GR

Original Submission

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Shrimp-Derived Chitosan GRAS Notification

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Volume 1

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April 25, 2005

Letter

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April 25, 2005

Dr. Robert Martin
US Food and Drug Administration
Center for Food Safety and Applied Nutrition
HFS 255
5100 Paint Branch Parkway
College Park, Maryland 20740

Dear Dr. Martin:

Re: GRAS Notification for Primex Shrimp-Derived Chitosan Resubmission

In accordance with the proposed rule for Substances Generally Recognized as Safe, which was published in the *Federal Register* at Vol. 62, No. 74 on April 17, 1997, Primex ehf, of Siglufjordur, Iceland would like to submit notice of a claim that the use of Primex Shrimp-Derived Chitosan as a food ingredient is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act, because such use is GRAS.

Chitosan is a polysaccharide, composed mainly of glucosamine, 2-amino-2-deoxy-β-D-glucose. Chitosan occurs widely in nature in the cell walls of fungi, and as a breakdown product of chitin. Commercially, chitosan is prepared by deacetylating chitin, which is the second most abundant natural polymer on earth. The fact that chitosan is a natural substance, and is derived from a natural substance, allows it to be a candidate for GRAS status as a food ingredient. The structure of the raw material, chitin, is similar to that of cellulose.

Chitosan has long been considered a source of dietary fiber, as it cannot be completely broken down in the upper human digestive tract. Chitosan has three functional groups, an amino group, as well as both primary and secondary hydroxyl groups, which form the basis for its functionality in foods.

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Primex ehf (Primex) of Siglufjordur, Iceland has developed a unique process to manufacture chitosan from the arctic shrimp, *Pandalus borealis*. A consistent

source of raw material and well-designed manufacturing practices have made it possible to use chitosan more widely as a food ingredient. The Notification which accompanies this letter contains the information required in proposed 170.36 to allow the FDA to evaluate whether the submitted notice provides a sufficient basis for a generally recognized as safe (GRAS) determination. This document is being submitted by Primex ehf, formerly Primex Ingredients ASA of Avaldsnes, Norway.

Regulatory Background for this Submission

An eleven-volume GRAS Report in support of the safe use of chitosan in foods was commissioned originally by Primex Ingredients ASA, and was prepared by Lee B. Dexter and Associates. The Report was reviewed by a Panel of Experts qualified by training and experience to assess the safety of food ingredients (June, 2000). The Experts concurred with Primex's determination that chitosan is safe for general use in foods. The Panel relied upon the results of numerous animal toxicology and human feeding studies, a history of safe consumption in Japan and other Pacific Rim nations, clinical studies sponsored by Primex, and a large body of published literature. A copy of the Expert Opinion is attached to this notice as Appendix I.

A GRAS Notification, GRN000073, was submitted to the Center for Food Safety and Applied Nutrition (CFSAN), by Primex on March 14, 2001 under proposed 21 CFR §170.36. Following their review of the submission, the management group of the GRAS review team at CFSAN agreed that chitosan was non-toxic to humans and other test animals, but questioned whether or not chitosan would interfere with fat-soluble vitamin and mineral status in humans, when the substance was consumed on a chronic basis as part of a general diet. Several questions were posed by Dr. George Pauli and others during a review meeting held March 4, 2002. The concerns about the effect of chitosan consumption on human nutritional status stemmed from the results of a 1995 publication by Deuchi *et al.*, in which rats were fed a high-fat diet containing 5% of a viscous preparation of chitosan. This report prompted CFSAN regulators to ask whether the effect of chitosan in rats would be predictive of its physiological effect in humans. The specific questions posed by the GRAS review team are listed below.

1. Might the physiological effect of chitosan consumption be similar to that of a well-known sucrose-ester fat-substitute, Olestra™, which has been shown to reduce the absorption of fat-soluble vitamins?
2. Would the consumption of chitosan result in sufficient fecal fat excretion, that the nutritional status of humans would be threatened by the loss of the fat-soluble vitamins A, D, E, and K?
3. Would mineral status be threatened, especially with regards to calcium?
4. Why did certain chitosan consumption studies in both humans and animals show that vitamin K levels increased? Would this in fact create an at-risk population for those taking prescribed blood-thinning drugs?

In order to answer these questions in detail, Primex ehf commissioned additional research and sponsored additional clinical studies. Six volumes of references were added to the original GRAS Report, and the Expert Opinions of two additional Experts were obtained. Subsequently, the agency requested updated Expert Opinions on these more recent studies. Primex has obtained the Expert Opinions of two of its original Panel members, Dr. Joseph Borzelleca (chairman), and Cleve Denny (retired from the National Food Processors Institute). The Expert Opinions of Dr. George Fahey (University of Illinois) and Dr. David Kritchevsky (of the Wistar Institute) have also been obtained. These Expert Opinions can be found in the Appendices. The Opinion of Dr. Kritchevsky will be sent as an amendment at a later date. The company has concluded that there are sound biological reasons why rats fed a high fat-high chitosan diet do not model the effects seen in humans, and that in recent studies have confirmed that Shrimp-Derived Chitosan can be considered GRAS for general use in foods .

This Notification of a claim for premarket exemption is based on a GRAS determination under proposed §170.36. Primex ehf has prepared a Notification document in triplicate, which accompanies this letter. A fourth copy has been prepared for review by USDA. The company would appreciate notice of the receipt of this document, and looks forward to any comments the agency would care to make on the Notification. If you have any questions regarding the content of the Notification, you may reach either myself at the number listed above or Lee B. Dexter at (512) 276-7408.

Sincerely,

Lee B. Dexter
Technical Consultant

CC: Bjarte Langhelle, Primex ehf
Steven Dillingham Strategro, International

Notification
Table of Contents

Primex ehf
GRAS Notification

Table of Contents

Page

Introduction 1

Section I. GRAS Exemption Claim

A. Notifier 3

B. Common or Usual Name 3

C. Applicable Conditions of Use 3

D. Basis of the GRAS Determination 4

E. Availability of Data and Information and Key to References 4

F. Signature of an Official for Primex Ingredients ASA 5

Section II. Chemical Identity

A. Common or Usual Name and Identity 1

B. Formal Names (IUPAC or Chemical Abstracts Names) 1

C. Synonyms, Other Common Names, Tradenames 1

D. Chemical Formulae, Structures and Molecular Weights 1

1. Empirical Formula 1

2. Structural Formula 2

3. Molecular Weight 2

E. Chemical Abstracts Service Registry Number (CAS Registry No.) 2

F. Quantitative Composition of Primex Chitosan 2

1. Product Identity 2

[redacted]

Page

Table 1: Final Product Specifications for Primex FG Chitosan 4

Table 2: Final Product Specifications for Primex FG95 Chitosan..... 5

Table 3: Final Product Specifications for Primex FGLV Chitosan 6

2. Analysis of Five Lots of Primex Ingredients Chitosan 7

Table 4: Product: Chitosan Made from Shrimp Shells, *Pandalus borealus* 9

Table 5: Microbiological Parameters 10

Table 6: Primex Chitosan Metals Analysis 10

Table 7: Mean Heavy Metal Values for 20 lots of Primex Chitosan produced in 2003 11

Table 8: Protein Values for Primex Chitosan 11

G. Manufacturing Process..... 11

1. Introduction 11

2. Production Process of Primex Chitosan 14

Table 9: Specific Production Steps for the Primex Chitosan Products..... 19

Table 10: Primex Chitosan Products Market and Properties..... 20

Figure 1: ChitoClear Product specific Processing Steps 21

3. HACCP Standard and Recall Tracking Program..... 22

4. Critical Control Points in ChitoClear FG Production 22

Figure 2: Primex Chitosan Production 24

5. Primex Tracking and Recall Program 25

Figure 3: Primex Tracking and Recall Program 26

H. Characteristic Properties of Chitosan 27

1. Introduction 27

2. Chitosan as an Absorbent of Free Fatty Acids in Palm Oil 27

3. Physical and Chemical Structure of Chitosan 28

I. Comparison of Primex Chitosan with Other Preparations 29

Table 11: Comparison of the Specifications of Commercial Chitosans 30

J. Chitosan in Nature 30

Table 12: Amounts Of Alkali-Insoluble Materials (AIM) and Chitosan Produced By *Mucoraceae* Strains;Viscosities and Degrees of Acetylation of Chitosan Isolates 33

K. Physicochemical Properties of Chitosan Which Form the Basis for its Potential Functionality in Food 34

L. Potential Uses of Chitosan in Foods 36

Table 13: Food Applications of Chitin, Chitosan and Their Derivatives In The Food Industry 37

1. Use of Chitosan as an Antimicrobial 38

2. Demonstrations of the Emulsification Properties of Chitosan in Food Models 47

Table 14: Behavior of Chitosan Emulsions 49

Table 15: Effect of Low-viscosity Chitosan Concentration on the Foaming of Whey Protein Isolate 50

3. Chitosan as a source of Dietary Fiber 51

4. Source of D-Glucosamine 52

5. Use of Chitosan as a Dietary Supplement in the US 52

M. Summary of the Current Market and Uses of Chitosan 53

1. Chitosan as an Anti-microbial 53

2. Use of Chitosan as a Dietary Supplement 54

3. Usage of Chitosan in Japan 54

4. Chitosan use in China 55

Section III. Information on Self-Limiting Levels of Use and Probable Consumption

A. Self-Limiting Levels of Use 1

Table 1: Use of Shrimp-derived Chitosan In the Food Categories Listed by the US FDA at 21 CFR §170.3 (n) 6

Table 2: Estimated Intakes of ChitoClear™ Chitosan from Selected Food Categories 7-8

Table 3: USDA Major Food Categories and Corresponding Categories from 21 CFR §170.3 (n) 9

B. Recently Developed Applications 10

Section IV. Detailed Summary of the Basis for the Determination that Primex Chitosan is GRAS

A. Introduction to the Section 1

1. Regulatory Background for this Submission 1

B. Questions Posed by CFSAN 2

1. Summarized Responses

a) Question 1. 3

b) Question 2. 4

Table 1: Nutritional Effects of Viscous Polysaccharide 4

Table 2: Apparent Digestibility of Energy, Protein, and Non-Starch Polysaccharides (NSP) in a High-Fiber Diet Rich in Fruits and Vegetables 7

Table 3: Differences in the Ability of Rats Versus Humans to Digest Fats 8

Table 4: Fecal Fat Loss in Humans 10

Table 5. Serum fat soluble vitamin and carotenoid Concentrations 12

c) Question 3. 13

Table 6: Summary of Mineral Analyses for Calcium in Humans Consuming Chitosan 15

d) Question 4 16

C. Conclusion to Summarized Responses 17

D. Supporting Discussion to Primex Responses 19

1. Question 1. 19

a) Comparison of the structures and of Chitosan and Olestra™. 20

b) Proposed Uses of Shrimp-Derived Chitosan and Olestra™.... 21

c) Physiological Effect of Olestra™ 22

d) Issues Associated with Olestra™ 24

e) Physiological Effect of Chitosan 25

2. Question 2. 26

a) Influence of Viscous Dietary Fibers on Fat Digestion 27

Table 7: Effect of Fiber on the Disappearance of Cholesterol and Triglyceride from the Small Intestine 28

Table 8: Lymphatic Recovery of Triglycerides and Cholesterol in Rats Adapted to Fiber 29

b) Quantifying the Effects of Viscous Polysaccharides on Fecal Fat Content in Humans 29

Table 9: Human Studies Reporting Changes in Fecal Fat Composition as a Result of Consuming Dietary Fiber 30

c) Mechanisms by which Dietary Fiber Can Impede Fat Absorption 31

d) Perspective on the Use of Rats as Models for Human Digestion 43

e) Comparative Digestibilities of Fibers and Fat between Rats and Humans 46

f) Results – Experiment 1: Diets Rich in Fruits and Vegetables 46

Table 10: Experiment 1: Apparent Digestibility of Energy, Protein, and Non-Starch Polysaccharides (NSP) in a Low-Fiber Control Diet and a Diet Rich in Fruits and Vegetables 47

g) Results - Experiment 2: Diets with Added Citrus Fruit DF 48

Table 11: Experiment 2: Apparent Digestibility of Energy, Protein, and Non-Starch Polysaccharides (NSP) in a Low-Fiber Control Diet and a Diet Rich in Citrus Fiber 49

h) Fecal Bulking Capacity in the Rat and Man 50

i) Factors Influencing the Differences in Fiber and Fat Digestibilities between Rats and Humans 51

j) Energy Values Derived from Non-Starch Polysaccharides: Comparative Studies in Humans and Rats 54

k) Human Experiments Conducted by Wisker, *et al.*, 1996 55

Table 12: Increase in Fecal Dry Matter Due to the Consumption of Non-Starch Polysaccharides (NSP) in Humans and Rats 59

Table 13: Net Energy Values of Non-Starch Polysaccharides (NSP) Calculated from NSP Fermentability and From Digestible Energy Balances in Humans And Rats 59

l) Relationship of these Studies to Regulatory Concerns about the Effect of Chitosan Consumption by Humans 61

Table 14: Fecal Fat Loss in Humans 65

m) Effect of chitosan on dietary lipid absorption in the rat 66

3. Question 3. Would mineral status be threatened, especially with regards to calcium? 69

Table 15: Tolerability parameters modifications during the two treatments 72

Table 16: Effect of Chitosan on Mineral Balance in Humans 73

Table 17: Tolerability parameters modifications during the two treatments 74

Table 18: Clinical results of chitosan treatments: Tolerability of the diet plus dietetic integrator 75

Table 19: 76

E. Primex Chitosan Human Study 77

Table 20. Pretrial characteristics of the study subjects. Mean (SD) 84

Table 21. Energy and nutrient composition of the background diet. Mean (SD) 85-87

Table 22. Scores for RAND-36 subscales. Mean (SD) 90-91

Table 23. Serum fat soluble vitamins and carotenoids concentrations during the study 93-94

Table 24. Hematology and clinical chemistry. Mean (SD) 95-96

Table 25. Body weight. Mean(SD) 97

Table 26. Blood pressure. Mean (SD) 98

Table 27. Defecation frequency. (SD) 99

Symptoms and subject dropouts 100

Conclusion 100-101

1. Question 4 102

F. Human Feeding Studies 109

000016

Human Safety 111

Table 28: Effect of Chitosan on Human Subjects 113

Table 29: Effect of Chitosan on Mineral Balance in Humans 119

Table 30: Effect of Chitosan on Hematological Parameters 120

Table 31: Effect of Chitosan on Hematological Parameters 128-129

Fate of Chitosan in the Gastrointestinal Tract 130

Table 32: Tablet Position and Radioactivity Released with Time After Administration to One Volunteer 131

3. Chitosan and other Foods as Sources of Glucosamine 136

4. Chitosan Degradation 139

5. Exposure 140

6. Technical Effects 141

7. Contaminants and Manufacturing 143

Table 33: Contaminant Analysis 144

Table 34: Dioxin Analysis 145

Table 35: Virus Analysis 146

G. Animal Safety and Toxicology Studies 146

Table 36: Toxicity Assessments of Chitosan in Animals 147-148

H. Other Regulatory Approvals 148

I. Summary 151

Appendix I: Expert Opinion

A. Introduction 152

B. Conclusion 153

Appendix II: Complete List of References

References 1-34

Appendix III: Symptoms and Dropouts

Table 1: The number of the subjects reporting symptoms 3-4

Table 2: The severity of reported abdominal symptoms by degreee (median) 5-6

Table 3: Defecation Frequency. Mean (ISD) 7

Study Dropouts 7

Table 4: Subjects withdrawing from study 10

Table 5: Association of Gastrointestinal symptoms with menses 11

Table 6: Mean severity of distressing GI symptoms rated on a 0 to 4 scale on a daily basis 11

Appendix IV

Emulsification and Foaming Agent Properties of Chitosan 1

Table 1: Influence of WPI Concentration on Foaming with Corn Oil 2

Table 2: Foaming properties of WPI/Chitosan with different lipids 3

Table 3: Effects of low-viscosity Chitosan and hydrolyzed chitosans 4

Section I

Section I GRAS Notification Claim

Primex ehf
GRAS Notification

Introduction

Chitosan is a polysaccharide, composed mainly of glucosamine, 2-amino-2-deoxy-β-D-glucose. Chitosan occurs widely in nature in the cell walls of fungi, and as a breakdown product of chitin. Commercially, chitosan is prepared by deacetylating chitin, which is the second most abundant natural polymer on earth [Shahidi, *et al.,* 1999 Vol 8 Tab 145]. The fact that chitosan is a natural substance, and is derived from a natural substance, allows it to be a candidate for GRAS status as a food ingredient. The structure of chitin is similar to that of cellulose, and chitosan has long been considered a source of dietary fiber, as it cannot be completely broken down in the upper human digestive tract [Omrod, 1998 Vol 7 Tab 112]. Chitosan has three functional groups, an amino group, as well as both primary and secondary hydroxyl groups, which are the basis for its functionality in foods [Shahidi, *et al.,* 1999 Vol 8 Tab 145].

Primex ehf (Primex) of Siglufjordur, Iceland has developed a unique process to manufacture chitosan from the arctic shrimp, *Pandalus borealis.* A consistent source of raw material and well-designed manufacturing practices have made it possible to use chitosan more widely as a food ingredient. This Notification document contains the information required in proposed 170.36 to allow the FDA to evaluate whether the submitted notice provides a sufficient basis for a generally recognized as safe (GRAS) determination. This document is being submitted by Primex ehf, formerly Primex Ingredients ASA of Avaldsnes, Norway.

In compliance with 21 CFR § 170.30, Primex determined that Primex chitosan can be considered GRAS when used in accordance with current Good Manufacturing Practices. Primex wishes to voluntarily notify the Center for Food Safety and Applied Nutrition (CFSAN) of that determination, and according to proposed § 170.36, the company is submitting the following GRAS exemption claim.

Section I GRAS Notification Claim

Originally, Primex Ingredients ASA prepared a twelve-volume GRAS Report, which formed the basis for the information found in this notification. The company also commissioned a panel of experts (Expert Panel), qualified by scientific training and experience to assess the safety of food ingredients, who critically evaluated the chitosan GRAS Report as well as other data and information relevant to the use and safety of this ingredient. In a meeting held on June 8, 2000, the panel concurred with the company's determination that Primex chitosan can be considered generally recognized as safe for general use in food. Based on the data and information contained in the Report and the opinion of the expert panel (which is attached to this notification), Primex explicitly accepts responsibility for the GRAS determination of Primex chitosan, known commercially as ChitoClear®. This Notification contains additional data and information from peer reviewed published literature and from studies sponsored by Primex ehf.

Section I
GRAS Exemption Claim

Primex ehf hereby notifies the U.S. Food and Drug Administration that the use of Primex chitosan (as a food ingredient is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act, because Primex has determined that such use is GRAS.

A. Notifier:

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580 Siglufjordur
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Tel.: +354 460 6900
Fax.: +354 460 6909

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124 S. Mountain Ave
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Phone: 973-783-7323
Fax: 973-509-2882
E-mail: info@strategro.com

B. Common or Usual Name:

Shrimp-derived Chitosan
Shrimp Chitosan

C. Applicable Conditions of Use:

Applications for chitosan include general use in foods as a multiple-use direct additive. The ingredient should be used under the conditions of current Good Manufacturing Practice. In order to classify the various effects ingredients may have in food, FDA has published a list of 32

physical or technical functional effects for which direct food ingredients may be added to food. These are codified at 21 CFR §170.3 (o) (1-32). Applications for chitosan are covered under several of the following terms as listed under 21 CFR §170.3 (o).

(2) "Antimicrobial agents": Substances used to preserve food by preventing the growth of microorganisms and subsequent spoilage, including fungistats, mold and rope spore inhibitors, and the effects listed by the National Academy of Sciences/National Research Council under "preservatives."

(3) "Antioxidants": Substances used to preserve food by retarding deterioration, rancidity, or discoloration due to oxidation.

(5) "Curing and pickling agents": Substances imparting a unique flavor and/or color to a food, usually producing an increase in shelf life stability.

(6) "Dough strengtheners": Substances used to modify starch and gluten, thereby producing a more stable dough, including the applicable effects listed by the National Academy of Sciences/National Research Council under "dough conditioner."

(8) "Emulsifiers and emulsifier salts": Substances which modify surface tension in the component phase of an emulsion to establish a uniform dispersion or emulsion.

(14) "Formulation aides": Substances used to promote or produce a desired physical state or texture in food, including carriers, binders, fillers, plasticizers, film-formers, and tableting aids, etc.

(16) "Humectants": Hygroscopic substances included in food to promote retention of moisture, including moisture-retention agents and antidusting agents.

(20) "Nutrient supplements": Substances which are necessary for the body's nutritional and metabolic processes.

(24) "Processing aids": Substances used as manufacturing aids to enhance the appeal or utility of a food or food component, including clarifying agents, clouding agents, catalysts, flocculants, filter aids, and crystallization inhibitors, etc.

(28) "Stabilizers and thickeners": Substances used to produce viscous solutions or dispersions, to impart body, improve consistency, or stabilize emulsions, including suspending and bodying agents, setting agents, jellying agents, and bulking agents, etc.

(29) "Surface-active agents": substances used to modify surface properties of liquid food components for a variety of effects, other than emulsifiers, but including solubilizing agents, dispersants, detergents, wetting agents, rehydration enhancers, whipping agents, foaming agents, and defoaming agents, etc.

Section I GRAS Notification Claim

(31) "Synergists": Substances used to act or react with another food ingredient to produce a total effect different or greater than the sum of the effects produced by the individual ingredients.
(32) "Texturizers": Substances which affect the appearance or feel of the food.

D. Basis of the GRAS Determination

The basis of the GRAS determination for Primex ehf chitosan was the use of scientific procedures.

E. Availability of Data and Information and Key to References

The data and information that are the basis of the GRAS determination for Primex ehf chitosan will be available at the address of the notifier listed above. However, throughout this Notification, citations to the published literature, which were included in the 15-volume GRAS Report are denoted as follows: [Author (*et al*), Year, Tab (number) Volume (number)]. In order to facilitate review of this document a complete list of references from the chitosan GRAS Report is included in Appendix 2 as a key. Recently identified references, which were not included in the original GRAS Report are listed as a continuation of the original list of references. The references within the text of this document and are given in a standard bibliographic form.

F. Signature of an official for Primex ehf

Officials for Primex ehf — Date

4/25/05

Lee B. Dexter, Technical Consultant

5/5/05

Bjarte Langhelle
Market Development Manager

Section II

Section II
Chemical Identity

A. Common or Usual Name and Identity

Shrimp-derived Chitosan
Shrimp Chitosan

B. Formal Names (IUPAC or Chemical Abstracts Names)

Chitosan

1. Polyglucosamine
2. Poly-[1→ 4] – ß-D-glucosamine
3. Poly 2-Amino-2-deoxy—D-Glucopyranose
4. Binary heteropolymer of ß (1→ 4)-2-Amino-2-deoxy—D-Glucopyranose and ß (1→ 4)-2-deoxy-2-acetamido-D-glucopyranose

Molecular Subunits

1. D-Glucosamine = 2-Amino-2-deoxy-D-Glucopyranose (GlcN)
2. N-Acetyl-D-glucosamine = 2-deoxy-2-acetamido-D-glucopyranose (GlcNAc)

C. Synonyms, Other Common Names, Tradenames

1. Trademark: ChitoClear® is a registered trademark name associated with Primex ehf chitosan.

2. Primex Chitosan

3. Deacetylated Chitin

D. Chemical Formulae, Structures and Molecular Weights

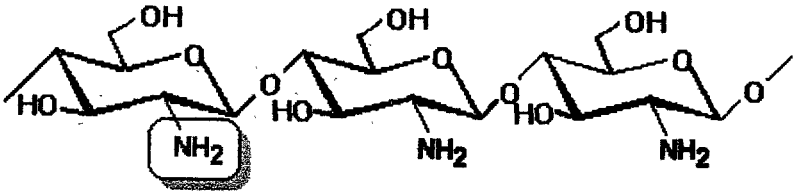
1. Empirical Formula $(GlcN)_n + (GNAc)_m$
$(C_6H_{11}\ N_1O_4)_n + (C_8H_{13}\ N_1O_5)_m$

Chitosan is a polysaccharide composed of GlcN and GlcNAc linked with ß (1→ 4)-glycosidic linkages.

000026

Section II Chemical Identity

2. Structural Formula



Chitosan

3. Molecular Weight

Most abundant species: ~1 million Daltons
[Wu, *et al.*, 1976, Vol 11 Tab 220]

E. Chemical Abstracts Service Registry Number (CAS Registry No.)

CAS Number 9012-76-4

F. Quantitative Composition of Primex Chitosan

1. Product Identity

The Primex chitosan product, ChitoClear™, is a binary heteropolymer of ß (1→ 4)-2-Amino-2-deoxy—D-Glucopyranose and ß (1→ 4)-2-deoxy-2-acetamido-D-glucopyranose produced by the deacetylation of chitin. Chitosan is obtained by the process of removing a sufficient number of acetyl groups (CH_3-CO) from chitin, so that the molecule becomes soluble in most diluted acids. This process, called deacetylation, releases amine groups (NH) and results in chitosan, a heteropolymer with cationic characteristics. Chitosans are typically characterized by two principal factors: viscosity and degree of deacetylation. Various investigators have suggested that chitosan and chitin be defined as soluble or insoluble in 0.1 M acetic acid, or by degree of deacetylation, where a substance with >20 % of deacetylation would be defined as chitosan [Shahidi *et al.*, 1998 Vol 8 Tab 144 and Shahidi, *et al.*, 1999 Vol 8 Tab 145].

The Primex product is composed of ≥ 90% dry matter, less than 1% ash, and negligible amounts of protein and fat. The percent deacetylation of the chitosan (as compared to chitin) is greater than 70%, and the turbidity rating is ≤50 NTUs. The product is further

000027

characterized by a solubility of ≥99% in 1.0 % acetic acid. Lead levels are <0.1ppm, and mercury levels are below the level of detection of 5 ppb. Microbial limits have been set to meet food grade specifications (Tables 1-3). The product occurs in several food grade forms characterized by their degree of deacetylation (>70 – >95%), and polymerization. Primex FG is a food grade product defined as having > 70% deacetylation. Primex FG95 has undergone extended deacetylation to yield a product with a degree of deacetylation >95%, and Primex FGLV is a low viscosity chitosan. Viscosity specifications for the FG and FG95 chitosan products have been set with upper and lower limits in order to encompass the range of products that has been either tested in human feeding studies, or is currently being sold commercially (See Section IV).

Table 1

Final Product Specifications for Primex FG Chitosan

Product: Off white chitosan made from fresh shrimp shells, *Pandalus borealis.*

Food Grade	
Chemical Parameters	
Dry Matter	>90 %
Moisture	<10 %
Ash	<1.0%
Degree of Deacetylation	>70%
Solubility	>99% (in 1% acetic Acid)
Turbidity of the Solution	< 50 NTUs
Viscosity	≥5 and ≤5000 (mPas (cPs)) 1% solutions in 1% acetic acid measured on a Brookfield LVT viscometer. 25°C. appropriate spindle at 30 rpm
Protein	<0.2%
Lead	<0.1 ppm
Mercury	<5 ppb
Flakes	0.1 – 6 mm
Micromilled Powder	Min. 100% < 60 mesh,
Microbiological Parameters	
Total Plate Count	≤ 1000 CFU/g
Eschericia coli	Negative
Coliform Organisms	Negative
Salmonella sp. 25 grams	Negative
Yeast and Mold	≤ 100 CFU/g

Table 2
Final Product Specifications for Primex FG95 Chitosan

Product: Off white chitosan made from fresh shrimp shells, *Pandalus borealis*.

Food Grade	
Chemical Parameters	
Dry Matter	>90 %
Moisture	<10 %
Ash	<1.0%
Degree of Deacetylation	>95%
Solubility	>99% (in 1% acetic Acid)
Turbidity of the Solution	< 50 NTUs
Viscosity	≥5 and ≤5000 (mPas (cPs)) 1% solutions in 1% acetic acid measured on a Brookfield LVT viscometer. 25°C. appropriate spindle at 30 rpm
Protein	<0.2%
Lead	<0.1 ppm
Mercury	<5 ppb
Flakes	0.1 – 6 mm
Micromilled Powder	Min. 100% < 60 mesh
Microbiological Parameters	
Total Plate Count	≤ 1000 CFU/g
Eschericia coli	Negative
Coliform Organisms	Negative
Salmonella sp. 25 grams	Negative
Yeast and Mold	≤ 100 CFU/g

Table 3
Final Product Specifications for Primex FGLV Chitosan
Product: Off white chitosan made from fresh shrimp shells, *Pandalus borealis*.

Food Grade	
Chemical Parameters	
Dry Matter	>90 %
Moisture	<10 %
Ash	<1.0%
Degree of Deacetylation	>70%
Solubility	>99% (in 1% acetic Acid)
Turbidity of the Solution	< 50 NTUs
Viscosity	5-50 (mPas (cPs)) 1% solutions in 1% acetic acid measured on a Brookfield LVT viscometer. 25°C. appropriate spindle at 30 rpm
Protein	<0.2%
Lead	<0.1 ppm
Mercury	<5 ppb
Flakes	0.1 – 6 mm
Micromilled Powder	Min. 100% < 60 mesh,
Microbiological Parameters	
Total Plate Count	< 1000 CFU/g
Eschericia coli	Negative
Coliform Organisms	Negative
Salmonella sp. 25 grams	Negative
Yeast and Mold	< 100 CFU/g

2. Analysis of Five Lots of Primex ehf Chitosan

Five (5) lots of Primex ehf chitosan, FG95, manufactured over a period of time, showed that product dry matter, as determined by a standardized drying method was greater than 95.0%. Ash content ranged from 0.1% to 0.6% [Table 4 below]. The degree of deacetylation was greater than 95.6%. The solubility in a 1.0% acetic acid solution was greater than 99.0%. Turbidity was less than 18 NTUs, and the viscosity was greater than 5 cps. Microbiological analyses showed that the product contained less than 1000 CFUs per gram, was negative for *E. coli,* coliform organisms, and *Salmonella sp.*(based on 25 grams), and yeast and mold counts were less than 100 per gram. Heavy metals, mercury and lead were measured on separate lots (Table 6) [Primex Ingredients ASA 2000, Vol 7 Tab 122]. Concentrations were calculated on a dry weight basis. Zinc, measured on 4 lots was present at < 1 μg/g. Arsenic measured on 8 lots totaled <0.1 μg/g. Cadmium, also measured on 8 lots was present in concentrations of< 0.005 μg/g, and lead on the basis of 10 lots was present at < 0.095 μg/g.

Table 7 presents mean levels of individual heavy metals for lots of Primex chitosan produced in 2003. Mean values for all metals were below the level of detection. Lead levels were less than 0.1 ppm, arsenic levels were less than 0.05 ppm, mercury levels were less than 5 ppb, and cadmium levels were less than 0.05 ppm.

Since chitosan is derived from a raw material that is potentially allergenic (shells of arctic shrimp), questions have been posed as to the protein content of chitosan. The harsh chemical treatment associated with deacetylation is known to degrade proteinaceous materials. Primex has confirmed through human clinical testing that its chitosan product is not allergenic (See Section IV). In an abundance of caution, Primex has also set a protein specification of less than 0.2% for its food grade products. In order to establish what level of protein may be present in any of its lots of chitosan, special testing methods must be used, since the normal Kjehldahl method for

testing proteins in foods, actually measures nitrogen content. Protein content is then determined by multiplying the amount of nitrogen present in a substance by a constant factor (usually 6.25). Since chitosan naturally contains nitrogen, using the Kjehldahl method would result in a calculated protein level that was both inaccurate and too high. Therefore, the chitosan industry has relied upon amino acid testing to establish the presence of protein in its products.

The actual amino acid content of Primex chitosan is presented in Table 8. The amino acid analyses were performed by Uppsala University Biomedical Center, in Sweden. Total protein was determined by summing the content of amino acids. These analyses confirmed that protein levels in the products are extremely low (<0.2%). The method used is included as Appendix 5.

The American Society for Testing and Materials (ASTM) has also adapted a sensitive testing procedure for measuring protein in chitosan salts destined for biomedical uses. These methods have been published in their *Standard Guide for Characterization and Testing of Chitosan Salts as Starting Materials Intended for Use in Biomedical and Tissue-Engineered Medical Product Applications*. This guide is also included in Appendix 5.

Table 4
Product: Chitosan Made from Shrimp Shells, *Pandalus borealus.*
Mean Analyses of 5 Lots[1]

Batch No.	Dry Matter	Ash	Degree of Deacetylation	Solubility[2]	Turbidity	Viscosity[3]
TM 807	96.8%	0.3%	97.5 % (Titration-Method)	>99.9% (in 1% acetic acid	13 NTUs	45 (mPa.S(cPs))
TM 808	96.8%	0.1%	95.6% (Titration-Method)	>99.9% (in 1% acetic acid	17 NTUs	68 (mPa.S(cPs))
TM 809	96.6%	0.2%	98.0% (Titration-Method)	>99.9% (in 1% acetic acid	14 NTUs	44 (mPa.S(cPs))
TM 813	95.2%	0.3%	96.4% (Titration-Method)	>99.9% (in 1% acetic acid	18 NTUs	66 (mPa.S(cPs))
TM 822	97.9%	0.6%	96.6% (Titration-Method)	>99.9% (in 1% acetic acid	15 NTUs	53 (mPa.S(cPs))
Mean Of Means	96.66%	0.3%	96.82%	>99.9%	15.4 NTUs	55.2 cPs

Form: Micromilled Powder: 100% less than 100 mesh, 50% less than 150 mesh

[1] Raw data is shown in Volume 7 Tab 121.
[2] 1% solutions in 1% acetic acid
[3] Measured on a Brookfield LVT viscometer, 25C, appropriate spindle at 30 rpm.

Table 5
Microbiological Parameters

Batch No.	Total Plate Count	*Escherichia coli*	Coliform Bacteria	*Salmonella* / 25 Grams	Mold and Yeast
TM 807	<1000/g	Negative	Negative	Negative	<100/g
TM 808	<1000/g	Negative	Negative	Negative	<100/g
TM 809	<1000/g	Negative	Negative	Negative	<100/g
TM 813	<1000/g	Negative	Negative	Negative	<100/g
TM 822	<1000/g	Negative	Negative	Negative	<100/g
Mean of Means	<1000/g	Negative	Negative	Negative	<100/g

Table 6
Primex Chitosan Metals Analysis

Element	No. of Lots	Mean Value (μg/g) dry wt.
Zinc	4	<1
Arsenic	8	<0.1
Cadmium	8	<0.005
Lead	10	0.095
Mercury	12	<0.005

Table 7
Mean Heavy Metal Values for 20 Lots of Primex Chitosan Produced in 2003

Lead	**< 0.1 ppm**
Arsenic	**< 0.05 ppm**
Mercury	**< 5 ppb**
Cadmium	**< 0.05 ppm**

Table 8
Protein Values for Primex Chitosan[1]

Lot. No	Date Submitted for Analysis	Protein % (w/w)
TM1274	18-Oct-02	0.0043
TM1213	6-Dec-02	0.1063
TM1214	6-Dec-02	0.0412
TM1222	6-Dec-02	0.0128
TM1239	6-Dec-02	0.0137
TM1360	15-Feb-03	0.0090
TM1410	24-Mar-03	0.0030
S030318-5HS	10-Apr-03	0.0000
S030320-1HS	11-Apr-03	0.0000

[1]As determined by Uppsala University Biomedical Center, Sweden

G. Manufacturing Process

1. Introduction

In general chitosan is prepared by deacetylation of chitin, which, other than cellulose, is the most abundant carbohydrate biopolymer on earth. The most common sources of chitin are crustacean shells, especially shrimp, crabs, and Antarctic krill. The reproducibility of the deacetylation process is affected not only by chemical reaction parameters but also by the biological origin of the raw material [Shahidi, *et al.*, 1999 Vol 8 Tab 145]. The properties of the nitrogen-

containing polycationic polysaccharide depend largely on the degree of deacetylation (DA), molecular weight, and chain conformation of the polymer. Knowledge of the degree of acetylation is an essential parameter in the characterization of chitosan.

Hwang, *et al.*, 1995, stated that the molecular weight and DA are greatly affected by the concentration, time and temperature of the deacetylation reaction [Hwang, *et al.*, 1995, Vol 5 Tab 58, and Wu, *et al.*, 1976, Vol 11 Tab 220]. Preparation of chitin from crab shell as practiced by Hwang, *et al.*, 1995 consists of demineralization with a 5% HCl treatment followed by deproteinization with 5% NaOH. Ash content and nitrogen content are measured to determine the quality of chitin after processing. A hot 50% NaOH solution is used for the deacetylation process of chitin, and the DA, molecular weight, and viscosity of the chitosan were measured by the researchers. The concentrated NaOH solution can be reused in some manufacturing schemes. Hwang, *et al.*, 1995, found that the degree of demineralization was not significantly different at 50°C and 70°C after 30 minutes of reaction time. Deproteinization decreased as process time increased. The nitrogen content reached 6.92% after 90 minutes at 80°C, which was similar to the theoretical nitrogen content of chitin. After 2 hours of reaction time, the degree of deacetylation reached by this process was 82.84% and the level continued to increase as the reaction time increased. Viscosity and molecular weight increased as the number of recycles of concentrated NaOH increased. The authors concluded that the chemical, biological, and physical properties of chitosan depend on the degree of deacetylation and the molecular size of the molecule [Hwang, *et al.*, 1995, Vol 5 Tab 58, and Wu, *et al.*, 1976, Vol 11 Tab 220].

The physical properties of chitosan relate to its polyelectrolyte and polymeric character. It is insoluble in water, alkaline solutions at pH levels above about 6.5, and organic solvents. However, chitosan dissolves readily in dilute solutions of many organic acids, including acetic, formic, tartaric, and citric acids [National Toxicological Survey, 2000, Vol 7 Tab 108].

Section II Chemical Identity

Data submitted to the United States Environmental Protection Agency for 1994 indicated that chitosan production was less than 10,000 pounds per year [National Toxicological Survey, 2000 Vol 7 Tab 108]. A world consumption estimate for the same year stated that there were more than 1,000 tons of chitin and chitosan being produced, with 800 tons of that amount being used in Japan. Primex estimates that there are about 2,000 tonnes of food grade chitosan being produced on an annual basis, and of that amount, about 50-100 tonnes is sold in the US.

2. Production Process of Primex Chitosan

Primex has developed a novel production process for chitosan, based on chitin from North Atlantic shrimp shells (*Pandalus borealis*). The company has secured a supply of this raw material through long term partnerships with the Icelandic shrimp industry and its adjacent peeling plant in Siglufjördur. North Atlantic shrimp have advantages in that they are not bottom dwellers like crabs, and therefore, are less likely to be contaminated with heavy metals.

Technological developments of new production processes has allowed the peeling industry to supply the shell raw material to Primex in full accordance with HACCP standards, ensuring high quality raw material.

The shrimp raw material is stored frozen immediately upon harvest at a temperature of less than -10°C on board the shrimp fishing vessels. The date and location of the harvest are also logged. The shrimp are stored frozen until they are thawed immediately prior to the peeling process. Once peeled, the shells are immediately sent to Primex' chitosan manufacturing plant for processing.

Primex's production processes are optimized with the aid of specialized production equipment. All quality control procedures are based on a single source of shrimp shell raw material. Primex personnel design all process equipment, and the entire process is fully automated from the receipt of the shrimp shells at Primex production plant to the final product. The product obtained from this process is currently being sold in Japan and Korea as a food ingredient or additive, and in other countries for non-food uses. It should be noted that all production raw materials are food grade quality and that there are a variety of critical control points used to ensure the wholesomeness of the product. Shrimp-derived chitosan produced by this process is now being sold into the more than 50

countries worldwide, including to the European Union, the US, and Japan as a dietary supplement.

The following is a basic description of the production process for Primex chitosan.

a) Collection of Raw Material

The shrimp shells are transferred via pipelines directly from the adjacent shrimp peeling factory to Primex' processing plant. The shells are kept at a constant refrigerated temperature and allowed to sit a maximum of 4 hours post peeling. Upon receipt, Primex quality control personnel perform a visual inspection of the raw material. Rejected material is discarded [Primex Ingredients ASA Production Process Vol 8 Tab 121]. Suitable shells are transferred to the automated processing equipment.

b) Deproteinization

The shrimp shells are stirred in specialized stainless steel tanks for a defined time in a solution of NaOH and water calculated to remove the protein from the shells. The water/NaOH solution is removed by passing the mixture through a mechanical filter.

c) Washing I

Fat and color are rinsed from the shrimp shell chitin with EU-classified tap water in specialized stainless steel tanks through sequential washing steps, which involve heating the shells and water to an appropriate temperature and adjusting the pH to a specific value.

d) Demineralization

The shrimp shell chitin is stirred in specially constructed stainless steel tanks in a solution of HCL and water at an appropriate pH and temperature. Process operators visually monitor the reaction. Laboratory analysis of ash content should not exceed 2.0 %.

e) Washing II

The demineralized chitin is washed with heated and pH adjusted tap water. The purpose of this rinse is to remove soluble fat and color. The pH value after washing should be >6.

f) Deacetylation

The chitin is added to a solution of NaOH of appropriately high concentration in order to remove the acetyl groups from the chitin. The chitin/NaOH solution is stirred at an appropriate temperature for an appropriate time in special stainless steel tanks. Analysis of the degree of deacetylation is conducted in the Primex laboratory. After deacetylation is completed the product is filtered through a mechanic filtering system.

g) Washing III

After the process of deacetylation, the mixture is rinsed with tap water in sequential steps to remove the NaOH and to reduce the pH value to less than 8. After washing is completed, the product is filtered through a mechanic filtering system.

h) Drying and Milling

The product can either be 1) dried directly into flake form appearance or 2) dried and milled in one step into powder appearance.

1. The chitosan wet flakes are dried in a Turbo dryer, until the desired moisture has been achieved. The moisture content of the chitosan is reduced to less than 10% before drying is completed.

2. The chitosan wet flakes are dried and milled in a one step process using a designer made Turbo drier/milling system, until the desired moisture and particle size is achieved. The moisture content of the chitosan is reduced to less than 10% before drying is completed.

i) Sieving and Packaging

After sieving through an appropriate wire filter screen, the product is collected in a hopper, and pumped into batch tanks. The production batches are then packed in large double lined bags, retainer samples are collected, and the product batches are stored until all the analyses are complete. Upon completion of the analyses, the product is packed in standardized cardboard cartons with inner polyethylene bags and labeled. When packaging is completed, the product is stored in accordance with food grade conditions at the Primex warehouse.

