other Relevant Animal Studies for Chitosan

Species (Strain), Sex, and Number of Animals	Route of Admin. and Study Duration	Test Article	Dose (mg/kg bw/d)	Parameters Measured Related to Safety	Reported Effects[a, b]	Reference
Mice (BALB/c), male and female,	Dietary, 28 days	Chitosan Source: Crab shell DAC: 80% Size: 3.6 µm in diameter	Group 1: 0 (control) Group 2: 0.5% (750[1]) Group 3: 5.0% (7,500[1])	• Body weight • Food consumption • Fecal bacteria	• After 4 weeks of feeding, group 3 had a statistically significant reduction in body weight • Average food consumption in week 4 was statistically lower in group 3 than control group • Facultative anaerobes, and lactobacillus concentrations were statistically lower in group 3 than control. Anaerobe colonies were higher in group 3 than controls. NSD in bifidobacterium and enterobacteraceae. NSD between group 2 and controls	Tanaka *et al.*, 1997
Mice (CF_1), female, approx 12/group	Dietary, 42 days	Low-molecular weight chitosan (LMWC), and high-molecular weight chitosan (HMWC) Source: NR DAC: 80 % Size: MW of 20,000 (LMWC) and 50,000 (HMWC)	Group 1: 0 (control) Group 2: 2% LMWC (3,000[1]) Group 3: 2% HMWC (3,000[1])	Body weight	Chitosan groups had lowered body weight, but HMWC was not statistically significant	Torzsas *et al.*, 1996
				Frequency of aberrant crypt foci	NSD in mice; HMWC decreased the number of aberrant crypt foci in azoxymethane-treated mice	

Table 3.8-1 Summary of Other Relevant Animal Studies for Chitosan

Species (Strain), Sex, and Number of Animals	Route of Admin. and Study Duration	Test Article	Dose (mg/kg bw/d)	Parameters Measured Related to Safety	Reported Effects[a, b]	Reference
Mice (transgenic homozygous apo E-deficient), mixed gender 10 /control 13/experimental	Dietary, 182 days (26 weeks)	Chitosan Source: prawn shells DAC: 78% Size: NR	Group 1: 0 Group 2: 5% (7,500[1])	Body weight	Chitosan-fed mice had significantly higher body weight on day 126 and 154 of study (improved growth)	Ormrod *et al.*, 1998
				General condition	NSD	
				Select organ weights	NSD in liver, epididymal, uterine horn fat pad weights	
				Food consumption	Food intake of all chitosan mice was marginally more than that of controls	
Guinea Pigs						
Guinea pigs (Hartley) 6/group	Dietary, 35 days	Chitosan Source: NR DAC: NR Size: NR	Group 1: 0 (control) Group 2: 5% (2,000[1])	Body weight, food intake, food efficiency ratio	NSD compared to controls	Jun *et al.*, 2010
				Relative organ weight and fat pad	NSD in relative organ weights NSD in fat pads except percentage of epididymal fat pad in chitosan group was significantly lower than control	
				Fecal excretion	Chitosan increased fecal weight, fecal fat excretion, fecal water excretion, fecal water content	
				Plasma cholesterol	Total cholesterol, LDL cholesterol, triacylglycerol decreased in chitosan	

Table 3.8-1 Summary of Other Relevant Animal Studies for Chitosan						
Species (Strain), Sex, and Number of Animals	**Route of Admin. and Study Duration**	**Test Article**	**Dose (mg/kg bw/d)**	**Parameters Measured Related to Safety**	**Reported Effects[a, b]**	**Reference**
					group	
				Lipid peroxide and GSH levels	GSH level in liver of chitosan group was higher	
Broiler Chickens						
Broilers, male 64/treatment	Dietary, 42 days	Chito-oligosaccharides Source: NR DAC: NR Size: Between 10^3 and 10^4 Da	Group 1: 0 (control) Group 2: 5% Group 3: 10% Group 4: 15%	Daily checks of disease and mortality	NSD in chitooligosaccharide groups	Huang *et al.*, 2005
				Body weight, food consumption	NSD for group 1, 2, 3 compared to control; Group 4 exhibited lower feed consumption in first three weeks, but NSD from week 3-6	
				Ileal digestibilities	Ileal digestibility for calcium and phosphorus increased with increasing chitooligosaccharide concentration, but decreased phosphorus digestibility for group 4. Ileal digestibility profile of individual amino acids changed	
Broilers (Arbor Acre), male, 42/treatment	Dietary, 42 days	Chitosan	Group 1: 0 (control Group 2: 0.02%	Average Daily Gain	NSD during weeks 0 to 3; increased quadratically (non-linear) during weeks 3 to 6; most effect noted at 0.05 and 0.1%	Shi *et al.*, 2005