Extended deacetylation

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For the production of ChitoClear FG95, which is a highly deacetylated form of chitosan, additional deacetylation

processing is required. After the deacetylation processing step described in section f) above, the chitosan is pumped into a separate processing tank, and "fresh" NaOH of a known concentration is added. The mixture of chitosan / NaOH is stirred at a known rpm, at an appropriate temperature and for an appropriate time. Analysis of the degree of deacetylation is conducted in the Primex laboratory. After deacetylation is complete, the product is filtered through a mechanical filtering system.

Depolymerization

For the production of ChitoClear FGLV, which is a low viscosity form of chitosan, an additional depolymerization step is required. The depolymerization step is performed after the third washing step described in step g). Dilute H_2O_2 at specific temperatures is used in sequential steps in the same procedure as described for the washing steps. After depolymerization, additional washing steps are performed to neutralize the pH.

The sequence of production steps specific for each of the Primex chitosan products is shown below in Table 9 and Figure 1 below. Specific market categories for the various products are shown below in Table 10.

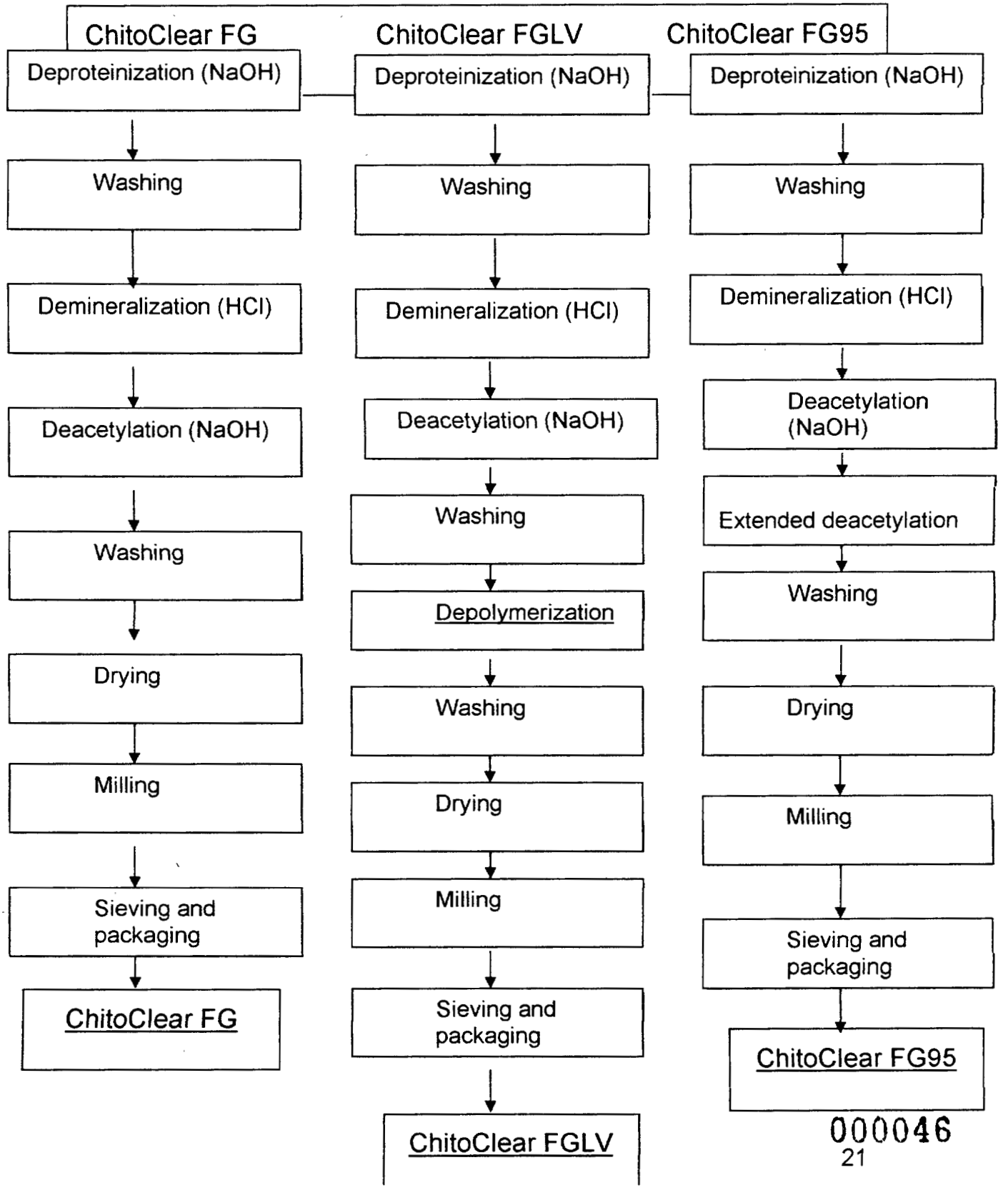
Table 9
Specific Production Steps for the Primex Chitosan Products

ChitoClear FG	Deproteinization, demineralization, deacetylation, drying/milling, sieving/packaging.
ChitoClear FG95	Deproteinization, demineralization, deacetylation, extended deacetylation, drying/milling, sieving/packaging.
ChitoClear FGLV	Deproteinization, demineralization, deacetylation, depolymerization, drying/milling, sieving/packaging.

Table 10
Primex Chitosan Products
Market and Properties

Primex Product Name	Properties of the Products	Market
ChitoClear FG	Highly deacetylated; Off-white powder/flakes; Used in the general food market	General Use in Foods; Dietary Supplements
ChitoClear FG95	Highly deacetylated; Off-white powder/flakes; Used as a dietary fiber	Dietary Supplements; Foods in General
ChitoClear FGLV	Depolymerized; Off-white powder/flakes; Low viscosity;	Used in Foods as an antimicrobial

Figure 1
Primex Chitosan Specific Processing Steps



3. HACCP Standard and Recall Tracking Program

The entire chitosan production process at Primex ehf is carried out according to the requirements of the HACCP standard. Primex has received a Quality Control Certificate from the Directorate of Fisheries stating that "Fish and Fishery products originating from this establishment have been handled and prepared or processed under a competent HACCP-and sanitary program consistently implemented and in accordance with US FDA's seafood HACCP regulation 21 CFR 123." Primex has provided written procedures for each specification parameter. The various analyses listed in Tables 1-3 are performed on each lot of final product. Methods for the various analyses can be found in Appendix 5.

References for test methods:

1. Dry matter content	: PRE-10-CP-001-03
2. Ash Determination	: PRE-10-CP-002-02
3. Colloidal titration-Degree of deacetylation	: PRE-10-CP-010-02
4. Solubility	: PRE-10-CP-006-02
5. Turbidity	: PRE-10-CP-003-02
6. Viscosity	: PRE-10-CP-004-02
7. Determination of Total Plate Count	: PRE-10-MB-003-02
8. Determination of Yeast and Mold	: PRE-10-MB-004-02
9. Determination of Coliform Bacteria	: PRE-10-MB-006-02
10. Determination of *E. coli*	: PRE-10-MB-005-02
11. Determination of *Salmonella*	: PRE-10-MB-008-02
12. Heavy Metal Content	: PRE-10-CP-514-02
13. Protein/Amino Acids	: Method of Uppsala University, Sweden

4. Critical Control Points in ChitoClear FG production:

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The Primex ehf chitosan plant has a certified Hazards Analysis Critical Control Point Program (HACCP) in place to assure product quality. Each segment of the Primex chitosan process is monitored

for quality control using critical control points, including raw materials procurement and storage.

Analytical methods are used to ensure that the final product parameters remain within food grade specifications. [Primex Ingredients Analytical Methods Vol 7 Tab 118].

The critical control points of the Primex production process are listed below and are shown in Figure 2.

1. Receipt of raw material: Visual control of shell. Check for odor.
2. Receipt of production chemicals: Visual control of package.
3. Deproteinization: pH level, temp, time of stirring, NaOH content, dry matter.
4. Demineralization: pH-level, temperature, time of stirring, acid content.
5. Deacetylation: NaOH content, temperature, time of stirring, dry matter.
6. Washing after deacetylation: pH-level, temperature
7. Packaging and storage: contaminants, temperature, humidity

Primex Chitosan Production	Description:	Quality Control Steps:
Collection of raw material Collection of shells at shrimp peeling plant, transported to processing plant.	Collection of shrimp shell raw material.	QC-steps: Visual quality control performed.
Deproteinization Shrimp shell chitin + NaOH are mixed and stirred at high temp.	Removal of protein.	QC-steps: Temp. time, NaOH-content, pH and dry matter content logged in prod. log. Visual control on color performed.
Washing Washing by tap water at high temp.	Removal of protein residuals and pH-level adjustment.	QC-steps: pH-level after each washing step logged in prod. log.
Demineralization Deproteinized chitin + HCl are mixed and stirred at medium temp.	Removal of minerals and protein residuals.	QC-steps: Temp., time, HCl-content and pH-level logged in prod. log.
Washing Washing by tap water at medium temp.	Removal of minerals and protein residuals and pH-level	QC-steps: Temp., pH-level, ash and dry matter content logged in prod. log.
Deacetylation Chitin + high concentration NaOH are mixed and stirred at high temp.	Removal of acetyl groups.	QC-steps: Temp., time, NaOH content and dry matter content logged in prod. log.
Washing Washing by tap water at medium and high temp.	Rinsing and neutralization.	QC-steps: Temp., dry matter content and pH-level after each washing step logged in prod. log.
Drying Direct drying of chitosan flakes, followed by sieving.	Drying.	QC-steps: Dry matter logged in prod. log. Visual control of color and feature performed.
Drying & Milling One step dry-milling process.	Drying/Milling.	QC-steps: Dry matter logged in prod. log. Visual control of color and feature performed.
Sieving & Packaging	Sieving &	QC-steps: Full analysis on all

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5. Primex tracking and recall program

Lot numbers are assigned to each batch produced from a given raw material. These lot numbers follow the batch throughout the production procedure. New lot numbers are given in chronological sequence when the final product is obtained. These lot numbers start with the designation TM (abbreviation for "tørket/møllet" in Norwegian - Dried / Milled, example "TM-XXXX"). The relation between the two lot number systems is kept on record for a minimum of 3 years. Accurate record keeping on the basis of the lot numbers assures that the product and all raw materials can be tracked from receipt to the retail level.

Adequate size retainer samples are stored for a minimum of 3 years from production in order to provide material for testing if necessary. Therefore, Primex ehf assures that it has the programs in place to execute a limited or general recall, in the event that its quality control system is breached. This program is described in a separate SOP (01-001-00).

Recall Program

The Primex recall program is designed to operate as follows:

-Non-conformance is recorded on a non-conformance report.
-The customer is asked to send a sample of the lot in question.
-The retainer lot is checked according to Primex procedures.
-The lot is traced back to the raw material and all production logs. Any potential non-conformance reports are investigated. Personnel involved in the production of the lot in question are "interviewed" to resolve any discrepancies.
-The customer is contacted to coordinate procedures at their warehouse.
-The complete lot is recalled from all customers if necessary.

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The Primex tracking and recall program is depicted below in Figure 3.

Figure 3
Primex tracking and recall program

Location		Documentation
1: Peeling factory	→	a: Log date and source of shrimp.
2: Raw material	→	b: Assign production lot number
3: Deproteinization	→	c: Record use of raw materials
4: Demineralization	→	d: Record use of raw materials
5: Deacetylation	→	e: Record use of raw materials
6: Drying / Milling		
7: Finished product	→	f: Retainer sample
		g: Assign final TM lot number

H. Characteristic Properties of Chitosan

1. Introduction

Comparison of the Physicochemcial Properties of Various Chitosans

Chitosan possesses certain physical properties, which form the basis for its functionality in foods. While the role of chitosan in food formulations will be discussed in Section II, several of the molecules basic properties are discussed below. Physicochemical characteristics and functional properties of five commercially available chitins and five chitosans were investigated by Shepard, *et al.,* 1997 [Shepard, *et al.,* 1997 Vol 8 Tab 146]. The physicochemical characteristics (nitrogen, ash, degree of deacetylation, bulk density, and viscosity) differed across the products tested. In functional properties, dye binding capacity differed depending on the products, although the average binding capacity (63%) of chitosans was higher that that (54%) of chitins [Shepard, *et al.,* 1997 Vol 8 Tab 146]. Water binding capacity ranged from 381 to 673% for chitins and from 458 to 805% for chitosans. Fat binding capacities of chitins were mostly similar (316-320%) except for one product (563%), whereas chitosans showed dissimilar binding capacities from 314 to 535%. However, the authors reported a significant correlation between water binding capacity and bulk density ($r = -0.89$, $P < 0.01$) and between fat binding capacity and viscosity ($r = 0.72$, $P < 0.05$) for chitin products. Both water and fat binding capacity of chitosan products were significantly correlated with ash ($r = 0.81$, 0.80) and with bulk density ($r = -0.98$, -0.95). The emulsifying capacity of egg yolk increased by addition of chitosan compared with the control. The authors stated that no differences in the emulsifying capacity of chitosan products was observed at any of the concentrations tested [Shepard, *et al.,* 1997 Vol 8 Tab 146].

2. Chitosan as an Absorbent of Free Fatty Acids in Palm Oil

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Ahmad *et al.,* 1997 studied the sorption capacities of low molecular weight chitosan. The purpose of the study was to determine whether

chitosan could absorb free fatty acids in oils, and thereby protect the oils from rancidity caused by the reaction of the free fatty acids with either oxygen or water. The authors' results showed that there are maximum sorption capacities of individual fatty acids towards chitosan; i.e., stearic (C_{18}:0), palmitic (C_{16}:0), myristic (C_{14}:0), oleic (C_{18}:1), and linoleic (C_{18}:2). Further, chitosans appeared to have a general pattern of absorption. Each fatty acid showed a maximum value of sorption to chitosan. The saturated fatty acids, which have longer carbon chains showed higher sorption capacities than did unsaturated fatty acids. In this study, stearic acid (C_{18}:0) had a higher sorption capacity than the other fatty acids tested, approximately 18 mg/g of chitosan. The lowest sorption capacities were given by myristic acid (C_{14}:0). The total sorption capacities of saturated acids decreased in the following order: C_{18}:0 (stearic) > C_{16}:0 (palmitic) > C_{14}: 0 (myristic) [Ahmad *et al.,* 1997 Vol 3 Tab 2]. The author concluded that chitosan might be useful in the treatment of oils to prevent rancidity.

Various investigators have established that chitosan is capable of absorbing oils and fatty acids [Ahmad *et al.,* 1997 Vol 3 Tab 2]. Ahmad *et al.,* 1997 has contributed the concept that chitosan has a greater affinity for absorbing saturated fatty acids, than those with less saturation. Such physical characteristics may be of great usefulness in healthy foods.

3. Physical and Chemical Structure of Chitosan

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Chitosan, a poly-glucosamine, is a natural substance found in the cell walls of fungi, and in soil, and sediments [Gooday *et al.,* 1990 Vol 4 Tab 45 and Shimahara, *et al.,* 1989, Vol 9 Tab 154]. In nature most chitosan is a breakdown product from the degradation of chitin by certain groups of bacteria, which produce deacetylase or chitosanase enzymes. Commercially, chitosan is also produced by the deacetylation of chitin. Primex ehf utilizes chitin from the Arctic shrimp *Pandalus borealis* as raw material for its chitosan production. Primex's commercial production of chitosan meets US HACCP standards, and results in a product, which is safe for human

consumption and can be shown to be lower in lead and other contaminants than other chitosans, or other marine food products (See Tables 4, 5, 6, and 9).

Chitosan is a binary heteropolymer of ß (1→ 4)-2-amino-2-deoxy—D-glucopyranose and ß (1→ 4)-2-deoxy-2-acetamido-D-glucopyranose. It's raw material, chitin is the (1→ 4)-β-linked homopolymer of N-acetyl-D-glucosamine, and is one of the most abundant organic materials on the planet. The individual polymer chains of chitin appear to be helixes, since each sugar unit is inverted in comparison to adjacent sugar units [Gooday *et al.,* 1990 Vol 4 Tab 45]. This configuration may account for the great stability and unique characteristics of chitin and chitosan.

Primex ehf has developed a set of food grade specifications for its chitosan products. The food grade specifications define products, which contain ≥ 90% dry matter, less than 1.0 % ash, and negligible amounts of protein and fat. The percent deacetylation of Primex chitosan is greater than 70%, and the turbidity rating is ≤50 NTUs. The product is further characterized by a solubility of ≥99% in 1.0 % acetic acid. Lead levels are very low (<0.1ppm), and microbial limits have been set to meet food grade specifications (Tables 1-3).

Analyses of 5 lots in duplicate show that Primex is capable of meeting its specifications, and of producing a consistent product (See Table 4). Primex ehf has a product recordation system in place, and maintains retainer samples, in the event a general recall is necessary.

I. Comparison of Primex Chitosan with Other Preparations 000054

Table 11 shows a comparison between the specifications of commercial chitosans. It should be noted that Primex chitosan contains less lead, has a higher degree of deacetylation, and provides a lower limit on microbial content than do the other chitosans. This may be due in part, because of the different source

organisms. Chitosan produced on the Pacific Rim is sourced from crab chitin, whereas that of Primex is sourced from arctic shrimp. It is logical to hypothesize, that the bottom-dwelling crab, would encounter more lead in its environment.

Table 11
Comparison of the Specifications of Commercial Chitosans

Variable	Japan*	Korea**	Kunpoong Bio	Primex
Description	White to bright yellowish	White to bright yellowish	White to bright yellowish	Off-White
Moisture	<12%	<15%	<7%	<10%
Deacetylation	>80%	>70%	>80%	>70%
Viscosity	>100 cPs (0.5%/0.5%)	-	-	≥5 and ≤5000 cPs (1%/1%)
Ash	<1.0%	<5.0%	<0.1%	<1.0%
Lead	<10 ppm	<20 ppm	<10 ppm	<0.1 ppm
CFUs/gram	<3,000			<1,000
Raw Material	Crab	Crab	Crab	Arctic Shrimp

* Japanese Health Food Association (JHFA) standard specifications
** Korean Food Additive standard specifications

J. Chitosan in Nature

Chitosan is a natural polysaccharide that occurs in nature in measurable amounts. It results in part from degradation of chitin from a variety of life forms (e.g. crustaceans) and from some species that produce chitosan directly. Chitosan is produced by several fungi, which utilize it in their cell walls as a structural component. Since there are vast amounts of fungi throughout nature with chitosan as an integral component, it is not surprising to learn that mankind has been unknowingly exposed to chitosan for millennia.

To illustrate this point, the fate of chitin in sediments in the Ythan Estuary, Aberdeen, Scotland has been studied by Hillman, Gooday, and Rosser (unpublished observations). The major input of chitin into this estuary is the productivity of the crustacean *Corophium volutator.*

Adults of this species contain 8.4% chitin [Gooday *et al.,* 1990 Vol 4 Tab 45].

The annual input from this source alone was estimated as 3.7g chitin per square meter. Chitin and chitosan were detected in the sediments. Levels of both were reported to be considerably higher in winter than summer. Values for chitin and chitosan in the top 2 cm of sediments were 4.3 and 55.8 mg (g dry sediments)-1, respectively, in summer, and 36.2 and 156.7 mg g-1 in winter. Chitin contents declined rapidly with depth, being very low in the anaerobic sediment below 5 cm. Chitosan levels showed little variation with dept in the upper sediment, but were lower in the anaerobic sediment. It was suggested that much of the chitosan represented degradation of chitin produced in the sediment, whereas much of the chitin was from surface deposition. Chitinase and chitin deacetylase activities were detected in the sediment, both being higher in summer than winter. Values for chitinase were 93 and 31 mg chitin hydrolyzed hr-1 (g dry sediment)-1, respectively, for summer and winter; for chitin deacetylase, values were 33 and 5 mg chitin deacetylated h-1 g-1 for summer and winter. All assays were carried out at 150°C. Chitinase activities showed little variation in depth whereas chitin deacetylase activities were highest in the upper sediment. The finding of considerable levels of chitosan and chitin deacetylase suggest, that in these sediments a considerable amount of chitin degradation occurs via deacetylase, as well as by hydrolysis to chito-oligosaccharides, but the major agents of this deacetylation have yet to be identified. Litterbag experiments (nylon, 20-p.m mesh), using purified chitin, chitosan, and cellulose for comparison, gave rates of degradation for 2 months in the summer as 10.3, 7.0 and 4.4 mg (g polysaccharides)-1 day -1, respectively. Hillman *et al.,* (1989) reported the operation of a model system designed to imitate the natural estuarine environment and the author used it to study chitin degradation. Results obtained were remarkably similar to those for the natural system; for example, relative rates for degradation of chitin =, chitosan, and cellulase for 1 month at 150°C were 2.4: 2.0:1,

compared with 2.3:1.6:1 in the Ythan Estuary [Gooday *et al.,* 1990 Vol 4 Tab 45].

In nature chitosans are degraded by chitosanases, which have generally been recognized as enzymes that attack chitosan but not chitin. The Enzyme Commission defines chitosanase (EC 3.2.1.123) as an enzyme catalyzing the endo hydrolysis of β-1,4-glycosidic linkages between GlcNAc and GlcN residues in a partially acetylated chitosan. This kind of activity, however, was also observed at significant levels for enzymes first identified as chitinases, lysozymes, cellulases or hemicellulases, proteases, lipases, and β-1,3-1,4-glucanase. The Enzyme Commission's initial definition of chitosanases was accompanied by a comment that chitosanases "act only on polymers with 30% to 60% acetylation." The definition of this enzyme no longer holds, according to more recent findings on chitosanases. From the viewpoint of enzyme definition alone, further information about the structure and function of the chitosanase is needed [Gooday *et al.,* 1990 Vol 4 Tab 45]. Work at the Norwegian Biopolymer Laboratory and elsewhere has shown that there are many chitinases and chitosanases with specific but varying specificities for linkages and monomer sequences (O. Smidsrød, personal communication).

Table 12 below summarizes results from Shimahara, *et al.,* 1989, who studied the production of chitosan by various fungi in the family *Mucoraceae* [Shimahara, *et al.,* 1989, Vol 9 Tab 154]. The results show that certain species of fungi produced up to 180 mg/200ml of medium [Shimahara, *et al.,* 1989, Vol 9 Tab 154]. These data illustrate that chitosan is widespread in nature.

Table 12

Amounts Of Alkali-Insoluble Materials (AIM) and Chitosan Produced by *Mucoraceae* Strains; Viscosities and Degrees of Acetylation of Chitosan Isolates

Genus, Species, and Strain		AIM	Chitosan	%Chitosan	Vis.
Ab.	*california NRIC* 1165	352	137	39	9.4
Ab.	*glauca NRIC* 1181	334	140	42	11
Ab.	*butleri Hut* 1001	416	-	43	16
Ab.	*glauca var. paradoxa NRIC* 1182	290	108	37	17
Ab.	*japonica AHU* 9006	275	124	45	8.4
Ab.	*orchidis HUT* 1034	328	163	50	17
Ac.	*elegans IFO* 4022	295	35	12	NT
C.	*minor HUT* 1077	180	38	21	6.3
C.	*mucoroides HUT* 1079	222	45	20	5.2
C.	*muscae HUT* 1086	92	43	47	4.5
M.	*alternans HUT* 1115	104	15	14	NT
M.	*dubius HUT* 1128	220	31	14	5.2
M.	*guilliermondii IFO* 9403	408	30	7	NT
M.	*inaequisporus IFO* 8624	35	13	37	NT
M.	*tuberculisporus IFO* 9256	292	43	15	3.4
P.	*nitens IFO* 5694	46	13	28	Nt
			129		
R	*acetoins*	330		39	12
R.	*chinnensiss*		66		
		144	115	45	2.4
R.	*nircinans*	212		54	5.1
R.	*delemar*		84		
		292	98	29	2.4
R.	*microsporous*	250	96	39	3.2
R.	*nodosus*	274		35	2.6
Z.	*californesis*	253	57	26	3.8
Z.	*expoens*	466	65	14	1.6

Vis = Viscosity of 0.1 % chitosan solution (in 0.5 % acetic acid).
DA = Degree of acetylation evaluated from IR spectrum.
Ab. = Absidia ; Ac. = Actinomucor ; C. = Circinella; M. = Mucor ;
P. = Phycomycetes ; R. = Rhizopus; Z. = Zygorhynchus.
NT = Not tested.

K. Physicochemical Properties of Chitosan Which Form the Basis for Its Potential Functionality in Food

Chitosan is a biocompatible cationic polysaccharide found indigenously in the cell walls of various fungi, crustaceans and insects [Illum, 1998 Vol 5 Tab 61]. It can also be chemically derived by deacetylating chitin, which is the second most abundant natural material on earth [Shahidi, *et al.,* 1999 Vol 8 Tab 145 and Shahidi *et al.,* 1998 Vol 8 Tab 144]. Chitin is similar to cellulose in structure, and is found, for example, in the exoskeletons of crabs and shrimps. The term chitosan refers to a family of polymers whose members differ in their degree of deacetylation (i.e., ratio of N-acetylated to deacetylated subunits) and molecular weight. These two characteristics are fundamental to the physicochemical properties of the chitosans and therefore have considerable influence on their biological activities [Illum, 1998 Vol 5 Tab 61 and Shahidi *et al.,* 1999 Vol 8 Tab 145].

Over the last decade, coinciding with the commercial production of higher purity chitosans, much interest and experimental activity has centered on the use of such chitosans in numerous food and biomedical applications. Chitosans have been found to have many beneficial properties of use to the food industry, including, antimicrobial activity, protein aggregation, emulsification capability, film-forming ability, clarifying ability, and fatty acid absorption [Knorr, 1991 Vol 6 Tab 76 and Shahidi *et al.,* 1999 Vol 8 Tab 145].

This unique set of characteristics has led the chitosans to be considered as an ingredient in a wide array of food materials. Much of the evidence for the applicability of chitosan comes from the patent literature, where applications involving chitosan have ranged from edible films, to a component of artificial sweetening systems [U.S. Patent No. 5,741,505 VOL. 10 Tab 194, U.S. Patent No. 5,762,992, VOL. 10 Tab 195, and U.S. Patent No. 5,736,179 VOL. 10 Tab 193].

Section II Chemical Identity

One of the most active areas of chitosan applications research has been directed towards its antimicrobial properties, and the mechanism by which chitosan exerts this technical effect was partially elucidated by Helander, *et al.*, 2001 in Finland [Helander, *et al.*, 2001 Vol 13 Tab 277]. This group found that chitosan disrupts the barrier properties of the outer membrane of gram-negative bacteria. Chitosan (250 ppm) at pH 5.3 induced significant uptake of a hydrophobic probe 1-N-phenylnaphthylamine (NPN) in *Escherichia coli, Pseudomonas aeruginosa* and *Salmonella typhimurium*. The effect was reduced (*E. coli, Salmonellae*) or abolished (*P. aeruginosa*) by MgCl2. No NPN uptake was observed during exposure of the *Salmonellae* to chitosan at pH 7.2. Chitosan also sensitized *P. aeruginosa* and the *Salmonellae* to the lytic effect of sodium dodecyl sulfate (SDS); such sensitization was not blocked by MgCl2 and was reversible by washing chitosan-treated cells prior to SDS exposure. Chemical and electrophoretic analyses of cell-free supernatants of chitosan-treated cell suspensions showed that interaction of chitosan with *E. coli* and the *Salmonellae* involved no release of lipopolysaccharide (LPS) or other membrane lipids. However, chitosan rendered *E. coli* more sensitive to the inhibitory action of dyes and bile acids used in selective media. Highly cationic mutants of *S. typhimurium* were more resistant to chitosan than the parent strains. Electron microscopy showed that chitosan caused extensive cell surface alterations and covered the outer membrane with vesicular structures. Chitosan thus appeared to bind to the outer membrane, explaining the loss of the barrier function. The authors concluded that this property makes chitosan a potentially useful indirect antimicrobial for food protection.

Chitosan has been identified as being biocompatible, non-antigenic, non-toxic, and biofunctional [ka *et al.*, 2002 Vol 16 Tab 347]. Both chitosan and its raw material chitin are able to form complexes with many of the transition metals, as well as some of those from groups 3 –7 of the periodic table [Kamil, *et al.*, 2002 Vol 14 Tab 289]. The heavy metal-polymer complexes are believed to form as a result of oxidative bonding with chitosan. This involves the donation of

nonbonding pairs of electrons from the nitrogen, and/or the oxygen of the hydroxyl groups, to a heavy metal ion. N,O-Carboxymethyl chitosan has been found to bond chemically with ions of numerous heavy metals, such as iron, copper, mercury and zinc, thus binding or sequestering them when present in even 10 –1000 ppm. The cupric ion seems to form one of the strongest complexes with chitosan in the solid state. Synthetic antioxidants and chelating agents may be added to food products in order to prevent lipid oxidation. However, the growing consumer demand for food devoid of synthetic antioxidants has focused reports on the discovery of new natural preservatives. Several sources of natural antioxidants are known, and some of them, such as those of rosemary and sage, are currently used in a variety of food products.

L. Potential Uses of Chitosan in Foods

Shahidi *et al.,* 1998 and 1999 summarizes the potential uses for chitosan in foods [Shahidi *et al.,* 1998 and 1999 Vol 8 Tabs 144 and 145]. The Table below was compiled by the authors of the two published reports.

In addition to the antimicrobial effects of chitosan, recent food application studies have shown that chitosan can contribute positive characteristics to baked goods through emulsification (moistness and fineness of grain). However, the product appears to have a self-limiting level which, when exceeded produces a final consumer product, whose total quality is less than comparable controls [Poole, 1989 Vol 11 Tab 225]. Chitosan is currently being used in dietary supplement preparations as a source of dietary fiber, and as a hypocholesteremic agent [Primex Ingredients ASA Product Labels, Vol 8 Tab 126].

Table 13
Food Applications of Chitin, Chitosan and Their Derivatives In The Food Industry

Area of application	Examples
Antimicrobial agent	Bactericidal Fungicidal Measure of mold contamination In agncultural commodities
Edible films	Controlled moisture transfer between Food and surrounding environment Controlled release of antioxidants Controlled release of nutrients, flavors and drugs Reduction of oxygen partial pressure Controlled rate of respiration Temperature control Controlled enzymatic browning In fruits Reverse osmosis membranes
Additive	Clarification and deacidification In fruits and beverages Natural flavor extender Texture controlling agent Emulsifying agent Food mimetic Thickening and stabilizing agent Color stabilization
Nutritional quality	Dietary fiber Hypocholesterolemic effect Livestock and fish feed additive Reduction of lipid absorption Production of single cell protein Antigastric agent Infant feed ingredient
Recovery of solid materials Food processing wastes	Affinity flocculation Fractionation of agar
Purification of water	Recovery of metal ions, Pesticides, phenols and PCB's Removal of dyes
Other applications	Enzyme immobilization Encapsulation of nutraceuticals Chromatography Analytical reagents

Section II Chemical Identity

1) Use of Chitosan as an Antimicrobial

Most recently, a number of studies have been published on the use of chitosan as an antimicrobial. Begin, *et al.,* 1999, of Agriculture and Agri-Food Canada reported on the types of antimicrobial films that may be produced from chitosan. In their laboratory, the authors prepared antimicrobial films by dissolving chitosan into hydrochloric, formic, acetic, lactic and citric acid solutions. Below 40 degrees C, the counter ions could be classified into two groups based on their effect on zero-shear-rate viscosity in 2% solutions of organic acids. Chloride and citrate produced solutions with much lower viscosities than formate, acetate and lactate. At higher temperatures, these differences vanished, and the activation energies of viscous flow were all similar between 40 and 60 degrees C. Films prepared from these solutions were evaluated in tension for Young's modulus, stress and elongation at yield and break points. Films made from hydrochloric, formic and acetic acids were hard and brittle, whereas those from lactic and citric acids were soft and could be stretched. The authors found a good correlation between Young's modulus and the volume of the counter ion. They explained that film properties are essentially governed by the volume of the counter ion and not by the interactions between a counter ion and the macromolecule under evaluation. Their results suggested that acetate has the maximum molecular volume above which the film strength decreases very rapidly Begin, *et al.,* 1999 Vol 3 Tab 14].

Ouattar, *et al.,* 2000 studied the inhibition of surface spoilage bacteria in processed meats with antimicrobial films prepared with chitosan at Agriculture Agri-Food Canada [Ouattar, *et al.,* 2000 Vol 15 Tab 323]. This study was undertaken to evaluate the feasibility of using antimicrobial films, designed to slowly release bacterial inhibitors, to improve the preservation of vacuum-packaged processed meats during refrigerated storage. The antimicrobial films were prepared by incorporating acetic or propionic acid into a chitosan matrix, with or without addition of

lauric acid or cinnamaldehyde. These films were tested with bologna, regular cooked ham, or pastrami. At various times during storage, the packages were opened and the levels of antimicrobial agents remaining in the chitosan matrix were measured. Regardless of film composition or meat product, propionic acid was nearly completely released from the chitosan matrix within 48 h of application, whereas release of acetic acid was more limited, with 2-22% of the acid remaining in chitosan after 168 hours of storage. Addition of lauric acid, but not cinnamaldehyde, to the chitosan matrix generally reduced the release of acetic acid significantly ($P <$ or $= 0.05$) and the release was more limited onto bologna than onto ham or pastrami.

In addition, the efficacies of the various films for inhibiting the growth of indigenous lactic acid bacteria, *Enterobacteriaceae*, *Lactobacillus sakei* or *Serratia liqueficiens*, which had been surface-inoculated onto the meat products were tested. Whereas lactic acid bacteria were not affected by the antimicrobial films under study, the growth of *Enterobacteriaceae* and *S. liquefaciens* was delayed or completely inhibited as a result of film application. The strongest inhibition was observed on drier surfaces (bologna), where acid release was slower, and with films containing cinnamaldehyde, since this compound showed greater antimicrobial activity under these conditions [Ouattar, *et al.*, 2000 Vol 15 Tab 323].

The antimicrobial properties of chitosan in mayonnaise and mayonnaise-based shrimp salads was reported by Roller, et al., in 2000 [Roller, *et al.*, 2000 Vol 15 Tab 331]. Mayonnaise containing 3 g/liter of chitosan combined with acetic acid (0.16%) or lemon juice (1.2 and 2.6%) was inoculated with log 5 to 6 CFU/g of *Salmonella enteritidis*, *Zygosaccharomyces bailii*, or *Lactobacillus fructivorans* and stored at 5 and 25 degrees C for 8 days. In mayonnaise containing chitosan and 0.16% acetic acid, 5 log CFU/g of *L. fructivorans* were inactivated, and numbers remained below the sensitivity limit of the plate counting technique for the

duration of the experiment. *Z. bailii* counts were also reduced by approximately 1 to 2 log CFU/g within the first day of incubation at 25 degrees C, but this was followed by growth on subsequent days, giving an overall growth delay of 2 days. No differences in counts of *Z. bailii* in mayonnaise stored at 5 degrees C or of *Salmonella enteritidis* stored at either temperature were observed.

In mayonnaise containing lemon juice at both 1.2 and 2.6%, no substantial differences were observed between the controls and the samples containing chitosan. In shrimp salads stored at 5 degrees C, the presence of a coating of chitosan (9 mg/g of shrimp) inhibited growth of the spoilage flora from approximately log 8 CFU/g in the controls to log 4 CFU/g throughout 4 weeks. However, at 25 degrees C, chitosan proved ineffective as a preservative. The results demonstrated that chitosan may be useful as a preservative when combined with acetic acid and chill storage in specific food applications [Roller, *et al.,* 2000 Vol 15 Tab 331].

In a study published one year later, Oh, *et al.,* 2001 evaluated the antimicrobial characteristics of chitosans against food spoilage microorganisms in mayonnaise and liquid media in Korea. Four different kinds of chitosans were prepared for this study. The antimicrobial activities of the chitosans were tested against four species of food spoilage microorganisms (*Lactobacillus plantarum, Lactobacillus fructivorans, Serratia liquefaciens*, and *Zygosaccharomyces bailii*). The initial effect of the chitosans was biocidal, and counts of viable cells were significantly reduced. After an extended lag phase, some strains recovered and resumed growth. The activities of chitosan against these microorganisms increased with the concentration. Chitosan-50 was most effective against *L. fructivorans*, but inhibition of *L. plantarum* was greatest with chitosan-55. The authors found no significant difference among the chitosans in their antimicrobial activity against *S. liquefaciens* and *Z. bailii*. The addition of chitosan to mayonnaise significantly decreased the viable cell

counts of *L. fructivorans* and *Z. bailii* during storage at 25 degrees C. These authors concluded that chitosan could be used as a food preservative to inhibit the growth of spoilage microorganisms in mayonnaise [Oh, *et al.,* 2001 Vol 15 Tab 318].

Rhoades, *et al.,* 2000, described the antimicrobial actions of degraded and native chitosan against spoilage organisms in laboratory media and foods. The objective of that study was to determine whether chitosan and hydrolysates of chitosan could be used as novel preservatives in foods. Chitosan was hydrolyzed by using oxidative-reductive degradation, crude papaya latex, and lysozyme. Mild hydrolysis of chitosan resulted in improved microbial inactivation in saline and greater inhibition of growth of several spoilage yeasts in laboratory media, however, highly degraded chitosan products exhibited no antimicrobial activity [Rhoades, *et al.,* 2000 Vol 15 Tab 330].

In pasteurized apple-elderflower juice stored at 7 degrees C, the addition of 0.3 g of chitosan per liter eliminated yeasts entirely for 13 days, while the total counts and the lactic acid bacterial counts increased at a slower rate in comparison to the control. The addition of 0.3 or 1.0 g of chitosan per kg had no effect on the microbial flora of hummus, a chickpea dip; in the presence of 5.0 g of chitosan per kg, bacterial growth but not yeast growth was substantially reduced compared with growth in control dip stored at 7 degrees C for 6 days. Improved antimicrobial potency of chitosan hydrolysates like that observed in the saline and laboratory medium experiments was not observed in juice and dip experiments. The authors concluded that native chitosan has potential for use as a preservative in certain types of food but that the increase in antimicrobial activity that occurs following partial hydrolysis is too small to justify the extra processing involved.

Roller, et al., 1999, from the School of Applied Science at South Bank University in London, UK investigated the antifungal properties of chitosan glutamate in laboratory media and apple

juice. The antimicrobial properties were investigated against 15 yeasts and moulds associated with food spoilage. Of the seven strains of filamentous fungi studied, chitosan reduced the growth rate of *Mucor racemosus* at 1 g/l, while concentrations of 5 g/l were required to completely prevent growth of three strains of *Byssochlamys spp.* on agar plates incubated at 25 degrees C for 3 weeks. Three strains of filamentous fungi were resistant to the antifungal effects of chitosan at 10 g/l. The presence of chitosan in apple juice (pH 3.4) at levels ranging from 0.1 to 5 g/l inhibited growth at 25 degrees C of all eight spoilage yeasts examined in this study. The initial effect of chitosan in apple juice was biocidal with viable numbers reduced by up to 3 log cycles. Following an extended lag phase, some strains recovered and resumed growth to levels similar to those observed in unsupplemented apple juice [Roller, et al., 1999 Vol 15 Tab 331].

The most sensitive strain was an isolate of *Zygosaccharomyces bailii* obtained from a spoiled carbonated beverage; this yeast was completely inactivated by chitosan at 0.1 and 0.4 g/l for 32 days of storage at 25 degrees C. The most resistant strain was *Saccharomycodes ludwigii*, an isolate from spoiled cider. A level of addition of 5 g/l of chitosan was required to inactivate this strain and to maintain yeast-free conditions in apple juice for 14 days at 25 degrees C. Growth inhibition and inactivation of filamentous moulds and yeasts, respectively, was determined to be concentration-, pH- and temperature-dependent. It was concluded that chitosan was worthy of further study as a natural preservative for foods prone to fungal spoilage [Roller, et al., 1999 Vol 15 Tab 331].

000067

Knowles, *et al.,* 2001, investigated the efficacy of chitosan, carvacrol, and a hydrogen peroxide-based biocide against foodborne microorganisms in suspension and adhered to stainless steel. The ability of natural compounds to inactivate foodborne organisms adhered to surfaces was investigated with the ultimate aim of replacing synthetic biocides by more

environmentally friendly, natural alternatives. The antimicrobial efficacy of 0.5, 1.0, and 2.0% chitosan and Spor-Klenz RTU (a commercial biocide based on hydrogen peroxide and peroxyacetic acid) and 0.5, 1.25, and 2.0 mM carvacrol was determined at 20 degrees C against *Listeria monocytogenes, Salmonella enterica serovar Typhimurium, Staphylococcus aureus*, and *Saccharomyces cerevisiae* adhered to stainless steel disks. The authors found that treatment with up to 2.0% chitosan reduced the viable cell count in the microbial films of the four test organisms by 2.4, 1.8, 2.3, and 0.9 log CFU/test surface (t.s.), respectively. By contrast, planktonic counts of the same organisms were reduced by 0.8 to 1.7 log CFU/ml at 2.0% chitosan. Treatment with 2 mM carvacrol reduced the viable counts of adhered *Listeriae, Salmonellae*, and yeasts by 2 to 3 log CFU/t.s. but *S. aureus* counts were reduced by only 0.9 log CFU/t.s. The efficacy of any single compound was species specific. In the case of microbial films prepared using *Listeriae* and *Salmonellae*, Spor-Klenz RTU was most biocidal, followed by carvacrol and then chitosan. However, dried films of *S. aureus* were most sensitive to chitosan and relatively resistant to carvacrol and Spor-Klenz RTU. By contrast, yeast films were most sensitive to carvacrol and least sensitive to chitosan. It was concluded that carvacrol and chitosan may have potential for use as natural biocides although optimization of conditions would be necessary [Knowles, *et al.,* 2001 Vol 14 Tab 295]

000068

A group of scientists working at the Food Research and Development Centre, Agriculture and Agri-Food Canada, and led by Savard, 2002 described the antimicrobial action of hydrolyzed chitosan against spoilage yeasts and lactic acid bacteria of fermented vegetables [Savard, *et al.,* 2002 Vol 15 Tab 335]. The antimicrobial properties of various chitosan-lactate polymers (ranging from 0.5 to 1.2 MDa in molecular weight) against two yeasts isolated from fermented vegetables and against three lactic acid bacteria from a mixed starter for sauerkraut were investigated on methylene blue agar (MBA) and in vegetable juice

medium (VJM). Chitosan-lactate reduced the growth of all microorganisms in solid MBA as well as in liquid (VJM) medium. In MBA, a concentration of 5 g/liter was needed to inhibit the growth of *Saccharomyces bayanus*, while 1 g/liter was sufficient to inhibit the growth of *Saccharomyces unisporus*. Lactic acid bacteria were also inhibited in this range of concentrations. The low-molecular-weight chitosan-lactate DP3 (0.5 kDa) was reported to be most efficient in solid medium (MBA), and inhibitory activities decreased with increasing hydrolysate lengths. In liquid medium (VJM), 0.5 g of chitosan-lactate per liter reduced the growth rates for both yeasts, but 10 g/liter was insufficient to prevent yeast growth. Intermediate-molecular-weight chitosan-lactate (5 kDa) was more efficient than chitosan of low molecular weight. Native chitosan (1.2 MDa) showed no inhibition in either medium. Microscopic examination of *S. unisporus* Y-42 after treatment with chitosan-lactate DP25 showed agglutination of a refractive substance on the entire cell wall, suggesting an interaction between chitosan and the cell wall. When chitosanase was added to the culture media containing chitosan-lactate, refractive substances could not be observed.

Kamil, *et al.*, 2002 studies the antioxidant efficacy of chitosans of different viscosity (14 cP, 57 cP and 360 cP) in cooked, comminuted herring flesh (Clupea harengus) [Janak, *et al.*, 2002 Vol 16 Tab 346]. The oxidative stability of treated fish flesh was determined and compared with fish treated with conventional antioxidants (at a level of 200 ppm), such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tert–butylhydroquinone (TBHQ). The progress of oxidation was monitored by employing the peroxide value, 2-thiobarbituric acid-reactive substances (TBARS) and static headspace gas chromatographic analysis. In general, all chitosans exhibited varying anti-oxidant activities in this fish flesh model system. The formation of hydroperoxides and TBARS, in herring samples containing 200 ppm 14 cP chitosan, was reduced after 8 days of storage by 61 and 52%, respectively. Among the different

viscosity chitosans, 14-cP chitosan was more effective than the higher viscosity chitosans in preventing lipid oxidation in the herring flesh model system.

The authors explained that the highly unsaturated fatty acids commonly found in seafoods are particularly sensitive to oxidative change during storage. Various authors have shown that the oxidation of muscle foods occurs in the order of fish >poultry >pork >lamb. Although the process of lipid oxidation is favored in meats, the direct reaction between oxygen and highly unsaturated lipids is kinetically hindered [Kamil, *et al.*, 2002 Vol 14 Tab 289]. Hence, an activating factor is necessary to initiate free radical chain reactions followed by self-propagation. The authors stated that it had been proposed that lipid oxidation in fish may be initiated and promoted by a number of mechanisms involving autoxidation, photo-sensitized oxidation, lipoxygenase, peroxidase, and microsomal enzymes. Researchers have identified several sources of protein-bound iron that exist in biological tissues, namely myoglobin, hemoglobin, ferritin, transferrin, and haemosiderin. Iron bound to these proteins may be released during post-harvest storage and cooking, activating oxygen and initiating lipid oxidation. Janak, *et al.*, stated that there is a range of concentrations of haematin compounds in muscles from different species of fish and that these are present in relatively large concentrations in the muscle of most fatty fish, especially their lateral band dark muscle. Autoxidation of oxymyoglobin and oxyhaemoglobin (both in the Fe 2+ oxidation state) may also result in the formation of superoxide anion, metmyoglobin and methaemoglobin (both in the Fe 3+ oxidation state). The formation of superoxide anion from oxymyoglobin/oxyhaemoglobin may be catalyzed by anions such as SCN ,OCN ,F and Cl. The hypothesis has been that increased oxidation of seafoods at lower humidities may be attributed to the concentration of prooxidants such as metal ions and hemoglobin. The main source of free iron or nonheme iron in cells is ferritin, which is a soluble iron storage protein found in liver, spleen and

skeletal muscle and has a molecular mass of 450 kDa and contains 4500 iron atoms when fully loaded. Researchers have observed that storage of unfrozen mackerel ordinary muscle at 4C for 7 days resulted in a 1.4-fold increase in low molecular weight iron-containing compounds from 0.16 to 0.23 m Fe/g muscle. A small amount of iron is also bound to molecules such as ATP, ADP, organic acids and DNA. These compounds are capable of decomposing hydroperoxides (ROOH) in order to form free radicals. Others have reported that metal ions such as those of copper and iron, can enhance lipid autooxidation to a greater extent in their lower valency states. Also reported, is that ferrous ion at 1 –10 ppm levels acts as a strong pro-oxidant in cooked fish muscles, and that the relative prooxidant activity of ions in fish muscle decreased in the order of Cu 2+ >Fe 2+ >Co 2+ >Cd 2+ >Li + >Ni 2+ >Mg 2+ >Zn 2+ >Ca 2+ >Ba 2+. Superoxide anion may be dismutated to form hydrogen peroxide, resulting in the formation of hydroxyl radicals via the reaction of H2 O2 with Fe 2+. In this study low viscosity chitosan, was identified as a versatile antioxidant for a broad range of seafood applications [Kamil, *et al.,* 2002 Vol 14 Tab 289].

The purpose of a study by Sagoo, *et al.,* 2002 was to develop novel preservation systems for chilled, comminuted pork products that are sold raw using chitosan [Sagoo, *et al.,* 2002 Vol 15 Tab 333]. *In vitro* testing had showed that viable numbers of *Saccharomyces ludwigii* were reduced by up to 4 log cfu/ml on exposure to 0.05%chitosan in 0.9% saline at pH 6 .2. Higher concentrations of chitosan (0.25 and 0.5%)were required to achieve similar levels of inactivation with *Lactobacillus viridescens*, *Lac. sakei* and *Listeria innocua. Torulaspora delbrueckii* and *Salmonella enteritidis* PT4 were resistant to chitosan at the concentrations tested in this study (up to 0 .5%).Trials in real foods showed that dipping of standard and skinless pork sausages in chitosan solutions (1.0%) reduced the native microflora (total viable counts, yeasts and molds, and lactic acid bacteria by approximatel 1 –3 log cfu/g for 18 days at 7 °C.

Chitosan treatment increased the shelf-life of chilled skinless sausages from 7to 15 days. Addition of 0.3and 0.6% chitosan to an unseasoned minced pork mixture reduced total viable counts, yeasts and molds, and lactic acid bacteria by up to 3 log cfu/g for 18 days at 4 °C compared with the untreated control. The results indicated that chitosan was an effective inhibitor of microbial growth in chilled comminuted pork products [Sagoo, *et al.,* 2002 Vol 15 Tab 333].

2) Demonstrations of the emulsification properties of chitosan in food models

The same principals that are operable in the human body, with respect to the fat emulsifying properties of chitosan can also be utilized in food formulations. Particularly interesting from the point of view of a food scientist is the ability of chitosan to form emulsions with lipids in an acid environment. The following tables summarize the work of Stephen Poole, 1989, and Rodriguez, 2000, who researched the emulsification and foaming properties of chitosan in model food systems [Poole, 1988 Vol 11 Tab 225 and Rodriguez, *et al.,* 2000 Vol 17 Tab 376]. Greater detail on the work of these two investigators can be found in Appendix IV.

a) Behavior of Chitosan Emulsions and Droplet Formation

According to Rodriguez, *et al.,* 2000, chitosan appears to be a promising emulsifier, because it provides both electrostatic and viscosifying stabilization [Rodriguez, *et al.,* 2000 Vol 1 7 Tab 376]. Due to its properties as a polymeric surfactant, chitosan participates in the following mechanisms:

i. Chitosan, like polymeric surfactants, may attach to a surface via several segments, which gives it a large free energy of adsorption, despite a low free energy of adsorption per segment.

ii. Unlike low molecular weight surface-active agents, chitosan offers steric stabilization of dispersions.

Together these mechanisms are referred to as electrostatic stabilization.

Rodriguez, *et al.*, 2000 determined the most efficient and economical shrimp-derived chitosan/oil ratios to yield stable emulsions. In a series of experiments, emulsions were prepared by adding a variable quantity of sunflower oil (20-120g) to a constant amount of 1% w/v chitosan in aqueous acetic acid (80g). Samples were stirred in a food processor for 3 minutes and a 65 ml aliquot was placed in a 100 ml cylinder. The elapsed time to phase separation was recorded. Three layers were observed when phase separation occurred: oil, white emulsion, and a dilute emulsion composed of very fine droplets. The researchers found that 86 g chitosan solution per 14 g oil and 80 g chitosan solution per 20 g oil (the second being the most economical) yielded the most stable emulsions (Table 14) [Rodriguez, *et al.*, 2000 Vol 17 Tab 376].

Table 14
Behavior of Chitosan Emulsions[1]

Chitosan solution (1%)/ oil Proportion, g/g	Stability (1 h)
92/8	Two phases separation: Concentrated and dilute emulsion
86/14	No phase separation
80/20	No phase separation
70/30	Two phases separation: Concentrated and dilute emulsion
60/40	Three phases separation: Oil, concentrated and Dilute emulsion
40/60	Three phases separation: Oil, concentrated and Dilute emulsion

[1]Chitosan was sourced from shrimp.

b) Foam-enhancing Properties of Low-viscosity Chitosan

Small basic (cationic) proteins are so effective at enhancing the foaming properties of acidic proteins that they can tolerate the inclusion of lipids, which would otherwise prevent the foaming of solutions of acidic proteins alone. Chitosan is basic and interacts with proteins, but high molecular weight chitosan with a high viscosity does not foam, because the interaction with protein is so strong that a precipitate is formed [Poole, 1988 Vol 11 Tab 225].

Poole determined whether low viscosity chitosan behaves like basic proteins, which enhance the foaming of acidic proteins in the presence of lipids. Table 15 shows the effect of low-viscosity chitosan (1% in 1% acetic acid) on the foaming of a 6% whey protein isolate (WPI) system stressed with corn oil. The lipid prevented foaming of the WPI until chitosan was

incorporated at a level of 0.6% or above. At that point, excellent foaming was observed [Poole, 1988 Vol 11 Tab 225]. Chitosan and the acidic protein were dissolved separately in 0.8% acetic acid. The oil or melted fat was emulsified with the protein solution and any carbohydrate added. The chitosan solution was then mixed with the protein solution and the pH was adjusted with 1M NaOH. The resulting mixture was whipped at full speed (200 rev/min) for 5 minutes in a Kenwood Chef Mixer. The prepared foam was then transferred to a graduated cylinder before the desired parameters were measured [Poole, 1988 Vol 11 Tab 225].

Table 15
Effect of Low-Viscosity Chitosan Concentration on the Foaming of Whey Protein Isolate
(WPI = 6.0%; corn oil = 10%; sucrose = 10%; pH = 6.0)

% Chitosan	%Foam Expansion	%Foam Volume Stability	%Foam Liquid Stability	Foam Description
0	120	60	15	Poor
0.1	140	65	15	Poor
0.2	100	60	20	Poor
0.4	100	60	20	Poor
0.6	550	100	90	Fairly Stiff
0.7	560	100	95	Very Stiff
0.8	560	100	95	Very Stiff

The lipid prevented foaming of WPI, but when chitosan was incorporated at a level of 0.6% (ratio = 1:10), foaming was excellent. As with basic protein, it is believed that this enhancing effect involves electrostatic interactions between the proteins molecules and chitosan at a pH value above the isoelectric point (pH 5.3) of the WPI [Poole, 1988 Vol 11 Tab 225].

Electrostatic interactions between acidic and basic species are by definition pH-dependent and foaming behavior reflects this,

according to the author. Foam enhancement occurred within a fairly narrow pH range (5.5-6.5). Above pH 6.5 excessive precipitation prevented foaming.

Below pH 5.3, the protein was positively charged and did not interact strongly with chitosan. Hence careful control of pH would be required in processing operations. However, this may be more than compensated by the benefits of being able to incorporate fat (e.g. cocoa or cocoa butter) in protein-based whipping formulations.

Additional studies in which the ability of chitosan to assist in foam formation was optimized can seen in Appendix IV.

3) Chitosan as a Source of Dietary Fiber

The current definition of dietary fiber consists of the remnants of edible plant cell walls, including: polysaccharides, lignin, and associated substances resistant to (hydrolysis) digestion by the alimentary enzymes of humans [Dreher, 1987 Vol 4 Tab 27]. Food scientists from around the world have recognized chitosan a source of dietary fiber [Furda, 2000, Vol 4 Tab 40, Dreher, 1987 Vol 4 Tab 27, and Institute of Medicine, 2001 Vol 17 Tab 373]. The Panel on the Definition of Dietary Fiber and the Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, of the Food and Nutrition Board, Institute of Medicine, has defined "Added Fiber" as "isolated, nondigestible carbohydrates that have beneficial physiological effects in humans" [Institute of Medicine, 2001 Vol 17 Tab 373]. According to the Panel's definitions, chitosan would contribute to "Total Fiber" intake in humans. While, certain professional organizations may continue to refine the definition of dietary fiber, chitosan nonetheless consists largely of polysaccharides that are not digested in the upper gastrointestinal tract of humans [AACC Report on Dietary Fiber, 2002 Vol 12 Tab 226].

Dreher in the Handbook of Dietary Fiber, classified chitosan as a non-traditional source of dietary fiber, and stated that chitin/chitosan were aminopolysaccharides. Dreher described chitin as being similar to cellulose, except that it contained N-acetylamine groups at each 2-carbon atom of the glucose molecule [Dreher, 1987 Vol 4 Tab 27]. As stated above, chitin is the second most abundant organic compound on earth [Knorr, 1991 Vol 6 Tab 76], and is found especially in marine invertebrates, insects, fungi, and yeasts. Chitin represents 14-27% and 13-15% of the dry weight of shrimp and crab-processing wastes, respectively. It is a rather inert material and is very insoluble, however, the deacetylation of chitin with a strong base yields chitosan, which is a more soluble material, especially in dilute acids [Shahidi *et al.,* 1998 Vol 8 Tab 144]. According to Dreher, chitin and chitosan appear to have great potential in food science [Dreher, 1987 Vol 4 Tab 27].

4) Source of D-Glucosamine

Today, one of the uses of chitosan is as a feedstock or source for glucosamine. Hirano indicated in 1989 that approximately 60 tons of chitosan per year went into the production of D-glucosamine [Hirano, 1990 Vol 5 Tab 54].

Chitosan can function as a source of glucosamine, when taken as a dietary supplement. Glucosamine is an anti-inflammatory compound, synthesized in the human body from glucose and incorporated into gylcosaminolycans and glycoproteins [Muzzarelli, 2000 Vol 7 Tab 105].

5) Use of Chitosan as a Dietary Supplement in the U.S.

Use of chitosan as a dietary supplement in the U.S. has been growing rapidly. According to Information Resources, Inc.,

approximately US . has been sold by the three leading brands since February, 1999. This translates to approximately 240,000 kilograms of consumption, at an average wholesale price of U.S. . per pound. Actual usage may be closer to 500,000 kilograms per year, since the *Natural Products Insider, 1999 Buyer's Guide* lists 72 suppliers/distributors of the chitosan in the U.S.

Consumer use is mainly for the substance's hypocholesteremic activity, and for its use as a dietary source of glucosamine [National Toxicology Program, 2000 Vol 7 Tab 108].

M. Summary of the Current Market and Uses of Chitosan

1) Chitosan as an Antimicrobial

Several recently published studies have demonstrated that chitosan has potential as an antimicrobial and fungistat in various food systems. It appears that the type of chitosan and its concentration will need to be optimized for each application. However, scientists performing food protection experiments on several continents have concluded that chitosan may be a natural alternative to reduce the level of contamination by pathogenic and spoilage organisms in foods [Roller, *et al.,* 2000 Vol 15 Tab 331, Oh, *et al.,* 2001 Vol 15 Tab 318, Rhoades, *et al.,* 2000 Vol 15 Tab 330, Knowles, *et al.,* 2001 Vol 14 Tab 295, and Savard, *et al.,* 2002 Vol 15 Tab 335].

2) Use of chitosan as a Dietary Supplement

In the U.S. the consumption of chitosan has been growing rapidly. Industry trade journals, such as the *Natural Products Insider, 1999 Buyer's Guide* lists 72 suppliers/distributors of the chitosan. Much of the chitosan sold is consumed for its effects as a dietary fiber, or for its ability to provide a source of glucosamine.

3) Usage of Chitosan in Japan

The Japan Times of April 11, 2000 reports that sales of dietary chitosan in Japan totaled total nearly US$1 billion per year [*Japan Times,* April 11, 2000 Vol 14 Tab 286]. However, a 1999 U.S. State Department Document entitled "Japan – Chitin and Chitosan Market – IMI990408" indicates that the current health food market for chitosan in Japan is slightly more than 200 metric tons per year. Total chitin production for the same period was estimated at 1,000 metric tons per year [U.S. State Department Report IMI990408, 1999 Vol 17 Tab 376]. Further, Japan exports approximately 200 MT of chitosan per year to the U.S. and Europe. Current prices for chitosan for food use were reported to be . yen/kilo (US . per pound, when 120 yen = US$1.00). This makes chitosan a very expensive ingredient in the Japanese market. According to the State Department Report, the total market for chitosan has dropped, due to competing products in the wastewater sector [U.S. State Department Report IMI990408, 1999 Vol 17 Tab 376]. According to the *Korea Times* nearly 50% of the chitosan manufactured in Japan is used in purifying drinking and wastewater streams [*Korea Times*, April 7, 1999 Vol 14 Tab 297].

The *Korea Times* also reports that Japan is the world's leader in the manufacture of chitosan, accounting for 90% of the

global chitosan market worth approximately 4 trillion yen (120 yen = 1 US dollar) [*Korea Times*, April 7, 1999 Vol 14 Tab 297]. The report indicated that there are ten firms manufacturing chitosan in Japan, and that 60-package chitosan into various products [*Korea Times*, April 7, 1999 Vol 14 Tab 297].

Currently, certain foods containing chitosan have been approved in Japan as Foods for Specific Health Use (FOSHU). Those foods include a biscuit (Healket™) by Nippon Kayaku Co., Ltd., a fish cake (Health Support Food "Marin" Including Chitosan™) by Ito Han Foods, Inc., and a biscuit from Nippon Menard Cosmetic Co., (Menard CholeToru Bar™). All three foods carry labeling for cholesterol reduction [National Toxicology Program Board of Scientific Counselors, 1999 Vol 7 Tab 108].

Other foods containing chitosan include: "Oppen Bifidus Chitosan" by Oppen Cosmetics. The product is a health food supplement, which is designed to maintain a healthy gastrointestinal tract. It contains chitosan which cleanses the intestines, lactic acid bacteria (*Bifidus bacteria* and *Lactobacillus faecalis*), which regulate the function of the intestines, "Kohki [*Engelhardtia chrysolepsis*] Extract", which decreases neutral fat and suppresses obesity, raffinose, which is a nutritional source for *Bifidus bacteria*, collagen, which is a substance reputed to be an effective skin moisturizer, and B group vitamins. The product is formed into a small triangular tablet, and has "an elegant prune flavor", according to the manufacturer. A package of 210 tablets costs Y5500 (US$ 45.83) [*Japanese Cosmetic and Household Goods Monitor*, 1999 Vol 17 Tab 374].

4) Chitosan Use in China

Section II Chemical Identity

Qindao Golden Yuan Biological Products Co. Ltd. has indicated in its product literature, that it is the largest chitosan manufacturer in China [Qindao Golden Yuan Biological Products Co. Ltd., 1999 Vol 8 Tab 130]. The company reports its plant capacity at 200 metric tons, most of which is exported to the United States and Europe. The product is derived from the Alaskan crab, and its uses in China according to a company representative are similar to those in Japan.

Section III

Section III
Information on Self-Limiting Levels of Use and Probable Consumption

A. Self-Limiting Levels of Use

While there are few published studies on the self-limiting levels of chitosan use in foods, several factors are expected to limit the use of the ingredient in food formulations. First, chitosan is a very expensive ingredient, with a value in excess of US $20 per pound. Second, studies by food scientists have shown that chitosan has been an effective emulsifier at percentages of use of less than 1.0% [Poole, 1988 Vol 11 Tab 225 and Rodriguez, *et al.,* 2000 Vol 12 Tab 237].

Excessive chitosan usage may produce aggregation of ingredients, depending on the synergies involved and the pH of the formulation [Poole, 1988 Vol 11 Tab 225]. Poole found that use of greater than 0.8% chitosan yielded a dense aggregate in a whey protein isolate-corn oil emulsion [Poole, 1988 Vol 11 Tab 225]. Further, Rodriguez *et al.,* 2000, stated that the affinity between chitosan and water decreased with increasing pH [Rodriguez, *et al.,* 2000 Vol 12 Tab 237].

Therefore, use of chitosan is expected to be limited by its physicochemical properties. Rodriguez *et al.,* 2000, concluded that the behavior of chitosan in systems containing different chitosan/oil ratios was due to certain properties of chitosan. The authors found that the surface tension of chitosan solutions (1% v/v acetic acid aqueous solution) was higher than that of the solvent (water). This implied that chitosan is strongly excluded from the air/solution interface. According to the researchers, the phenomena are consistent with expectations derived from the theoretical behaviors of the cationic $-NH_3^+$ and alcoholic –OH groups distributed on the molecules structure [Rodriguez *et al.,* 2000 Vol 12 Tab 237].

000083

Primex submits that food processors will be involved in a great deal

of applications development, in order to determine those most economical uses for chitosan.

B. Probable Consumption

A Realistic Estimate of Exposure to Shrimp-Derived Chitosan

Primex is providing a realistic estimate of exposure to Shrimp-Derived Chitosan. The FDA's guidance document "*Estimating Exposure to Direct Food Additives and Chemical Contaminants in the Diet*", USDA's *Continuing Survey of Food Intakes by Individuals* (CSFII, 1996), and recent market surveys were used as references to estimate the realistic exposure to Shrimp-Derived Chitosan produced by Primex [Enns, *et al.,* 1997 Vol 4 Tab 36].

1. Exposure Estimate

In order to estimate the probable human exposure to Shrimp-Derived Chitosan on a continual daily basis, Primex has relied upon examples of commercial formulations from Asia and Europe, examples of potential Shrimp-Derived Chitosan use from recent published studies, the patent literature, and the results of its own food technology research. Exposure to Shrimp-Derived Chitosan was estimated in accordance with the agency's guidance document, cited above, "*Estimating Exposure to Direct Food Additives and Chemical Contaminants in the Diet*" [FDA, September 1995]. Use of the material was estimated for the 94 NAS food categories, on a working Table titled "GRAS Food Additive Categories and Sub-Categories" (data not shown). Use levels as a percent of the final food products were then estimated across food sub-categories to yield averages for the major food groups. For instance, all of the 16 sub-categories in the Baked Goods category were averaged to yield a "Baked Goods" use level that was entered into Table 1. Table 1 lists the 43 Food Categories at 21 CFR §170.3 (n). Since neither the "GRAS Food Additive

Categories and Sub-categories", nor the "43 Food Categories" include food intake data, the average use levels for the sub-food categories shown on Table 1 were then re-averaged, if necessary, so that they were consistent with the food categories from USDA's *Continuing Survey of Food Intakes by Individuals* (CSFII) [Enns, *et al.,* 1997 Vol 2 Tab 11]. The CSFII food categories, along with Shrimp-Derived Chitosan use level, food sub-category, mean intake, and Shrimp-Derived Chitosan intake are shown in Table 2. This Table presents mean intake data for adults 20 years and over (eaters only).

For example, three food sub-categories (White Bread and Rolls, Dark Bread and Rolls, and Bread, Other) from the NAS list were averaged to yield the average use level for Yeast Bread and Rolls shown on Table 2 (the CSFII categories). Since the CSFII intake data is statistically valid, percentage use levels for Shrimp-Derived Chitosan may be multiplied by the mean intakes indicated in the CSFII database to yield an estimated exposure to Shrimp-Derived Chitosan from a given food category. The resulting totals for each food category were then summed to yield a total mean daily intake.

Note that zeros were entered for Shrimp-Derived Chitosan for certain food categories where Shrimp-Derived Chitosan use is unlikely, e.g., in coffee and teas. The total daily mean intake from Table 2 was doubled to estimate the 90th percentile (in accordance with the guidance document).

The results of these calculations and potential exposure from other sources are summarized at the bottom of Table 2. The estimated daily mean exposure derived from Table 2 was 1.98 grams of Shrimp-Derived Chitosan per day. The 90th percentile is then estimated to be 3.96 grams per day. Contributing to these totals were 0.267 grams per day from bakery goods, 0.25 grams per day from grain products, 0.081 grams per day from fruit products, 0.2315 from meat products, and 0.339 grams per day from milk

products. The 90th percentile is well within the range of recently published studies in which humans have been fed chitosan, and with the range of a study sponsored by Primex ehf, in which subjects consumed 4.5 and 6.75 grams of chitosan per day [Tapola, *et al.*, 2004 Vol 16 Tab 348, Gades, *et al.*, 2002 Vol 13 Tab 260, Gades, *et al.*, 2002 Vol 13 Tab 261, and Schiller, *et al.*, 2001 Vol 14 Tab 309].

Primex ehf is also aware that certain individuals may experience additional exposure to Shrimp-Derived Chitosan from its use as a source of dietary fiber in dietary supplement form [Enns, *et al.*, 1997 and Langhelle, 1999 Vol 8 Tab 126]. The recommended dose of chitosan as a dietary supplement is 1.5 to 3.0 grams per day. This dosage has been recommended based on the results of clinical trials with human volunteers [Colombo, *et al.*, 1996, Vol 3 Tab 24].

While the percentage of use assigned to each food subcategory was considered realistic, this exposure estimate remains conservative because 100% market penetration was assumed, and Shrimp-Derived Chitosan was assumed to be included in all products in a given subcategory. Additionally, the values presented below assume that 100% of consumers are "eaters" of chitosan-containing products within a given food category.

In reality, chitosan is expected to be used in only certain products within a given subcategory. Further, use within a subcategory will be limited by economics and organoleptic considerations. Market development in the US is likely to be directed towards the functional properties of chitosan, and certain functional properties may not be appropriate for a given consumer product. An example of this is that chitosan tends to increase viscosity in batter or fluid food systems. This may not create a desirable product in all cases.

Additionally, Primex Shrimp-Derived Chitosan is a very expensive food ingredient, costing US$ 20-30 dollars per kilogram. Therefore, those applications in which it will be cost effective will need to be carefully chosen, and will most likely dictate that chitosan will be used at the minimum level necessary to produce a given technical or nutritional effect.

Table 1 below shows the realistic percentages of use assigned to each of the forty-three food categories listed at 21 CFR § 170.3 (n). The correlation between the major food categories derived from the USDA food survey, and those listed at § 170.3 (n) is shown in Table 3.

The mean and the 90th percentile of estimated exposure to Primex Shrimp-Derived Chitosan remains lower than the recommended levels of intake for dietary fiber (25-30 grams per day) in the United States [Enns, *et al.,* 1997 Vol 4 Tab 36]. Additionally, dietary fiber intake in the US averaged for men and women has been estimated by USDA at 16.1 grams per day [Enns, *et al.,* 1997 Vol 4 Tab 36]. This value still represents a deficit in relation to levels established for good health. It is possible that consumption of chitosan may provide health benefits from additional dietary fiber intake. Further, no untoward effects have been reported in multiple studies involving chitosan consumption by human volunteers consuming similar amounts of chitosan [See Section IV, Safety]. Therefore, Primex submits that the exposure to chitosan represented by this estimate will pose no threat to the public health.

Section III Information on Self-Limiting Levels of Use and Probable Consumption

Table 1
Use of Shrimp-Derived Chitosan In the Food Categories Listed by the US FDA At 21 CFR §170.3 (n)

	Food Categories	Realistic Percentage of Use in the US Market
01	Baked Goods. Baking Mixes	0.035%
02	Beverages alcoholic	0%
03	Beverages and beverage bases. non-alcoholic	0.625%
04	Breakfast Cereals	0.032%
05	Cheeses	0.56%
06	Chewing Gum	0%
07	Coffee and tea	0%
08	Condiments and relishes	0.3%
09	Confections and frostings	0
10	Dairy product analogs	0.56%
11	Egg Products	0.213%
12	Fats and oils	0.22%
13	Fish Products	0.02%
14	Fresh eggs	0%
15	Fresh fish	0.02%
16	Fresh fruits and fruit juices	0.46%
17	Fresh Meats	0.025%
18	Fresh poultry	0.025%
19	Fresh vegetables	0.21%
20	Frozen dairy desserts and mixes	0.77%
21	Fruit and water ices	0%
22	Gelatins. puddings and fillings	0%
23	Grain products and pastas	0.026%
24	Gravies and Sauces	0%
25	Hard candy	0%
26	Herbs. seeds. spices. seasonings. blends	0%
27	Jams and jellies. home prepared	0%
28	Jams and jellies commercial	0.25%
29	Meat products	0.45%
30	Milk. whole and skim	0.2%
31	Milk products	0.77%
32	Nuts and Nut products	0%
33	Plant protein products	2.2%
34	Poultry products	0.45%
35	Processed fruits and fruit juices	0.46%
36	Processed vegetables and vegetable juices	0.21%
37	Snack foods	0.75%
38	Soft Candy	0.5%
39	Soups. home prepared	0%
40	Soups and soup mixes	0.85%
41	Sugar. white granulated	0%
42	Sugar substitutes	0%
43	Sweet Sauces. toppings. and syrups	0%

Section III Information on Self-Limiting Levels of Use and Probable Consumption

Table 2
Estimated Intake of Shrimp-Derived Chitosan in Selected Food Categories

Major Food Codes	Food Category	Chitosan Solids Use Level (%)	Food Intake (g)	Chitosan Solids Intake (g)	CSFII
	Baked goods, baking mixes				
51	Yeast Breads and Rolls	0.0256	50	0.0128	1996
52, 55	Quick breads, pancakes, etc.	0.04	20	0.1	1996
53	Cakes, cookies, pastries, pies[1]	0.04	38	0.0152	1996
58	Mixtures mainly grain	0.153	107	0.139	1996
	Breakfast cereals			0.267	
571 - 574, 578	Ready-to-eat cereals	0.032	17	0.085	1996
	Grain products and pastas				
562	Rice	0.0256	19	0.076	1996
561	Pasta	0.0256	21	0.084	1996
54	Snack foods (crackers, chips)	0.75	12	0.09	1996
	Total Vegetables		132		1996
	Fresh vegetables[2]	0.21	45	0.0945	1996
	Processed vegetables, juices	0.21	14	0.0294	1996
	Dark Green Vegetables	0.21	13	0.0273	
	Total Fruits				1996
612	Citrus juices[3]	0.46	17.7	0.0814	1996
621	Dried fruits	0	1	0	1996
641,642	Non-citrus juices and nectars	0	26	0	1996
	Fruits and mixtures	0	0	0	1996
	Milk products				
114	Yogurt	0.77	8	0.062	1996
131,132, 133, 134	Milk desserts[4]	0.77	24	0.096	1996
140-147	Cheese	0.56	8	0.0448	1996
116, 118	Milk-Based Beverages[5]	0.2	34	0.136	1966

000089

Section III Information on Self-Limiting Levels of Use and Probable Consumption

Table 2 (continued)

	Meat Products				
252	Sausages, processed meats	0.45	21	0.0945	1996
	Fresh Meat	0.25	34	0.085	1996
	Poultry	0.25	20	0.05	1996
	Fish	0.02	11	0.002	1996
	Eggs				
321,323,324, 33,34	Egg Products[6]	0.213	9	0.0192	1996
	Legumes				
	Legumes[7]	2.2	1.8	0.0396	1996
	Nuts and Nut Products				
	Nuts and Nut Products	0	3	0	1996
	Fats and Oils				
832	Salad dressings	0.22	8	0.00176	1996
	Total sugars and sweets				
917,918	Candy (Soft and Hard)	0.5	7	0.035	1996
911 - 916	Other Sugar Products[8]	0.25	15	0.0375	1996
	Beverages Non-Alcoholic				
	Coffee	0	254	0	1996
923	Teas	0	128	0	1996
925	Low Cal. Fruit Drinks and Ades	1	18	0.18	1996
92400100	Low Cal. Carbonated Drinks	0	74	0	1996
	Total Mean Intake (grams)			**1.98**	
	90th Percentile			**3.96**	
	Potential Dietary Supplement Use				**1.5-3.0**
	1) Includes dietetic bars		5) Includes only skim milk products		
	2) Includes tomatoes and lettuce products		6) Includes processed egg		
	3) Includes citrus juices with pulp		7) Includes only Tofu and si		
	4) Includes low fat ice creams etc.		8) Includes Jams, Jellies, et		

000090

Table 3
USDA Major Food Categories And Corresponding Categories From 21 CFR 170.3 (n)

USDA Food Category[1]	Categories Listed In 170.3 (n)[2]
Total Grain Products	01, 04, 23, 37
Dietary Supplement; Source of Soluble Fiber	
Vegetables and Vegetable Dishes	36
Fruits and Fruit Preparations	16, 35
Milk Desserts/Cheese Products	5, 20, 22, 31,10
Fish and Fish Products	13, 15
Total Fats and Oils	12
Legumes and Legume Dishes	33, 36
Sugars and Sweets	06, 09, 25, 28, 38, 42, 43
Nuts and Seeds	32
Total Non-Alcoholic Beverages (excluding coffee and fruit juices)	16, 35, 03

1. Major food categories are those used in USDA's Continuing Survey of Food Intake by Individuals [Enns, *et al.,* 1997 Vol 4 Tab 36].
2. Column 2 contains the number assigned to the various food categories as listed in 21 CFR § 170.3 (n), which are contained within those reported by Enns, *et al.*, 1997 [Enns, *et al.,* 1997 Vol 4 Tab 36].

Recently Developed Applications

One of the most cost effective uses of Shrimp-Derived Chitosan identified by Primex, is its use as an antimicrobial to retard spoilage in various foods (Rhoades, *et al.*, 2000, *Applied and Environmental Microbiology* (66): No 1). Rhoades, *et al.*, 2000 reported that the addition of 0.3g/L of chitosan (either native or degraded), when added to refrigerated items with short shelf lives, such as chickpea hummus or pasteurized apple-elderberry juice was able to reduce the microbiological content of these products by approximately 3 to 4 log CFU/ml. Yeast levels were completely reduced by the addition of chitosan to the products, and remained below the level of detection even after several days of storage.

Several research groups have published results on the use of chitosan to produce antimicrobial films (Begin, *et al.*, 1999, *International Journal of Biological Macromolecules*: (1):63-67; Nunthanid, *et al.*, 2001, *Drug Development and Industrial Pharmacology* (2):143-57; Ouattar, *et al.*, 2000, *International Journal of Food Microbiology*: 62(1-2):139-48; Tsai, *et al.*, 1999, *Journal of Food Protection* 62(3):239-243; and Roller, *et al.*, 2000, *Journal of Food Protection* 63(2):202-9. The films contained either chitosan alone or chitosan in combination with other antimicrobials, such as propionic acid. Low levels of chitosan solids were used to produce the films (0.5% to 2.0%) in acetic or other food grade acids.

000092

These applications trials by the authors cited above, as well as others indicated that low levels of chitosan solids either as a film or a powder could be used to increase the shelf life of foods susceptible to microbial degradation. For instance, the investigation by Roller, *et al.*, 2000 showed that 3g/L of chitosan in combination with 0.16% acetic acid slowed growth of *Lactobacillus fructivorans*, and that a coating of chitosan (9mg/g

of shrimp) reduced the growth of spoilage microorganisms from 8 CFU/g to 4 CFU/g in shrimp salad, when stored at 5°C. Tsai, *et al.*, 1999 suggested that the antibacterial mechanism of shrimp chitosan may be due to a cross-linkage between the polycations of chitosan, and the anions on the surface of bacteria, such as *E. coli*, thus causing leakage of glucose and lactate dehydrogenase from the cell. Primex is aware that other foods, such as fresh fruits and vegetables may become candidates for chitosan film coverings to retard spoilage and preserve appearance. Based on the expanding knowledge base surrounding these types of applications for Shrimp-Derived Chitosan, as well as its recent market survey, Primex believes that the mean consumption of chitosan will not exceed 1.98 grams per day in a normal diet.

Section IV

Shrimp-Derived Chitosan
GRAS Notification

Section IV

Table of Contents

Table of Contents:
Section IV Detailed Summary of the Basis for the Determination that Primex Chitosan is GRAS

A. Introduction to the Section 1
1. Regulatory Background for this Submission 1
B. Questions Posed by CFSAN 2
1. Summarized Responses
a) Question 1. 3
b) Question 2. 4
Table 1: Nutritional Effects of Viscous Polysaccharide 4
Table 2: Apparent Digestibility of Energy, Protein, and Non-Starch Polysaccharides (NSP) in a High-Fiber Diet Rich in Fruits and Vegetables 7
Table 3: Differences in the Ability of Rats Versus Humans to Digest Fats 8
Table 4: Fecal Fat Loss in Humans 10
Table 5. Serum fat soluble vitamin and carotenoid concentrations 12
c) Question 3. 13
Table 6: Summary of Mineral Analyses for Calcium in Humans Consuming Chitosan 15
d) Question 4 16
C. Conclusion to Summarized Responses 17
D. Supporting Discussion to Primex Responses 19
1. Question 1. 19
a) Comparison of the structures and of Chitosan and Olestra™ .. 20
b) Proposed Uses of Shrimp-Derived Chitosan and Olestra™ 21
c) Physiological Effect of Olestra™ 22
d) Issues Associated with Olestra™ 24
e) Physiological Effect of Chitosan 25

Table of Contents:
Section IV Detailed Summary of the Basis for the Determination that Primex Chitosan is GRAS

2. Question 2. 26

a) Influence of Viscous Dietary Fibers on Fat Digestion 27

Table 7: Effect of Fiber on the Disappearance of Cholesterol and Triglyceride from the Small Intestine..... 28

Table 8: Lymphatic Recovery of Triglycerides and Cholesterol in Rats Adapted to Fiber 29

b) Quantifying the Effects of Viscous Polysaccharides on Fecal Fat Content in Humans..... 29

Table 9: Human Studies Reporting Changes in Fecal Fat Composition as a Result of Consuming Dietary Fiber 30

c) Mechanisms by which Dietary Fiber Can Impede Fat Absorption. 31

d) Perspective on the Use of Rats as Models for Human Digestion..... 43

e) Comparative Digestibilities of Fibers and Fat between Rats and Humans 46

f) Results – Experiment 1: Diets Rich in Fruits and Vegetables..... 46

Table 10: Experiment 1: Apparent Digestibility of Energy, Protein, and Non-Starch Polysaccharides (NSP) in a Low-Fiber Control Diet and a Diet Rich in Fruits and Vegetables..... 47

g) Results - Experiment 2: Diets with Added Citrus Fruit DF 48

Table 11: Experiment 2: Apparent Digestibility of Energy, Protein, and Non-Starch Polysaccharides (NSP) in a Low-Fiber Control Diet and a Diet Rich in Citrus Fiber 49

Table of Contents:
Section IV Detailed Summary of the Basis for the Determination that Primex Chitosan is GRAS

h) Fecal Bulking Capacity in the Rat and Man 50

i) Factors Influencing the Differences in Fiber and Fat Digestibilities between Rats and Humans 51

j) Energy Values Derived from Non-Starch Polysaccharides: Comparative Studies in Humans and Rats 54

k) Human Experiments Conducted by Wisker, *et al.*, 1996 55

Table 12: Increase in Fecal Dry Matter Due to the Consumption of Non-Starch Polysaccharides (NSP) in Humans and Rats.....59

Table 13: Net Energy Values of Non-Starch Polysaccharides (NSP) Calculated from NSP Fermentability and From Digestible Energy Balances in Humans And Rats 59

l) Relationship of these Studies to Regulatory Concerns about the Effect of Chitosan Consumption by Humans 61

Table 14: Fecal Fat Loss in Humans. 65

m) Effect of chitosan on dietary lipid absorption in the rat 66

3. Question 3. Would mineral status be threatened, especially with regards to calcium? 69

Table 15: Tolerability parameters modifications during the two treatments 72

Table 16: Effect of Chitosan on Mineral Balance in Humans 73

Table 17: Tolerability parameters modifications during the two treatments. 74

Table 18: Clinical results of chitosan treatments: Tolerability of the diet plus dietetic integrator.....75

Table of Contents:
Section IV Detailed Summary of the Basis for the Determination that Primex Chitosan is GRAS

Table 19:.....76

E. Primex Chitosan Human Study..... 77

Table 20. Pretrial characteristics of the study subjects. Mean (SD) 84

Table 21. Energy and nutrient composition of the background diet. Mean (SD)..... 85-87

Table 22. Scores for RAND-36 subscales. Mean (SD)..... 90-91

Table 23. Serum fat soluble vitamins and carotenoids concentrations during the study 93-94

Table 24. Hematology and clinical chemistry. Mean (SD) 95-96

Table 25. Body weight. Mean(SD)..... 97

Table 26. Blood pressure. Mean (SD) 98

Table 27. Defecation frequency. (SD)..... 99

Symptoms and subject dropouts..... 100

Conclusion 100-101

1. Question 4..... .102

F. Human Feeding Studies 109

Table of Contents:
Section IV Detailed Summary of the Basis for the Determination that Primex Chitosan is GRAS

1. Human Safety 111

Table 28: Effect of Chitosan on Human Subjects 113

Table 29: Effect of Chitosan on Mineral Balance in Humans 119

Table 30: Effect of Chitosan on Hematological Parameters 120

Table 31: Effect of Chitosan on Hematological Parameters 128-129

2. Fate of Chitosan in the Gastrointestinal Tract 130

Table 32: Tablet Position and Radioactivity Released with Time After Administration to One Volunteer 131

3. Chitosan and other Foods as Sources of Glucosamine 136

4. Chitosan Degradation 139

5. Exposure 140

6. Technical Effects 141

7. Contaminants and Manufacturing 143
Table 33: Contaminant Analysis 144

Table 34: Dioxin Analysis 145

Table 35: Virus Analysis 146

G. Animal Safety and Toxicology Studies 146

Table 36: Toxicity Assessments of Chitosan in Animals 147-148

H. Other Regulatory Approvals 148

I. Summary 151

000101

GR

Summary to
FDA Questions

000102

Section IV:

Detailed Summary of the Basis for the Determination That Primex Chitosan is GRAS

A. Introduction to the Section

This section of the GRAS Notification in support of shrimp-derived chitosan contains a brief discussion of the relevant safety information presented previously in GRAS Notice No. 000073. In addition newly identified toxicity studies are also discussed below. However, the majority of this section will be devoted to answering several specific questions posed by reviewers from the Center for Food Safety and Applied Nutrition (CFSAN). These questions concerned the nutritional effects of consuming shrimp-derived chitosan on a chronic basis as part of a normal diet.