Table 3.8-1 Summary of Other Relevant Animal Studies for Chitosan						
Species (Strain), Sex, and Number of Animals	**Route of Admin. and Study Duration**	**Test Article**	**Dose (mg/kg bw/d)**	**Parameters Measured Related to Safety**	**Reported Effects[a, b]**	**Reference**
		Source: NR DAC: NR Size: NR	Group 3: 0.05% Group 4: 0.1% Group 5: 0.3% Group 6: 0.5%	Average Daily Feed	NSD during weeks 0 to 6	
				Feed Conversion Efficiency	Tended to increase ($p = 0.057$) during weeks 0 to 3; increased quadratically (non-linear) during weeks 3 to 6; most effect noted at 0.05 and 0.1%	
Hens (White leghorn) Broilers Rabbits	Dietary, 239 days (34 weeks)	Chitosan Source: crab shells DAC: NR Size: NR	Hens and Broilers: Group 1: 0 Group 2: 1.4 Group 3: 3.6-4.2, Group 4: 14-18 Rabbits: Group 1: 0 Group 2: 0.8	Growth, appetite, appearance	All animals normal, except hens fed the large dose of chitosan due to incomplete digestion. Constriction was found in part of the jejunum, but no visible injury was found on the surface of mucous membranes and liver weight and color were normal Physiological trouble of hens were reversed within 1 week of chitosan-free feeding	Hirano *et al.*, 1990
				Hypolipidemic activity	Chitosan suppressed elevations in cholesterol and triacylglycerol due to high-cholesterol diet	

♂ = male animals; ♀ = female animals; DAC: Degree of deacetylation; HDL = high-density lipoprotein; LDL = low-density lipoprotein; NR: not reported; NSD = no significant difference; RBC = red blood cell; VLDL = very low-density lipoprotein; WBC = white blood cell. [1]Doses of dietary exposure to chitosan were estimated when possible according to guidelines set by the Food and Drug Administration (U.S. FDA, 1993)

3.9 Human Clinical Studies

Studies evaluating the administration of chitosan to healthy human subjects have been widely reported in the literature. Although primary endpoints monitored in these studies relate to effects of chitosan on management of body weight and cholesterol metabolism, controlled placebo controlled dose-response investigations assessing the safety and tolerance in healthy subjects consuming up to 6.5 g of chitosan per day have been reported (Bokura and Kobayashi, 2003; Tapola *et al.*, 2008). These studies are summarized in Table IV.G-1. In general, chitosan consumption was well tolerated at levels typically ranging from 1 to 6 g per day, for durations as long as 24 weeks. The most commonly reported adverse effects related to chitosan consumption were transient and mild, and were primarily gastrointestinal in nature (*e.g.,* constipation, nausea, bloating, indigestion, and abdominal pain).

In a systematic review of 14 randomized controlled trials published before 2005, chitosan was found to significantly reduce body weight and total serum cholesterol. However, the effects were small and the clinical relevance is questionable (Mhurchu *et al.*, 2005). Only 2 studies measured fecal fat excretion; however, no conclusions could be drawn about the effects of chitosan consumption on this parameter as different measures were used in the study and both contained small sample sizes. When studies that reported adverse events were analyzed, no differences in the frequency of adverse events were found between the chitosan intervention groups compared to controls.