1. Regulatory Background for this Submission

In order to assure the public of the safety of shrimp-derived chitosan, Primex EHF of Norway, now Primex ehf (Primex), conducted a GRAS self affirmation process on behalf of the safety of its products. An eleven-volume GRAS Report was reviewed by a five-member Expert Panel in accordance with 21 CFR §170.30. The Expert Panel concurred with the findings of Primex EHF, that the company's shrimp-derived chitosan was safe for general use in foods, assuming that it met a set of published specifications and was used under current Good Manufacturing Practice [Expert Opinion, 2000 Appendix II]. Subsequently, a GRAS Notification, GRN000073, was submitted to the Center for Food Safety and Applied Nutrition (CFSAN), by Primex on March 14, 2001 under proposed 21 CFR §170.36. Following their review of the submission, the management group of the GRAS review team at CFSAN agreed that chitosan was non-toxic to humans and other test animals, but questioned whether or not chitosan would interfere with fat-soluble vitamin and mineral status in humans, when the substance was consumed on a chronic basis as part of a general diet. These questions were posed by Dr. George Pauli and others during a review meeting held March 4, 2002. The concerns about the effect of chitosan consumption on human nutritional status stemmed from the results of a 1995 publication by

Deuchi *et al.*, in which rats were fed a high-fat diet containing 5% chitosan [Deuchi *et al.*, 1995 Vol 4 Tab 29]. The results of the Deuchi study showed that rats consuming this type of diet experienced significant reductions in fat digestibility, and therefore reduced reserves of fat soluble vitamins, A, D, and E, and certain minerals, such as calcium, magnesium, and iron. Vitamin K levels, however, remained unaffected. This report prompted CFSAN regulators to ask whether the effect of chitosan in rats would be predictive of its physiological effect in humans. The specific questions posed by the GRAS review team is listed below.

B. Questions Posed by CFSAN

1. Might the physiological effect of chitosan consumption be similar to that of a well-known sucrose-ester fat-substitute, Olestra™, which has been shown to reduce the absorption of fat-soluble vitamins?
2. Would the consumption of chitosan result in sufficient fecal fat excretion, that the nutritional status of humans would be threatened by the loss of the fat-soluble vitamins A, D, E, and K?
3. Would mineral status be threatened, especially with regards to calcium?
4. Why did certain chitosan consumption studies in both humans and animals show that vitamin K levels increased? Would this in fact create an at-risk population for those taking prescribed blood-thinning drugs?

For clarity the paragraphs immediately below, will repeat each question in order, and present a summarized response. The reader will then be directed to other sub-sections for a more detailed discussion of each answer. Primex submits that it has responded to each question posed by CFSAN, and that the review continues to support the safety of shrimp-derived chitosan both from a toxicological and a nutritional perspective. Newly identified toxicological data will follow the detailed responses to the questions posed.

1. Summarized Responses

a) Question 1. Might the physiological effect of chitosan consumption be similar to that of a well-known sucrose-ester fat-substitute, Olestra™, which has been shown to reduce the absorption of fat-soluble vitamins?

A response to this question was presented to CFSAN on October 2, 2002 during a Pre-Notification meeting.

Olestra™ is a mixture of sucrose-esters, formed from the addition of 6, 7, or 8 fatty acids to the available 8 free hydroxyl moieties of sucrose. This ingredient is now regulated as a food additive with the requirement that fat-soluble vitamins be added to the preparation to compensate for any loss through excretion [Olestra Final Rule, *Federal Register,* 1996 Vol 13 Tab 256]. In contrast chitosan is a polysaccharide derived from the chitinous exoskeletons of crustaceans such as shrimp and crabs, and its chemical and physical properties are closely related to other viscous polysaccharides, such as glucomannan, xanthan gum, and pullulan. While both Olestra™ and chitosan have been reported to reduce fat absorption, Olestra™ is substantially inert in the body (>98.5% recovery). It reduces fat absorption by means of substituting for the fat used to make savory snacks. Therefore, total fat intake for eaters of savory snacks may be reduced in proportion to the amount of sucrose-ester used. Based on 8-week human studies as well as other data, the agency concluded that losses of fat soluble vitamins A, D, E, and K were significant [Olestra Final Rule, *Federal Register,* 1996 Vol 13 Tab 261].

Chitosan on the other hand interacts with the body on many levels. Like other viscous polysaccharides it effects the immune system, liver and cholesterol metabolism, the production and availability of digestive enzymes, and gut microflora. Additionally, whereas Olestra™ is non-fermentable by colonic microflora, chitosan is fermented to glucosamine, which ultimately enters the blood stream [Han, *et al.,* 1999 Vol 5 Tab 51, Furda, 2000 Vol 4 Tab 40 and Ebihara, *et al.,* 1989 Vol 4 Tab 35]. At the conclusion of the presentation, CFSAN reviewers concurred that the two

substances would elicit different physiological responses in the human digestive tract. No further questions on this subject were posed by the agency. For further information see sub-section D.

Question 2. Would the consumption of chitosan result in sufficient fecal fat excretion, that the nutritional status of humans would be threatened by the loss of the fat-soluble vitamins A, D, E, and K?

Chitosan behaves in the body as a viscous polysaccharide. As such its physiological effects are no different than other viscous polysaccharides in the diet [Cummings, 1978, Vol 12 Tab 247, Oku, 1982 Vol 15 Tab 319 and Slavin, 1980 Vol 16 Tab 346, and Schneeman, 1996 Vol 15 Tab 338]. Fiber researchers recognized very early that there was a trade-off between the benefits of fiber consumption and its effect on nutrient absorption [Cummings, 1978, Vol 12 Tab 247; Oku, 1982, Vol 14 Tab 219]. The Complementary Medicines Evaluation Committee in Australia has also recognized that there is a known interference between fiber and nutrient absorption [Complementary Medicines Evaluation Committee (7), 1998 Vol 12 Tab 245]. Table 1 lists the documented nutritional effects of viscous polysaccharides.

Table 1: Nutritional Effects of Viscous Polysaccharides

Nutritional Effects of Viscous Polysaccharides
▪ **Decrease protein digestibility / Kelsay, *et al.*, 1979**
▪ **Decrease fat digestibility / Furda, 2000**
▪ **Decrease disaccharidase digestibility / Thomsen, *et al.*, 1982**
▪ **Decrease calcium absorption / Oku, *et al.*, 1982**
▪ **Decrease vitamin A absorption / Khokhar and Kapoor, 1990**
▪ **Decrease vitamin E absorption / Schaus, *et al.*, 1985**

The nutritional concerns surrounding the consumption of chitosan were mainly derived from its effects in rats, who, in certain studies, experienced significant fecal fat loss, and consequently, significant fat-soluble vitamin loss, when chitosan consumption reached 5% of the diet [Deuchi, *et al.*, 1994, 1995a and 1995b Vol 4 Tabs 28, 29 and 30]. In

contrast several human studies, in which humans consumed gram quantities of chitosan, did not reveal significant fecal fat loss (See Table 2 below) [Schiller, *et al.,* 2001 Vol 15 Tab 337, Guerciolini, *et al.,* 2001 Vol 13 Tab 273, Gades, *et al.*, 2002 Vol 13 Tab 266, and Gades, *et al.*, 2003 Vol 13 Tab 267]. Other human studies have shown that blood levels of fat soluble vitamins and minerals remained unaffected following chitosan consumption [Girola, *et al.,* 1996 Vol 4 Tab 42, Guistina, *et al.,* 1995 Vol 4 Tab 43, Colombo, *et al.*, 1996 Vol 3 Tab 24, Sciutto, *et al.,* 1995 Vol 8 Tab 141, Veneroni, *et al.,* 1996 Vol 11 Tab 214, and Wuolijoki, *et al.,* 1999 Vol 11 Tab 221]. Likewise, a chemist with the Complementary Medicines Evaluation Committee in Australia advised that there was no evidence that the fat absorbing properties of chitosan would cause a selective loss of vitamin E or essential fatty acids [Complementary Medicines Evaluation Committee (7), 1998 Vol 12 Tab 245].

These divergent results led Primex to question whether the results in rats might represent a "worst-case scenario" for chitosan consumption in humans, or whether species differences in fiber and fat digestion might exist. Further investigation revealed that the high fat-high fiber test diets used in the rat studies were not good predictors of results in humans. Published studies have shown that rats and humans do not digest fat and fiber to the same extent. Rats have no gall bladder, and hence cannot emulsify high fat content meals for thorough digestion, and further, rats have a much shorter transit time than humans, which effects their ability to digest dietary fiber [Bach-Knudsen, et al., 1994 Vol 12 Tab 236 and Wisker, et al, 1997 Vol 16 Tab 367].

Recently, a European research team compared the abilities of humans and rats to digest common dietary fibers, when both species were consuming the same mixed diet [Bach-Knudsen, et al., 1994 Vol 12 Tab 236 and Bach-Knudsen, et al., 1991 Vol 12 Tab 235]. The research team noted that, similar fiber digestibilities had been observed for humans and rats when isolated fiber sources had been tested [Nyman *et al.* 1986 Vol 14 Tab 314]. On the basis of these results, the rat was considered to be a suitable model for humans for the estimation of

energy values from non-starch polysaccharide (NSP) isolates (British Nutrition Foundation, 1990). However, Knudsen and Whisker, *et al.*, stated that no evidence had been presented to establish whether this was also the case when NSP were fed with a mixed diet.

When high-fat diets are used, as in the Deuchi studies on chitosan, Knudsen and Wisker *et al.*, pointed out that there are significant anatomical differences between humans and rats that may need to be taken into consideration [Wisker, *et al.*, 1996c Vol 15 Tab 64]. For instance, rats have no gallbladder, cannot concentrate bile and have a nearly constant flow of diluted bile into the duodenum. Rats may therefore be unable to emulsify large meals of fat sufficiently, which potentially may lead to higher losses of fat and thereby energy to the feces [Wisker, et al., 1996c Vol 15 Tab 332]. In a series of several experiments the research team found that an increased fat intake caused higher fecal fat excretion in both species, and that humans were able to digest non-starch polysaccharides significantly better than were rats. The authors explained that in man, but probably also in the rat; most of the fecal fat is due to the bacterial mass. Table 2 shows that the difference in the ability to digest NSP between man (0.734) and the rat (0.597) when both were consuming a high-fiber diet was quite significant. On the high fiber diet all the constituent sugars with the exception of arabinose and galactose were degraded more completely in man than in the rat (data not shown) [Bach-Knudsen, *et al.*, 1994 Vol 12 Tab 236]. The investigators stated that even when both species were fed high-fiber diets, the rat moves ingesta through the gut in 54-58% of the time required by humans, thus leading to lower digestibilities.

Table 2: Apparent Digestibility of Energy, Protein, and Non-Starch Polysaccharides (NSP) in a High-Fiber Diet Rich in Fruits and Vegetables

(Means and standard deviations for eight humans and five rats per diet)

Diet	High-Fiber				
	Humans		Rats		*P* value
	Mean	SD	Mean	SD	
Energy	0.897	0.022	0.865	0.012	0.008
Protein	0.841	0.031	0.858	0.018	0.265
Total NSP	0.734	0.097	0.597	0.063	0.011

From Knudsen, *et al.*, 1994

The studies of Knudsen and Wisker, *et al.*, have raised several issues that are important to the consideration of the use of orally administered chitosan in humans. Various regulatory agencies have expressed concern that the increase in fecal fat observed in rats consuming high fat-high-chitosan diets might translate to reduced nutritional status in humans due to the loss of fat-soluble vitamins and certain minerals [Complementary Medicines Evaluation Committee, 1998 Vol 12 Tab 245]. Yet human feeding studies in which chitosan was fed at up to 5.25 grams per day with or without a high-fat diet have failed to consistently show significant loss of fat through the feces [Schiller, *et al.*, 2001 Vol 15 Tab 336 and Gades, et al., 2002 Vol 13 Tab 266]. Until now, this discrepancy in fecal fat loss between species had remained largely unexplained, leaving regulators to assume that the data from rats might represent the worst case scenario for humans who were consuming chitosan at or above the 90th percentile (Estimated to equal 3.2 g/day in the Primex GRAS Notification 000073).

Several reasonable conclusions can be drawn from this body of work. First, rats are less likely than humans to be able to digest viscous polysaccharides, like chitosan, which are known to interfere with fat digestion. Additionally, rats will be further hindered from emulsifying large quantities of fat in the diet, because rats have no gall bladder.

Therefore, the feeding of large quantities of fat along with viscous polysaccharides puts the rat at a distinct disadvantage in comparison to humans (See Table 3 below). This makes the rat an inappropriate model to predict the nutritional effects of high-fat-chitosan containing diets in humans.

Second, humans have a greater ability to digest polysaccharides than do rats, because of more microbial capacity in the colon, and longer transit times (about 40% longer than rats). In one study in which a radiolabelled chitosan coating was tracked in a human volunteer, only 11.3% of the radioactive activity remained once the chitosan had reached the transverse colon [Macleod, *et al.,* 1999 Vol 6 Tab 97].

Thirdly, humans have a greater ability to emulsify fat than do rats, because they have the advantage of bile acid secretions from the gall bladder.

Finally, the test material used in several of the rat studies was extremely viscous (2,000 to 4,000 cPs [200-450 cPs in 0.5% solution]), exacerbating the problem. Whereas the more highly refined chitosan used in the human studies had a maximum viscosity of 155 cPs [Primex, 2002 unpublished data, Deuchi, *et al.,* 1995 Vol 4 Tab 29, and Wada, *et al.,* 1997 Vol 11 Tab 217].

Table 3: Differences in the Ability of Rats Versus Humans to Digest Fats

Rats	Humans
No gallbladder	Gallbladder
Cannot concentrate bile	Can concentrate bile
Nearly constant flow of diluted bile into the duodenum	Concentrated bile is delivered to the duodenum on demand
Unable to emulsify large meals of fat	Can emulsify fats
Faster transit time (54-58% of humans)	Transit time is ~ 40% longer

Taken together the data and information discussed in this section would seem to explain why humans have not experienced excessive fat

excretion following consumption of chitosan. As a species humans are better equipped to emulsify fats in the diet, and to ferment polysaccharides than are rats. Thus, there is no evidence to suggest that chitosan would pose a risk to fat soluble vitamin status in humans, especially at the exposure level proposed (3.2 gram per day at the 90th percentile).

This question was considered earlier by the Complementary Medicines Evaluation Committee in Australia [Complementary Medicines Evaluation Committee (6), 1998 Vol 12 Tab 245]. It was determined that many Australians consume 60 - 80 grams of fat per day. Therefore, loss of even 10 – 20 grams of fat through the use of 2 grams of chitosan theoretically capable of absorbing 5 -10 grams of fat/gram would still leave sufficient fat to provide fat soluble vitamins. Further, the Committee pointed out that fat digestion begins in the mouth, and that for maximum efficiency, chitosan would need to be mixed with food. Additionally, Shils and Young reported in 1988 that increased fecal fat loss with high fiber diets has been observed, but that the increases seldom exceed 7 grams more than normal fecal fat loss [Complementary Medicines Evaluation Committee (6), 1998 Vol 12 Tab 245].

In the US Americans consume an average of 74.3 grams of fat per day, according to USDA's *Continuing Survey of Food Intakes by Individuals* [Enns, *et al.*, 1997 Vol 4 Tab 36]. Table 4 summarizes the fecal fat loss from individuals consuming chitosan in controlled studies. These individuals consumed between 76 and 135 grams of fat per day, while taking 2.7 to 5.25 grams of chitosan per day. Fecal fat losses in comparison to the control ranged from – 1.09 g/d to + 5.27 g/d. This equates to a worse case (most fat loss / least fat intake) loss of 7% of fat intake per day. Fine and Fordtran, 1992 found that mean fecal fat loss in normal individuals was 3.4 g/d, while persons with diarrhea experienced a fecal fat loss of 13.6 g/d [Fine, *et al.*, 1992 Vol 13 Tab 263]. If mean fat loss for normal persons is 3.4 g/d and average fat intake is 74.3 grams, then normal fecal fat loss is 4.6% of fat intake. The worst case increase in fecal fat resulting from chitosan consumption by humans was therefore 2.4% (7% - 4.6%). While this value may represent an increase,

it is not large enough to be of nutritional significance. Table 4 contains excerpts from a 2001 review by Dr. John Cummings on the effect of dietary fiber on fecal composition. Based on this lengthy review, it becomes clear that there is general consensus among scientists in the field that dietary fibers in general, and viscous fibers in particular increase fecal fat. However, those increases reported amount to tenths of grams to less than 10 grams. Table 4 shows that fecal fat increases due to chitosan fall within that range.

Table 4: Fecal Fat Loss in Humans

Guerciolini, *et al.*, 2001 12 healthy subjects 2.67g chitosan/day	**Fecal Fat Results** Baseline: 1.36 g/d (0.67-2.03) With chitosan: 0.27 g/d (-1.49-2.07)
Schiller, *et al.*, 2001 59 mildly obese subjects 2.7g chitosan/day	**Fecal Fat Results** Fecal fat measured on 4 test subjects and 3 placebo subjects g/day Test Placebo Baseline 2.93 5.97 8 Weeks 8.90 3.63
Gades and Stern, 2002 7 healthy males, high fat (135 g/d) 5.25 g chitosan	**Fecal Fat Results** 6.9 g/d with no chitosan 6.8 g/d with chitosan
Gades and Stern, 2003 15 healthy males, high fat (133 ± 23 g/d) 4.5 g chitosan	**Fecal Fat Results** 6.1 g/d with no chitosan 7.2 g/d with chitosan
Fine and Fordtran, 1992 Normal subjects Mild to severe diarrhea	**Fecal Fat Results** 3.4 g/d (Mean) 13.6 g/d

000112

To confirm the effect of chitosan on human mineral and fat soluble vitamin nutrition, Primex ehf sponsored a study in 2003, which was conducted by Oy Foodfiles Ltd in Finland. The title of the study was "Effect of Chitosan on Serum Fat-Soluble Vitamins and Plasma Cholesterol Concentrations in Mildly Hypercholesterolemic Subjects" [Tapola, *et al.,* 2003 Vol 16 Tab 357]. The chitosan administered in the study was manufactured by Primex ehf from the arctic shrimp *Pandalus borealis*. The study was designed as a randomized, single blind, placebo controlled parallel trial, whose primary objective was to determine the effect of two different doses of Primex chitosan (4.5 g and 6.75 g) on serum concentrations of fat-soluble vitamins (vitamin A, α-carotene, β-carotene, vitamin E and vitamin D), and plasma cholesterol concentrations as compared with a placebo and glucomannan as a positive control.

In this study sixty five (65) subjects were randomized and fifty-six (56) male and female subjects completed the trial. The subjects were randomly assigned to one of four groups. The subjects were given 0, 4.5, or 6.75 g /day of Primex chitosan or 6.75 g/day of glucomannan for eight weeks. The tablets were taken with water 15 minutes before the three main meals of the day [Tapola, *et al.,* 2003 Vol 16 Tab 357].

The results showed that there was no significant difference in serum fat soluble vitamin levels after intervention with Primex ehf chitosan at either 4.5 or 6.75 grams per day. Table 5 summarizes the results and presents the comparisons with both the placebo and glucomannan, another viscous soluble fiber.

For additional discussion of the human studies shown above and for species and test material differences, see sub-section E.

Section IV Detailed Summary of the Basis for the Determination that Primex Chitosan is GRAS

Table 5. Serum Fat Soluble Vitamin and Carotenoid Concentrations

Mean (SD)	Chitosan 4.5 g (n = 15)	Chitosan 6.75 g (n = 12)	Glucomannan 6.75 g (n = 15)	Placebo (n = 14)	p-value[a]	p-value[b]
Serum vitamin A (µmol/l)						
0 wk	3.51 (0.89)	3.65 (0.86)	3.74 (0.99)	3.68 (0.55)	NS	NS
8 wk	3.98 (1.11)	3.81 (1.07)	3.75 (0.98)	3.87 (0.74)		
p-value[c] *(0.029)*	NS	NS	NS	NS		
Serum α-carotene (µmol/l)						
0 wk	1.20 (0.75)	1.24 (1.07)	1.43 (1.78)	1.17 (0.54)	NS	NS
8 wk	0.90 (0.53)	1.07 (0.94)	1.12 (1.52)	1.07 (0.47)		
p-value[c]	NS	NS	NS	NS		
Serum β-carotene (µmol/l)						
0 wk	2.11 (1.17)	2.13 (1.55)	2.53 (3.09)	2.22 (1.40)	NS	NS
8 wk	2.05 (1.58)	2.16 (1.43)	2.19 (2.83)	2.15 (1.36)		
p-value[c] (NS)						
Serum vitamin E (µmol/l)						
0 wk	35.18 (4.18)	33.08 (4.42)	34.95 (6.33)	33.92 (6.02)	NS	NS
8 wk	35.14 (3.87)	32.22 (5.77)	34.27 (7.06)	34.66 (7.84)		
p-value[c] (NS)						
Serum 25(OH)D (nmol/l)						
0 wk	63.0 (23.1)	61.5 (31.2)	56.1 (27.6)	60.6 (20.8)	NS	NS
8 wk	67.5 (19.9)	74.1 (31.1)	69.8 (33.1)	65.4 (18.4)		
p-value[c] (0.001)	NS	NS	NS	NS		

[a] The significance of the overall difference among the study groups analyzed with the GLM for repeated measures (vitamin A, β-carotene, vitamin E and 25(OH)D) or with the Kruskal-Wallis test (α-carotene) adjusted with the Bonferroni correction .

[b] The significance of the time*group interaction analyzed with the GLM for repeated measures.

[c] The significance of the within-group difference analyzed with the GLM for repeated measures (vitamin A, β-carotene and vitamin E) or with the Wilcoxon test (α-carotene) adjusted with the Bonferroni correction.

Question 3. Would mineral status be threatened, especially with regards to calcium?

Chitosan, when fed in large quantity to rats, has been reported to reduce the absorption of calcium [Deuchi, *et al.,* 1995 Vol 4 Tab 29 and Wada, *et al.,* 1997 Vol 11 Tab 217]. The mechanism by which viscous dietary fibers may impede the absorption of certain minerals, has been studied previously [Oku, *et al.,* 1982 Vol 15 Tab 320]. Oku, *et al.,* measured calcium transport in the everted duodenal sac of rats fed 10 or 20% cellulose, glucomannan, or pullulan for 7 or 8 weeks. The authors found that calcium binding activity was significantly reduced in all groups fed unavailable carbohydrates. Interestingly, the activities of certain enzymes, such as, sucrase and alkaline phosphatase were also considerably diminished with the intake of these fibers. These results indicated that the inhibitory effect of unavailable carbohydrates on intestinal calcium absorption is partially due to the loss of calcium binding protein caused by large amounts of undigested substances transiting through the gastrointestinal tract.

Several authors have shown that both the positive and negative effects of fiber consumption may be accentuated by viscous polysaccharides, such as guar gum, xanthan gum, pectin, or glucomannan [Ikegami, *et. al.,* 1990 Vol 13 Tab 280 and Schneeman, 1996 Vol 15 Tab 338]. These studies have largely been conducted in test animals consuming a large portion of their diet as indigestible substances, and in the case of chitosan, with substances that were much higher in viscosity than that specified by Primex for its food grade products (55 versus ~2000 cPS) [Primex, 2002 unpublished data, Deuchi, *et al.*, 1995 Vol 4 Tab 29 and Wada, *et al.*, 1997 Vol 11 Tab 217].

000115

The effect of chitosan consumption on the mineral nutritional status of humans has been measured [Girola, *et al.,* 1996 Vol 4 Tab 42, Guistina, *et al.,* 1995 Vol 4 Tab 43, Colombo, *et al.*, 1996 Vol 3 Tab 24, Sciutto, *et al.,* 1995 Vol 8 Tab 141, and Veneroni, *et al.,* 1996 Vol 11 Tab 214] (See Table 6 below) No adverse effect in human mineral status has been noted, when the test subjects were consuming chitosan in gram

quantities. There were no significant differences between baseline and after the subjects had consumed chitosan for 30 days. This may be due to the fact that the humans were consuming a more moderate amount of chitosan in comparison to their body weight than were the rats used in the Deuchi studies, and it is also very likely due to the physiological differences between rats and humans. Humans have a much larger capacity to ferment dietary fibers in the colon, than do rats in the cecum. Therefore, at the levels of intake proposed, there is no evidence to support the notion that the consumption of chitosan would result in negative mineral status in humans. Significantly, another study, in which chitosan was fed at the 5% level was conducted by Gordon and Williford in 1983. The authors concluded that there was no effect of chitosan on mineral status in rats at levels of 5% or below in diets. Unfortunately, no information was provided on the viscosity of the test material in those studies.

As in Question 2 above Primex ehf confirmed the effect of chitosan on human calcium in a sponsored study in 2003, which was conducted by Oy Foodfiles Ltd in Finland. The title of the study was "Effect of Chitosan on Serum Fat-Soluble Vitamins and Plasma Cholesterol Concentrations in Mildly Hypercholesterolemic Subjects" [Tapola, *et al.,* 2003 Vol 16 Tab 357]. The chitosan administered in the study was manufactured by Primex ehf from the arctic shrimp *Pandalus borealis*. The study was designed as a randomized, single blind, placebo controlled parallel trial, whose primary objective was to determine the effect of two different doses of Primex chitosan (4.5 g and 6.75 g) on serum concentrations of fat-soluble vitamins (vitamin A, α-carotene, β-carotene, vitamin E and vitamin D), plasma cholesterol concentrations, and certain clinical chemistry and hematological parameters. The analyses performed included measurements for serum calcium levels. These data were compared with a placebo and glucomannan as a positive control.

In this study sixty five (65) subjects were randomized and fifty-six (56) male and female subjects completed the trial. The subjects were randomly assigned to one of four groups. The subjects were given 0, 4.5, or 6.75 g /day of Primex chitosan or 6.75 g/day of glucomannan for

eight weeks. The tablets were taken with water 15 minutes before the three main meals of the day [Tapola, *et al.,* 2003 Vol 16 Tab 357].

The results in this study showed that serum calcium levels dropped slightly (0.04 – 0.1 mmol/L), but remained within the normal range after all the interventions, including the placebo and glucomannan. The results are summarized in Table 6.

For additional discussion of the human studies shown above and for species and test material differences, see sub-section E.

Table 6: Summary of Mineral Analyses for Calcium in Humans Consuming Chitosan

Study	Baseline Calcium	After Chitosan
Colombo, *et al.*,1996, Vol 3 Tab 24	2.66±0.32 Meq/L	2.61±0.31 Meq/L
Girola, *et al.*, 1996, Vol 4 Tab 42	9.08±0.76 mg/dL	9.08±0.76 mg/dL
Giustina, *et al.*, 1995, Vol 4 Tab 43	9.28±0.72 mg/dL	9.11±0.59 mg/dL
Sciutto, *et al.*, 1995, Vol 8 Tab 141	9.07±0.81 mg/dL	9.09±0.79 mg/dL
Veneroni, *et al.*, 1996, Vol 11 Tab 214	9.07±0.79 mg/dL	9.08±0.82 mg/dL
Tapola, *et al.,* 2003 Vol 16 Tab 357 (4.5g/d Primex Chitosan)	2.31±0.06 mmol/L	2.27±0.07 mmol/L
Tapola, *et al.,* 2003 Vol 16 Tab 357 (6.75g/d Primex Chitosan)	2.37±0.07 mmol/L	2.30±0.07 mmol/L

d. **Question 4. Why did certain chitosan consumption studies in both humans and animals show that vitamin K levels increased? Would this in fact create an at-risk population of those taking prescribed blood-thinning drugs?**

Regulatory reviewers have noticed that vitamin K levels have been reported to increase in both rats and humans when consuming chitosan [Pittler, *et al.,* 1999 Vol 7 Tab 117 and Deuchi, *et al.,* 1995 Vol 4 Tab 29]. Vitamin K is a fat soluble vitamin, and an increase in vitamin K levels seemed to be counterintuitive with concerns that fecal fat loss would lead to depletion of fat-soluble vitamins. Further, if vitamin K levels did increase with chitosan consumption, and vitamin K increases clotting ability, would persons taking anticoagulant drugs constitute an at-risk group?

Fairfield and Fletcher, publishing in a recent issue of the *Journal of the American Medical Association* have indicated that there is no known toxicity state for vitamin K [Fairfield, *et al., 2002* Vol 13 Tab 260]. According to the authors, vitamin K is synthesized by intestinal bacteria. This fact lends additional credence to the assertion that chitosan is degraded in the intestine, as a dietary fiber [Chae, *et al.,* 2000 Vol 12 Tab 243]. The current RDI for vitamin K is 80 μg/L, a level that is rarely attained even in healthy subjects. According to the authors, individuals taking anti-coagulants should be given clear instructions as to diet, as increases in vitamin K may occur with any increase in indigestible carbohydrate [Fairfield, *et al.,* 2002 Vol 13 Tab 260].

Several studies have been conducted to investigate the interaction between bacterially generated vitamin K and warfarin, an anticoagulant drug [Paiva, *et al.,* 1998 Vol 15 Tab 324]. Within these studies, no significant differences were observed when comparisons were made between groups. The investigators concluded that the data did not support the hypothesis that bacterial synthesis of menaquinones in patients with bacterial overgrowth due to

conditions, such as atrophic gastritis conferred resistance to the effect of warfarin [Camilo *et al.*, 1988 Vol 12 Tab 242].

For additional details, the reader may wish to see sub-section E.

C. Conclusion to Summarized Responses

Primex has conducted a thorough review of the literature surrounding the physiological effects of viscous polysaccharides in general and chitosan in particular in order to answer the questions posed by the agency. Published studies have shown that humans have not been observed to experience significant fecal fat loss, even when chitosan has been consumed at more than five grams per day [Gades, *et al.*, 2002 Vol 13 Tab 266]. If significant fecal fat is not lost to the feces, then logic would dictate, that there would be no significant change in fat-soluble vitamin status. In fact, the nutritional status of the fat-soluble vitamins A, D, and E has been measured in human subjects. No change in vitamin status has been observed, even when the subjects were consuming a limited diet, and therefore, would presumably have limited access to additional vitamin sources [Colombo, *et al.*, 1996 Vol 3 Tab 24 and Sciutto, *et al.,* 1995 Vol 8 Tab 141]. Studies support the view that results in rats may not be predictive of those in humans, when the physiological effects of viscous polysaccharides are determined.

Since the late 1970s and early 1980s, researchers have known that dietary fibers may decrease serum calcium levels [Oku, *et al.,* 1982 Vol 15 Tab 319]. This effect would not be limited to chitosan, and humans consuming chitosan have not experienced significant calcium loss.

Primex ehf has supported its conclusions with a 56-subject placebo controlled single blind human clinical study, in which either 4.5 or 6.75 grams of Primex chitosan was consumed per day for 8 weeks [Tapola, *et al.,* 2003 Vol 16 Tab 357]. Data from this study are being prepared for publication as of the date of this submission. These data confirm that humans consuming chitosan derived from *Pandalus borealis* maintained their fat soluble vitamin and calcium status over an 8 week period. Further, they did so without experiencing changes in

other clinical chemistry or hematological parameters. These results were consistent with those seen for the placebo and another viscous soluble fiber.

Vitamin K status has been shown to increase when both humans and test species consumed chitosan. Unlike the other fat-soluble vitamins, vitamin K can be synthesized by colonic bacteria, and chitosan consumption has been shown to increase the amount of fermentable carbohydrate reaching the colon. Therefore, an increase in the numbers of colonic microflora would indicate that vitamin K levels might rise [Fairfield, *et al.,* 2002 Vol 13 Tab 260 and Chae, *et al.,* 2000 Vol 12 Tab 239]. While most of the population consumes inadequate levels of vitamin K, Fairfield, *et al.,* 2002 has suggested that persons taking anticoagulants may need to be counseled if they intend to increase their consumption of any indigestible carbohydrate [Fairfield, *et al.,* 2002 Vol 12 Tab 255]. Kamali, *et al.,* 2000 concluded that the combined effects of age and vitamin K appear to account for much of the inter-individual variability in anticoagulant dosage requirements [Kamali, *et al.,* 2000 Vol 14 Tab 288]. Researchers working at a number of well respected nutritional laboratories have concluded that recent data are consistent with a human dietary vitamin K requirement of approximately 1 microgram/kg body weight per day. [Suttie, *et al.,* 1988 Vol 15 Tab 340].

Therefore, a balanced review of the published literature and unpublished information contained in this document concerning chitosan supports the conclusion that shrimp-derived chitosan is a safe food ingredient for human consumption. Various animal and human studies have shown that chitosan is a non-toxic polysaccharide that is well tolerated in the diet. Likewise, its metabolism within the human body produces metabolites that are non-toxic and are similar to the metabolites of other dietary ingredients [Kelly, 1998 Vol 14 Tab 291].

Question I

D. Supporting Discussion to Primex Responses

1. Question 1. Might the physiological effect of chitosan consumption be similar to that of a well-known sucrose-ester fat-substitute, Olestra™, which has been shown to reduce the absorption of fat-soluble vitamins?

The results of a publication by Deuchi, which described a significant decrease in fat digestibility and subsequent loss in fat-soluble vitamins, when chitosan was consumed as 5% of a rat diet, led CFSAN to question whether similar results would occur in human beings. Historically, CFSAN had reviewed another substance, Olestra™, as a food additive. Olestra™ was designed as a fat substitute, and was approved for use in the preparation, of savory snacks. The agency issued a final rule on Olestra™ on January 30, 1996 [*Federal Register* January 30, 1996 Vol 13 Tab 261]. In a series of extensive clinical evaluations, Olestra™ was shown to carry fat-soluble vitamins out of the gastrointestinal tract, and hence, the petitioner agreed to enrich Olestra™ with vitamins E, A, D, and K.

Chitosan has also been reported to reduce fat absorption, and to reduce the levels of fat-soluble vitamins and minerals under certain circumstances in rats [Deuchi, *et al.,* 1995, Vol 4 Tab 28 and 29. Because of the potential similarity in physiological effects between Olestra™ and chitosan, reviewers at CFSAN suggested that Primex further investigate the effect of the substance on human vitamin and mineral status in the case where chitosan would be consumed as a part of a normal diet.

This section will discuss the physiological effect of chitosan on the nutritional status of rats and humans, and will compare the effects of chitosan with those of Olestra™. During the course of the discussion, chitosan will also be compared to substances other than Olestra™. The company will present data and information suggesting that chitosan belongs to a group of polysaccharides, termed, viscous dietary fibers. The nutritional and physiological effects of chitosan will be compared with

those of similar polysaccharides. For purposes of this discussion the term "chitosan" will often be substituted for "shrimp-derived chitosan".

a. Comparison of the structures and of Chitosan and Olestra™

Chitosan is a polysaccharide derived from the exoskeletons of crustaceans such as shrimp and crabs. Chemically, chitosan is a binary heteropolymer of β 1-4-2-amino-2-deoxy-D-glucopyranose and β 1-4-2-deoxy-2-acetamido-D-glucopyranose. The product is produced by the deacetylation of chitin. Chitin is an abundant natural material, which is commercially produced from shrimp and crab processing. Chitosan is obtained by the process of removing a sufficient number of acetyl groups from chitin, so that the molecule becomes soluble in most dilute acids. This is the process called deacetylation. Deacetylation releases amine groups, (NH), and results in chitosan, a heteropolymer, with cationic characteristics. Chitosan has been used, particularly in Asian countries, for many decades, and is currently produced by a number of companies worldwide [Lian, *et al.,* 1998. Vol 14 Tab 300].

Olestra™ is the common name for the mixture of sucrose-esters, formed from the addition of 6, 7, or 8 fatty acids to the available 8 free hydroxyl moieties of sucrose. Both saturated and unsaturated fatty acids with chain links ranging from C12 – C20 and higher can be used to manufacture Olestra™. Many toxicology studies were conducted to show the safety of Olestra™, however, the substance was also shown to impede fat-soluble vitamins and mineral absorption in humans.

The identity of sucrose octaester as the principle component of Olestra™ has been verified by infrared, mass, and nuclear magnetic spectrometry. The methyl esters used to prepare Olestra™ are obtained by the reaction of refined triglyceride oils with methanol in the presence of sodium methoxide or from esterification of their fatty acids. The resulting esters are washed with water to remove residual methanol, dried under vacuum, and distilled. The fats and oils can be derived from a variety of edible oil sources, such as soybean, palm,

coconut, fully hydrogenated rapeseed or cottonseed. Sucrose and the methyl esters are mixed with an alkaline metal soap of a long chain fatty acid. A small amount of trans-esterification catalyst, such as an alkaline metal, like sodium or potassium, is added and the mixture is heated under vacuum to withdraw the volatile methanol by-product. Following the reaction, excess methyl esters and free methanol are removed by evaporation under vacuum. Standard steam deodorization is used to remove the free fatty acids and odors. The structures of the two compounds are shown below.

[Schematics of structures temporarily removed]

b. Proposed Uses of Shrimp-Derived Chitosan and Olestra™

Olestra™ was proposed for use as a calorie-free replacement for up to 100% of the conventional fats and oils used in the preparation of savory snacks, such as flavored and unflavored chips and crisps, flavored and unflavored extruded snacks and crackers. These uses include substitution for fat for frying as well as sources of fat in dough conditioners, oil sprays, and flavors. The agency concluded that an adjusted lifetime average for the estimated daily intake of Olestra™ was justified. The value for the 90th percentile was estimated to be 7.0 grams per person per day.

Chitosan was proposed for use as an: antioxidant, curing and pickling agent, dough strengthener, emulsifier, formulation aide, humectant, nutrient supplement, processing aid, stabilizer and thickener, surface-active agent, synergist, and texturizer. The estimated intake at the 90th percentile was approximately 3.2 grams per person per day, and the company acknowledged that between 1.5 and 3.0 grams per day could be consumed as a dietary supplement.

c. Physiological Effect of Olestra™

Preliminary studies of Olestra™ in which rats were intravenously injected with Olestra™ radiolabeled with ^{14}C on the sucrose portion of the molecule showed that the radiolabeled Olestra™ initially deposited in the liver and to a lesser extent, in the spleen. By 21 days, the concentration of Olestra™ in the liver had dropped to about 50% of the three-day level. Olestra™ was excreted unchanged via the biliary and fecal routes. According to the sponsor, these results demonstrated that the Olestra™ that accumulated in the liver following intravenous injection was not metabolized because radiolabel was not accumulated in other tissues, which would have occurred if Olestra™ had been hydrolized by hepatic enzymes. The absence of Olestra™'s metabolites was confirmed by thin layer chromatography, which showed intact Olestra™ in the bile and feces. The half-life of Olestra™ in the liver was found to be about 5 days.

Procter and Gamble conducted a series of studies in male and female rats to determine the fate of penta-, hexa-, hepta-, and octa-ester preparations of Olestra™ administered by gavage. Fat balance studies conducted in the rat demonstrated that enzymatic hydrolysis can convert mono- through penta-ester formulations of Olestra™ to sucrose and fatty acids, while hexa- through octa-ester formulations are not absorbed. In order to detect the absorption of a very small amount of the administered dose, Olestra™ of high-chemical and radio- chemical purity and high specific activity (1 mCi per gram), was dosed at high levels (0.1 mCi per rat). Tissues were collected, combusted, and analyzed for radio labelled CO_2, or the lipid fraction was extracted and analyzed for intact Olestra™ via HPLC. Urine, feces, expired CO_2, and the carcass were analyzed for ^{14}C. The urine was analyzed for ^{14}C-sucrose to assess whether or not Olestra™ had been absorbed and metabolized. A variety of Olestra™ preparations were tested in this study, and the mean recovery of unabsorbed radiolabel from the rat feces, GI tract and contents, animal wipes and animal rinse solutions, and cage wipes and cage rinse solutions, was

greater than 98.5% of the administered dose regardless of the radiolabeled Olestra™ formulations studied. This recovered amount represents Olestra™ that was not absorbed. The majority of the absorbed radioactivity was found in the expired CO_2 and urine. Analysis of liver lipids for intact Olestra™ and urine for ^{14}C-sucrose did not show any radiolabeled carbon. These data demonstrated that most of the ingested Olestra™ remained intact and was not absorbed, but was excreted intact in the feces.

Procter and Gamble conducted two sub-chronic toxicity studies in rats. The first sub-chronic Olestra™ feeding study in rats showed no adverse effects, but used unheated Olestra™. The second study used heat-abused Olestra™, which was fed at 0, 1, 5, and 10 percent of the diet. Three different control groups were used. Groups one and two were chow controls, while group 6 control rats were maintained on a diet that contained 10% previously heated triglyceride. Diets for groups 2 through 5 were supplemented with vitamins A, D, and K at five times the NRC requirement. Olestra™ fed rats compensated for the decrease in caloric intake due to Olestra™ having zero calories by consuming more than control rats. No adverse treatment related affects were observed. The results indicated that heated Olestra™ was non-toxic when fed to rats at levels as high as 10% of their diet for a period of 90 days.

The petitioner concluded that Olestra™ would not interfere with normal intestinal fermentation of dietary fiber. Procter and Gamble stated that the pivotal studies that demonstrated that Olestra™ was not metabolized by microflora in the GI tract were a clinical study in humans and rat absorption and metabolism studies. The clinical studies showed no production of radiolabelled metabolic breakdown products and no changes in either Olestra™ fatty acid composition or ester distribution following incubation of radiolabelled Olestra™ with fecal microflora from humans who had consumed 7 grams per day of Olestra™ for up to 31 days.

A published study demonstrated that Olestra™ was not metabolized by the microflora of the GI tract. Likewise, both published and unpublished studies in humans and animals demonstrated that consumption of Olestra™ would have no meaningful effect on the absorption, synthesis, or the excretion of bile acids.

d. Issues Associated with Olestra™

The petitioner, Procter and Gamble, had hypothesized that Olestra™ would interfere with the absorption of fat-soluble nutrients if the nutrients partitioned into Olestra™ in the GI tract. If partitioning occurred, that portion of the nutrients that were present in the Olestra™ phase would be unavailable to the micelle-mediated transport system in the gut, and rather than being absorbed by the body, those nutrients would be excreted in the feces along with the Olestra™. Neither existing Olestra™ data nor the partitioning mechanism suggested that water-soluble nutrients would be affected by Olestra™. However, certain water-soluble nutrients, such as folate and vitamin B_{12} (which are hard to absorb), are absorbed in multi-step processes. The sponsors suggested that the multi-step nature of the processes might allow an opportunity for Olestra™ to interfere with key steps, such as binding or cleavage reactions. Calcium, zinc, and iron were known to be limited in the US diet, thus any affect on their absorption might increase the risk of nutritional inadequacy.