Jull *et al.* (2008) included 15 human clinical studies in a meta-analysis of effect of chitosan on obesity for interventions lasting longer than 4 weeks (Jull *et al.*, 2008). Thirteen of the 15 studies were included in the previous systematic review by Mhurchu *et al.* (2005). Chitosan treatment resulted in significantly greater weight loss (weighted mean difference -1.7 kg; 95% confidence interval (CI) -2.1 to -1.3 kg, P <0.00001), decrease in total cholesterol (-0.2 mmol/L [95% CI -0.3 to -0.1], P <0.00001), and a decrease in systolic (-6 mm Hg [95% CI -7 to -5], P <0.00001) and diastolic (-3 mm Hg [95% CI -4 to -2], P <0.00001) blood pressure compared with placebo. However, the authors noted that many of the included studies included were of poor quality, and that results obtained from high quality trials suggest the effect of chitosan on body weight is minimal and unlikely to be of clinical significance. Similar to the previous analysis, no clear conclusions could be drawn regarding the effect of chitosan on fecal fat excretion, and that the frequency of adverse events did not differ between chitosan treatment and placebo control groups.

Overall, the data provided by these studies indicate that chitosan consumption is safe and well tolerated at high dietary doses (up to 6.5 g/person/day). These doses exceed estimated exposure to chitosan from under the proposed uses by several orders of magnitude.

Table 3.9-1 Summary of Human Studies on Chitosan

Number and Characteristics of Subjects	Route of Administration, Study Duration, and Study Design	Test Article and Properties	Dose (g/d)	Parameters Measured Related to Safety	Reported Effects	Reference
Healthy Subjects						
10 subjects, healthy volunteers, not taking antioxidants (such as vitamin E or C) during the 3 months before inclusion in the study	Oral preparation, 4 weeks, Open-label, placebo-controlled, cross-over study	Water-soluble chitosan Source: NR DAC: 95% Size: average MW of 20 kDa	Group 1: 0 Group 2: 0.54	• Blood pressure, BMI • HDL and LDL cholesterol, triglycerides • Atherogenic index • Calcium and phosphorous levels • Plasma antioxidant capacity	• NSD in blood pressure, BMI, levels of total cholesterol, phosphorous, or calcium • Decrease in levels of plasma glucose, and atherogenic index after 2 weeks and persisted until the end of study • Concentration of HDL cholesterol increased during treatment period; no significant difference in LDL-cholesterol • Lowered the ratio of oxidized to reduced albumin, and increased total plasma antioxidant activity	Anraku *et al.*, 2009
24 subjects, healthy males and females	Oral capsule, 12 days Double-blind, placebo-controlled, cross-over study	Chitosan Source: NR DAC: NR Size: NR	Group 1: 0 Group 2: 2.5	• Food intake • Weight • Fecal fat content	• NSD in weight or food intake • Very small increase in fecal fat content in men, but NSD in women • No adverse effects reported	Gades and Stern, 2005

Table 3.9-1 Summary of Human Studies on Chitosan

Number and Characteristics of Subjects	Route of Administration, Study Duration, and Study Design	Test Article and Properties	Dose (g/d)	Parameters Measured Related to Safety	Reported Effects	Reference
8 subjects, healthy male volunteers	Oral biscuits, 14 days	Chitosan Source: sea crab shells DAC: NR Size: NR	Week 1: 0 Week 2: 3 Week 3: 6 Week 4: 0	• Mean energy and nutrient intake • Fecal microbiota, bacterial metabolites, fecal weight, moisture content, pH value	• Decrease in lecithinase-negative clostridia ("may lead to improvement in intestinal environment") • Decrease in fecal ammonia • Chitosan inhibits putrefactive activity of intestinal microbiota and may contribute to reduction of factors that lead to disease states	Terada *et al.*, 1995
8 subjects, healthy males	Biscuits, 14 days Random, placebo-controlled cross-over study	Chitosan Source: NR DAC: 90.5% Size: 500 kDa	Group 1: 0 Group 2: Week 1: 3 Week 2: 6	• Body weight • Nutrition survey • Serum lipid • Bile acid and neutral cholesterol in feces	• Intake of energy, protein, fat, and cholesterol did not change • Average total serum cholesterol level decreased, serum HDL-cholesterol increased, NSD in serum triglyceride and phospholipid • NSD in bile acid excretion, amount of secondary bile acid excreted as lithocholic acid significantly decreased • Excreted amount of metabolite of cholesterol,	Maezaki *et al.*, 1993