The agency and its consultants ultimately concluded that there was no concern about the consumption of Olestra™ and water-soluble nutrients. The petitioners submitted several published and unpublished studies in animals and humans to demonstrate that consumption of Olestra™ would have no meaningful effect on the absorption, synthesis, or excretion of bile acids. The agency found no evidence to indicate that the consumption of Olestra™ resulted in adverse health conditions. With respect to whether Olestra™'s potential to cause diarrhea–like symptoms or loose stools, FDA noted that the soft stool and diarrhea appeared to be caused by disruption of the fecal matrix, and were not associated with the

clinical signs of fluid loss, which is the case in classical diarrhea. Therefore, FDA concluded, that there was no basis that any sub-populations would be at risk due to the consumption of Olestra™.

The totality of the data on Olestra™ showed that the substance was basically inert in the gastrointestinal tract, and that it did not interact with the body to any significant degree, other than to exert physical effects on the partitioning of fat-soluble nutrients.

e. Physiological Effect of Chitosan

Unlike Olestra™, chitosan interacts with the body, producing a number of physiological effects, which have been well recognized, and are common to the effects of other viscous polysaccharides. These effects can be related to physical properties, which include:

- Chitosan is cationic in nature
- Chitosan is viscous in mild acids
- Chitosan binds to proteins
- Chitosan is indigestible in the upper GI Tract
- Chitosan is fermentable in the colon
- Chitosan entranes fats
- Chitosan is degradable into glucosamine
- Chitosan interacts with the immune system
- Chitosan effects bile acid metabolism
- Chitosan is bioadhesive
- Chitosan is abrasive to tissues

[Furda, 2000, Vol 4 Tab 40, Gordon, *et al.*, 1984, Vol 4 Tab 45, Landes, *et al.*, 1976 Vol 6 Tab 89, and Kelly, G.S., 1998, Vol 14 Tab 291].

Question 2

2. **Question 2. Deuchi, *et al.*, (1995), reported that rats fed 5% of their diet as chitosan experienced a significant decrease in fat digestibility with concurrent loss of certain fat soluble vitamins and minerals such as calcium. Reviewers at the Center for Food Safety and Applied Nutrition (CFSAN) questioned whether chitosan consumption by humans would result in a similar lowering of nutritional status for fat soluble vitamins or minerals, or put another way, would the consumption of chitosan result in sufficient fecal fat excretion, that the nutritional status of humans would be threatened by the loss of the fat-soluble vitamins A, D, and E?**

In order to answer the question posed above, this section will briefly review:

- What is known about the effects of viscous polysaccharides on nutrient digestibility in general
- What has been reported about the effect of specific viscous polysaccharides on fat digestibility
- The historical use of rats as models for human digestion
- Species differences in the ability to digest fats and dietary fibers when present as part of a mixed diet
- Relationship of these studies to regulatory concerns about the effect of chitosan consumption by humans

Finally, the section will conclude with a discussion of whether or not data involving chitosan consumption by rats can appropriately be extrapolated to humans.

As discussed above, many feeding studies have been conducted in which the physiological and nutritional effects of various chitosan products were tested in rats [Deuchi, *et al.*, 1994, 1995a and 1995b Vol 4 Tabs 28, 29 and 30, Gordon, *et al.*, 1983, 1984 Vol 4 Tabs 46 and 47, and Lian, *et al.*, 1998 Vol 14 Tab 300]. In one study chitosan had been identified from among 23 fiber materials tested as a fiber that might benefit human nutrition, by preferentially hindering fat as opposed to protein digestibility [Deuchi, *et al.*, 1994 Vol 4 Tab 30]. Scientists had recognized the adverse effect of fiber intake on protein digestibility since the late 1970s [Eggum, 1995 Vol 13 Tab 257]. British researchers, while

touting the benefits of dietary fiber consumption, also recognized that those benefits had trade-offs, because of the negative effects fiber consumption had on the absorption of other types of nutrients [Cummings, 1978, Vol 12 Tab 247]. Deuchi, *et al.*, among others hypothesized that if certain dietary fibers could reduce only the fat digestibility, then consumption of those fibers would achieve a reduction in caloric absorption without effecting protein nutrition; a concept thought to benefit those persons on western diets, where excess calories is often undesirable [Deuchi, *et al.*, 1994 Vol 4 Tab 30].

Deuchi, *et al.*, designed several studies to test this hypothesis, by feeding rats as test species high-fat diets along with various percentages of chitosan preparations. The results of these studies have been extrapolated to humans. Yet, little if any comparative data is available to suggest that rats and humans would digest fats and chitosan, a polysaccharide, to the same extent, or that the two species would experience the same physiological effects.

a. Influence of Viscous Dietary Fibers on Fat Digestion

The influence of viscous dietary fibers on fat digestion has been reviewed by Schneeman (1995) [Schneeman, 1996 Vol 15 Tab 338]. Schneeman reported that the ability of sources of dietary fiber to interfere with rapid digestion and absorption of lipids has been well documented. The viscous polysaccharides can interfere with micelle formation or disrupt mixing within the small intestinal contents. Schneeman stated that viscous polysaccharides slow the disappearance of lipids from the digestive tract, and studies with ileostomy patients have indicated that lipid in the ileostomy fluid increases by about 50% in patients fed guar gum, and by about 36% in patients given pectin [Higham, *et al.*, 1992 and Sandberg *et al.*, 1983]. In contrast, an insoluble fiber, such as wheat bran did not significantly increase fat excretion in ileostomy fluid [Sandberg, *et al.*, 1981]. Primex submits that consumption of chitosan should have no more effect on fat digestion, and hence fecal fat loss than any other

viscous polysaccharides, such as guar gum, glucomannan, pectin, xanthan gum, psyllium, oat gum, or carrageenan.

In animals the consumption of viscous polysaccharides delays the disappearance of lipid from the small intestine. Schneeman reported that labeled triolein and cholesterol were significantly higher in the intestinal contents of rats and significantly lower in the tissue of their small intestines 2.5 hours after a test meal containing viscous polysaccharides instead of cellulose (See Table 7 below) [Schneeman, 1996 Vol 15 Tab 338].

Table 7: Effect of Fiber on the Disappearance of Cholesterol and Triglyceride from the Small Intestine

	^{3}H-Cholesterol		^{14}C-Triolein	
	(Percent of Label Emptied from the Stomach)			
	Small Intestine Contents	Small Intestine Tissue	Small Intestine Contents	Small Intestine Tissue
Cellulose	33^{a}	57^{c}	7^{a}	20^{c}
Guar Gum	43^{b}	45^{b}	9^{b}	11^{b}
Glucomannan	58^{c}	27^{a}	12^{c}	1^{a}

$^{a, b, c}$ Values with different superscripts differ significantly ($p < 0.05$).
[Schneeman, 1996 Vol 14 Tab 287]

Likewise, Vahouny *et al.*, (1988) found that oleic acid uptake in rats who were adapted to fiber was much lower when viscous fibers, such as psyllium or guar gum were fed in comparison to cellulose or diets without fiber (See Table 8 next page). Thus the ability of many types of fiber to interfere with fat digestion in both humans and animals has been well established [Vahouny, et al., 1983 Vol 11 Tab 211].

Table 8: Lymphatic Recovery of Triglycerides and Cholesterol in Rats Adapted to Fiber

	Cholesterol		Oleic Acid	
	(Percent of Dose Infused in the Intestine)			
	4 Hours	24 Hours	4 Hours	24 Hours
No Fiber	18.9	49.0	48.5	70.0
Cellulose	8.3*	37.5	37.0	67.0
Pectin	5.0*	18.0*	26.0*	55.0
Guar Gum	6.1*	28.0*	32.5*	50.0
Psyllium	3.2*	12.0*	19.0	27.5*

*Values differ significantly from the fiber free control ($p < 0.05$).
[Schneeman, 1996 Vol 15 Tab 338]

b. Quantifying the Effects of Viscous Polysaccharides on Fecal Fat Content in Humans

While it has been established that many types of fiber are able to interfere with fat digestion in both humans and animals the real question is how much fat is lost to the feces in humans, and is this amount significant enough to impair nutritional status. The results cited above indicate that viscous indigestible polysaccharides have similar effects on fat uptake. In the CRC Handbook of Dietary Fiber in Human Nutrition (2001), John Cummings reviews the effect of dietary fiber consumption on human fecal weight and composition. His chapter contains a very long table, which includes relevant studies in which changes in fecal fat are reported. Dr. Cummings' table breaks out the changes in fecal composition by fiber type, which is of particular significance to this submission. The Table below is excerpted from Dr. Cummings' review. Fibers similar in physiological effect to chitosan are listed along with the increase in fecal fat caused by their consumption. The amount of each fiber consumed in grams is listed along with the number of human subjects in each

study. Significantly, most of the studies reviewed were conducted in the late 1970s and 1980s. Thus, this information which was widely known at one time may have been forgotten as the decades have passed.

Table 9: Human Studies Reporting Changes in Fecal Fat Composition as a Result of Consuming Dietary Fiber

Fiber	Grams of Fiber Consumed/Day	Increase in Fecal Fat/Day in Grams	Number of Subjects per Study
Pectin	12.0	4.8	9
	37.0	0.8	9
	36.0	1.2	5
	10	1.2	65
	15 g	-0.2	8
Isphagula	10	3.7	47
Psyllium Powder	20 g	9.0	50
Plantago ovata	25	1	
Gum Karaya	10.5 g	-0.3 g	5
Oat bran (Toasted)	12	0.8	6
Oat bran (plain)	12.0	0.7	6
Rolled Oats	110-160 g	-6.0 g	10
Mixed fiber	8.7	3.6	6
Mixed fiber	33	1.3	20-27
Guatemalan Diet	89	0.7	6
Fruit and Vegetables	24	1.5	12
Fruit and Vegetables	26	0.8	12
Carboxymethyl-cellulose	15	2.5	5
Xanthan Gum	10.4 to 12.9	0.3	5
Gum Tragacanth	9.9	3.1	5

Table 9 above contains data from studies listed by Cummings, in which both data for behfl fecal fat and data for fecal fat following fiber ingestion were listed. The effects of cereal fibers were not listed in this Table. The data demonstrate that fecal fat increased slightly in each study listed above, irrespective of the amount of fiber ingested, except for one study with pectin, one for rolled oats, and one for gum Karaya. In those three studies, fecal fat actually decreased in relation to a behfl diet [Cummings, 2001Vol 12 Tab 246].

Fecal fat increased when viscous polysaccharides were consumed. as well as when fiber from fruits and vegetables was increased. Most notable is the fact that increases in fecal fat were modest, ranging from 0.3 grams for Xanthan gum to 9.0 grams per day for psyllium powder. On average fecal fat increased by 0.12 grams for every gram increase in dietary fiber consumption. It is thus clear that all fibers are capable of increasing fecal fat, but that the increase experienced by humans is not nutritionally significant to those consuming a western diet [Cummings, 2001Vol 12 Tab 246]. As will be discussed later in this section, most of the increase in fecal fat actually originates from an increase in bacterial lipids, because fiber stimulates greater bacterial numbers in the colon [Bach-Knudsen, *et al.*, 1994 Vol 12 Tab 236 and Wisker, *et al.,* 1988 Vol 16 Tab 368].

c. Mechanisms by which Dietary Fiber Can Impede Fat Absorption.

It has been claimed that consumption of chitosan can increase fecal fat excretion in humans. The mechanism proposed for this effect was discussed in the Primex GRAS Report (GRN 000073). Dr. Ivan Furda (2000) reviewed the proposed mechanisms, which have been put forward to explain the hypocholesterolemic and lipid lowering effects of chitosan [Furda 2000, Vol 4 Tab 40]. These effects were established in *in vitro* assays and in animal trials, and they include:

- Viscosity effect
- Ionic interaction
- Entrapment of mixed micelles and bile acids and fatty acids
- Entrapment of dietary triglycerides
- Inhibition of pancreatic lipases and other digestive enzymes

The effects listed above are common to other viscous polysaccharides. The following review has been included to establish that these effects have been observed for other viscous polysaccharides, which are now common to the American diet. Much of this work was conducted in animal models, and it may be prudent to point out that not all the data has been confirmed for humans. However, for this discussion, it will be assumed that the animal

models will be generally indicative of the potential effects exerted by viscous fibers in the human digestive system.

Consumption of Viscous Indigestible Polysaccharides Leads to Changes in the Exocrine Pancreatic-Biliary Function and May Depress the Process of Digestion and Absorption

Ikegami, *et al.,* 1990 studied the effect of viscous indigestible polysaccharides on pancreatic - biliary secretion and digestive organs in rats. The authors stated that it was well known that certain dietary fibers can impair the digestion and absorption of several nutrients. At the time the article was written, the current knowledge about the physiological responses to fiber-rich foods and to individual types of fiber components suggested that there are certain potentially important mechanisms by which fibers can alter the function of digestive organs. These include primary actions such as:

- Altering the rate of transit
- Altering the pH of intraluminal contents
- Altering the viscosity of intraluminal contents
- Modification of digestive enzyme activities
- Interaction of fibers with the intestinal surface

These primary actions can change the physiological function of digestive organs by interfering with the digestion and absorption of nutrients. Previous studies had demonstrated in rats that indigestible materials, in particular highly viscous fibers, decrease the accessibility of absorbable nutrients to the mucosal surface. Furthermore, dietary fiber was thought to cause morphological changes in intestinal surfaces, resulting in alterations of enzyme activities, secretion and turnover of mucins and gastrointestinal hormone release [Ikegami, *et al.,* 1990 Vol 13 Tab 280].

In the study conducted by Ikegami, *et al.,* growing rats were fed a nonfiber diet or a diet containing approximately 5% of one of the following fibers: apple pectin, γ-carrageenan, locust bean gum, gum xanthan, guar gum, or sodium alginate. Pancreatic-bile secretion was

found to be elevated in rats fed the highly viscous polysaccharides, sodium alginate, locust bean gum, gum xanthan and guar gum for a period of 2 weeks. The polysaccharides may have interfered with the digestion and absorption of nutrients, resulting in a decreased digestibility and an enlargement of digestive organs. Interestingly, when alginic acid and calcium alginate, insoluble polysaccharides that did not contribute to viscosity, were given to rats, they had no effect on pancreatic and biliary secretion compared with sodium alginate. The results demonstrated that consumption of viscous indigestible polysaccharides leads to changes in the exocrine pancreatic-biliary function and may depress the process of digestion and absorption. Rats may compensate for the inefficiency of digestion and absorption with a hyperplasia/hypertrophy of digestive organs and an increased secretion of digestive juice [Ikegami, *et al.*, 1990 Vol 13 Tab 280].

The addition of viscous indigestible polysaccharides such as sodium alginate, γ-carrageenan, pectin and konjac mannan (glucomannan) to the diet was found to cause an enlargement of digestive organs and a reduction in the apparent digestibility of nutrients in rats. Recently, the authors observed that long-term administration of sodium alginate and konjac mannan to rats increased pancreatic weight and the secretion of amylase and protease; similarly, the volume of secreted juice from the common bile duct and the output of total bile acids were increased. These effects were not observed with agar. Because a single dose of sodium alginate and konjac mannan did not affect pancreatic-bile secretion, the hypersecretion observed over a prolonged period of feeding these fibers may be the result of an adaptation of the animals to viscous indigestible polysaccharides [Ikegami, *et al.*, 1990 Vol 13 Tab 280].

Dietary Fiber Consumption Inhibits Enzyme Activity

Isaksson *et al.* conducted a series of studies in the early 1980s, which were designed to investigate the relationship between dietary fiber and digestive activity. In the team's first study trypsin, amylase, lipase and phospholipase activities were assayed in buffer solutions and in human duodenal juice after incubation with different types of dietary fiber. In buffer solutions, trypsin activity was slightly reduced and amylase activity heavily reduced by low methoxy pectin (LM). Lipase activity was markedly reduced by the LM pectin and also moderately reduced by high methoxy pectin. The activities of pancreatic enzymes in human duodenal juice were examined after *in vitro* incubation with pectins, guar gum, wheat bran and Isphagula. Isphagula did not affect the enzymes except for lipase activity, which was moderately inhibited. The other fiber preparations reduced amylase activity by 35-100% lipase by 40-95% and trypsin by 40-85% at fiber concentrations of up to 1.5 g%. LM pectin had the strongest inhibitory effect among the different fiber preparations studied. Phospholipase activity was only influenced by the pectins, which caused a 75% reduction. The authors concluded that dietary fiber of different kinds has the capacity to inhibit pancreatic enzyme activities. This inhibitory effect was found to be dependent on the type of fiber, and different fibers affected the enzymes differently. Interestingly, the inhibition seemed to be more pronounced when exerted in human duodenal juice than in conventional buffer systems [Isaksson *et al.,* 1982a Vol 13 Tab 281, Isaksson *et al.,* 1982b Vol 13 Tab 282, Isaksson *et al.,* 1983a Vol 13 Tab 283,Isaksson *et al.,* 1984a Vol 13 Tab 284, and Isaksson *et al.,* 1984b Vol 13 Tab 285].

Increasing the Viscosity of Duodenal Juice Causes a Reduction in Enzyme Activity; The inhibitory effects of fibers on enzyme activities can be attributed to effects on viscosity, pH, and adsorption

In a second study the importance of various factors for dietary fiber effects on pancreatic enzyme activities was studied *in vitro*. High methoxy pectin (HM) and guar gum, reduced enzyme activities, particularly lipase activity, and increased the viscosity of duodenal juice. Increasing the duodenal juice viscosity by the addition of polyethylene glycol also caused a reduction in enzyme activity, particularly of lipase. LM pectin, which reduced amylase and lipase activities especially, significantly lowered duodenal juice pH. Likewise, the lowering of duodenal juice pH by adding hydrochloric acid caused a reduction of amylase and lipase activities particularly. Pretreating fibers with acid pH tended to increase their enzyme-inhibiting properties. The authors found that trypsin adsorption to wheat bran was partly reversible by buffered washings. Changing the ionic strength and incubation time appeared relatively unimportant for the effects of the fiber on enzymes. It was assumed that the inhibitory effects of the fibers on enzyme activities could be attributed *inter alia* to the effects on viscosity, pH, and adsorption, and that gastric acidification of fiber and conditions lowering intestinal pH may enhance these effects [Isaksson *et al.*, 1982b Vol 13 Tab 282].

Viscous Dietary Fibers Cause Digestive Organ Hypertrophy and Increased Enzyme Secretions

Isaksson *et al.* stated that it was well established that oral administration of trypsin inhibitor stimulated the secretion and growth of the rat pancreas. In a third study, trypsin inhibitor (Trasylol) as well as dietary fiber such as LM pectin (37%) and wheat bran were found to stimulate pancreatic enzyme secretion in acute experiments in conscious rats with bile-pancreatic fistulae. Feeding for 10 days with wheat bran resulted in increased pancreatic weight and in increased protein and trypsinogen content. Administration of HM pectin (73%) caused increased pancreatic protein content and LM pectin increased pancreatic trypsinogen activity/milligram tissue. The results suggested that pectin and wheat bran might interfere with the feedback regulation of pancreatic enzyme secretion exerted by intraluminal trypsin, and, like trypsin inhibitor, have a secretagogue and trophic effect on the pancreas [Isaksson *et al.,* 1983a Vol 13 Tab 283].

To further investigate the effect of viscous polysaccharides on pancreatic function, ileostomized rats were fed diets with different fiber contents in a continuation of this series of studies. The addition of 5% pectin to the diet caused an increase in the wet weight, fat content, amylase activity per gram, and lipase output of the ileostomy evacuates. Twenty percent wheat bran in the diet increased weight, fat and nitrogen content, and trypsin output of the evacuates. In normal rats pectin added to a meal containing 3H-labeled triolein increased the isotope activity of the feces, indicating an impaired fat absorption. In rats where the pancreatic ducts were surgically occluded with a tissue glue, the fat absorption was, however, not significantly affected by pectin. The results of this study showed that fiber can cause a change in the intestinal enzymatic milieu of ileostomized rats and can cause steatorrhea, which could be at least partly explained, by malabsorption [Isaksson *et al.,* 1983b Vol 13 Tab 284].

Isaksson *et al* had shown that dietary fiber inhibits pancreatic enzyme activity--i.e., trypsin, lipase and amylase--in buffer solutions and in human duodenal juice *in vitro*. The researchers stated that it was well established that oral administration of trypsin inhibitor stimulates the secretion and growth of the rat pancreas. In another study, the trypsin inhibitor (Trasylol) as well as dietary fibers such as low methoxy pectin (37%) and wheat bran were found to stimulate pancreatic enzyme secretion in acute experiments in conscious rats with bile-pancreatic fistulae. Feeding for 10 days with wheat bran resulted in increased pancreatic weight and in increased protein and trypsinogen content. Administration of high methoxy pectin (73%) caused increased pancreatic protein content and administration of low methoxy pectin increased pancreatic trypsinogen activity/milligram tissue. The results suggested that pectin and wheat bran may interfere with the feedback regulation of pancreatic enzyme secretion exerted by intraluminal trypsin, and, like the trypsin inhibitor, have a secretagogue and trophic effect on the rat pancreas [Isaksson *et al.,* 1983b Vol 13 Tab 284].

In Pancreatectomized Humans Dietary Fiber May Induce Fat Malabsorption and Inhibit Digestive Enzyme Activities

In totally pancreatectomized humans breath $^{14}CO_2$ excretion after ingestion of ^{14}C-labeled triolein was significantly increased by a granulated pancreatic enzyme preparation and was reduced when pectin was added to the enzyme supplement. In the same subjects pectin reduced trypsin, lipase, and amylase activities of jejunal aspirates after a test meal supplemented with a pancreatic enzyme substitute, which was shown to give good enzyme activities in the intestine. In patients with chronic pancreatitis, breadth $^{14}CO_2$ excretion was reduced by wheat bran, which also caused a reduction in lipase and amylase activities of duodenal aspirates after a test meal. The findings demonstrated the efficiency of treatment with a granulated pancreatic enzyme preparation in restoring intraluminal enzyme activities and fat absorption in patients with pancreatic

insufficiency. They also showed that pectin and wheat bran may induce fat malabsorption and inhibit digestive enzyme activities *in vivo* [Isaksson *et al.*, 1984 Vol 13 Tab 285].

Humans Cannot Consume the Quantities of Viscous Compounds Fed to Animal Models. The Amounts Administered Seldom Exceed 10 g Per Day

To simulate the effects of more reasonable levels of consumption, Marie-Anne Levrat-Verny, *et al.*, 2000, studied the effect of low levels of viscous hydrocolloids in rats. She reasoned that experiments with hydrocolloids have frequently been carried out using a relatively high percentage in the diet (seldom less than 5%), to achieve substantial modifications of intestinal digestion and lipid variables. However, humans cannot consume such quantities of viscous compounds, and the amounts administered seldom exceed 10 g per day, which corresponds to about 2.5% of the diet [Levrat-Verny, *et al.*, 2000, Vol 14 Tab 299].

According to the authors, the effect of various fibers or related compounds on cholesterol metabolism had been extensively studied, and it was generally accepted that viscous water-soluble fractions are the most effective in lowering plasma cholesterol concentrations. In this area, various types of polysaccharides, especially gums, and particularly guar gum (GG), have been investigated. The authors stated that GG is a galactomannan extracted from the endosperm of leguminous guar, which is extensively used as a gelling agent in food industry. This viscous fiber is rapidly fermented by colonic microbiota and, subsequently, high intake can cause gastrointestinal intolerance in humans [Todd *et al.* 1990, Vol 16 Tab 359]. Xanthan gum (XG) was described as a biosynthetic edible gum produced by the bacterium *Xanthomonas campestri*. It is composed of glucose, mannose and glucuronic acid, and is characterized by a coiled backbone and tightly packed side groups. Konjac mannan (KM) on the other hand is obtained from the tubers of *Amorphophagus konjac,* and it is a linear polymer of mannose and glucose (Southgate, 1976). These two compounds are also viscous fibers

that are fermented in the large intestine [Osilesi *et al.* 1985, Vol 15, Tab 321 and Vorster *et al.* 1985, Vol 16, Tab 363].

By Increasing the Viscosity of the Digesta and the Thickness of the Unstirred Layer in the Small Intestine, Fibers Can Decrease Cholesterol and Bile Acid Absorption

According to Marie-Anne Levrat-Verny, *et al.,* the ability of soluble fibers to lower plasma cholesterol appeared to be due to different mechanisms. By increasing the viscosity of the digesta and the thickness of the unstirred layer in the small intestine, fibers can decrease cholesterol and bile acid absorption [Anderson *et al.*, 1994, Vol 12 Tab 230, Evans *et al.* 1992, Vol 13 Tab 259, Gee *et al.* 1983, Vol 13 Tab 268]. Disturbance of the enterohepatic cycling of bile acids was considered to be important in the cholesterol-lowering effect of fibers. Increased fecal losses of bile acids were thought likely to induce bile acid synthesis in the liver and hence accelerate cholesterol oxidation. This situation was generally accompanied by an induction of hydroxymethylglucaryl CoA reductase (HMGR, a rate-limiting enzyme of cholesterol synthesis), which is a response to the channeling of hepatic cholesterol toward oxidation and/or excretion [Arimandi *et al.* 1992, Vol 12 Tab 232, Levrat *et al.* 1996, Vol 14 Tab 298, Kamil *et al.* 2002, Vol 14 Tab 389]. In parallel, short-chain fatty acids (SCFA) resulting from microbial fermentation of soluble fibers in the large bowel are absorbed in the portal vein, and a major part of these are metabolized by the liver, where propionate especially can affect various metabolic processes [Remesy *et al.* 1989, Vol 15 Tab 328]. It has been proposed that propionate could depress cholesterol synthesis. Although this effect is relatively well documented *in vitro*, its relevance *in vivo* remained unclear according to the authors [Demign, *et al.* 1995 Vol 12 Tab 252, Stark, *et al.*,1993, Vol 16 Tab 350, Stephen 1994, Vol 16 Tab 352, Wolever *et al.* 1995, Vol 16 Tab 370].

Hydrocolloids have been proposed as cholesterol-lowering agents, but their viscosity limits their use in human nutrition. Therefore, a low level (1.0 %) of hydrocolloids (guar gum, (GG); xanthan gum, (XG);

and konjac mannan) was investigated in rats fed 0.2 g/100 g cholesterol diets. The purpose of the Levrat-Verny, *et al.,* study was to determine if low levels of hydrocolloids in rats fed semi-purified diets would affect plasma lipid concentrations, and whether this depended on changes in the digestive balance of sterols and bile acids. All the diets were enriched with 0.2% cholesterol, and diets containing 1% GG, XG or KM were compared to a control fiber-free diet.

Food intake and body weight gain were not altered by the diets. Bile flow and cholesterol bile flux were not modified by diet, whereas the bile acid flux was greater in rats fed hydrocolloid diets. The cecal pool of bile acids was greater than control rats only in rats fed the XG diet (+71 %, $P < 0.001$). The fecal excretion of neutral sterols was stimulated in rats fed the hydrocolloid diets; cholesterol apparent digestibility (60% in controls) was reduced to 30-36% in rats fed hydrocolloids. Bile acid fecal excretion was not altered by diet treatment. As a result, apparent steroid balance was about +40 μLmol/d in controls and only +10 to +20 μLmol/d in rats fed hydrocolloids. Both plasma cholesterol and triglycerides were significantly lower than controls in rats fed XG, but only cholesterol was lower in rats fed the GG diet. These effects were essentially found in the d < 1.040 kg/L fraction. Liver cholesterol content was significantly lower than in controls in rats fed the GG or XG diets. Liver HMG CoA reductase was not affected by the hydrocolloid diets. The authors concluded that, a low percentage of viscous hydrocolloids lowers plasma cholesterol in cholesterol-fed rats. They speculated that inhibition of intestinal cholesterol absorption may be the primary mechanism [Levrat-Verny, *et al.,* 2000 Vol 14 Tab 299].

The Ability of Gums to Form Semisolid Gels at Low Concentrations May Be Associated with the Effectiveness of Those Gums to Influence Lipid Content and Enzyme Activities of the Liver

Osilesi, *et al.,* 1988 Vol 15 Tab 322, of the Carbohydrate Nutrition Laboratory, Beltsville Human Nutrition Research Center, Agricultural

Research Service, studied hepatic responses to edible gums during refeeding of starved rats. The authors had previously reported that edible gums have many biological effects, some of which are physiologically important. The feeding of gums affects gastrointestinal physiology and eventually alters the histology of the digestive tract. Over time, gums lower serum levels of cholesterol and increase the fraction of serum cholesterol carried by high-density lipoproteins. Of special interest was the ability of some gums to improve the control of blood glucose in diabetic people [Osilesi, *et al.,* 1988 Vol 15 Tab 322].

The authors reported that, when an aqueous suspension of xanthan gum was added to a nutritionally adequate, high carbohydrate diet and fed to starved rats, the gum attenuated the increase usually seen with refeeding in total lipid and in the activity of two lipogenic enzymes in the liver. The question arose whether the short-term feeding of starved rats might be a sensitive and useful model for studying the effects of edible gums. Five experiments were reported in their publication. Experiment 1 tested the ability of xanthan gum to depress enzyme activities and total lipids in the liver when the gum was added as a dry powder in place of cellulose. Experiment 2 examined the ability of agar to affect hepatic parameters when it was added to the diet as a dry powder or as a semirigid gel. In experiments 3 and 4, changes in total liver lipids and enzyme activities during varying periods of refeeding were charted when xanthan gum was or was not included in the diet. Experiment 5 dealt with the hepatic effects of feeding any one of seven gums with highly variable physical properties. Hepatic responses to the gums were compared with measurements of the viscous and gel-forming properties ofz the gums [Osilesi, *et al.,* 1988 Vol 15 Tab 322].

The ability of edible gums to depress total liver lipids and activities of two hepatic enzymes (glucose-6-phosphate dehydrogenase and NADP-linked malic enzyme) was examined during the refeeding of 2 day-starved rats. Gums were fed as 4% of dry ingredients, occasionally with added water, in otherwise identical high glucose,

nutritionally adequate diets. Feeding of xanthan gum for 1 or 2 days reduced the rise in the two enzyme activities and in total liver lipids, but after 4 or 7 days only total liver lipids were affected. Agar, which is insoluble at room temperature, was effective only when incorporated in the diet as a stiff gel. Guar, carrageenan. karaya and pectin reduced the change in at least one hepatic parameter, but acacia and gum ghatti, which impart little viscosity to water, were without effect. The ability of seven gums to form semisolid gels at low concentrations was measured and appeared to be associated with the variable effectiveness of those gums in influencing lipid contents and enzyme activities of the liver [Osilesi, *et al.,* 1988 Vol 15 Tab 322].

Indigestible Polysaccharides Inhibit the Activity of Disaccharidases in the Gut

Oku and Kwon, 1988 reported on the existence of disaccharidases and an enzyme that hydrolyzes maltitol in the large intestine of rats. In addition, the properties of disaccharidases were studied in the cecum and colon with hyperplasia induced by the ingestion of non-digestible carbohydrates such as maltitol and glucomannan. Maltase activity was detected in the cecal and colonic mucosa of rats fed a regular diet, although a very low level was found as compared with that in the small intestinal mucosa. Maltitol hydrolysis was notably lower in the cecum and colon than in the small intestine. The Km of maltose was 5.56 mM in the small intestine and 5.59 mM in the cecum, while that in the colon was 2.56 mM. The Vmax of maltose was at very low levels in the cecum (0.38 mumol/mg protein/h) and colon (0.37 mumol/mg protein/h) in comparison with that in the small intestine (30.3 mumol/mg protein/h). With regard to the maltitol hydrolyzing enzyme, Km and Vmax were 2.00 mM and 2.51 mumol/mg protein/h in the small intestine, respectively. Km and Vmax in the cecum and colon could not be measured because the level was too low. The tissue weights of the cecum and colon increased significantly in both the maltitol ($p < 0.01$, $p < 0.05$) and glucomannan ($p < 0.01$, $p < 0.05$) groups in comparison with that of

the control group. The specific activity of maltase decreased significantly in the small intestine of the maltitol ($p < 0.05$) and glucomannan ($p < 0.01$) groups. However, maltase activity in the cecum and colon was not lowered by maltitol ingestion, although it decreased significantly in the cecum of the glucomannan group ($p < 0.01$). Sucrase activity in the small intestine and cecum decreased significantly when maltitol ($p < 0.05$, $p < 0.01$) or glucomannan ($p < 0.01$, $p < 0.01$) were fed, whereas it did not decrease in the colon. Maltitol hydrolyzing activity did not decrease significantly in the small intestine of the maltitol group, although, according to the authors, the activity in the cecum and colon was not measured by exactly the same methods as used in the rest of the study [Oku, *et al.*, 1998 Vol 15 Tab 320].

Thus as reviewed above viscous dietary fibers, including chitosan, have been observed by many investigators to have an array of similar effects not only on the digestive process, but also on the digestive organs and the enzymes they secrete. These effects are both chemical and physical in nature, and they appear to be common among the viscous fibers now added to the human diet. The combined result of these physiological effects is to reduce in part the digestibility of the major nutrients, including fat. Again, however, the reduction in apparent fat digestibility has not been shown to exceed a few grams in humans, and this may be taken as beneficial to the entire well being of the consumer, as the positive effects of dietary fiber consumption have been shown by health authorities to far outweigh the loss of calories or nutrients [Vinik, *et al.*, 1988 Vol 16 Tab 362].

d. Perspective on the Use of Rats as Models for Human Digestion

Do the data from the animal studies reviewed above translate to human physiological responses? In actuality very little information has been available to allow a comparison of the extent of fiber digestibility between rat and man. Recently, however, a European research team has compared the digestibilities of common dietary fibers between human subjects and rats, when both species were

consuming a mixed diet [Bach-Knudsen, *et al.*, 1994 Vol 12 Tab 236 and Wisker, *et al.*, 1997 Vol 16 Tab 367]. Knudsen, *et al.*, recognized the need for appropriate animal models when studying questions in human nutrition, citing that under many experimental conditions and situations it is difficult or impossible to use human subjects. Furthermore, studies with humans are generally complicated, time-consuming and expensive. The rat has been the most frequently used animal model for studying digestibility in humans and much valuable information has been produced using the rat in these types of studies (Food and Agriculture Organization/World Health organization (FAO/WHO), 1991) [Bach-Knudsen, *et al.*, 1994 Vol 12 Tab 236]. The great value of the rat model has been the low cost associated with the relatively simple research facilities required. This has allowed the use of large numbers of animals and the rapid production of results.

Knudsen, *et al.*, 1994 stated that one major advantage is that the rat is omnivorous, thus allowing studies on a wide variety of foods. This has made it possible to test a nutrient source as the sole dietary component and avoid associated effects, which are inevitable, if the food is provided in a mixture [Bach-Knudsen, *et al.*, 1994 Vol 12 Tab 236]. The rat can thus, with high accuracy, predict how much of a nutrient in a food source is biologically available, and earlier data demonstrated good agreement between man and the rat (FAO/WHO, 1991). Since differences in the nutritive value of foods are, to a great extent, due to differences in digestibility, the rat has helped to bridge the gap between chemical measurements and costly biological experiments performed on humans. In light of this, a joint FAO/WHO expert committee recommended the use of the rat balance method for predicting protein digestibility in man (FAO/WHO, 1991) [Bach-Knudsen, *et al.*, 1994 Vol 12 Tab 236].

Additionally, because human balance studies are expensive and time consuming, such values are often measured in rats [Livesey *et al.*, 1990 Vol 14 Tab 302 and Livesey *et al.*, 1995 Vol 14 Tab 305]. Studies in rats are easier to perform, require less dietary material,

cost less, and the results can be obtained more rapidly than in humans.

In the past rat studies have allowed the investigation of fiber sources not yet approved for human diets. Nonetheless, Wisker, *et al.*, points out that knowledge of how well the information obtained using rats applies to humans is limited. The research team noted that for several isolated fiber sources, a similar extent of fiber digestibility was observed in humans and rats [Nyman *et al.*, 1986 Vol 14 Tab 314]. On the basis of these results, the rat was considered to be a suitable model for humans for the estimation of energy values of non-starch polysaccharide (NSP) isolates [British Nutrition Foundation 1990, in Livesey *et al.*, 1990 Vol 14 Tab 302 and Livesey *et al.*, 1995 Vol 14 Tab 305]. However, it was not established whether this was also the case with NSP contained in mixed diets.

In a study with young adults, growing pigs and Sprague-Dawley rats Forsum *et al.* (1982) measured the true protein digestibility of three different diets [Bach-Knudsen, *et al.*, 1994 Vol 12 Tab 236]. The agreement between man and the rat was extremely good while the values obtained with pigs was more variable.

Wisker, *et al.*, 1996c noted that when high-fat diets are used however, there are significant anatomical differences between humans and rats that may need to be taken into consideration [Wisker, *et al.*, 1996c Vol 15 Tab 332]. Rats have no gallbladder, cannot concentrate bile and have a nearly constant flow of diluted bile into the duodenum [Bjornhag, *et al.*, 1992 Vol 12 Tab 238]. Rats may therefore be unable to emulsify large meals of fat sufficiently, which potentially may lead to higher losses of fat and thereby energy to the feces [Wisker, *et al*, 1996c Vol 15 Tab 332].

e. Comparative Digestibilities of Fibers and Fat between Rats and Humans

To study potential species differences, Knudsen, *et al.*, compared the digestibility in man and the rat of energy, protein, fat, and non-starch polysaccharide in mixed diets with added dietary fiber (DF) from:

- **Fruits and Vegetables (Experiment 1)**
- **Citrus Fruits (Experiment 2) and,**
- **Barley (Experiment 3).**

The studies in humans took place in Kiel, Germany, while the rat studies were performed at Foulum, Denmark [Bach-Knudsen, *et al.*, 1994 Vol 12 Tab 236]. It is worth noting, when considering the results of these experiments (discussed below), that the rat diets tested by Deuchi *et al.*, typically contained 20% corn oil, while the mixed diets tested by Knudsen, *et al.*, typically contained from 13.1 to 19.5% total fat.

f. Results – Experiment 1: Diets Rich in Fruits and Vegetables

Importantly, the researchers found that in a diet low in DF digestible energy (DE) was the same for both rat and man, while with a diet high in DF dietary DE was significantly ($P < 0.01$) higher for man than for the rat (see Table 10 next page). However, apparent protein digestibility (ADP) did not differ between man and rat on either diet irrespective of the DF level. The researchers also observed a lower digestibility, although not significant, of total NSP in the rat than in man on the low DF diet, but the difference between man (0.734) and the rat (0.597) on the high DF diet was significantly greater. On the high DF diet all the constituent sugars with the exception of arabinose and galactose were degraded more completely in man than in the rat (See Table 11) [Knudsen, *et al.*, 1994 Vol 12 Tab 236].

000151

Table 10
Experiment 1: Apparent Digestibility of Energy, Protein, and Non-Starch Polysaccharides (NSP) in a Low-Fiber Control Diet and a Diet Rich in Fruits and Vegetables

(Means and standard deviations for eight humans and five rats per diet)

Diet	Low Fiber					High-Fiber				
	Humans		Rats		*P* value	Humans		Rats		*P* value
	Mean	SD	Mean	SD		Mean	SD	Mean	SD	
Energy	0.947	0.012	0.937	0.004	0.067	0.897	0.022	0.865	0.012	0.008
Protein	0.900	0.021	0.915	0.006	0.110	0.841	0.031	0.858	0.018	0.265
NSP										
Arabinose	0.844	0.029	0.850	0.020	0.646	0.811	0.033	0.792	0.042	0.367
Xylose	0.859	0.041	0.712	0.077	0.001	0.747	0.064	0.538	0.055	0.001
Mannose	0.781	0.174	0.745	0.059	0.636	0.773	0.156	0.592	0.062	0.021
Galactose	0.838	0.044	0.807	0.027	0.157	0.776	0.052	0.732	0.080	0.237
Glucose	0.658	0.257	0.527	0.069	0.249	0.592	0.199	0.382	0.080	0.032
Uronic acids	0.912	0.047	0.879	0.053	0.104	0.915	0.034	0.764	0.080	0.011
Total NSP	0.802	0.106	0.720	0.050	0.104	0.734	0.097	0.597	0.063	0.011

From Bach Knudsen, *et al.*, 1994

000151

g. Results - Experiment 2: Diets with Added Citrus Fruit DF

Following a diet containing DF from citrus fruit, DE was the same for both species when a low DF level was fed, but at a high level of DF intake, DE was significantly lower for the rat than for man with values of 0.906 in the rat compared with 0.931 in man ($P < 0.01$; Table 10). Again, there was no difference with regard to the digestibility of protein between the two species. Digestibility of fat with citrus fiber was not significantly different between man and the rat at the low DF intake level, but on the high DF intake level digestibility was 0.948 in man compared with 0.959 in the rat ($P < 0.01$). However, the digestibility of total NSP and its residues was consistently and significantly ($P < 0001$) lower in the rat than in man. For the low DF diet the digestibility of total NSP was 0.774 in man compared with only 0.501 in the rat. The biggest difference in the digestibility of the component sugars was seen for xylose and the smallest difference for arabinose [Bach-Knudsen, *et al.*, 1994 Vol 12 Tab 236].

Table 11
Experiment 2: Apparent Digestibility of Energy, Protein, and Non-Starch Polysaccharides (NSP) in a Low-Fiber Control Diet and a Diet Rich in Citrus Fiber

(Means and standard deviations for ten humans and six rats per diet)

Diet	Low Fiber					High-Fiber				
	Humans		Rats		*P* value	Humans		Rats		*P* value
	Mean	SD	Mean	SD		Mean	SD	Mean	SD	
Energy	0.941	0.015	0.933	0.010	0.251	0.931	0.012	0.906	0.013	0.002
Protein (N x 6.25)	0.891	0.029	0.909	0.009	0.173	0.873	0.024	0.886	0.018	0.265
Fat	0.954	0.012	0.958	0.007	0.500	0.948	0.009	0.959	0.003	0.009
NSP										
Arabinose	0.694	0.071	0.579	0.069	0.007	0.860	0.030	0.714	0.136	0.005
Xylose	0.762	0.066	0.403	0.090	0.001	0.794	0.054	0.499	0.127	0.001
Mannose	0.858	0.046	0.577	0.115	0.001	0.892	0.030	0.459	0.109	0.001
Galactose	0.770	0.046	0.537	0.104	0.001	0.836	0.033	0.601	0.134	0.001
Glucose	0.723	0.124	0.483	0.142	0.021	0.834	0.055	0.251	0.152	0.001
Uronic acids	0.908	0.027	0.627	0.130	0.001	0.955	0.013	0.640	0.138	0.001
Total NSP	0.774	0.063	0.501	0.106	0.001	0.885	0.026	0.517	0.125	0.001

From Bach Knudsen, *et al.*, 1994

000153

h. Fecal Bulking Capacity in the Rat and Man

The fecal dry weight increment due to DF was higher in the rat than in man for all the DF sources except when the barley DF was fed at the low protein level. On a barley DF-high protein diet there was a higher fecal protein excretion in the rat than in man, whereas on a barley DF-low protein diet and the citrus DF diet fecal protein losses were lower in the rat than in man. On the fruit and vegetable diet there were no differences in the increment in fecal protein due to the ingestion of DF. The increase in fecal fat in response to adding fiber was about the same in the two species for the particular fibers tested.