Table 3.9-1 Summary of Human Studies on Chitosan

Number and Characteristics of Subjects	Route of Administration, Study Duration, and Study Design	Test Article and Properties	Dose (g/d)	Parameters Measured Related to Safety	Reported Effects	Reference
					coprostanol, was significantly lower	
Hypercholesterolemic Subjects						
56 subjects, mild hypercholesterolemia	Oral tablets 55 days Parallel, placebo-controlled, single-blind trial	Chitosan (ChitoClear fg 95 chitosan) Source: NR DAC: >95% Viscosity: <500 mPa·s	Group 1: 0 (placebo) Group 2: 4.5 Group 3: 6.75	• Hematology: blood count, plasma creatinine, urate, γ-glutamyl transferase, calcium, serum ferritin • Serum: *alpha*- and *beta*-carotene, vitamin A, vitamin E, 25-hydroxyvitamin D • Plasma total and HDL-cholesterol, total triglyceride concentrations • Body weight, blood pressure • RAND 36-item Health Survey • Incidence and severity of gastrointestinal, skin and other symptoms	• NSD in hematology, serum biochemistry, plasma lipids, body weight • Association in incidence of constipation, heartburn, nausea in first 4-week period in chitosan groups (not significant between groups after performing pair-wise comparisons) • Three subjects in chitosan group and 1 subject in placebo group reported skin symptoms	Tapola *et al.*, 2008
95 subjects, mild or moderate hypercholesterolemia	Oral tablet 12 weeks Multicenter, placebo-controlled, randomized study	HEP-40, low-molecular weight chitosan Source: NR DAC: 93% Size: 40 kDa	Group 1: 0 (placebo) Group 2: 1.2 Group 3: 1.6 Group 4: 2.4	• Blood cholesterol levels • Incidence of adverse events • Serum parameters	• NSD in non-serious adverse events • No serious adverse events reported • No clinically important changes in any laboratory safety parameters • NSD in serum 25(OH)D • HEP-40 reduced serum LDL-cholesterol and	Jaffer and Sampalis, 2007

Table 3.9-1 Summary of Human Studies on Chitosan

Number and Characteristics of Subjects	Route of Administration, Study Duration, and Study Design	Test Article and Properties	Dose (g/d)	Parameters Measured Related to Safety	Reported Effects	Reference
					total cholesterol at weeks 4, 8. • At 12 weeks, NSD in lipid profile parameters	
90 women, Mild to moderate hypercholesterolemia	Oral capsules 8 weeks, Double-blind, placebo-controlled, randomized study	Chitosan Source: NR DAC: 89.5% Viscosity: 160 mPa·s	Group 1: 0 (placebo) Group 2: 1.2	• Serum chemistry profiles • Complete blood counts • Changes in physical findings and signs • Blood pressure	• NSD in body weight, BMI, blood pressure, food consumption • Chitosan therapy produced statistically significant reduction in total cholesterol at 8 weeks • NSD in HDL cholesterol, triglyceride levels	Bokura *et al.*, 2003
Overweight Subjects						
12 subjects, obese, without diabetes mellitus	Oral tablet 3 months Placebo-controlled, randomized, double-blind trial	Chitosan (Vitamin World, 750 mg chitosan) Source: NR DAC: NR Size: NR	Group 1: 0 (placebo) Group 2: 2.25	• Serum glucose, total cholesterol, HDL cholesterol, triglycerides	• NSD serum glucose levels, lipid profile • Significant decrease in triglycerides • No adverse events with interventions • Insulin sensitivity increased significantly	Hernandez-Gonzalez *et al.*, 2010
30 subjects, overweight, hyperlipemic, under physical training	Oral tablet 4 months Double-blind, placebo-controlled	Low molecular weight chitosan, polyglucosamine	Group 1: 0 (placebo) Group 2: 2	• Anthropometric measures • Blood pressure • LDL and HDL-cholesterol, blood glucose and triacylglycerol	• More significant reduction in body weight, waist circumference, LDL-cholesterol, triacylglycerol than placebo control • HDL increase was higher than placebo	Cornelli *et al.*, 2008

Table 3.9-1 Summary of Human Studies on Chitosan

Number and Characteristics of Subjects	Route of Administration, Study Duration, and Study Design	Test Article and Properties	Dose (g/d)	Parameters Measured Related to Safety	Reported Effects	Reference
					control • Metabolic syndrome was reduced in 12 cases in the supplement group	
134 subjects, Overweight adults, 83% women	Oral capsules 60 days Double-blind, placebo-controlled study	Chitosan Source: NR DAC: NR Size: NR	Group 1: 0 (placebo) Group 2: 3	• Body composition • Blood chemistries • Tracking forms (daily caloric intake, activity levels)	• Significant reduction in mean scale weight, fat mass • NSD in total cholesterol, HDL, LDL, or bone mineral density	Kaats *et al.*, 2006
250 subjects, Overweight adults, 82% women	Oral capsule, 24 weeks Randomized, double-blind, placebo-controlled trial	β-Chitosan Source: squid pens DAC: 75.5% Size: NR	Group 1: 0 (placebo) Group 2: 3	• Body weight • Blood pressure • Waist circumference • Serum lipids • Plasma glucose • Fat-soluble vitamins in serum • Fecal fat losses • Health-related quality of life questionnaire	• NSD in BMI, waist circumference, body fat, blood pressure, fat-soluble vitamins, fecal fat loss • Statistically significant decrease in total cholesterol levels, LDL-cholesterol, but not clinically significant • NSD in HDL-cholesterol • NSD in health-related quality of life questionnaire answers	Mhurchu *et al.*, 2004
68 subjects, Normoglycemic obese individuals	Oral tablet 12 weeks Randomized, double-blind, placebo controlled	Absorbitol, a salt of chitosan Source: shellfish DAC: NR Size: NR	Group 1: 0 (placebo) Group 2: 3	• Body weight • Waist/hip ratio • Blood pressure • Bioelectric impedance analysis • Serum total cholesterol, triglyceride, HDL cholesterol, glucose	• NSD in adverse effects reporting • NSD in weight, body composition, blood composition, blood pressure, lipid profile, fasting insulin levels	Ho *et al.*, 2001

Table 3.9-1 Summary of Human Studies on Chitosan

Number and Characteristics of Subjects	Route of Administration, Study Duration, and Study Design	Test Article and Properties	Dose (g/d)	Parameters Measured Related to Safety	Reported Effects	Reference
59 subjects, overweight, mildly obese, females	Oral capsule, 8 weeks, Randomized, double-blind, placebo-controlled	Rapidly-soluble chitosan, LipoSan Ultra™ Source: NR DAC: > 78% Size: > 100 kDa	Group 1: 0 (placebo) Group 2: 3	• Body weight • Waist/hip ratio • Symptom Observational Survey questionnaire • Routine calorie and dietary fat intake; exercise diary • Fasting serum lipid levels • Fecal fat	• NSD in calorie and dietary fat intake • NSD in total Symptom Observational Survey results, though chitosan group reported more incidences of gastrointestinal discomfort, mild nausea, and heartburn; were alleviated by increasing water consumption • In placebo group, mean weight increased significantly by 1.5 kg while treatment group decreased mean weight by 1.0 kg • BMI was lower in chitosan group • Chitosan group exhibited an increasing trend in fecal fat excretion, but no statistical conclusion (sample size too small)	Schiller *et al.*, 2001
30 subjects, overweight volunteers	Oral capsules, 28 days Randomized, double-blind, placebo-controlled	Chitosan Source: NR DAC: NR Size: NR	Group 1: 0 (placebo) Group 2: 2	• Body mass index • Blood pressure • Quality of life • Serum cholesterol • Serum triglycerides • Vitamin A, D, E, *beta*-carotene	• NSD in body mass index, serum cholesterol, serum triglycerides, vitamin A, D, E, *beta*-carotene • Small increase in vitamin K after 4 weeks	Pittler *et al.*, 1999