In comparison with fecal protein, losses of fat were measurable but small both in man and in the rat and appeared to depend mainly on the amount and sometimes also on the type of fat ingested [Bach-Knudsen, *et al.*, 1994 Vol 12 Tab 236]. An increased fat intake caused higher fecal fat excretion in both species, but when citrus fiber, barley fiber, or fiber from fruits and vegetables was consumed, the digestibility of dietary fat was not dependent on the DF content of the diet [Walker *et al.* 1973, Vol 16 Tab 364; Apgar *et al.* 1987, Vol 12 Tab 231]. However, according to Knudsen, *et al.*, non-starch polysaccharides, which form viscous solutions, as well as some other DF, can cause higher fecal lipid losses both in man and in the rat and thereby a decrease in fat digestibility [Southgate & Durnin, 1970, Vol 16 Tab 349; Mokady, 1973, Vol 14 Tab 310; Prynne & Southgate, 1979, Vol 15 Tab 326]. In contrast to protein digestibility, the influence of DF on apparent fat digestibility appeared unrelated to fiber fermentation, but instead was found to be related to an interference with the digestion and absorption of dietary fat in the small intestine [Chen, *et al.*, 1987, Vol 12 Tab 244; and Bach-Knudsen, *et al.*, 1994 Vol 12 Tab 236]. This observation confirmed the findings of Isaksson *et al.* and others discussed above.

i. Factors Influencing the Differences in Fiber and Fat Digestibilities between Rats and Humans

Bach-Knudsen, *et al.*, 1994, Vol 12 Tab 236; and Wisker, *et al.*, 1996, Vol 16 Tabs 365, 366, explained that the lower digestibility of NSP residues in rats as compared to humans can be related to differences in gut physiology and consequently transit-time (TT). The rat is mainly a cecum fermenter, which is in contrast to man where the fermentative capacity of the cecum is insignificant compared with that of the colon. It is also well established that the rat, like other small animals, needs more energy per unit body-weight, which will inevitably influence the TT through the large bowel, thus leaving less time for microbial fermentation of NSP residues (Van Soest *et al.* 1982, Vol 16 Tab 363) [Bach-Knudsen, *et al.*, 1994 Vol 12 Tab 236]. Mean transit time (MTT) through the whole gut for the human subjects given the low DF control diets in the Knudsen study was approximately 58-76 hours; comparable to that found in other studies using typical western-style diets (Cummings *et al.* 1976; Spiller *et al.* 1986; Stevens *et al.* 1987). This figure may decrease to 38-59 hours when the DF intake reaches 35-50 g/d (Cummings *et al.* 1976; Spiller *et al.* 1986; Stevens *et al.* 1987) [Bach-Knudsen, *et al.*, 1994 Vol 12 Tab 236]. Most current data indicate that MTT in the rat is lower; 32-52 hours at a DF intake of 0.4-0.5 g/d (Raczynski *et al*, 1982; Bach Knudsen *et al.* 1991; Hansen *et al.* 1992) and 22-32 hours at a DF intake of 1.5-1.7 g/d (Raczynski *et al*, 1982; Bach Knudsen *et al.* 1991; Hansen *et al.* 1992).

The DF source in Experiment 2 was citrus, which has a high pectin content, whereas in Experiment 3 insoluble barley DF in the form of cellulose and hemicellulose was used to increase the DF level. *In vitro* and *in vivo* fermentation studies have shown that isolated pectin is an easily degradable DF source for the microflora (Nyman & Asp, 1982) while insoluble barley DF in the

form of cellulose and hemicellulose is far less degradable (Bach Knudsen *et al.* 1991).

Species differences in level and composition of the microflora between man and the rat may be another factor that may influence the digestibility of NSP residues. The responses of the individual species of the gut flora in different animal species to changes in nutrient supply are largely unknown, primarily because of the complexity of this ecosystem. The microbial population that inhabits the human colon is a large (about 10^{11} viable counts /g feces) and a highly complex community comprising 400-500 bacterial species (Macfarlane & Cummings, 1991). The bacterial population in the cecum and colon of the rat is not so well described but it would probably reach the same level as in man (about 10^{11} viable counts / g feces; B. O. Eggum, B.B. Jensen and K.E. Bach Knudsen, unpublished results). However, at the species level, the flora of the rat is probably different to that of man. According to Moore *et al.* (1987) there is now increasing evidence that each animal species including man carries a flora distinct from that found in other animals. The authors believed that this is a result of natural selection, which provides protection for different animals. The fewer the bacterial species that different animals share, the less the likelihood of disease transmission between animal species [Bach-Knudsen, *et al.*, 1994 Vol 12 Tab 236].

Nutritional factors that are known to influence large bowel fermentation are the type and amount of residues passing into the large bowel. It has been shown in both man and the rat that compared to other nutrient sources; dietary fiber elicits a more extensive microbial fermentation in the colon, followed by a higher excretion of microbial fecal N (Mason & Palmer, 1973; Stephen & Cummings, 1980). In good agreement with these findings the authors found the lowest apparent digestibility of protein in man, who also was able to digest NSP to the greatest extent.

The authors explained that in man (Stephen & Cummings, 1980), but probably also in the rat; most of the fat found in the feces is due to the bacterial mass. In studies with rumen bacteria it has been shown that the lipid content of bacteria showed species differences and that it was also dependent on the amount of fat fed (Czerkawski, 1976). If the same is true for bacteria of the large intestine, the fat content of the colonic microflora may depend on the amount of fat remaining unabsorbed from the small intestine. Therefore, unlike fecal protein, the amount of fat found in the feces will not correlate with the fermentation of DF and the amount of bacteria resulting from this process. The authors stated that this was in accordance with their results on the fibers tested, as they found only small differences in apparent digestibility of dietary fat between man and the rat despite the greater extent of DF fermentation in man (See Table 11) [Bach-Knudsen, *et al.*, 1994 Vol 12 Tab 236].

The research team reported being somewhat disconcerted to find that for several of the diets the rats digested NSP residues much less well than man. This has a direct effect on the bulking capacity and apparent digestibility of protein, both of which are higher in the rat than in man, and on the amount of digestible energy. The greatest agreement between man and the rat was found for the digestibility of fat with the fibers tested. The results of the study, according to the authors, favored the view of Van Soest *et al.* (1982), that larger animals such as man and the pig digest polysaccharides to a greater extent than smaller animals such as the rat. The results suggested that absolute digestibility values have to be estimated on the species in question. It is, however, noticeable that the rank of the different diets followed the same order for rat and man. It should also be stressed that in this study, together with most of the literature cited, there is very good agreement between man and the rat when considering energy, protein and fat digestibility. The similar capacity of man and rat to digest protein supports the recommendation made by FAO/WHO

(1991) that the rat may be used for predicting true protein digestibility in man [Bach-Knudsen, *et al.*, 1994 Vol 12 Tab 236].

j. Energy Values Derived from Non-Starch Polysaccharides: Comparative Studies in Humans and Rats

In a later study, the same research team investigated the energy values that could be derived from non-starch polysaccharides by humans and rats [Wisker, *et al.,* 1996c Vol 15 Tab 332]. The authors stated that an increase in dietary fiber intake is associated with a concomitant loss of energy to feces. Besides the higher energy loss caused by unfermented dietary fiber, an increased intake of dietary fiber is associated with additional losses of energy in the form of protein and fat derived mainly from bacterial matter. Livesey, (1990) calculated from published human balance studies that, on average, 30% of the energy of fermented carbohydrate in mixed human diets is converted to bacterial energy and lost to the feces [Wisker, *et al.*, 1996c Vol 15 Tab 332].

In agreement with the concept of Kleiber (1975), the energy values given for NSP are actually partial digestible energy values. These are less than apparent digestible energy values because of the effect of dietary fiber on fecal protein and fat losses. However, for some fiber sources, especially whole grain cereal fibers, partial digestible energy values were lower than expected based on the extent of NSP fermentation (Livesey 1990, Wisker *et al.* 1988 and 1996b) [Wisker, *et al.,* 1996c Vol 15 Tab 332].

As stated above, this group had found that the apparent digestibility of NSP in mixed diets was lower in rats than in humans [Wisker, *et al.,* 1996c Vol 15 Tab 332]. Therefore, the aim of this later study was to compare the energy values in humans and rats of various NSP sources contained in high fiber diets. The NSP sources studied were a mixture of fruits and vegetables, a citrus fiber concentrate, a barley fiber concentrate (insoluble fiber)

at two levels of protein intake, and coarse and fine whole meal rye bread.

k. Human Experiments Conducted by Wisker, *et al.*, 1996

Thirty-seven healthy, free-living female students 22-31 years old took part in three experiments of 21 days each. Each experimental period was separated by three weeks. During the experimental periods, the subjects consumed a controlled diet that maintained their weight in a range of ± 1.0 kg. The studies were approved by the Ethics Committee of the Medical Faculty of the University of Kiel. Normal energy intake of each subject was calculated from a 7-d prestudy record using German food tables (Deutsche Forshungsanstalt fur Lebensmittelcemie 1986 and 1990) [Wisker, *et al.*, 1996c Vol 15 Tab 332]. Groups of five or six Wistar male rats were fed the same mixed diets as the human subjects. A total of 10 experimental diets were investigated during the course of four studies. Additional fiber intake ranged from 14.1 grams per day for barley fiber to 31.5 grams per day for fruits and vegetables.

The results showed that fecal dry matter excretion after intake of NSP from fruits and vegetables, the citrus fiber concentrate and the barley fiber concentrate at high protein intake was significantly higher in rats than in humans, whereas it was similar in both species after intake of NSP from barley fiber at low protein intake and from coarse and fine whole meal rye bread (Table 12) [Wisker, *et al.*, 1996c Vol 15 Tab 332].

Partial digestible energy values of NSP calculated from fermentation and from digestible energy balances are given in Table 13. In the human subjects, partial digestible energy values of NSP calculated from fermentation were 4.4 to 11.4 kJ/g NSP. Consistent with the lower values in rats than in humans for fermentation of NSP from fruits and vegetables, citrus fiber concentrate, and partial digestible energy values of these NSP

were significantly lower in rats than in humans [Wisker, *et al.*, 1996c Vol 15 Tab 332].

The researchers explained that when fermentability and energy values of NSP are studied in rats, semipurified behfl diets are used as control diets, and these typically are fiber-free. When test fibers are added to these diets, the fiber is either added at the expense of available carbohydrates, or is added on top of the available carbohydrates. Therefore, these diets contain only one fiber, whose fermentation can easily be calculated from intake and excretion values. However, this procedure does not reflect common dietary practices in humans: few people consume fiber-free diets, and different fiber sources are present in the diet. In addition, diets with very low levels of dietary fiber seemed to be rather effective in causing additional energy losses in the form of protein and fat, resulting in comparatively low energy values. However, above a daily intake of about 20g/d of dietary fiber from natural food sources in mixed diets, the energy value of most dietary fibers depended only on their fermentability (Livesey 1990). Therefore it has been recommended that energy values of fiber supplements should be determined by adding them to diets that already contain quantities of unavailable carbohydrates of about 20g/d (British Nutrition Foundation 1990, Livesey 1990 and 1991b). In these investigations, NSP intake of humans during the consumption of the low fiber control diets (i.e., behfl diets) was in this range (16.7 to 20.2 g/d) and thus was consistent with Livesey's (1990 and 1991b) proposals. Accordingly, the experimental diets used in the Wisker studies differed from those used in other studies in rats. The low fiber control diets contained 37 to 50 g NSP/kg diet dry matter compared with 69 to 120 g in the high fiber diets [Wisker, *et al.*, 1996c Vol 15 Tab 332].

Key and Mathers (1993b) calculated by multiple linear regression whether beans as additional fiber sources for rats altered the fermentation of NSP from whole meal bread in the behfl diet. The proportion of whole meal bread was kept constant whereas the

contribution of beans increased from 0 to 450 g/kg at the expense of sucrose and casein, leading to an increase in the dietary NSP content of from 27.9 to 96.6 g/kg. They found that the presence of additional bean NSP had no effect on the fermentation of NSP in bread. A similar study in pigs also showed that additional pea NSP did not affect the fermentation of wheat NSP contained in a behfl diet [Goodlad, *et al.*, 1991 Vol 13 Tab 271 and Wisker, *et al.*, 1996c Vol 15 Tab 332].

The main factor influencing the availability of the energy provided by fiber is fermentability. The additional NSP consumed during the high fiber diet periods was in most cases less fermented in rats than in the human subjects with the exception of NSP from coarse whole meal rye bread, which was degraded to a somewhat greater extent in rats. These results are consistent with the author's pervious findings that the apparent digestibility of total NSP in nine of the 10 experimental diets was lower in rats than in humans [Bach Knudsen *et al.* 1994, Vol 12 Tab 236 and Wisker *et al.*, 1996c Vol 15 Tab 330]. In these studies, differences between species were greater with the high fiber diets than with low fiber diets, with the exception of the high fiber diet containing coarse whole meal rye bread. In addition to probable species differences in the capacity of the microflora to ferment certain fiber sources in the large intestine, the lower digestibility of the additional dietary NSP in rats compared with humans may have been due to species differences in colonic transit time, which influences the degree of fermentation [Bach-Knudsen *et al.* 1994, Vol 12 Tab 236 and Van Soest *et al.* 1982 Vol 16 Tab 361]. Several studies have shown that transit times are shorter in rats than in humans, leaving less time for bacterial degradation of NSP in rats [Cummings *et al.* 1976, Vol 12 Tab 248 Raczynski *et al.* 1982 Vol 15 Tab 327]. Although in both species transit time can be shortened by an elevated fiber intake, the magnitude of this effect may differ between species, with consequences for the extent of fermentation [Wisker, *et al.*, 1996c, Vol 15 Tab 332 and

Cummings *et al.* 1976, Vol 12 Tab 248 and Raczynski *et al.* 1982 Vol 15 Tab 327].

Section IV Detailed Summary of the Basis for the Determination that Primex Chitosan is GRAS

Table 12: Increase in Fecal Dry Matter Due to the Consumption of Non-Starch Polysaccharides (NSP) in Humans and Rats[1]

Study	Fiber Source	Humans	Rats	P value
		g/g additional NSP		
1	Fruits and vegetables	0.7 ± 0.2 (8)	1.1 ± 0.2 (5)	0.007
2	Citrus fiber	0.3 ± 0.2 (10)	0.8 ± 0.4 (6)	0.009
3	Barley fiber			
	High protein intake	0.9 ± 0.3 (12)	1.5 ± 0.4 (6)	0.003
	Low protein intake	1.1 ± 0.3 (12)	1.2 ± 0.4 (6)	0.47
4	Whole meal rye bread			
	Coarse	1.1 ± 0.3 (7)	0.8 ± 0.2 (5)	0.086
	Fine	1.0 ± 0.1 (7)	0.9 ± 0.3 (6)	0.534

[1] Values are mean ± SD, with *n* in parentheses.

Table 13: Net Energy Values of Non-Starch Polysaccharides (NSP) Calculated from NSP Fermentability and From Digestible Energy Balances in Humans And Rats[1]

Study	Fiber Source	Humans	Rats	P value	Humans	Rats	P value
		kJ/g NSP ingested			kJ/g NSP ingested		
1	Fruits and vegetables	6.0 ± 0.09 (8)	4.1 ± 0 9 (5)	0.0011	0.7 ± 0.2 (8)	1.1 ± 0.2 (5)	0.007
2	Citrus fiber	8.3 ± 0.5 (10)	4.2 ± 2.3 (6)	0.0001	0.3 ± 0.2 (10)	0.8 ± 0.4 (6)	0.009
3	Barley fiber						
	High protein intake	3.6 ± 1.6 (12)	1.6 ± 2.4 (6)	0.045	0.9 ± 0.3 (12)	1.5 ± 0 4 (6)	0.003
	Low protein intake	3.2 ± 1.3 (12)	1.8 ± 1.5 (6)	0.056	1.1 ± 0.3 (12)	1.2 ± 0.4 (6)	0.47
4	Whole meal rye bread						
	Coarse	4.9 ± 1.1 (7)	5.5 ± 1.0 (5)	0.301	1 1 ± 0.3 (7)	0.8 ± 0.2 (5)	0.086
	Fine	5.2 ± 0.4 (7)	4.5 ± 1.2 (6)	0.161	1.0 ± 0.1 (7)	0.9 ± 0.3 (6)	0.534

[1] Values are mean ± SD, with *n* in parentheses.

According to the authors, losses between 20% and 40% have been found in rats fed different sources of unavailable carbohydrates [Livesey *et al.* 1990 Vol 14 Tab 302 and Davies *et al.* 1991 Vol 12 Tab 250]. An average conversion of fermented energy to fecal energy

of 0.3 was regarded as suitable for rats as well as humans [Livesey, *et al.*, 1991a Vol 14 Tab 303 and Wisker, *et al.*, 1996c Vol 15 Tab 332].

With the exception of the value for citrus fiber in humans, partial digestible energy values calculated from energy balances were lower than those estimated from NSP fermentability in both humans and rats. In humans these differences were greatest for coarse whole meal bread. However, for NSP from fruits and vegetables, citrus fiber, and barley fiber at high or low protein intake, the differences between these energy values where higher in rats than in humans. The investigators stated that this finding was consistent with the greater increase in fecal dry matter losses in rats than in humans. The increase in fecal dry matter losses was due in large part to a higher excretion of unfermented dietary fiber and of residues (mostly Klason lignin) in rats than in humans [Bach-Knudsen *et al.* 1994, Vol 12 Tab 236]. The species differences in fecal dry matter excretion were most likely related to shorter transit times in rats. Shorter transit times lead to a lower degree of NSP fermentation (Van Soest *et al.* 1982) and also result in a higher yield of microbial cells per gram of fermented carbohydrate because of a diminished requirement of bacteria for maintenance energy (Cummings 1984) [Wisker, *et al.*,1996c Vol 15 Tab 332].

With respect to the results obtained in rats, the study findings differed from those of [Livesey *et al.*, 1995 Vol 14 Tab 305]. These authors found a good agreement in rats in energy values calculated either from NSP fermentation or from conventional digestible energy balances when several starch-free or low starch fiber supplements were added to fiber-free semipurified diets. In the studies describe above both rats and humans consumed additional fiber along with a mixed diet, which already contained about 20 g/d of dietary fiber.

With regard to nutrient types, the investigators found that calculated per gram of additional NSP ingested, fecal protein losses tended to be higher in humans than in rats, except for

barley fiber at high protein intake. This finding was thought to be consistent with the higher fermentation of NSP in humans than in rats [Bach-Knudsen *et al.* 1994, Vol 12 Tab 236, Wisker *et al.,* 1996a, Vol 15 Tab 330 and Wisker *et al.,* 1996c Vol 15 Tab 332] Further, for the fibers studies fecal losses of fat were very similar in humans and rats as calculated per gram of additional NSP (Bach Knudsen *et al.* 1994, Wisker *et al.* 1996a). In addition, there seems to be no effect of fat content of rat diets on NSP digestibility. The authors concluded that the rat is not always a suitable model for the prediction of energy values of NSP in mixed diets consumed by humans.

I. Relationship of these Studies to Regulatory Concerns about the Effect of Chitosan Consumption by Humans

The information presented in this section has raised several issues that are important to the consideration of the use of chitosan for human consumption. Various regulatory agencies have expressed concern that the increase in fecal fat observed in rats consuming high fat-high-chitosan diets might translate to reduced nutritional status in humans due to the loss of fat-soluble vitamins and certain minerals [Complementary Medicines Evaluation Committee, 1998 Vol 12 Tab 245]. Yet human feeding studies in which chitosan was fed at up to 5.25 grams per day with or without a high-fat diet have failed to show significant loss of fat through the feces [Schiller, *et al.,* 2001 Vol 15 Tab 337 and Gades, *et al.*, 2002 Vol 13 Tab 266]. To date this discrepancy in fecal fat loss between species has remained largely unexplained.

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The studies discussed above do shed some light on this matter, however. Using study protocols in which both rats and humans consumed the same mixed diets Knudsen, *et al.*, 1994 and Wisker, *et al.*, 1996 have established that fecal dry weight due to NSP was higher in the rat than in man for all the NSP sources tested, except when insoluble dietary fiber from barley was fed along with a low protein diet. When additional fiber from fruit and vegetable sources were added to the diets, no differences in fecal

protein were observed between the species due to the ingestion of dietary fiber. The increase in fecal fat in response to adding fiber was about the same in the two species [Bach-Knudsen, *et al.*, 1994 Vol 12 Tab 236 and Wisker, *et al.,* 1996a Vol 15 Tab 330].

In comparison with fecal protein, losses of fat are small both in man and in the rat and appear to depend mainly on the amount and sometimes also on the type of fat ingested [Bach-Knudsen, *et al.*, 1994 Vol 12 Tab 236]. An increased fat intake caused higher fecal fat excretion in both species. However, according to Knudsen, *et al.*, non-starch polysaccharides, which form viscous solutions, such as chitosan, can cause higher fecal lipid losses both in man and in the rat and therefore a decrease in fat digestibility. This was shown to be the case for many viscous polysaccharides in the review of the effect of dietary fiber on fecal composition by Cummings [Cummings, 2001 Vol 12 Tab 246].

In contrast to protein digestibility, the influence of dietary fiber on apparent fat digestibility was said to be related to an interference with the digestion and absorption of dietary fat in the small intestine [Bach-Knudsen, *et al*, 1994, Vol 12 Tab 236]. Knudsen, *et al.*, 1994 and Wisker, *et al.*, 1996 explained that in man and most probably also in the rat, most of the fecal fat is due to the bacterial mass.

The lower digestibility of NSP residues in rat than in man can be related to differences in gut physiology and consequently transit-time (TT). The researchers explained that the rat is mainly a cecum fermenter, in contrast to man where the fermentative capacity of the cecum is insignificant compared with that of the colon. It is also well established that the rat, like other small animals, needs more energy per unit body-weight, which will inevitably influence the TT through the large bowel, thus leaving less time for microbial fermentation of NSP residues [Bach-Knudsen, *et al.*, 1994 Vol 12 Tab 236]. The investigators stated

that even when both species were fed high-fiber diets, the rat moves ingesta through the gut in 54-58% of the time required by humans. These types of results suggested that absolute digestibility values have to be estimated on the species in question [Bach-Knudsen, *et al.*, 1994 Vol 12 Tab 236; and Wisker, *et al.,* 1996a Vol 15 Tab 330].

Species differences in level and composition of the microflora between man and the rat may be another factor that may influence the digestibility of NSP residues. It has been shown in both man and the rat that compared to other nutrient sources, dietary fiber elicits a more extensive microbial fermentation in the colon, followed by a higher excretion of microbial fecal N. In good agreement with these findings the authors found the lowest apparent digestibility of protein in man, who also was able to digest NSP to the greatest extent [Bach-Knudsen, *et al.*, 1994 Vol 12 Tab 236].

Several reasonable conclusions can be drawn from this body of work. First, rats are less likely than humans to be able to digest viscous polysaccharides, like chitosan, which are known to interfere with fat digestion. Additionally, rats will be further hindered from emulsifying large quantities of fat in the diet, because rats have no gall bladder. Therefore, the feeding of large quantities of fat along with viscous polysaccharides puts the rat at a distinct disadvantage in comparison to humans, making data from rat studies of questionable value, when predicting the nutritional effects of a high-fat-chitosan containing diet in humans.

Second, humans have a greater ability to digest polysaccharides than do rats, because of more microbial capacity in the colon, and longer transit times. In one study in which a radiolabelled chitosan coating was tracked in a human volunteer, only 11.3% of the radioactive activity remained once the chitosan had reached the transverse colon [Macleod, *et al.,* 1999 Vol 6 Tab 97]. Thirdly, humans have a greater ability to emulsify fat than do rats,

because they have the advantage of bile acid secretions from the gall bladder.

Taken together the data and information discussed in this section would seem to explain why humans have not experienced excessive fat excretion following consumption of chitosan. As a species humans are better equipped to emulsify fats in the diet, and to ferment polysaccharides than are rats. Thus, again, it would seem that when polysaccharides are added to a mixed diet, data from human studies should be used to determine the physiological effects of such substances. Table 14 below summarizes recently published studies, which show that humans consuming a chitosan of comparative viscosity to that produced by Primex, lost very little fat in their feces. In fact fecal fat content was similar to that reported for other fibers [Cummings, 2001Vol 12 Tab 242]. Gades and Stern, 2003 concluded that the effect of chitosan on fat absorption was clinically negligible [Gades, *et al.,* 2003 Vol 13 Tab 267].

Section IV Detailed Summary of the Basis for the Determination that Primex Chitosan is GRAS

Table 14: Fecal Fat Loss in Humans

Guerciolini, *et al.*, 2001 Randomized open label Two-period design 12 healthy subjects for 7 days 2.67g chitosan/day 2,500 kcal/d with 30% fat Standardized meals	Fecal Fat Results Baseline 1.36 g/d (0.67-2.03) With chitosan 0.27 g/d (-1.49-2.07)
Schiller, *et al.*, 2001 Randomized double-blind -Placebo controlled 59 mildly obese subjects for 8 weeks 2.7g chitosan/day 2,086 kcal/d aver. 76 g fat aver. Normal meals 1.0 kg weight loss Conclusion: 1.0 kg weight loss = ~ 7,700 kcal Total fecal fat loss over the period = 3008.9 kcal (~53 kcal/d) Fecal fat accounted for 39% of weight loss	**Fecal Fat Results** Fecal fat measured on 4 test subjects and 3 placebo subjects **g/day Test Placebo** Baseline 2.93 5.97 (Mean) 8-Weeks Fecal fat increased by 5.97g/d in test subjects Fecal fat of placebo group dropped 2.34g/d Total fecal fat for placebo group = 4.5% Total fecal fat for chitosan group = 11.7%
Gades and Stern, 2002 7 healthy males consumed 135g fat per day for 12 d 5.25 g chitosan was taken on days 6-9	**Fecal Fat Results** Fecal fat did not vary between the two periods 6.9 g/d with no chitosan 6.8 g/d with chitosan Fecal fat was 5.1% of baseline fat consumption
Gades and Stern, 2003 15 males consumed 4.5 g chitosan/d for a 4 days supplement period during a 12 d test Subjects consumed 133 +/- 23 g of fat/d	Fecal fat increased by 1.1 +/- 1.8 g/d (p = 0.02), from 6.1 +/- 1.2 to 7.2 +/- 1.8 g/d.

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m. Effect of chitosan on dietary lipid absorption in the rat

The discussions above have established that fecal fat data from the rat cannot be extrapolated to model the effects of viscous polysaccharide consumption by humans. Researchers have established that rats do not digest fat in the same manner as humans, because their transit time is shorter, and they have no gall bladder to dispense bile acids, hence, they cannot emulsify fats, nor can they digest fibers to the same degree. However, study conclusions have differed, even when the rat has been used as an experimental animal. Appendix II contains a comparison of two studies in which rats were fed chitosan at the 5% level and above. Deuchi *et al.,* (1995) concluded that chitosan fed at the 5% level reduced the absorption of certain elements, while Gordon and Williford (publishing about a decade earlier) concluded that at dietary levels below 5%, neither chitin not chitosan appeared to compromise element absorption [Gordon, *et al.*, 1984 Vol 4 Tab 46]. Appendix II also contains a review of a study by Lian, *et al.*, 1998, in which the group presented data indicating that the effect of chitosan on fat digestibility by the rat was dependent on its viscosity [Lian, *et al.*, 1998 Vol 14 Tab 300].

It appears that the differing results among the research teams may have been due to physical differences in the chitosan being studied. Deuchi *et al.,* (1995) have shown that as the *in vitro* viscosity of chitosan preparations having comparable degrees of deacetylation increased, the apparent fat digestibility by rats fed a high fat diet decreased. While the apparent fat digestibility in the presence of chitosans with viscosities of 5 -10 cP was 70%, the fat digestibility in the presence of chitosan with viscosities of 450-500 cP was 50%. It is important for the reader to note, that these viscosity values were measured in a 0.5% solution. They correspond to approximately 50 cPS and 4,000 cPs, when measured in a 1.0% solution as specified by Primex. The material

used in the Gordon and Williford study was only characterized by particle size (0.50 – 0.25 mm), and therefore, may have been a more crude preparation with lower viscosity.

The Deuchi results were unexpected for two reasons, according to the authors. First, as mentioned above, chitosan precipitates in the small intestine at neutral pH, loosing its gastric viscosity near absorptive sites. Second, Kanauchi *et al.*, (1995) attributed the enhancing effects of ascorbic acid on the reduction of fat absorption by chitosan to the reduction of its gastric viscosity [Kanauchi, *et al.*, 1995 Vol 5 Tab 70]. It is possible, however, that the precipitation of chitosan in the small intestine (duodenum) of rats due to a gradual increase in pH is not necessarily spontaneous but rather gradual, and that chitosan remains viscous in the initial segments of the duodenum [Furda, 2000 Vol 4 Tab 40]. This apparently affects the fat absorption mechanism in rats more than cholesterol absorption.

While the intestinal cholesterol absorption in lymph-cannulated rats was reduced by low viscosity chitosan hydrolyzates as well as by viscous chitosan, the absorption of oleic acid and triglycerides was also reduced by viscous chitosan, but only delayed by chitosan hydrolyzates [Vahouny *et al.*, 1983 Vol 11 Tab 212 and Ikeda *et al.*, 1989 Vol 5 Tab 59]. This again indicated that effective reduction of lipid absorption by chitosan, unlike reduction of cholesterol absorption, might require higher initial viscosity in the small intestine, particularly in the duodenum [Furda, 2000 Vol 4 Tab 40]. This type of behavior would then be species specific, and dependent on transit time, the degree of fat emulsification, and the level of enzyme secretion.

The studies and data reviewed above support the view that all viscous polysaccharides impair the digestion of fat by a number of mechanisms, ranging from physically hindering the absorption process, to interfering with digestive enzyme activity. However, increases in fecal fat in humans as a consequence of dietary fiber

consumption, amounted to only 12.5% of total fat intake (9.0 grams [Reported by Cummings for psyllium]/72.0 total fat intake [Reported by the CSFII, 1996]) at the highest level found in this review. In persons consuming a Western diet, this has not been considered a level high enough to threaten fat soluble vitamin status [Complementary Medicines Evaluation Committee (6), 1998 Vol 12 Tab 245 and Dr. George C. Fahey, Jr. Expert Opinion, Appendix III]. Fecal fat loss in humans consuming chitosan at up to 5.25 grams per day is in fact lower than that reported for many other viscous polysaccharides [Cummings, 2001 Vol 12 Tab 246] [See Table 14]. Therefore, there is no reason to suppose that chitosan consumption would pose any more of a threat to fat soluble vitamin or mineral status than any other viscous polysaccharide.

Question 3

3. **Question 3. Would mineral status be threatened, especially with regards to calcium?**

Chitosan, when fed in large quantity to rats, has been reported to reduce the absorption of calcium [Deuchi, *et al.,* 1995 Vol 4 Tab 29 and Wada, *et al.,* 1997 Vol 11 Tab 217]. The mechanism by which dietary fibers impede the absorption of certain minerals, has been studied previously [Oku, *et al.,* 1982 Vol 15 Tab 319]. Oku, *et al.,* measured calcium transport in the everted duodenal sac of rats fed 10 or 20% cellulose, glucomannan, or pullulan for 7 or 8 weeks. The absorption of calcium was not effected by the cellulose, but decreased significantly in the 20% glucomannan group. Further, calcium binding activity was significantly reduced in all groups fed unavailable carbohydrates. Interestingly, the activities of certain enzymes, such as, sucrase and alkaline phosphatase were also considerably diminished with the intake of these fibers. These results indicated that the inhibitory effect of unavailable carbohydrates on intestinal calcium absorption is partially due to the loss of calcium binding protein caused by large amounts of undigested substances transiting through the gastrointestinal tract.

During their discussion, the authors pointed to both the positive and negative effects of dietary fiber consumption. They stated that the beneficial effects of unavailable carbohydrates in the diet have received much attention, in part because fibers help to reduce the absorption of harmful substances associated with diseases such as colon cancer, diabetes, atherosclerosis and diverticular disease [Oku, *et al.,* 1982 Vol 15 Tab 319]. However, the authors explained that similar mechanisms might affect the absorption of desirable nutrients. While this effect was cited as detrimental for malnourished children in developing countries or patients with nutritional disorders, the authors felt that it might be beneficial for people living on refined foods. Several authors have shown that both the positive and negative effects of fiber consumption may be accentuated by viscous polysaccharides, such as guar gum, xanthan gum, pectin, or

glucomannan [Ikegami, *et al.,* 1990 Vol 13 Tab 280]. These studies have largely been conducted in test animals consuming a large portion of their diet as indigestible substances, and in the case of chitosan, with substances that were of high viscosity in comparison to the food grade specifications of Primex (55 versus ~2000 cPS) [Primex, 2002 unpublished data, Deuchi, *et al.*, 1995 Vol 4 Tab 29 and Wada, *et al.*, 1997 Vol 11 Tab 217].

Several studies have measured the effect of chitosan consumption on the mineral nutritional status of humans [Wuolijoki, *et al.,* 1999 Vol 11 Tab 221 and Colombo, *et al.*, 1996 Vol 3 Tab 24]. No adverse effect in humans has been noted, when the test subjects were consuming chitosan at the gram level. This may be due to the fact that the humans were consuming a more moderate amount of chitosan in comparison to their body weight than were the rats used in other studies, and it is also very likely due to the physiological differences between rats and humans. Humans have a much larger capacity to ferment dietary fibers in the colon, than do rats in the cecum. Therefore, at the levels of intake proposed, there is no evidence to support that the consumption of chitosan would result in negative mineral status in humans.

A study by Veneroni, *et al.,* 1996 showed that 40 subjects consuming 4 tablets (1600 mg) of chitosan divided into 2 doses along with a restricted (1000 calorie) diet for 4 weeks experienced no loss in calcium, sodium, potassium, or iron status (See Table 15 below) [Veneroni, *et al.,* 1996 Vol 11 Tab 214].

Likewise, Colombo, *et al.*, 1996 found that 43 obese subjects, ages 30-69 (Mean 48.3 ± 12.5) consuming 4 tablets per day (1600 mg) in 2 doses for 4 weeks along with a low calorie diet, showed no change in mineral status [Colombo, *et al.*, 1996 Vol 3 Tab 24] (See Table 16 below].

Guistina, *et al.,* 1995 and Girola, *et al.,* 1996 described similar findings [Girola, *et al.,* 1996 Vol 4 Tab 42 and Guistina, *et al.,* 1995

Vol 4 Tab 43]. In the Guistina study 50 obese subjects, ages 26-65 consumed 4 tablets per day of chitosan for 4 weeks (1,600 mg), while maintaining a low (1100) calorie diet. Table 17 shows that calcium levels for both the placebo group and the test group varied between 9.09 and 9.28 mg/dL, and that there was no significant difference between the groups.

Table 18 shows that similar results were observed by Girola, *et al.,* no matter whether the subjects were consuming 1 or 2 chitosan caplets per day [Girola, *et al.,* 1996 Vol 4 Tab 42]. The chitosan used in the studies depicted in Tables 15 -18 is described in the specifications shown in Table 19. Note that the viscosity falls within the range of that described for Primex chitosan.

Section IV Detailed Summary of the Basis for the Determination that Primex Chitosan is GRAS

Table 15
Tolerability parameters modifications during the two treatments
[Veneroni, *et al.*, 1996 Vol 11 Tab 214]

Treatment	Parameter	Baseline	After 4 Weeks	Statistics*
Diet + Placebo	Sodium (mEq/L)	138.2± 12.61	138.9±13.54	N.S.**
	Potassium (mEq/L)	4.53± 0.25	4.53±0.27	N.S.**
	Calcium (mg/dL)	9.05± 0.80	9.08±0.77	N.S.**
	Magnesium (mEq/L)	1.94± 0.23	1.95±0.24	N.S.**
	Ferritin (ng/ml)	102.3 ±11.96	104.0±13.23	N.S.**
	Iron (ug/dL)	113.4 ±12.20	114.8±13.16	N.S.**
Diet + Chitosan	Sodium (mEq/L)	139.5±13.48	139.0±12.93	N.S.**
	Potassium (mEq/L)	4.51±0.24	4.52±0.25	N.S.**
	Calcium (mg/dL)	9.07±0.79	9.08±0.82	N.S.**
	Magnesium (mEq/L)	1.96±0.25	1.97±0.28	N.S.**
	Ferritin (ng/ml)	103.6±12.77	105.2±13.04	N.S.**
	Iron (ug/dL)	109.1±13.21	111.4±12.63	N.S.**

Table 16
Effect of Chitosan on Mineral Balance in Humans
[Colombo, *et al.*, 1996 Vol 3 Tab 24]

Treatment	Parameter	Normal Val.	Before	After	Statistics*
Diet + Chitosan	Sodium (meq/L)	137-145	140.16±15.21	140.14±15.18	N.S.**
	Potassium (meq/L)	2.7-3.9	3.12±0.29	3.13±0.30	N.S.**
	Calcium (meq/L)	2.1-3.0	2.66±0.32	2.61±0.31	N.S.**
	Magnesium (meq/L)	2.0-2.5	2.31±0.27	2.30±0.28	N.S.**
	Zinc (Hg/dL)	75-120	94.06±9.11	92.78±9.51	N.S.**
	Iron (Hg/dL)	80-180	101.68±11.67	100.44±10.62	N.S.**
	Copper (Hg/dL)	70-140	106.11±9.41	102.57±10.55	N.S.**
	Vitamin A (Hg/dL)	20-100	55.32±6.83	51.40±7.02	N.S.**
	Vitamin D (nmol/L)	20-100	63.59±7.21	60.64±7.02	N.S.**
	Vitamin E ((Hg/dL)	1.5-4	2.67±0.31	2.53±0.42	N.S.**
Diet + Placebo	Sodium (meq/L)	137-145	142.33±15.75	141.62±16.37	N.S.**
	Potassium (meq/L)	2.7-3.9	3.19±0.28	3.18±0.29	N.S.**
	Calcium (meq/L)	2.1-3.0	2.64±0.34	2.63±0.33	N.S.**
	Magnesium (meq/L)	2.0-2.5	2.32±0.29	2.31±0.29	N.S.**
	Zinc (Hg/dL)	75-120	93.15±10.06	91.22±9.22	N.S.**
	Iron (Hg/dL)	80-180	102.78±11.29	100.03±11.96	N.S.**
	Copper (Hg/dL)	70-140	113.52±10.96	111.08±9.45	N.S.**
	Vitamin A (Hg/dL)	20-100	58.21±6.76	56.32±6.31	N.S.**
	Vitamin D (nmol/L)	20-100	62.67±6.93	64.28±7.61	N.S.**
	Vitamin E ((Hg/dL)	1.5-4	2.74±0.36	2.70±0.39	N.S.**

*Comparison before-after with Student's t-test
** N.S. = difference not significant

Section IV Detailed Summary of the Basis for the Determination that Primex Chitosan is GRAS

Table 17
Tolerability parameters modifications during the two treatments.

[Guistina, *et al.*, 1995 Vol 4 Tab 43]

Treatment	Parameter	Baseline	After 4 Weeks	Statistics*
Diet + Chitosan	Sodium (mEq/L)	139.4±12.5	138.2±11.7	N.S.**
	Potassium (mEq/L)	4.53±0.20	4.54±0.23	N.S.**
	Calcium (mg/dL)	9.28±0.72	9.11±0.59	N.S.**
	Magnesium (mEq/L)	1.95±0.22	1.97±0.28	N.S.**
	Ferritin (ng/ml)	100.1±14.66	102.6±15.2	N.S.**
	Iron (ug/dL)	115.5±12.4	117.5±12.4	N.S.**
Diet + Placebo	Sodium (mEq/L)	139.4±12.5	138.2±11.7	N.S.**
	Potassium (mEq/L)	4.55±0.24	4.54±0.22	N.S.**
	Calcium (mg/dL)	9.09±0.81	9.10±0.78	N.S.**
	Magnesium (mEq/L)	1.96±0.20	1.95±0.22	N.S.**
	Ferritin (ng/ml)	103.6±11.4	104.2±12.9	N.S.**
	Iron (ug/dL)	109.8±11.6	111.8±10.9	N.S.**

Section IV Detailed Summary of the Basis for the Determination that Primex Chitosan is GRAS

Table 18
Clinical results of chitosan treatments:
Tolerability of the diet plus dietetic integrator

[Girola, *et al.*, 1996 Vol 4 Tab 42]

Treatment	Parameter	Baseline	After 4 Weeks	Statistics*
Diet + Placebo	Sodium (mEq/L)	138.4±13.5	137.7±14.1	N.S.**
	Potassium (mEq/L)	4.58±0.20	4.52±0.27	N.S.**
	Calcium (mg/dL)	9.05±0.84	9.07±0.81	N.S.**
	Magnesium (mEq/L)	1.94±0.23	1.92±0.24	N.S.**
	Ferritin (ng/ml)	108.1±13.23	104.6±14.0	N.S.**
	Iron (ug/dL)	111.2±13.1	110.9±12.8	N.S.**
Diet + Int. 1 cpa/d	Sodium (mEq/L)	139.5±14.3	138.7±13.9	N.S.**
	Potassium (mEq/L)	4.68±0.33	4.71±0.30	N.S.**
	Calcium (mg/dL)	9.09±0.72	9.07±0.78	N.S.**
	Magnesium (mEq/L)	1.95±0.21	1.92±0.26	N.S.**
	Ferritin (ng/ml)	105.6±12.8	107.2±11.9	N.S.**
	Iron (ug/dL)	107.1±11.9	109.2±12.0	N.S.**
Diet + Int. 2 cpa/d	Sodium (mEq/L)	139.9±14.0	139.2±13.7	N.S.**
	Potassium (mEq/L)	4.55±0.29	4.60±0.31	N.S.**
	Calcium (mg/dL)	9.08±0.76	9.08±0.79	N.S.**
	Magnesium (mEq/L)	1.94±0.23	1.95±.024	N.S.**
	Ferritin (ng/ml)	104.9±12.2	105.2±12.6	N.S.**
	Iron (ug/dL)	106.8±12.4	107.3±11.5	N.S.**

Section IV Detailed Summary of the Basis for the Determination that Primex Chitosan is GRAS

Table 19

PRODUCT SPECIFICATIONS* Product Name: SEACURE CL 110 Generic Name: Poly D-glucosamine hydrochloride Product Code: 27170110		
Variable	Range	Measurement
Viscosity	0 – 20	Mpas
pH	4.0 – 6.0	
Loss on drying	< 10	%
Insolubles	< 0.5	%
Specific weight	0.2 – 0.6	G/cc
Deacetylation	> 70	%
Ash content	< 1.0	%
Acid blend	10 – 20	% hydrochloric acid
Particle size	95% through 60	Mesh
Heavy metals	< 200	Ppm
(Cd, Cr, Cu, Mn, Pb, Ni,		
Zn, Hg)		
As	< 1	Ppm
Pb	< 20	Ppm
Fe	< 200	Ppm
Total viable count	< 1,000	Cfu/g
Yeast and mold	< 200	Cfu/g

*SME: Pronova Biopolymer Standard Method
Methods available upon request
Drammen, January 1, 1994
Pronova Biopolymer

000181

E. Primex Chitosan Human Study

1. Evaluation of Chitosan as a Possible Compound Causing Immediate Hypersensitivity Reactions

Primex ehf sponsored a study to investigate whether its commercial chitosan (Chito-Clear) could induce immediate allergic reactions in a large sample of subjects undergoing routine skin-prick tests for the diagnosis of suspected allergy or skin disease. Another aim of the study was to evaluate whether or not there was any cross-reactivity between chitosan and shrimp or fish [Harvima, *et al.*, 2002 Vol 13 Tab 276].