Table 3.9-1 Summary of Human Studies on Chitosan

Number and Characteristics of Subjects	Route of Administration, Study Duration, and Study Design	Test Article and Properties	Dose (g/d)	Parameters Measured Related to Safety	Reported Effects	Reference
					in chitosan group compared with placebo • Minor adverse events reported in 9 subjects in chitosan group to be constipation	
Diabetic (Type 2) Subjects						
18 subjects, dyslipidemic type 2 diabetic subjects	Dietary supplementation, 12 weeks, Random, placebo-controlled	Chitosan Source: NR DAC: 90% Size: 1,000 kDa	Group 1: 0 Group 2: 1.8	• Body weight • Plasma cholesterol • HDL-cholesterol, LDL-cholesterol, triglyceride • Adverse events	• NSD in cholesterol, triglyceride concentration • Increase in HDL-cholesterol, concomitant reduction in LDL-cholesterol • Mild digestive discomfort	Ausar *et al.*, 2003

BMI: body mass index; DAC: degree of deacetylation; HDL: high-density lipoprotein; LDL: low-density lipoprotein; NR: not reported; NSD: no significant difference

4 Other relevant safety studies

Other safety and toxicology data collected on chitosan of fungal source (*A. bisporus*) by means of *in vitro* and *in vivo studies* conducted on animals or humans to examine the toxicity of chitosan for different purposes (pharma, cosmetics,...) are summarized in Table 4-1 below. These data have been generated either directly on the product (as a powder), or on its aqueous polar extracts or on n cosmetic formulation (cream) containing chitosan.

4.1 Information Pertaining to the Safety of Residual beta-1,3-glucans in the processing aid

Fungal chitosan contain *beta*-1,3-glucans at maximum concentrations of 10 to 15% on a w/w% basis. Since shellfish derived chitosan preparations do not contain *beta* glucans, ancillary safety data on the toxicity of *beta*-1,3-glucans is provided. Jonker *et al.* (2010) reported on the repeated consumption of an insoluble *A. niger* derived chitin/*beta*-1,3-glucan preparation (KiOnutrime-CG) in Wistar rats in a Good Laboratory Practice (GLP)-compliant subchronic toxicity study. The study was conducted in accordance with the following guidelines: OECD Guideline for Testing of Chemicals No. 408 (OECD, 1998); U.S. Food and Drug Administration (FDA) IV.C.4.a (U.S. FDA, 2000); and EC Guideline No. B.26, EEC Directive 2001/59/EC, Official Journal of the European Communities, No. L225, 21.8.2001 (EC, 2001). Groups of male and female Wistar rats (20/sex/group) [Crl:WI(WU)] were administered chitin-glucan as a dietary admixture at concentrations of 0 (control), 1, 5, or 10% (equivalent to 0, 632, 3,217, and 6,589 mg/kg body weight/day, respectively, for males and 0, 684, 3,437, and 7,002 mg/kg body weight/day, respectively, for females) for a period of 13 weeks. Food intake in high-dose rats was significantly increased with no changes in body weight, in comparison to control rats. The author considered this finding to be toxicologically irrelevant due to the lower energy content of the high-dose diet compared to the control diet. A significant increase in the absolute weight of the full and empty cecum of mid- and high-dose males and high-dose females, and a significant increase in the full and empty cecum weights relative to body weight in the high-dose males and females were reported compared to controls. Cecal enlargement occurs in rodents administered large dietary quantities of non-digestible polysaccharides/polyols, and is an effect that is not considered relevant to humans (WHO, 1987). The authors concluded that under the conditions of the study, the NOAEL for KiOnutrime-CG was 10% in the diet, the highest concentration tested, which was equivalent to an overall estimated daily intake of 6,589 mg/kg body weight/day for males and 7,002 mg/kg body weight/day for females.

Similar findings have been reported in other toxicity studies evaluating the effect of orally administered insoluble fungal derived *beta*-glucan preparations in rodents (Feletti *et al.*, 1992; Babícek *et al.*, 2007). In a GLP and OECD No. 408 compliant subchronic toxicity study, a NOAEL of 100 mg/kg body weight (the maximum deliverable gavage dose) was derived for Fisher-344 rats administered a *Saccharomyces cerevisiae* derived *beta*-1,3-glucan preparation on a repeated basis over a period of 91 days (Babícek *et al.*, 2007). The chronic (52 weeks) toxicity of a *Candida albicans* derived *beta*-1,3-D-glucan insoluble isolate was evaluated by Feletti *et al.* (1992). Groups of Sprague-Dawley rats (20/sex/group) were randomized to treatment groups receiving gavage dose of *beta*-glucan at 0 (saline), 50, 100, or 200 mg/kg body weight/day. Similar to findings reported by Jonker *et al.* (2010), animals randomized to the high-dose male and female treatment groups (200 mg/kg body weight/day) experienced soft stools,

diarrhea, and fecal enlargement with variable hyperplasia of the colon mucosa. A NOAEL of 200 mg/kg body weight per day, the highest dose tested, can be determined from this study.

Table 4-1 Safety studies for Chitosan of fungal source			
In vitro studies	**Cell type;**	**Conditions, concentration**	**Conclusion**
Cytotoxicity according to the ISO 10993-5 standard : Biological Evaluation of Medical Devices: Tests for in vitro cytotoxicity	L-929 mouse fibroblasts	Aqueous extracts at 0.2g/ml	Under the conditions of this study, the chitosan extract showed no cytotoxicity (KitoZyme 2008d)
Mutagenicity- AMES test according to OECD 471 and test method B13/B14 of Directive 2000/32/EC	4 *Salmonella typhimurium* strains and one *Escherichia coli* WP2 strain	Aqueous extracts at 0.2g/ml chitosan	Based on the results obtained in this study, the chitosan extracts were found to be non- mutagenic and non-promutagenic under the test conditions (KitoZyme 2008c)
Irritant potential by HETCAM (Hen's Egg Chorio- Allantoic Membrane) test Luepke et al. Fd Chem Toxic 23, 287 (1985), JORF 26/12/1996)	Chorio-allantoic membrane of a hen's egg	Aqueous extracts at 0.2g/ml chitosan-0.3ml applied pur.	Under the conditions of this study, the chitosan after polar extraction, must be classified "practically non irritant" according to classification established in the JORF (26/12/1996) (Kitozyme 2008e)
In vivo studies	**Animal types**	**Conditions, concentration**	**Conclusion**

Acute oral toxicity according to OECD 423 (24/04/2002) and test method B1tris of directive 2004/73/EC	Rat	Chitosan powder at 2000 mg/kg body weight	The LD50 of chitosan is higher than 2000 mg/kg body weight by oral route in the rat ; no mortality; no clinical signs related to administration of tested product ; the body weight evolution of the animals remained normal ; no treatment-related changes (Kitozyme 2008a)
Acute toxicity after intravenous administration according to ISO 10993-11 concerning biological evaluation of medical devices	Mouse	Aqueous extracts at 0.2g/ml chitosan; injection of 50ml/kg body weight	The LD50 of the polar extract is higher than 50 ml/kg body weight by intravenous route in mouse; no mortality; no clinical signs related to administration of tested product (kitoZyme 2008b)
Human studies	**Nbre of volunterrs**	**Conditions, concentration**	**Conclusion**
Skin sensitizing potential according to Marzulli-Maibach method under dermatological control	50 adult healthy volunteers with normal skin	Cosmetic formulation at 5% chitosan	Under these study conditions, the product can be considered non irritant and non-sensitizing to the skin and can be labeled as hypoallergenic (kitoZyme 2008f)

Acute cutaneous tolerance according to single patch test method under dermatological control	10 adult healthy volunteers with normal skin	Cosmetic formulation containing chitosan	Under these study conditions, the product is considered as non-irritating to the skin (Kitozyme 2008g)
Environemental studies	**Species**	**Conditions, concentration**	**Conclusion**
Environmental acute toxicity – Daphnia mobility inhibition assay realized according to OECD 202.	*Daphnia magna*	Dispersion of chitosan in water	The inhibition level (IL_{50}) is > 10588 mg/l (kitozyme 2011)
Environmental acute toxicity – Growing inhibition of algae according to OECD 201.	*Pseudokirchner -iella subcapitata*	Dispersion of chitosan in water	The inhibition levels is > 10316 mg/l (Kitozyme 2011)