The test material was ChitoClear® FG 95 Lot TM762 as manufactured by Primex ehf. The chitosan was derived from the fresh chilled shells of the arctic shrimp (*Pandalus borealis*). The preparation was a white powder consisting of micromilled fine particles. This powder was (was mixed with a 0.9 % NaCl solution (0.15 g chitosan per 0.6 ml 0.9 % NaCl) to make a thick chitosan mash which was included in the basic series of allergens in the prick-test. A new chitosan mash was prepared every working day.

A random sample of 221 people (132 women and 89 men) between 18 and 80 years of age were prick-tested with the basic series of common allergens routinely used in the Allergy Unit of the Department of Dermatology, Kuopio University Hospital, as well as with commercial fish, shrimp, and non-commercial chitosan. The mean age of subjects was 43.5 ± 13.7 years.

Prick-tests were single-blind open tests with internal controls (10 mg/ml histamine- and control-solutions) on the test procedure. Blood samples for subjects showing a positive reaction to chitosan and/or fish and/or shrimp were collected for the determination of serum total and allergen-specific IgE levels [Harvima, *et al.*, 2002 Vol 13 Tab 276].

000182

The protocol was designed so that subjects who reacted positively to chitosan in the first prick-test were to be retested using diluted chitosan preparations. In addition, reacting subjects were to take part in a peroral challenge with chitosan in the in-patient ward of the Department of Dermatology, Kuopio

University Hospital. The challenge was to be performed with a ChitoClear® capsule and a placebo capsule. However, because no positive reactions for chitosan occurred in the prick-tests, further prick-tests with diluted preparations of chitosan or challenge tests were not performed [Harvima, *et al.,* 2002 Vol 13 Tab 276].

A positive skin-prick test was indicated by the development of a wheal at the puncture site 3 mm or larger in diameter than the wheal caused by the diluent control. All wheals were to be measured within 15 minutes of antigen challenge. A positive reaction to the prick-tests for one or more allergens occurred in 101 subjects. The number of positive reactions varied between 1 and 13 per subject. The mean number of positive reactions in the basic series was 1.73 allergens. In addition to the basic series, some of the subjects were prick-tested for other allergens. Eight of them reacted positively for certain food allergens and three for other allergens. Four subjects reacted positively for fish and eight for shrimp. All subjects reacted negatively for chitosan.

One out of four fish-positive and five out of eight shrimp-positive subjects had elevated levels of fish-specific or shrimp-specific IgE RAST-tests (> 0.3 IU/l can be considered an indication of possible hypersensitivity). None of the serum samples obtained from 11 fish/shrimp positive subjects showed chitosan-specific IgE in an immunospot analysis when a water dilution of Chitoclear was used as the allergen. The total serum IgE levels were above normal limits, in all fish/shrimp positive subjects - except one who demonstrated a positive shrimp prick-test [Harvima, *et al.,* 2002 Vol 13 Tab 276].

The principal investigators of the study felt that it was noteworthy that 221 allergy prone subjects, suffering from one or more of the following symptoms:

- Asthma
- Rhinitis
- Conjunctivitis or eye symptoms
- Atopic dermatitis
- Urticaria
- Unspecific itching
- Unspecific dermatitis

000183

reacted positively to chitosan, including those found to be allergic to fish or shrimp. In a further analysis, no chitosan-specific IgE was found in the serum of eleven fish- or shrimp-positive subjects. Based on these results, the investigators concluded that the probability for immediate IgE-mediated hypersensitivity reactions in the general population to chitosan derived from *Pandalus borealis* is very small [Harvima, *et al.,* 2002 Vol 13 Tab 276].

2. Effect of Chitosan on Serum Fat-Soluble Vitamins and Plasma Cholesterol Concentrations in Mildly Hypercholesterolemic Subjects

To confirm the effect of chitosan on human mineral and fat soluble vitamin nutrition, Primex ehf sponsored a study in 2003, which was conducted by Oy Foodfiles Ltd in Finland. The title of the study was "Effect of Chitosan on Serum Fat-Soluble Vitamins and Plasma Cholesterol Concentrations in Mildly Hypercholesterolemic Subjects" [Tapola, *et al.,* 2003 Vol 16 Tab 357]. The chitosan administered in the study was manufactured by Primex ehf from the arctic shrimp *Pandalus borealis*. The study was designed as a randomized, single blind, placebo controlled parallel trial.

1. Objective

The primary objective of this study was to determine the effect of two different doses of Primex chitosan (4.5 g and 6.75 g) on serum concentrations of fat-soluble vitamins (vitamin A, α-carotene, β-carotene, vitamin E and vitamin D), and plasma cholesterol concentrations as compared with a placebo and glucomannan as a positive control in mildly hypercholesterolemic subjects.

2. Subjects

Seventy nine (79) subjects were screened for the study. Sixty five (65) subjects were randomized and fifty-six (56) mildly hypercholesterolemic male and female subjects completed the trial. After a two-week run-in period the subjects were randomly assigned to one of four groups. Female and male subjects were randomized separately. The subjects were given 0, 4.5, or 6.75 g /day of chitosan or 6.75 g/day of glucomannan in the form of tablets. The tablets were taken with main meals for eight weeks. The mean duration of the intervention period was 55 days (minimum 50 and maximum 58) in the chitosan 4.5 g group, 55 days (minimum 53 and maximum 56) in the chitosan 6.75 g group, and 54 days (minimum 50 and maximum 58) in the glucomannan group [Tapola, *et al.,* 2003 Vol 16 Tab 357].

Subjects had to be between the ages of 18 and 55 years old and had to have a total plasma cholesterol concentration of 5-6.5 mmol/l and have a total plasma triglyceride concentration less than 4 mmol/l. The exclusion criteria included the presence of liver or kidney disease or disorders, severe lactose-intolerance, gastrointestinal diseases leading to malabsorption of fat-soluble vitamins, a history of unstable coronary artery disease (myocardial infarction, unstable angina pectoris, coronary artery bypass graft (CABG), percutaneous transluminal coronary angioplasty (PTCA) within the previous 6 months), stroke, temporal ischemic attack, malignant disease, other serious illness or the history of severe allergic reactions (anaphylactic reaction) when exposed to fish or crustaceans. Use of lipid lowering medications, laxatives, guar gum or glucomannan, psyllium or other dietary supplements with laxative or bulking effect, use of dietary supplements or drugs providing fat-soluble vitamins, chronic use of drugs having the potential to interfere with vitamin absorption and excessive consumption of alcohol (> 45 g ethanol per day) [Tapola, *et al.*, 2003 Vol 16Tab 357]. In total 79 subjects were screened for the study.

3. Test products and instructions

Chitosan and glucomannan were provided in tablets. The subjects were instructed to take six tablets three times a day with a glass of water 15 minutes before meals in the morning, at noon and in the evening. No other dietary restrictions were imposed. The subjects followed their habitual diet.

Each chitosan tablet contained 750 mg Primex FG 95 chitosan. The chitosan 4.5 g group were required to take 6 chitosan tablets and 12 placebo tablets and the chitosan 6.75 g group took 9 chitosan and 9 placebo tablets per day. Each glucomannan tablet contained 375 mg of glucomannan. The glucomannan group took 18 glucomannan tablets and the Placebo group took 18 placebo tablets per day. The subjects were instructed to return any unused tablets. Compliance was assessed by counting the returned tablets.

4. Materials Tested

The test material was Primex FG 95 chitosan with a degree of deacetylation of more than 95 % and a viscosity of less than 500 mPas. Glucomannan was derived from konjac root (*Amorphallus konjac*). The placebo tablets contained

cellulose, lactose and small amounts of ferroxide and magnesium stearate [Tapola, *et al.*, 2003 Vol 16 Tab 357].

5. Methods

Venous blood samples were collected after an overnight (12 hour) fast. Laboratory samples for routine hematology and basic laboratory tests (plasma creatinine, plasma urate, plasma gamma-glutamyl transferase, plasma calcium, serum ferritin) were analyzed with standardized methods at the Clinical Chemistry Unit, Kuopio University Hospital.

Serum samples of α- and β-carotene, vitamin A, E and 25-hydroxyvitamin D (25(OH)D) were stored at - 70°C until analyzed at end of the study. Serum α- and β-carotene, vitamin A and E were analyzed simultaneously by HPLC with internal 4-point calibration. Serum 25(OH)D concentrations were determined by an RIA-method (Radioimmunometric method, DiaSorin Inc, USA) [Tapola, *et al.*, 2003 Vol 16 Tab 357].

Plasma concentrations of lipids were analyzed at the Clinical Chemistry Unit, Kuopio University Hospital using lithium-heparin. Plasma total and HDL-cholesterol and total triglyceride concentrations were measured using commercial reagents (Konelab CHOLESTEROL, code 981368, Konelab HDL-CHOLESTEROL, code 981655 and Konelab TRIGLYCERIDES, code 981301N) with a Konelab 60i Clinical Chemistry Analyzer (Labsystems CLD, Konelab Finland). Plasma LDL-cholesterol concentration was calculated by the Friedewald formula [Tapola, *et al.*, 2003 Vol 16 Tab 357].

Body weight was measured twice at every visit using a digital scale (Scale Seca 707, Vogel & Falke GmpH & Co, Hamburg, Germany). The mean value of the two measurements was used in the analyses. Blood pressure measurements were performed after a ten minute rest in a sitting position with an automatic sphygmomanometer (Omron 711 automatic IS, Omron Matsusaka Co, Ltd, Japan) in triplicate on every occasion. The mean value of the last two measurements was used in the analyses.

The composition of the diet was monitored by a four-day food record taken once during the run-in period, and once during the intervention period. The

subjects recorded their dietary intake for four consecutive days in food records, including one weekend day or day off from work. The serving sizes were estimated with a portion size picture booklet [Tapola, *et al.,* 2003 Vol 16 Tab 357]. Oral and written instructions about how to record foods were given to each subject. At the study visits a nutritionist checked the food records for completion, thus clarifying any missing information. The energy and nutrients were calculated using Micro-Nutrica® (version 2.5) dietary analysis software (developed by the Social Insurance Institution, Turku, Finland), which is based on Finnish and international food composition data.

The RAND-36 Item Health Survey 1.0 (Hays *et al,* 1993; Aalto *et al,* 1999) was used to assess the following eight health concepts: 1) general health; 2) physical functioning; 3) mental health; 4) social functioning; 5) vitality; 6) bodily pain; 7) role functioning/physical and 8) role functioning/emotional.

Each subject completed a structured form twice covering the incidence and severity of gastrointestinal (diarrhea, flatulence, abdominal pain, abdominal bloating, constipation, heartburn, nausea, abdominal rumbling, bowel cramps) skin and other symptoms. The severity of symptoms was determined with a four-point category scale.

6. Statistical analyses

Statistical analyses were performed with the SPSS Base and Advanced models 10.1 statistics program (SPSS Inc, Chicago, U.S.A). Normal distribution of variables was checked with the Shapiro-Wilk test. P-values less than 0.05 were regarded as statistically significant.

The power of the study was calculated only for the primary outcome measure; serum β-carotene concentration. The power of the study was 0.80 based on an assumption of being able to detect a 512 nmol/l difference in serum β-carotene concentration, when the standard deviation is 500 nmol/l with 60 subjects and the probability for type I error $\alpha = 0.05$.

7. Treatment compliance

The mean chitosan intake was 95.1 ± 6.9 % (minimum 73.7 %) and 95.3 ± 5.6 % (minimum 79.9 %) of the target amount in the Chitosan 4.5 and 6.75 gram

groups, respectively. The mean glucomannan intake was 95.6 ± 4.4 % (minimum 88.5 %) of the target amount in the glucomannan group. In other words, the mean intake of chitosan was 4.28 ± 0.31 g in the Chitosan 4.5 group and 6.43 ± 0.38 g in the Chitosan 6.75 group. The mean intake of glucomannan was 6.46 ± 0.29 g in the glucomannan group.

During the run-in period, there were some differences in the nutrient intake among the groups. The fat intake was lowest in the glucomannan group and fiber intake was lowest in the chitosan 4.5 g group. However, there were no statistically significant differences in energy and nutrient intake among the groups during the intervention period.

8. Results

The investigators stated that serum α-carotene concentration seemed to decrease in every study group during the intervention period, but that this change was not statistical significant (Table 23). There were no significant differences in serum vitamin A, α-carotene, β-carotene, vitamin E or 25-hydroxyvitamin D concentrations among the study groups and there were no significant differences among the groups in serum fat-soluble vitamin and carotenoids after standardization for serum total lipid concentration (Table 23). However, standardized serum 25-hydroxyvitamin D concentration increased during the intervention period in the glucomannan group.

There were no significant differences in clinical chemistry or hematology measurements among the study groups (Table 24). However, hematocrit increased in the glucomannan group. Plasma calcium concentration decreased in every study group, but remained within the normal range.

a. Body weight and blood pressure

Body weight remained stable during the intervention period in every study group (Table 25). Blood pressure did not change significantly from the baseline in any of the study groups (Table 26).

Table 20. Pretrial characteristics of the study subjects. Mean (SD)

	Chitosan 4.5 g (n = 15)	Chitosan 6.75 g (n = 12)	Glucomannan (n = 15)	Placebo (n = 14)	p-value[a]
Male/Female	7/8	7/5	8/7	5/9	NS
Age (y)	46 (5)	45 (6)	49 (3)	45 (8)	NS
Body mass index[1]	25 (3)	26 (4)	26 (3)	26 (4)	NS
Plasma[2] total cholesterol (mmol/l)	5.8 (0.6)	5.8 (0.5)	5.8 (0.7)	5.8 (0.5)	NS
Plasma[2] LDL-cholesterol (mmol/l)	3.60 (0.65)	3.71 (0.41)	3.49 (0.66)	3.52 (0.81)	NS
Plasma[2] HDL-cholesterol (mmol/l)	1.55 (0.44)	1.40 (0.44)	1.62 (0.40)	1.57 (0.44)	NS
Plasma[2] total triglyceride (mmol/l)	1.38 (0.62)	1.53 (0.89)	1.48 (0.76)	1.46 (0.68)	NS

[1] Body weight (kg) / height^2 (m)

[2] Lithium-heparin-plasma

[a] The significance of the difference among the study groups analyzed with the Chi-Square test of independence (sex), with the univariate analysis of variance (body mass index and plasma lipids) or with the Kruskal-Wallis test (age).

000189

Section IV Detailed Summary of the Basis for the Determination that Primex Chitosan is GRAS

Table 21. Energy and nutrient composition of the background diet. Mean (SD)

	Chitosan 4.5 g (n = 15)	Chitosan 6.75 g (n = 12)	Glucomannan (n = 15)	Placebo (n = 14)	p-value[a]	p-value[b]
Energy (MJ) Run-in period Intervention period p-value[c] (NS)	7.7 (1.9) 7.9 (1.8)	9.1 (2.4) 8.6 (2.6)	8.8 (2.2) 8.3 (2.4)	7.8 (2.2) 8.1 (2.3)	NS	NS
Protein (E-%) Run-in period Intervention period p-value[c] (NS)	16.4 (3.2) 16.1 (2.9)	17.4 (2.9) 18.1 (4.8)	18.0 (3.3) 18.6 (4.5)	18.1 (2.5) 18.9 (4.3)	NS	NS
Fat (E-%) Run-in period Intervention period p-value[c]	34.5 (5.2)[e] 32.2 (5.7) NS	32.8 (5.2) 33.7 (4.7) NS	27. 9 (6.0) 32.8 (6.0) 0.028	32.4 (3.6) 31.8 (4.1) NS	0.012[d] NS[d]	0.008
Saturated fatty acids (E-%) Run-in period Intervention period p-value[c]	14.3 (2.5) 13.0 (2.8) NS	12.7 (2.7) 14.1 (2.5) NS	11.5 (2.4) 13.2 (2.5) NS	13.2 (2.5) 12.8 (2.6) NS	NS[d] NS[d]	0.020

000190

Section IV Detailed Summary of the Basis for the Determination that Primex Chitosan is GRAS

Table 21 continued

	Chitosan 4.5 g (n = 15)	Chitosan 6.75 g (n = 12)	Glucomannan (n = 15)	Placebo (n = 14)	p-value[a]	p-value[b]
Monounsaturated fatty acids (E-%)						
Run-in period	11.6 (2.4)	10.3 (2.2)	8.9 (2.7)	10.8 (1.4)	0.038[d]	0.032
Intervention period	10.7 (2.9)	10.7 (2.1)	10.9 (2.8)	10.6 (1.6)	NS[d]	
p-value[c]	NS	NS	0.044	NS		
Polyunsaturated fatty acids (E-%)						
Run-in period	4.8 (1.0)	5.8 (2.1)	4.2 (1.1)	5.1 (1.0)	NS	NS
Intervention period	5.0 (1.4)	5.1 (1.6)	5.1 (1.4)	5.1 (1.4)		
p-value[c] (NS)						
Carbohydrates (E-%)						
Run-in period	46.2 (5.6)	48.7 (4.8)	50.3 (7.0)	46.7 (4.7)	NS[d]	0.002
Intervention period	50.0 (7.5)	46.8 (5.7)	44.9 (6.3)	45.5 (6.1)	NS[d]	
p-value[c]	NS	NS	NS	NS		
Alcohol (E-%)						
Run-in period	2.9 (2.8)	1.0 (2.1)	3.9 (5.4)	2.0 (4.1)	NS[f]	
Intervention period	1.8 (2.5)	1.3 (1.5)	3.7 (4.4)	3.8 (5.1)	NS[f]	
p-value[g]	NS	NS	NS	NS		

000191

Section IV Detailed Summary of the Basis for the Determination that Primex Chitosan is GRAS

Table 21 continued

	Chitosan 4.5 g (n = 15)	Chitosan 6.75 g (n = 12)	Glucomannan (n = 15)	Placebo (n = 14)	p-value[a]	p-value[b]
Cholesterol (mg) Run-in period Intervention period p-value[g]	257 (112) 278 (151) NS	258 (77) 264 (106) NS	233 (84) 252 (95) NS	228 (51) 254 (65) NS	NS[f] NS[f]	
Fiber (g)* Run-in period Intervention period p-value[c] (0.046)	19 (7)[h] 20 (6) NS	27 (7) 23 (7) NS	26 (6) 24 (8) NS	24 (7) 23 (6) NS	0.031 0.024[d] NS[d]	NS
Vitamin A (µg) Run-in period Intervention period p-value[c] (NS)	1516 (2358) 919 (614)	1728 (1789) 1384 (1121)	1441 (1549) 784 (264)	793 (298) 1171 (1200)	NS	NS
Vitamin D (µg) Run-in period Intervention period p-value[g]	6.2 (3.1) 5.4 (2.5) NS	7.7 (3.1) 6.0 (2.5) NS	9.1 (4.6) 8.8 (8.5) NS	5.4 (3.5) 5.5 (3.5) NS	NS[f] NS[f]	
Vitamin E (mg) Run-in period Intervention period p-value[c] (NS)	8.0 (3.0) 8.0 (2.5)	10.9 (3.0) 9.4 (2.4)	8.7 (3.0) 9.4 (4.0)	8.7 (2.1) 8.6 (2.8)	NS	NS

000192

[a] The significance of the overall difference among the study groups analyzed with the GLM for repeated measures.
[b] The significance of the time*group interaction analyzed with the GLM for repeated measures.
[c] The significance of the within-group difference analyzed with the GLM for repeated measures.
[d] The significance of the difference among the study groups analyzed with the Oneway analysis of variance (ANOVA) adjusted with the Bonferroni correction.
[e] Indicates the significant difference ($p < 0.05$) compared to the glucomannan group.
[f] The significance of the difference among the study groups analyzed with the Kruskal-Wallis test adjusted with the Bonferroni correction.
[g] The significance of the within-group difference analyzed with the Wilcoxon test adjusted with the Bonferroni correction.
[h] Indicates the significant difference ($p < 0.05$) compared to the Chitosan 6.75 g and glucomannan groups.
* not including chitosan and glucomannan

000193

b. RAND-36

The scores for eight RAND-36 subscales (i.e. General health, Physical functioning, Mental Health, Social functioning, Vitality, Bodily pain, Role functioning/physical, Role functioning/ emotional) are presented in the Table 25. There were no differences among the groups, or significant changes within-groups.

Section IV Detailed Summary of the Basis for the Determination that Primex Chitosan is GRAS

Table 22. Scores for RAND-36 Subscales. Mean (SD)

	Chitosan 4.5 g (n = 15)	Chitosan 6.75 g (n = 12)	Glucomannan (n = 15)	Placebo (n = 14)	p-value[a]
General health					
0 wk	77.0 (16.7)	79.6 (10.5)	73.0 (16.9)	76.4 (16.1)	NS
8 wk	82.0 (12.1)	82.5 (10.8)	77.3 (13.6)	83.6 (9.5)	NS
p-value[b]	NS	NS	NS	NS	
Physical functioning					
0 wk	97.0 (5.3)	95.8 (6.0)	96.7 (4.5)	97.1 (5.8)	NS
8 wk	96.3 (5.8)	96.7 (4.9)	96.3 (5.5)	97.9 (5.4)	NS
p-value[b]	NS	NS	NS	NS	
Mental health					
0 wk	83.5 (11.4)	86.7 (8.4)	80.3 (8.3)	87.7 (5.5)	NS
8 wk	78.9 (9.5)	86.7 (10.3)	82.4 (11.1)	83.4 (15.7)	NS
p-value[b]	NS	NS	NS	NS	
Social functioning					
0 wk	91.7 (13.1)	95.8 (8.1)	88.3 (15.3)	94.6 (8.1)	NS
8 wk	92.5 (10.4)	97.9 (4.9)	90.0 (13.5)	93.8 (16.1)	NS
p-value[b]	NS	NS	NS	NS	

000195

Section IV Detailed Summary of the Basis for the Determination that Primex Chitosan is GRAS

Table 22 Continued

	Chitosan 4.5 g (n = 15)	Chitosan 6.75 g (n = 12)	Glucomannan (n = 15)	Placebo (n = 14)	p-value[a]
Vitality					
0 wk	78.0 (14.0)	81.3 (12.6)	72.7 (11.2)	81.1 (7.6)	NS
8 wk	79.3 (13.7)	80.8 (12.0)	75.3 (11.7)	77.5 (13.0)	NS
p-value[b]	NS	NS	NS	NS	
Bodily pain					
0 wk	80.2 (23.1)	86.5 (10.8)	82.0 (21.5)	84.1 (18.3)	NS
8 wk	87.2 (14.3)	88.5 (16.6)	84.3 (18.7)	92.5 (12.0)	NS
p-value[b]					
Role functioning/Physical					
0 wk	93.3 (14.8)	100 (0)	93.3 (14.8)	92.9 (15.3)	NS
8 wk	93.3 (20.0)	100 (0)	96.7 (8.8)	98.2 (6.7)	NS
p-value[b]					
Role functioning/Emotional					
0 wk	84.4 (30.5)	100 (0)	93.3 (18.7)	92.9 (14.2)	NS
8 wk	84.4 (30.5)	97.2 (9.6)	88.9 (30.0)	90.5 (27.5)	NS
p-value[b]					

[a] The significance of the difference among the study groups analyzed with the Kruskal-Wallis test.

[b] The significance of the within-group difference analyzed with the Wilcoxon test.

c. Fat soluble vitamins

Chitosan did not have any significant effect on serum concentrations of vitamin A, α-carotene, β-carotene, vitamin E and 25-hydroxyvitamin D during 8 weeks of consumption. This observation was found to be consistent with the results of the studies reported by [Pittler, *et al.,* 1999 Vol 7 Tab 117 and [Colombo, *et al.*, 1996 Vol 3 Tab 24]. In both studies serum vitamin A, β-carotene, vitamin D and E concentrations remained stable in overweight subjects during a 4-week period of chitosan ingestion with both a normal or a hypocaloric diet. In the present study, serum 25-hydroxyvitamin D concentration increased slightly, but not statistically significantly in all study groups. This thought to be due to the seasonal variation of vitamin D synthesis in the skin. The present study was conducted from April to June and vitamin D_3 synthesis in the skin is less in spring than in summer [Tapola, *et al.,* 2003 Vol 16 Tab 357].

d. Calcium concentrations

Plasma calcium concentration decreased after the ingestion of chitosan, glucomannan and placebo tablets, in other words, in all study groups. However, the mean plasma calcium concentration for all study groups was within the normal range 2.20- 2.65 mmol/l. None of the additional safety parameters measured, including clinical chemical parameters, hematolocigal parameters, or blood pressure were affected by chitosan (Tables 25 and 26).

Section IV Detailed Summary of the Basis for the Determination that Primex Chitosan is GRAS

Table 23. Serum fat soluble vitamins and carotenoids concentrations during the study. Mean (SD)

	Chitosan 4.5 g (n = 15)	Chitosan 6.75 g (n = 12)	Glucomannan (n = 15)	Placebo (n = 14)	p-value[a]	p-value[b]
Serum vitamin A (µmol/l)						
0 wk	3.51 (0.89)	3.65 (0.86)	3.74 (0.99)	3.68 (0.55)	NS	NS
8 wk	3.98 (1.11)	3.81 (1.07)	3.75 (0.98)	3.87 (0.74)		
p-value[c] (0.029)	NS	NS	NS	NS		
Serum α-carotene (µmol/l)						
0 wk	1.20 (0.75)	1.24 (1.07)	1.43 (1.78)	1.17 (0.54)	NS	
8 wk	0.90 (0.53)	1.07 (0.94)	1.12 (1.52)	1.07 (0.47)	NS	
p-value[c]	NS	NS	NS	NS		
Serum β-carotene (µmol/l)						
0 wk	2.11 (1.17)	2.13 (1.55)	2.53 (3.09)	2.22 (1.40)	NS	NS
8 wk	2.05 (1.58)	2.16 (1.43)	2.19 (2.83)	2.15 (1.36)		
p-value[c] (NS)						
Serum vitamin E (µmol/l)						
0 wk	35.18 (4.18)	33.08 (4.42)	34.95 (6.33)	33.92 (6.02)	NS	NS
8 wk	35.14 (3.87)	32.22 (5.77)	34.27 (7.06)	34.66 (7.84)		
p-value[c] (NS)						

000198

Section IV Detailed Summary of the Basis for the Determination that Primex Chitosan is GRAS

Table 23 continued

	Chitosan 4.5 g (n = 15)	Chitosan 6.75 g (n = 12)	Glucomannan (n = 15)	Placebo (n = 14)	p-value[a]	p-value[b]
Serum 25(OH)D (nmol/l)						
0 wk	63.0 (23.1)	61.5 (31.2)	56.1 (27.6)	60.6 (20.8)	NS	NS
8 wk	67.5 (19.9)	74.1 (31.1)	69.8 (33.1)	65.4 (18.4)		
p-value[c] (0.001)	NS	NS	NS	NS		

[a] The significance of the overall difference among the study groups analyzed with the GLM for repeated measures (vitamin A, β-carotene, vitamin E and 25(OH)D) or with the Kruskal-Wallis test (α-carotene) adjusted with the Bonferroni correction .

[b] The significance of the time*group interaction analyzed with the GLM for repeated measures.

[c] The significance of the within-group difference analyzed with the GLM for repeated measures (vitamin A, β-carotene and vitamin E) or with the Wilcoxon test (α-carotene) adjusted with the Bonferroni correction.

000199

Table 24. Hematology and clinical chemistry. Mean (SD)

	Chitosan 4.5 g (n = 15)	Chitosan 6.75 g (n = 12)	Glucomannan (n = 15)	Placebo (n = 14)	p-value[a]	p-value[b]
Hemoglobin (g/l) - 2 wk 8 wk p-value[c] (NS)	139 (13) 140 (15)	140 (15) 139 (13)	141 (14) 144 (13)	134 (10) 133 (11)	NS	NS
Hematocrit - 2 wk 8 wk p-value[c] (< 0.001)	0.40 (0.04) 0.41 (0.04) NS	0.40 (0.04) 0.41 (0.04) NS	0.41 (0.04) 0.42 (0.04) 0.032	0.39 (0.02) 0.40 (0.03) NS	NS	NS
Leucocytes (10^9/l) - 2 wk 8 wk p-value[e]	6.9 (2.4) 5.8 (1.5) NS	6.0 (1.6) 5.9 (1.6) NS	5.5 (1.1) 5.3 (1.5) NS	5.3 (0.9) 5.5 (1.0) NS	NS[d] NS[d]	
Plasma creatinine (µmol/l) - 2 wk 8 wk p-value[c] (NS)	85 (11) 84 (11)	89 (10) 88 (9)	86 (9) 87 (11)	86 (10) 83 (10)	NS	NS
Plasma urate (µmol/l) - 2 wk 8 wk p-value[c] (NS)	269 (74) 278 (90)	295 (77) 276 (85)	287 (80) 281 (95)	269 (47) 264 (64)	NS	NS

000200

Table 24 continued

	Chitosan 4.5 g (n = 15)	Chitosan 6.75 g (n = 12)	Glucomannan (n = 15)	Placebo (n = 14)	p-value[a]	p-value[b]
Plasma gamma-glutamyl transferase (U/l)						
- 2 wk	29 (18)	33 (11)	33 (26)	32 (20)	NS	NS
8 wk	29 (16)	32 (12)	32 (22)	32 (19)		
p-value[c] (NS)						
Serum ferritin (µg/l)						
0 wk	77 (38)	86 (73)	128 (156)	79 (79)	NS	NS
8 wk	72 (43)	84 (70)	129 (167)	73 (71)		
p-value[c] (NS)						
Plasma calcium (mmol/l)						
0 wk	2.31 (0.06)	2.37 (0.07)	2.36 (0.09)	2.36 (0.08)	NS	NS
8 wk	2.27 (0.07)	2.30 (0.07)	2.29 (0.07)	2.26 (0.06)		
p-value[c] (< 0.005)	0.020	0.016	< 0.005	< 0.005		

[a] The significance of the overall difference among the study groups analyzed with the GLM for repeated measures.
[b] The significance of the time*group interaction analyzed with the GLM for repeated measures.
[c] The significance of the within-group difference analyzed with the GLM for repeated measures.
[d] The significance of the difference among the study groups analyzed with the Kruskal-Wallis test adjusted with the Bonferroni correction.
[e] The significance of the within-group difference analyzed with the Wilcoxon test adjusted with the Bonferroni correction.

000201

Section IV Detailed Summary of the Basis for the Determination that Primex Chitosan is GRAS

Table 25. Body weight. Mean (SD)

	Chitosan 4.5 g (n = 15)	Chitosan 6.75 g (n = 12)	Glucomannan (n = 15)	Placebo (n = 14)	p-value[a]	p-value[b]
Body weight (kg)						
0 wk	70.1 (13.6)	79.5 (15.0)	72.8 (12.1)	74.0 (11.1)	NS	NS
4 wk	70.5 (13.6)	79.0 (15.4)	72.7 (12.1)	74.2 (11.4)		
8 wk	70.2 (13.4)	78.8 (15.5)	72.2 (11.9)	74.3 (11.3)		
p-value[c] (NS)						

[a] The significance of the overall difference among the study groups analyzed with the GLM for repeated measures.
[b] The significance of the time*group interaction analyzed with the GLM for repeated measures.
[c] The significance of the within-group difference analyzed with the GLM for repeated measures.

000202

Table 26. Blood pressure. Mean (SD)

	Chitosan 4.5 g (n = 15)	Chitosan 6.75 g (n = 12)	Glucomannan (n = 15)	Placebo (n = 14)	p-value[a]	p-value[b]
Diastolic blood pressure (mmHg)						
0 wk	79 (9)	87 (11)	80 (10)	88 (13)	NS	NS
4 wk	80 (9)	86 (9)	84 (9)	85 (10)		
8 wk	80 (10)	86 (9)	83 (10)	84 (13)		
p-value[c] (NS)						
Systolic blood pressure (mmHg)						
0 wk	125 (14)	135 (19)	127 (14)	137 (21)	NS	NS
4 wk	125 (14)	135 (13)	129 (13)	132 (16)		
8 wk	125 (13)	135 (13)	127 (12)	131 (20)		
p-value[c] (NS)						

[a] The significance of the overall difference among the study groups analyzed with the GLM for repeated measures.
[b] The significance of the time*group interaction analyzed with the GLM for repeated measures.
[c] The significance of the within-group difference analyzed with the GLM for repeated measures.

000203

Table 27. Defecation frequency. Mean (SD)

	Chitosan 4.5 g (n = 15)	Chitosan 6.75 g (n = 12)	Glucomannan (n = 15)	Placebo (n = 14)	p-value[a]
Defecation frequency (times per day)					
habitual	1.1 (0.6)	1.0 (0.4)	1.1 (0.5)	1.2 (0.6)	NS
0-4 wk	1.2 (0.6)	1.2 (0.5)	1.2 (0.5)	1.2 (0.6)	NS
4-8 wk	1.3 (0.6)	1.3 (0.7)	1.1 (0.6)	1.3 (0.6)	NS
p-value[b]	NS	NS	NS	NS	

[a] The significance of the difference among the study groups analyzed with the Kruskal-Wallis test adjusted with the Bonferroni correction.
[b] The significance of the within-group difference analyzed with the Friedman test adjusted with the Bonferroni correction.

000204

Symptoms and Subject Dropouts

Common gastrointestinal symptoms were reported to some extent by all groups (See Appendix III). Interestingly, there was no significant difference reported by the test subjects for their Physical Functioning, Vitality, Role Functioning/Physical or Bodily Pain at the end of the trial as measured by the RAND-36 [Tapola, *et al.,* 2003 Vol 16 Tab 357].

Nine subjects withdrew from the study. One male withdrew from the chitosan 4.5 gram per day group for unknown reasons, and two males withdrew from the placebo group; one for poor compliance, and one due to a screening failure. A total of six perimenopausal females withdrew from the high fiber groups (glucomannan 6.75 grams/day (2) and chitosan 6.75 grams per day (4)). This age group of females has been reported to have a greater incidence of gastrointestinal symptoms, which may be exacerbated by a sudden increase in dietary fiber intake [Georges, *et al.,* 1994 Vol 13 Tab 270 and Jarret *et al.,* 1996 Vol 14 Tab 287]. By chance these two groups also began the study with a 3-4 gram per day higher level of dietary fiber intake in comparison to the other groups. This level was however, statistically non-significant. A detailed report of the gastrointestinal symptoms experienced by all the groups can be found in Appendix III.

A few subjects reported skin symptoms during the study. These symptoms did not seem to follow a pattern related to the test materials. Additionally, the extensive allergenicity study conducted by Primex has shown that Primex chitosan does not initiate allergic symptoms. A detailed report of the skin symptoms reported can also be found in Appendix III.

Conclusion

000205

In the present study two different doses (4.5 g and 6.75 g) of Primex chitosan derived from the arctic shrimp *Pandalus borealis* in the form of tablets, as a part of the habitual Finnish diet, did not effect serum concentrations of vitamin A, α-carotene, β-carotene, vitamin E, 25-hydroxyvitamin D or plasma

cholesterol concentrations in 56 mildly hypercholesterolemic subjects, nor did the levels of these fat soluble vitamins change in comparison to the controls, when normalized for cholesterol level. Calcium levels in each group, including the placebo dropped slightly over the study, but all levels remained within the normal range. Other hematological and clinical chemistry parameters remained within the normal range for both chitosan groups, including ferritin, creatinine, and white blood cells.

In this study body weight remained constant across all groups. There was an inclination for a mild drop (-4.3%) in total cholesterol in the two highest treatment groups in comparison to the placebo group, whose cholesterol increased by 1.8%, however, these differences were not statistically significant. It is worth noting, that this chitosan product is relatively low in viscosity, while higher viscosity fiber preparations, like guar gum, have been more closely associated with lowered cholesterol levels [Schneeman, 1996 Vol 15 Tab 338]. Interestingly, another Finish research group feeding 15 grams per day of guar gum to 30 menopausal women also found a non-significant 5% reduction in serum cholesterol after a 6-month study duration [Makkonen, *et al.,* 1993 Vol 14 Tab 306].

Importantly, this study confirms that Primex chitosan does not lower human fat-soluble vitamin levels, nor does it lower calcium levels below the normal range for serum. Therefore, Primex chitosan does not compromise human nutrition, even when consumed at the 4.5-6.75 gram per day level. It is also important to note, that the levels of chitosan fed in this study are approximately 100-150% higher, than the Primex exposure estimate for the 90th percentile of approximately 4.0 grams per day.

GR

Question 4

000207

4. **Question 4. Why did certain chitosan consumption studies in both humans and animals show that vitamin K levels increased? Would this in fact create an at-risk population of those taking prescribed blood-thinning drugs?**

Regulatory reviewers have noticed that vitamin K levels have been reported to increase in both rats and humans when consuming chitosan [Pittler, *et al.,* 1999 Vol 7 Tab 117 and Deuchi, *et al.,* 1995 Vol 4 Tab 29]. Vitamin K is a fat-soluble vitamin, and an increase in vitamin K levels seemed to be counterintuitive. Further, if vitamin K levels did increase with chitosan consumption, and vitamin K increases clotting ability, would persons taking anticoagulant drugs constitute an at-risk group?

Fairfield and Fletcher, publishing in a recent issue of the *Journal of the American Medical Association* have indicated that there is no known toxicity state for vitamin K [Fairfield, *et al.,* 2002 Vol 13 Tab 260]. According to the authors, vitamin K is synthesized by intestinal bacteria. This fact lends additional credence to the assertion that chitosan is degraded in the intestine, as a dietary fiber. The current RDI for vitamin K is 80 μg/L, a level that is rarely attained even in healthy subjects. *Individuals taking anti-coagulants should be given clear* instructions as to diet, as increases in vitamin K may occur with any increase in indigestible carbohydrate [Fairfield, *et al.,* 2002 Vol 13 Tab 260].

The fact that chitosan increases intestinal bacteria was observed by Chae, *et al.,* in pigs. Pigs, weaners and growers, consumed 0.1 to 0.4% of the diet in the form of a chitooligosaccharide [Chae, *et al.,* 2000 Vol 12 Tab 243]. This intake level would be equivalent to a human consuming chitosan at the 90th percentile proposed by Primex (3.2 grams per day). After a 54 day feeding period Chae, *et al.,* showed that the test material did not change the pH in any area of the

digestive tract. but that both total and beneficial bacterial populations increased, while populations of *E. coli* were diminished. An increase in colonic microflora, may then explain the increase in serum vitamin K levels reported in both rats and humans by Pittler and Deuchi [Pittler, *et al.,* 1999 Vol 7 Tab 117 and Deuchi, *et al.,* 1995 Vol 4 Tab 29].

Several studies have been conducted to investigate the interaction between bacterially generated vitamin K and warfarin, an anticoagulant drug. Investigators agree that menaquinones (bacterially produced) contribute to vitamin K nutriture during dietary phylloquinone restriction, but not in sufficient quantity to restore normal vitamin K status [Paiva, *et al.,* 1998 Vol 15 Tab 324]. Camilo *et al.*, 1988 concluded that their data did not support the hypothesis that bacterial synthesis of menaquinones in patients with bacterial overgrowth due to atrophic gastritis conferred resistance to the effect of warfarin [Camilo *et al.*, 1988 Vol 12 Tab 242]. Further, Kamali, *et al.,* 2000 concluded that the combined effects of age and vitamin K appear to account for much of the inter-individual variability in warfarin dosage requirements [Kamali, *et al.,* 2000 Vol 14 Tab 288]. Therefore, no evidence was found to support the hypothesis that additional vitamin K generated by intestinal bacteria would have any adverse effect on the efficacy of anti-coagulant drugs. Several studies covering this area are reviewed below.

Paiva, *et al.,* working at the Jean Mayer US Department of Agriculture Human Nutrition Research Center on Aging at Tufts University in Boston, Massachusetts studied the Interaction between vitamin K nutriture and bacterial overgrowth in hypochlorhydria induced by omeprazole. The authors explained that subjects taking a hydrogen pump blocking agent (omeprazole) develop bacterial overgrowth of the small intestine. They tested the hypothesis that this bacterial overgrowth produces menaquinones, which would meet the

vitamin requirement in situations of vitamin K deficiency. In a crossover-type design, 13 healthy volunteers eating a phylloquinone-restricted diet for 35 days were randomly assigned to take omeprazole during the first period of study or starting on day 15 until the end of the study. Coagulation times, serum osteocalcin [total osteocalcin and undercarboxylated osteocalcin (ucOC)], plasma phylloquinone, urinary gamma-carboxyglutamic acid, and plasma undercarboxylated prothrombin (PIVKA-II) were measured. Plasma phylloquinone concentrations declined 82% with dietary phylloquinone restriction ($P < 0.05$) and were not significantly different in the period when the diet was combined with omeprazole treatment ($P > 0.05$). The mean value for PIVKA-II during the phylloquinone-restricted diet significantly increased 5.7-fold from the baseline ($P < 0.05$); however, the combination of omeprazole treatment and the phylloquinone-restricted diet significantly reduced PIVKA-II values by 21% ($P < 0.05$) compared with the diet period alone. There were no alterations in total or percentage ucOC concentrations during the phylloquinone-restricted diet or during the period of diet plus omeprazole treatment. The authors stated that their study data supports the hypothesis that bacterial overgrowth results in the synthesis and absorption of menaquinones. These menaquinones contribute to vitamin K nutriture during dietary phylloquinone restriction, but not in sufficient quantity to restore normal vitamin K status [Paiva, *et al.*, 1998 Vol 15 Tab 324].