4. Information on the toxicity of the chemical processing aid and if necessary, its major metabolites:

Information on the toxicity is available on section 3 here above

5. Safety assessment reports by international agencies or other national government agencies:

There is no safety assessment report prepared by international agencies or other government agencies expect the one reported in section B1 and B2 above.

C. Information related to the safety of an enzyme processing aid

Not applicable. Fungal chitosan is not an enzyme processing aid

D. Additional information related to the safety of an enzyme processing aid derived from a microorganism

Not applicable. Fungal chitosan is not an enzyme processing aid.

F. Information related to the dietary exposure of the processing aid

1. A list of food groups likely to contain the processing aid or its metabolites

Fungal chitosan from *A. niger* is proposed for use as a processing-aid in the manufacture of wine, beer, cider and spirits, as well as grain and beet derived food grade ethanol.

Chitosan derived from *A. niger*, was shown to be chemically and structurally equivalent to shellfish derived chitosan. The principal difference between the two chitosan preparations is the presence of small quantities of *beta*-1,3-glucans in *A. niger* sources of chitosan that are not present in shellfish chitosan. Therefore, data establishing the safety of shellfish-derived chitosan are considered relevant to the safety evaluation of fungal chitosan for the proposed food uses described herein.

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 in wine/alcoholic beverage processing for the GRAS determination.

A number of animal, human, and *in vitro* studies relevant to the safety of shellfish chitosan, which has a long history of safe use in the food supply, have been published. 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.

Shellfish derived chitosan has a long history of safe use in the food supply. It is currently approved for use as a natural food additive for general food use in Japan and Korea (Japan Food Chemical Research Foundation, 2011; KFDA, 2011), and has widespread use as a dietary supplement product in the United States, the European Union, and other regulatory jurisdictions throughout the world. Finally, fungal chitosan (derived from *Agaricus bisporus* and *Aspergillus niger* sources) has been granted Novel Food approval by the European Commission, for use in supplement products in the European Union based on its substantial equivalence to existing shellfish derived chitosan products that are currently in the market[8].

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 chitosan 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. Since chitosan is insoluble at slightly acidic to neutral pH levels, as well as in aqueous and ethanol solutions, it is unlikely that any residual chitosan will remain in the treated products. High-performance liquid chromatography (HPLC) analyses for residual chitosan in wine processed with chitosan indicate that the final product is free from chitosan carry-over products up to the limit of detection of the analysis method (10 mg/L). Therefore, the estimated intake of chitosan 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

APPENDIX

Appendix 1 Report on Industrial test on Chitosan Fungal Auxiliary on must and wine.

Appendix 2 CELABOR HPLC Chitosan

Appendix 3 Resolution OIV-oeno 336A-2009

Appendix 4 Resolution OIV-oeno 337A-2009

Appendix 5 Resolution OIV-oeno 338A-2009

Appendix 6 Resolution OIV-oeno 339A-2009

Appendix 7 Resolution OIV-oeno 368-2009

Appendix 8 Commission Regulation CE 53/2011

Appendix 9 GRAS Notice n°397 Response 12192011

Appendix 10 Argentina approval resolution

Appendix 11 EFSA Scientific Opinion on the safety of 'Chitin-glucan' as a Novel Food ingredient

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Table of CFR Sections Referenced (Title 21—Food and Drugs)		
Part	**Section §**	**Section Title**
170—Food additives	170.30	Eligibility for classification as generally recognized as safe (GRAS)
173—Secondary direct food additives permitted in food for human consumption	173.120	Carbohydrase and cellulase derived from Aspergillus niger
	173.280	Solvent extraction process for citric acid
184—Direct food substances affirmed as generally recognized as safe	184.1005	Acetic acid
	184.1033	Citric acid
	184.1763	Sodium hydroxide

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