Camilo *et al.*, 1988 described the interaction between vitamin K nutriture and warfarin administration in patients with bacterial overgrowth due to atrophic gastritis. Atrophic gastritis patients have intestinal bacterial overgrowth which could produce menaquinones. The purpose of the author's study was to evaluate the interaction between a diet low in phylloquinone and minidoses of warfarin in subjects with and without bacterial overgrowth. Subjects with atrophic gastritis (indicated by serum

pepsinogen ratio) and healthy volunteers were studied while fed a restrictive phylloquinone diet and while receiving a minidose of warfarin. Coagulation times, serum osteocalcin, serum undercarboxylated osteocalcin, plasma phylloquinone, plasma K-epoxide, plasma undercarboxylated prothrombin (PIVKA II) and urinary gamma-carboxyglutamic acid (Gla) were measured. At baseline, there were no differences between the groups for any of the variables measured. Comparisons between baseline and post intervention in both groups, showed significant increases in circulating levels of K-epoxide, PIVKA II, and undercarboxylated osteocalcin. However, no differences were observed when comparisons were made between groups. The authors concluded that their data did not support the hypothesis that bacterial synthesis of menaquinones in patients with bacterial overgrowth due to atrophic gastritis conferred resistance to the effect of warfarin [Camilo *et al.*, 1988 Vol 15 Tab 324].

Another group of researchers lead by Bach, *et al.,* 1996 from the University of Wisconsin assessed vitamin K status in human subjects administered "minidose" warfarin. The authors stated that vitamin K is required to convert specific glutamyl residues in a limited number of proteins to gamma-carboxyglutamyl residues. The response of various markers of vitamin K insufficiency to the administration of 1 mg/day of the vitamin K antagonist warfarin was studied in two groups of nine older (55-75 y) or younger (20-28 y) subjects. The most consistent and extensive effects observed were an increase in the concentration of serum under-gamma-carboxylated osteocalcin followed by an increase in plasma under-gamma-carboxylated prothrombin (PIVKA-II), and then by a decrease in urinary excretion of gamma-carboxyglutamic acid. Plasma concentrations of prothrombin were altered by the administration of warfarin, but prothrombin times, factor VII activity, prothrombin F-1 x 2 concentrations, and one less sensitive assay for under-gamma-carboxylated prothrombine

were not. The concentration of serum under-gamma-carboxylated osteocalcin was found to be reduced when subjects consumed 1 mg vitamin K/day than when they consumed their normal diet [Bach, *et al.,* 1996 Vol 12 Tab 234].

Kamali, *et al.,* 2000 studied the influence of of two different types of warfarin ((R) and (S)), vitamin K and vitamin K epoxide upon warfarin anticoagulation. The contribution of (R)- and (S)-warfarin enantiomers, vitamin K and vitamin K epoxide and patient factors to inter-individual variability in daily warfarin requirements were examined in a group of 73 patients. A simple correlation analysis showed that there was a significant positive relationship between individual requirements and plasma (S)-warfarin concentrations ($r = 0.25$; $p = 0.038$). Multivariate analysis for relationships with individual requirements demonstrated a highly significant positive relationship between those requirements, (S)-warfarin ($p = 0.004$) and plasma vitamin K epoxide concentrations ($p = 0.028$), and a significant negative relationship between individual requirements and plasma vitamin K concentrations ($p = 0.034$). Twenty five percent of the inter-individual variation in could be explained by these variables (adjusted $R2 = 0.25$). Correlation analysis of data showed that warfarin dosage was significantly and negatively correlated with patient age ($r = -0.42$; $p < 0.0001$). Patient age accounted for 25% of variation in warfarin dosage requirements ($R2 = 0.25$). The authors concluded that the combined effects of age and vitamin K appear to account for much of the inter-individual variability in warfarin dosage requirements [Kamali, *et al.,* 2000 Vol 14 Tab 288].

Another research group at the University of Wisconsin studied Vitamin K deficiency as it occurred from dietary vitamin K restriction in humans [Suttie, *et al.,* 1988 Vol 16 Tab 356]. The authors stated that Vitamin K is required for the maintenance of normal hemostatic function. In their study, they selected ten

college-aged male subjects who were fed diets restricted in vitamin K content for 40 d. Median phylloquinone intakes based on analysis of food composites dropped from 82 micrograms/d during the prestudy period to 40 and 32 micrograms/d after days 9 and 27 of dietary restriction. Serum phylloquinone concentrations fell from a mean of 0.87 to 0.46 ng/mL during the first 21days of vitamin K restriction. When the subjects received a supplement of 50 micrograms of phylloquinone/day for 12 days, serum phylloquinone rose to 0.56 ng/mL, and supplementation with 500 micrograms of phylloquinone/day increased serum phylloquinone to 1.66 ng/mL (about twice the daily requirement). The results of the study showed that Vitamin K restriction resulted in alterations in a functional clotting assay that detects undercarboxylated prothrombin species in plasma and in a decrease in urinary gamma-carboxyglutamic acid. Supplementation with either 50 or 500 micrograms of phylloquinone restored both these markers to near normal values. The conclusion was that these data were consistent with a human dietary vitamin K requirement of approximately 1 microgram/kg body weight per day.

Booth, *et al.,* 1999 of the Jean Mayer US Department of Agriculture Human Nutrition Research Center on Aging at Tufts University, Boston, Massachusetts looked at age and the response of vitamin K status to different intakes and sources of phylloquinone-rich foods. Phylloquinone, found in dark-green vegetables and certain plant oils, is the primary dietary source of the fat-soluble vitamin K. Limited data suggest that the relative bioavailability of phylloquinone from vegetables is lower than that from a supplement. This finding is relevant to the maintenance of optimal vitamin K status. The objective of their study was to compare, in younger and older adults, the relative bioavailability of phylloquinone from a vegetable with that of a fortified oil. In a crossover design with three 15-day residency periods in a metabolic unit, younger and older men and women

(n = 36) consumed a mixed diet containing 100 micrograms phylloquinone/day. During 2 residency periods, the mixed diet was supplemented for 5 days with either broccoli (377 micrograms phylloquinone/day; broccoli diet) or phylloquinone-fortified oil (417 micrograms/day; oil diet). The relative bioavailability of phylloquinone was defined by the difference in plasma phylloquinone, percentage serum undercarboxylated osteocalcin (%ucOC), and urinary gamma-carboxyglutamic acid in response to 5 days of supplementation. For both younger and older adults, plasma phylloquinone concentrations were higher ($P < 0.001$) and %ucOC values were lower ($P = 0.001$) after the broccoli and oil diets than after the mixed diet only. Overall, the response to broccoli supplementation was not significantly different from the response to the fortified oil in either age group. Urinary gamma-carboxyglutamic acid did not change in response to supplementation. The authors concluded that there was no significant difference in the relative bioavailability of phylloquinone, as evidenced by the lack of a significant difference in plasma phylloquinone and %ucOC between the 2 groups after either the broccoli or oil diets for younger and older adults [Booth, *et al.*, 1999 Vol 12 Tab 240].

Taken together, these studies indicate that many individuals in the US do not get enough vitamin K in their diets, that the addition of a fiber, particularly chitosan to the diet increases bacterial fermentation in the gut, and therefore the production of vitamin K. Humans with bacterial overgrowth in the gut have not been shown to negate the effect of warfarin. Warfarin dosages have been shown to vary with vitamin K status and with age. Therefore, individuals taking warfarin with any fiber should, as advised by Fairfield, *et al.*, discuss their dietary choices with a medical care giver [Fairfield, *et al.*, 2002 Vol 13 Tab 260].

F. Human Feeding Studies

In addition to providing responses to CFSAN's specific questions regarding chitosan consumption and nutritional status, this report reviews several pre-clinical and clinical studies that have been preformed to examine the safety of chitosan ingestion in various species including man. Twelve human studies published in peer-reviewed journals are discussed below, including several with placebo controlled double blind designs. Results of these studies are summarized in Table 28 and else where in the text.

A number of published toxicity assessments on chitosan have been conducted within the context of biomedical applications. Where appropriate, data from those studies has been included in separate sections to support the conclusion that chitosan does no harm to body tissues or processes. The totality of the published animal and human studies on chitosan suggest that there are no significant or consistent untoward effects attributable to its consumption, even when the substance is consumed at levels equivalent to several percent of the diet.

Both chitosan, and its raw material, chitin are biocompatible and are found indigenously in the cell walls of various fungi, crustaceans and insects [Illum, 1998 Vol 5 Tab 61]. Chemically, chitosan, a cationic polysaccharide, is the N-deacetylated product of chitin. The structure of chitosan is mainly that of a polymer of D-glucosamine and N-acetyl-D-glucosamine [Aspden, *et al.,* 1997a Vol 3 Tab 10]. Both chitin and chitosan are considered to be nitrogenous polysaccharides. The structure of the chitin molecule is similar to that of cellulose, but it is composed of units of 2-acetylamino-2-deoxy-D-glucopyranose, while chitosan is composed of ß (1 → 4)-2-amino-2-deoxy—D-glucopyranose and -acetylamino-2-deoxy -D-glucopyranose bound [1 → 4] by glycosidic bonds [Chobot, *et al.,* 1995 Vol 3 Tab 22]. These polysaccharides occur particularly in the fungal kingdom, in

invertebrate animals, and to a small degree in higher plants [Chobot, *et al.,* 1995 Vol 3 Tab 22].

Chitosan can also be chemically derived by deacetylating its raw material, chitin. Commercially, chitin is obtained from the cuticles of sea animals, which have been discarded as waste products from food industry. The technologies for obtaining chitin from lower fungi [e.g. the genus Aspergillus] have been elucidated [Chobot, *et al.,* 1995 Vol 3 Tab 22], but they are not considered economically viable.

The term chitosan refers to a family of polymers whose members differ in their degree of deacetylation (i.e., ratio of N-acetylated to N-deacetylated subunits) and molecular weight. These two characteristics are fundamental to the physicochemical properties of the chitosans and therefore have considerable influence on their biological activities [Aspden, *et al.,* 1997a Vol 3 Tab 10].

Because of the high cost of producing chitosan, it has not been historically used as a food or food ingredient. However, modern humans, continue to consume small quantities of chitosan as part of a normal diet. Natural sources of chitosan in the diet include various fungi and animals [Illum, 1998 Vol 5 Tab 61]. Both chitin and chitosan are inherent to a large extent in food fungi, e.g. *Pleurotus ostreatus* [Chobot, *et al.,* 1995 Vol 3 Tab 22]. Knowledge of ancient diets suggests that the amount of chitosan consumed by our ancestors was much greater than at present. However, the presence in the human intestinal system of certain enzymes that specifically cleave chitosan into its component chemical units (chitinase) strongly suggests that humans have the capacity to safely consume chitosan at much greater concentrations than are common in a Western diet [Muzzarelli, 1997, Vol 7 Tab 104]. The fact that the human body has evolved physiological processes to metabolize chitosan, the results from several recent animal toxicology studies, and a history of commercial consumption of chitosan in Japan, convincingly support the position that the consumption of chitosan as a macronutrient would pose no risk to the consumer.

Over the last decade, coinciding with the commercial production of higher purity chitosans, much interest and experimental activity has centered on the use of such chitosans in a great diversity of novel food and biomedical applications. Chitosans have been found to have many beneficial effects, including immunostimulation, cholesterol reduction, anticoagulant (e.g. sulfated derivatives) and hemostatic activities, and wound-healing and anti-microbial properties [Aspden, *et al.,* 1997a Vol 3 Tab 10]. All of these properties have been found to be based on the natural chemistry and structure of the material.

1. Human Safety

Overweight and obesity is a prevalent and costly threat to public health. Compelling evidence links overweight and obesity with serious disorders such as cardiovascular diseases and diabetes. However, dietary regimens are notoriously burdened with poor compliance. Chitosan is promoted in the US and other countries as an oral remedy to reduce fat absorption and has now been incorporated as a major constituent into several dietary supplements. For these reasons, several studies have recently been published, which have investigated the effect of chitosan on weight and cholesterol reduction in humans.

A number of recent human studies have examined the effect of daily doses of 1.0 to 6.0 grams of chitosan, when taken as a dietary supplement. These studies have measured important physiological parameters related to vitamin and mineral balance. No significant untoward effects were noted in over 310 individuals tested, even when chitosan was fed for periods of up to four weeks.

Table 28 summarizes the effects of chitosan on both healthy overweight individuals, and those with compromised health conditions. Interestingly, a group of human studies conducted

in Italy utilized the same source of chitosan as a supplement to the diet. The studies differed in their caloric restrictions, from no dietary restriction to a 1000 calorie/day diet. In those studies, volunteers consumed from 1.0 to 1.6 grams of chitosan per day (see below). Results of the several studies showed that chitosan intake did not alter mineral or hematological balances in the human subjects as compared to the controls. The authors also concluded that chitosan was more effective at contributing to weight loss, when accompanied by a low-calorie diet.

The effects of chitosan were investigated in eighty patients with renal failure undergoing long-term hemodialysis treatment. The patients were tested after a control treatment period of 1 week. Half were fed 30 chitosan tablets [45 mg chitosan/ tablet] three times a day (a total of 1.350 g/day). Ingestion of chitosan effectively reduced total serum cholesterol levels [from 10. 14 ± 4.40 to 5.82 ± 2.19 mM] and increased serum hemoglobin levels [from 58.2 ± 12.1 to 68 ± 9.0 g/L]. Significant reductions in urea and creatinine levels in serum were observed after 4 weeks of chitosan ingestion. The feeling of physical strength, the appetite and the sleep of patients in the treatment group had improved significantly after 12 weeks of ingestion, compared with those of patients in the control group. During the treatment period, no clinically problematic symptoms were observed. These data suggest that chitosan might be effective in reducing hypercholesteremia for renal failure patients, although the authors stated that the mechanism of the effect should be investigated further [Jing, *et al.*, 1997 Vol 5 Tab 67].

Section IV Detailed Summary of the Basis for the Determination that Primex Chitosan is GRAS

Table 28: Effect of Chitosan on Human Subjects

SUBJECTS	DOSAGE of CHITOSAN	DURATION	EFFECT	REFERENCE
40 Humans with Renal Failure	10 (45 mg) Tablets 3 X per day	12 weeks	Reduced Serum cholesterol 42.6% and Increased Serum Hemoglobin Levels 16.8%; No Problematic Symptoms Were Observed	Jing. *et al.*, 1997
Human Males; 8 Volunteers; Ages 20-23	3-6 g/day	2 weeks	Decrease in serum cholesterol Of 6.4% and 9.8% increase in HDL	Maezaki, *et al.*, 1993
15 Over weight Human Volunteers; Ages 18-60	1.0 Gram/day with Unrestricted Diet	28 Days	No change in BMI, serum cholesterol, triglycerides, Vitamins A, D, E, or β carotene. Vitamin K was significantly increased. No serious adverse effects were reported	Pittler *et al.*, 1999
51 Healthy Obese Women	1.2 grams/day of Microcrystalline Chitosan with no change in eating habits	8 Weeks	Chitosan was well-tolerated and no serious adverse events or changes in safety parameters, including fat-soluble vitamins A and E, and serum iron and transferrin were noted.	Wuolijoki, *et al.*, 1999
20 Obese Subjects; Ages 30-80	4 Chitosan Tablets per day (1.0 g twice daily) With and Without Dietary Restriction; Low calorie diet was 1200 calories	One Month	Tolerance was good, no side effects or abnormal blood chemistry were reported; Some constipation reported; administration of laxatives was rarely required	Macchi, 1996
50 Obese Patients; Ages 26-65	4 Tablets per day of Chitosan With Low (1100) Calorie Diet	4 Weeks	Adverse Effects Mild and transient; Not significantly different from controls, 4.2% with Chitosan, 6.4% with placebo	Giustina, *et al.*, 1995
45 Obese Subjects; Ages 25-70; Mean 46.7 ± 13.2	4 Tablets per day of Chitosan (1600 mg) in 2 doses; With Low Calorie Diet	4 Weeks	Mild and Transient Effects in 2.3% of Chitosan Subjects; Not Statistically Different from the Control	Sciutto, *et al.*, 1995
40 Subjects; Ages 32-70; Mean 52.4 ± 16.7	4 Tablets; 1600 mg Chitosan divided into 2 doses; With 1000 cal. diet	4 Weeks	Adverse effects (2.6%) were mild and transient nausea; Compared to constipation (5.4%) with placebo; No pathological or clinical change in blood chemistry or hematological data	Veneroni, 1996
7 Healthy Men; Arterial Blood	5 grams of Chitosan Powder	Single Dose	No Adverse Effects Reported; Chitosan Prevented Blood Pressure increase following a high-salt meal	Kato, *et al.*, 1994
43 Obese Subjects; Ages 30-69; Mean 48.3 ± 12.5	4 Tablets per day (1600 mg) in 2 doses; of Chitosan; With Low Calorie Diet	4 Weeks	Mild and Transient Adverse Effects in 5% of the Chitosan Subjects (flatulence); Not statistically different from the controls.	Colombo, *et al.*, 1995

000219

More recently, the primary aim of a study by Pittler, *et al.,* 1999 was to investigate the clinical effectiveness of oral chitosan for body weight reduction. Thirty-four overweight volunteers were included in a randomized placebo-controlled double-blind trial. Subjects were assigned to receive either four capsules of chitosan or indistinguishable placebo twice daily for 28 consecutive days. Measurements were taken at baseline, and after 14, and 28 days of treatment. Subjects maintained their normal diet and documented the type and amount of food consumed. Adverse effects were assessed and compliance was monitored. Data from 30 subjects were entered into an intention-to-treat analysis. After four weeks of treatment, body mass index, serum cholesterol, triglycerides, vitamins A, D, E and β-carotene were not significantly different in subjects receiving chitosan compared to those receiving placebo. Vitamin K was significantly increased after four weeks in the chitosan group compared with the placebo group [P<0.05]. Compliance was 91.5% and 96.0% for the chitosan and placebo groups, respectively. In conclusion, the data suggested that chitosan in the administered dosage, but without dietary alterations, did not reduce body weight in overweight subjects. No serious adverse effects were reported [Pittler, *et al.,* 1999 Vol 7 Tab 117].

Peroral microcrystalline chitosan [MCCh; 3 capsules, each 400 mg b.i.d.] or placebo was given for 8 weeks in a double-blind manner to 51 healthy obese women just before routine hospital and home meals. Weight records, serum lipids [total, LDL and HDL cholesterol, triglycerides] and safety laboratory parameters were monitored before the trial and at 4, 6 and 8 weeks of treatment. In a subgroup of subjects with a body mass index > or = 30, who had not changed their eating habits, serum LDL cholesterol decreased 0.57 ± 0.72 mmol/l [n = 11] at 4 weeks in the MCCh group and 0.10 ± 0.60 mmol/l [n = 14] in the placebo group [p < 0.05]. At 8 weeks, LDL cholesterol reduction was 0.48 ± 0.91 mmol/l in the MCCh group and 0.26 ± 0.57 mmol/l in the placebo group [p > 0.1]. In all subjects, the reduction in LDL cholesterol at 4 weeks was 0.48 +/- 0.72 mmol/l [n = 24], in

MCCh subjects, and 0.18 ± 0.58 mmol/l [n = 27] in placebo subjects [p = 0.057], and 0.52 ± 0.69 mmol/l and 0.31 ± 0.63 mmol/l, respectively, at 8 weeks [p > 0.1]. MCCh did not significantly alter serum total and HDL cholesterol [p > 0.1], but slightly increased serum triglycerides compared to placebo [p = 0.015- 0.06]. No reductions in weight were observed in any treatment group. Chitosan was well tolerated and no serious adverse events or changes in safety laboratory parameters were noted including serum fat-soluble vitamins A and E, and serum Fe++ and transferrin [Wuolijoki, *et al.*, 1999 Vol 11 Tab 221].

A very important trial was conducted to determine the weight loss and the lipid-lowering effects obtained with a new chitosan dietary fiber (a mixture of chitosan, guar's meal, ascorbic acid and other micronutrients) and a caloric restriction in a weight-reducing program for 90 obese subjects [Colombo, *et al.*, 1996 Vol 3 Tab 24]. In the randomized, double blind, placebo controlled trial, subjects were treated with a hypocaloric diet plus 4 tablets/day of chitosan dietary fiber or with a hypocaloric diet plus 4 tablets/day of placebo for 4 weeks. The chitosan content of each tablet was 400 mg.

The subjects in the study discussed above consisted of 90 adult patients (age range from 25 to 70 years; mean 46.7 + 13.2 years) of both sexes (45 men and 45 women). Inclusion criteria included ambulatory subjects aged between 20 an 70 years, mild obesity (overweight between 10% and 25% as regards the normality weight-height tables), mild hypertension, and hyperlipoproteinemia. Exclusion criteria included patients with known or presumed hypersensitivity to any component of the supplement (active substances and excipients); those who did not guarantee total adherence to the trial protocol; and patients with severe hepatic or gastrointestinal diseases, renal insufficiency, severe chronic disease, pregnancy, or concomitant administration of drugs that could interfere with the results of the study. If these drugs were taken previously, they were stopped 30 days before the new treatment schedule.

The patients were randomized (random number tables) into two groups containing the same number of subjects [Colombo, *et al.*, 1996 Vol 3 Tab 24]. The groups were normalized for sex composition, mean age, body weight, body height, and percentage of overweight, systolic and diastolic pressures, arterial pressure, serum total cholesterol, HDL cholesterol, LDL cholesterol and triglycerides. No significant differences using Chi-square and Student's t-test appeared between the treatment groups [Colombo, *et al.*, 1996 Vol 3 Tab 24].

The study showed a statistically significant reduction in body weight, overweight, arterial systolic and diastolic pressure, total and LDL cholesterol, and triglycerides and an augmentation of HDL cholesterol in both the groups. However, with the diet, which included chitosan, the variations were statistically greater than in the placebo group.

Body weight reduction was 7.19 kg in the chitosan group and 3.36 kg in the placebo group, overweight reduction was 1.45% in the chitosan group and 3.8% in the placebo group. Arterial systolic pressure reduction was 12.1 mm Hg in the chitosan group and 2.8 mm Hg in the placebo group.
Arterial diastolic pressure reduction was 12.4 mm Hg in the chitosan group and 3.8 mm Hg in the placebo group. Total cholesterol reduction was 25.3% in the chitosan group and 11.1% in the placebo group. LDL cholesterol reduction was 32.1% in the chitosan group and 13.6% in the placebo group. HDL cholesterol augmentation was 11.8% in the chitosan group and 4.6% in the placebo group.

Mild and transient side effects were reported in 2.3% of the chitosan treated subjects (nausea) and in 6.8% of the subjects in the placebo group (nausea or constipation), without any statistically significant difference between the two groups. No pathologic or clinically

significant changes in blood chemistry or hematological assay were observed.

The authors concluded that the data from this study suggested that diet plus a chitosan dietary fiber was a very useful treatment for overweight in obese subjects, and that their weight loss was associated with an evident reduction in hypertension and hyperlipoproteinemia [Colombo, *et al.*, 1996 Vol 3 Tab 24]. Significantly, subjects consuming chitosan maintained normal mineral and hematological parameters throughout the trial (See Tables 29 and 30 below).

Section IV Detailed Summary of the Basis for the Determination that Primex Chitosan is GRAS

Table 29
Effect of Chitosan on Mineral Balance in Humans

Treatment	Parameter	Normal Val.	Before	After	Statistics*
Diet + Chitosan	Sodium (meq/L)	137-145	140.16±15.21	140.14±15.18	N.S.**
	Potassium (meq/L)	2.7-3.9	3.12±0.29	3.13±0.30	N.S.**
	Calcium (meq/L)	2.1-3.0	2.66±0.32	2.61±0.31	N.S.**
	Magnesium (meq/L)	2.0-2.5	2.31±0.27	2.30±0.28	N.S.**
	Zinc (Hg/dL)	75-120	94.06±9.11	92.78±9.51	N.S.**
	Iron (Hg/dL)	80-180	101.68±11.67	100.44±10.62	N.S.**
	Copper (Hg/dL)	70-140	106.11±9.41	102.57±10.55	N.S.**
	Vitamin A (Hg/dL)	20-100	55.32±6.83	51.40±7.02	N.S.**
	Vitamin D (nmol/L)	20-100	63.59±7.21	60.64±7.02	N.S.**
	Vitamin E ((Hg/dL)	1.5-4	2.67±0.31	2.53±0.42	N.S.**
Diet + Placebo	Sodium (meq/L)	137-145	142.33±15.75	141.62±16.37	N.S.**
	Potassium (meq/L)	2.7-3.9	3.19±0.28	3.18±0.29	N.S.**
	Calcium (meq/L)	2.1-3.0	2.64±0.34	2.63±0.33	N.S.**
	Magnesium (meq/L)	2.0-2.5	2.32±0.29	2.31±0.29	N.S.**
	Zinc (Hg/dL)	75-120	93.15±10.06	91.22±9.22	N.S.**
	Iron (Hg/dL)	80-180	102.78±11.29	100.03±11.96	N.S.**
	Copper (Hg/dL)	70-140	113.52±10.96	111.029.45	N.S.**
	Vitamin A (Hg/dL)	20-100	58.21±6.76	56.326.31	N.S.**
	Vitamin D (nmol/L)	20-100	62.67±6.93	64.287.61	N.S.**
	Vitamin E ((Hg/dL)	1.5-4	2.74±0.36	2.700.39	N.S.**

*Comparison before-after with Student's t-test
** N.S. = difference not significant

Section IV Detailed Summary of the Basis for the Determination that Primex Chitosan is GRAS

Table 30
Effect of Chitosan on Hematological Parameters

Treatment	Parameter	Normal val.	Before	After	Statistics
Diet + Chitosan	Hemoglobin (g/dL)	12-18	14.02±1.33	14.14±1.82	N.S.**
	Hematocrit (%)	38-54	42.86±5.16	42.36±5.66	N.S.**
	Leukocytes (no.)	4500-11000	8930±1920	9210±2150	N.S.**
	Erythrocytes (millions/ml)	4.2-6.2	4.66±0.51	4.69±0.61	N.S.**
	Glucose (mg/dL)	70-110	97.4±93.52	95.22±9.64	N.S.**
	Urea nitrogen (mg/dL)	8-23	20.11±2.94	19.23±2.87	N.S.**
	Creatine (mg/dL)	0.6-1.2	0.92±0.33	0.95±0.31	N.S.**
	Bilirubin (mg/dL)	0.1-1.2	0.58±0.36	0.56±0.30	N.S.**
	AST (U/L)	5-30	22.06±3.28	24.39±3.46	N.S.**
	ALT (U/L)	5-35	26.61±3.07	25.44±3.19	N.S.**
	Gamma-GT (U/L)	5-50	21.17±2.54	20.72±2.77	N.S.**
Diet + Placebo	Hemoglobin (g/dL)	12-18	14.21±1.92	14.17±1.78	N.S.**
	Hematocrit (%)	38-54	42.81±6.02	43.02±5.56	N.S.**
	Leukocytes (no.)	4500-11000	7790±2420	8760±2210	N.S.**
	Erythrocytes (millions/ml)	4.2-6.2	4.45±0.50	4.52±0.55	N.S.**
	Glucose (mg/dL)	70-110	94.08±10.12	96.71±9.66	N.S.**
	Urea nitrogen (mg/dL)	8-23	19.65±2.71	19.74±2.59	N.S.**
	Creatine (mg/dL)	0.6-1.2	0.91±0.36	0.93±0.32	N.S.**
	Bilirubin (mg/dL)	0.1-1.2	0.55±0.24	0.57±0.28	N.S.**
	AST (U/L)	5-30	26.323.77	23.11±3.86	N.S.**
	ALT (U/L)	5-35	28.343.06	26.78±3.11	N.S.**
	Gamma-GT (U/L)	5-50	22.422.79	21.56±2.82	N.S.**

*Comparison before-after with student's t-test.
** N.S. = difference not significant.

000225

The effects of the introduction of the same chitosan dietary fiber preparation were tested over a four-week weight-reducing regimen for the treatment of obesity in a randomized, double blind, placebo controlled trial performed with 100 obese subjects. Group 1 (50 patients) was treated with a low caloric diet plus 4 tablets/day of placebo. Group 2 (50 patients) was treated with a low caloric diet plus 4 tablets/day of the chitosan preparation. Each tablet contained 400 mg of chitosan [Guistina, *et al.,* 1995 Vol 4 Tab 43].

The subjects in this trial were informed verbally and in writing about their rights, the aims and possible risks of the study, and each gave written consent. 100 adult patients (age range from 26 to 65 years; mean 43.9 =12.1 years) of both sexes (54 men and 46 women) were admitted to the trial.

The groups were normalized for sex composition, mean age, body weight, body height, percentage of overweight, systolic and diastolic pressures, arterial pressure, and heart and respiratory rate. No significant differences were calculated, using Chi-square and Student's t-test, between the treatment groups [Guistina, *et al.,* 1995 Vol 4 Tab 43]. The dose was two tablets twice a day, at the main meals (lunch and dinner) for four weeks. The weight-reducing regimen was a low caloric diet of about 1000-1100 kcal based on 34% fats, 41% carbohydrates and 25% proteins [Guistina, *et al.,* 1995 Vol 4 Tab 43].

At the end of the study Guistina observed a statistically significant reduction in body weight, overweight, arterial, systolic, and diastolic pressures, and respiratory rate in both the groups. In the chitosan treated subjects, the reductions were statistically greater than in placebo group. Body weight decreased from 83.6 Kg to 76.3 Kg in the chitosan group and from 82.3 to 79.3 kg in the placebo group. Overweight decreased from 17.2% to 7.3% in the chitosan group, and from 16.3% to 12.4% in the placebo group. Arterial systolic

pressure decreased from 145.3 mm Hg to 135.8 mm Hg in the chitosan group and from 146 to 142.9 in the placebo group. Arterial systolic pressure dropped from 92.6 mm Hg in the chitosan group to 84.2 mm Hg, and from 92.5 to 90.0 in the placebo group. Respiratory rate dropped from 27.6/min to 21.2/min in the chitosan group, and from 28.1/min to 26.3/min in the placebo group. [Guistina, *et al.,* 1995 Vol 4 Tab 43].

Adverse effects (always mild and transient) were reported in very few patients (4.2% in the chitosan treated group and 6.4% in the placebo group, without any statistically significant difference between the two groups). On the basis of what the authors considered to be a good level of statistical confidence in the clinical evidence, diet plus a chitosan dietary fiber was found to be very useful for the treatment of obesity and the secondary disorders of hypertension dyspnea. The authors concluded that chitosan in addition to a low caloric diet was more effective than a low caloric diet alone [Guistina, *et al.,* 1995 Vol 4 Tab 43].

Inclusion criteria involved ambulatory subjects with ages between 20 and 70 years, mildly obese subjects (overweight between10% to 25% in comparison to normality weigh-height tables), and subjects with mild hypertension in the study. Exclusion criteria were eliminated patients with known or presumed hypersensitivity to any component of the supplement (active substances or excipients); subjects who did not guarantee a total adherence to the trial protocol; and those with severe hepatic or gastrointestinal diseases, renal insufficiency, severe chronic disease, pregnancy, and concomitant administration of drugs that could interfere with the results of the study. If these drugs were taken previously they were discontinued 30 days before the new treatment schedule. The subjects were randomized (random number tables) into two groups containing the same number of subjects [Guistina, *et al.,* 1995 Vol 4 Tab 43].

Macchi, 1996, indicated that it was common knowledge that diabetes increases the risk of premature death, hyperlipidemia, arterial blood hypertension and ischemic heart diseases. Both for medical and aesthetic considerations, the need for safe and natural methods to improve the weight reducing process was obvious in particular if the substance enhanced the loss of body fat with a saving of muscular mass [Macchi, 1996 Vol 6 Tab 96]. Macchi stated that a new chitosan dietary fiber (CT), which was indigestible, and had a very low toxicity profile had been demonstrated to have antihyperlipidemic effects. The fiber appeared to interfere with fat absorption. After ingestion CT was solubilized in the acid environment of the stomach, where it entrapped fatty acids by means of ionic bonding, and neutral fats by means of hydrophobic forces, thereby decreasing their intestinal absorption [Macchi, 1996 Vol 6 Tab 96].

Macchi, 1996 conducted a study, whose purpose to evaluate the effects of CT associated with a low calorie diet or with a habitual free diet, compared to placebo (P), on body weight (BW), body mass index (BMI), body fat (BF), skinfolds (S), arterial blood pressure (BP) and blood chemistry analysis. Thirty obese subjects were studied in a randomized double blind controlled trial for a period of one month. Group A was treated with 4 tablets/day of CT plus a 1200 calorie diet; group B with the same hypocaloric diet and 4 tablets/day of placebo; group C with 4tablets /day of CT without dietary restriction. No side effects or abnormal modifications of blood chemistry analysis were noted at the end of the study. Subjects taking CT appeared satisfied with the treatment, but some complained of constipation. The study showed a statistically significant ($p<0.001$) reduction of BW, BMI, BF, S in all the groups, but it was greatest in group A (BW= - 4Kg; BMI= -1/7 Kg/cm; BF= - 4%; S= -17 mm in group A: BW= -2.6Kg; BMI = -0.6 Kg/cm; BF= -1.2%; S= -5 mm in group B; BW = -2.8 Kg; BMI= -1.1 Kg/cm; BF = -3.3%; S = -11 mm in group C).

000228

The investigators considered it worth emphasizing, that even if BW reduction was similar in groups B and C, subjects treated with CT

(group C) showed a statistically significantly greater skinfold decrease. It can be argued that subjects treated with CT (alone or in association with a hypocaloric diet) experienced a greater loss of adipose tissue in comparison to subjects only on diet restriction [Macchi, 1996 Vol 6 Tab 96].

Diastolic blood pressure, significantly decreased in groups A (-7 mm Hg) and C (-9 mm Hg) (p<0,001), but not in group B (-1 mm Hg n.s.). The authors hypothesized that the greater loss in BF may be responsible for the effect [Macchi, 1996 Vol 6 Tab 96].

Plasma cholesterol and triglyceride levels decreased significantly (p<0.01) in all groups (-26 mg/dl and - 27mg/dl in group A; -15 mg/dl and -26 mg/dl in group B; -26mg/dl and-27mg/dl in group C), but there was a significant increase in HDL-cholesterol (=11mg/dl, p <0,001) only in group C.

In conclusion Macchi's study demonstrated that the dietary fiber CT was useful in the treatment of obesity, because it enhanced the loss of body fat and acted to normalize plasma cholesterol and triglycerides levels, perhaps with a concomitant increase in HDL-cholesterol. Greater effects were obtained when CT was associated with a hypocaloric diet [Macchi, 1996 Vol 6 Tab 96].

In another randomized, double-blind, placebo-controlled trial body weight loss and lipid-lowering effects obtained with a caloric restriction and the same dietary fiber (a mixture of chitosan, guar's meal, ascorbic acid and others micronutrients) as used by diet Macchi, 1996 and Colombo, 1996 were investigated in a weight-reducing program for 80 obese adult subjects with hyperlipidemia [Veneroni, *et al.,* 1996 Vol 11 Tab 214]. The subjects were treated with a hypocaloric diet (1000 calories) plus 4 tablets/ day of chitosan dietary fiber (1.6 g) or with a hypocaloric diet plus 4 tablets/ day of a placebo for 4 weeks. At the end of the study period a statistically significant reduction in body weight and overweight, triglycerides and

total LDL cholesterol, and an augmentation of HDL cholesterol were observed in both groups but in the chitosan treated group the differences were substantially greater than in placebo group [Veneroni, *et al.,* 1996 Vol 11 Tab 214].

Body weight reduction was 7.9 Kg in the chitosan group and 3.4 Kg in the placebo group, overweight reduction was 11.2% in the chitosan group and 4.2% placebo group, total cholesterol reduction was 23.9% in the chitosan group, and 10.4% in the placebo group, LDL cholesterol reduction was 33.4% in the chitosan group and 12.1% in the placebo group, triglyceride reduction was 23.5% in the chitosan group and 9.3% in the placebo group. HDL augmentation was 10.2% in the chitosan group and 3.5% in the placebo group.

Adverse events were reported in 2.6% of the chitosan treated subjects (mild and transitory nausea) and in 5.4% of the subjects in the placebo group (constipation) without any statistically significant difference between the two groups [Veneroni, *et al.,* 1996 Vol 11 Tab 214].

No pathologic or clinically significant changes in blood chemistry or hematological data were observed. From the results obtained in the study, the diet plus a chitosan dietary fiber appeared to be a useful treatment (more effective than diet alone) of the overweight and hyperlipidemia obese subjects [Veneroni, *et al.,* 1996 Vol 11 Tab 214].

80 adult patients (age ranging from 32-70 years mean; 52.4 ± 16.7 years) of both sexes (37 men and 43 women) were admitted to the trial. Inclusion criteria included subjects who filled the following criteria: ambulatory, age between 20 and 70 years, mildly obese (overweight between 10% to 25% as compared to normality weight-height tables) and hyperlipidemic. Exclusion criteria included subjects with known or presumed hypersensitivity to any component of the supplement (active substances or excipients), subjects who did not

guarantee total adherence to the trial protocol, severe hepatic or gastrointestinal diseases, renal insufficiency, severe chronic disease, pregnancy, concomitant administration of drugs that could interfere with the results of the study. If these drugs were taken previously they were stopped 30 days before the new treatment schedule [Veneroni, *et al.,* 1996 Vol 11 Tab 214].

A trial was conducted to evaluate the efficiency and tolerability of the same chitosan dietary fiber tested by Veneroni, 1995 and Macchi, 1996 (a mixture of chitosan, Guar's meal, ascorbic acid and others micronutrients) in association with a low caloric diet in a weight-reducing program for 86 obese subjects. In the randomized, double blind, placebo controlled trial, subjects were treated with the hypocaloric diet plus 4 tablet/day of chitosan dietary fiber (1.6 g/d) or with a hypocaloric diet plus 4 tablets/day of placebo for 4 weeks [Sciutto, *et al.,* 1995 Vol 8 Tab 141].

At the end of the study a statistically significant reduction in body weight, overweight, total and LDL cholesterol, and triglycerides was observed in both groups. An increase in HDL cholesterol in the group treated with diet + chitosan was also observed, but in diet + chitosan group the variations were statistically greater than in diet + placebo group.

In order to verify the possible interference of chitosan with intestinal absorption of mineral salts or fat-soluble vitamins the authors determined blood concentrations of sodium, potassium, calcium, magnesium, zinc, iron, copper, and vitamins A, D, and E.

No significant changes were noted in blood concentrations of these substances after the treatment and there was no difference between the two treatment groups [Sciutto, *et al.,* 1995 Vol 8 Tab 141].

Mild and transient adverse effects were reported in 5% of the chitosan-treated subjects (flatulence) and in 18.4% of subjects in the

placebo group (nausea and/or constipation), without any statistical differences between the two groups [Sciutto, *et al.,* 1995 Vol 8 Tab 141].

The authors' findings suggest that diet plus the new chitosan dietary fiber is a very useful treatment of the overweight in obese subjects and that the weight loss is associated with an evident reduction in hyperlipidemia without any significant interference of intestinal absorption of mineral salts or fat-soluble vitamins (See Table 31 below) [Sciutto, *et al.,* 1995 Vol 8 Tab 141].

The studies discussed in the section above have indicated that human subjects are able to tolerate chitosan at the levels given, even when engaging in a restricted diet, without adverse effects. It is significant to note that subjects receiving placebo experienced mild and transient effects from the low calorie diets alone.

Section IV Detailed Summary of the Basis for the Determination that Primex Chitosan is GRAS

Table 31
Effect of Chitosan on Hematological Parameters

Treatment	Parameter	Before	After	Statistics*
Diet + Chitosan	Hemoglobin (g/dL)	14.28±1.46	14.34±2.06	N.S.**
	Hematocrit (g)	43.38±5.64	43.51±6.07	N.S.**
	Leukocytes (no.)	7840±1866	7756±1904	N.S.**
	Erythrocytes (millions/ml)	4.51±0.53	4.62±0.59	N.S.**
	Glucose (mg/dL)	92.57±10.37	91.65±9.02	N.S.**
	Urea Nitrogen (mg/dL)	19.78±2.76	19.67±2.50	N.S.**
	Creatinine (mg/dL)	0.89±0.25	0.90±0.29	N.S.**
	Bilirubin (mg/dL)	0.56±0.32	0.510.29	N.S.**
	AST (U/L)	28.41±3.55	25.443.11	N.S.**
	ALT (U/L0	25.09±3.88	23.193.26	N.S.**
	Gamma-GT (U/L)	19.04±2.72	18.56±2.59	N.S.**
	Sodium (mEq/L)	137.7±13.0	138.9±13.8	N.S.**
	Potassium (mEq/L)	4.55±0.24	4.54±0.21	N.S.**
	Calcium (mEq/dL)	9.07±0.81	9.09±0.79	N.S.**
	Magnesium (mEq/L)	1.92±0.20	1.91±0.25	N.S.**
	Ferritin (ng/ml)	106.4±12.71	105.1±14.7	N.S.**
	Iron(ng/dL)	109.0±11.9	110.2±12.3	N.S.**

Section IV Detailed Summary of the Basis for the Determination that Primex Chitosan is GRAS

Table 31 (continued)

	Hemoglobin (g/dL)	13.91±2.16	14.07±1.94	N.S.**
	Hematocrit (g)	43.78±5.11	43.66±5.01	N.S.**
Diet +	Leukocytes (no.)	6905±2086	7125±1945	N.S.**
PLACEBO	Erythrocytes(millions/ml)	4.38±0.41	4.49±0.48	N.S.**
	Glucose (mg/dL)	96.12±9.27	94.33±9.82	N.S.**
	Urea Nitrogen (mg/dL)	19.43±2.66	19.28±3.09	N.S.**
	Creatinine (mg/dL)	0.90±0.30	0.88±0.29	N.S.**
	Bilirubin (mg/dL)	0.53±0.19	0.54±0.20	N.S.**
	AST (U/L)	27.84±3.57	26.78±3.14	N.S.**
	ALT (U/L0	22.41±2.93	21.49±2.78	N.S.**
	Gamma-GT (U/L)	19.38±2.23	19.11±2.15	N.S.**
	Sodium (mEq/L)	140.2±13.1	139.1±12.6	N.S.**
	Potassium (mEq/L)	4.50±0.21	4.53±0.26	N.S.**
	Calcium (mg/dL)	9.05±0.77	9.08±0.73	N.S.**
	Magnesium (mEg/L)	1.98±0.22	1.96±0.23	N.S.**
	Ferritin (ng/ml)	104.1±13.3	105.7±12.4	N.S.**
	Iron (ng/dL)	105.2±12.0	107.9±11.8	N.S.**

*Comparison before-after with Student's t-test.

**N.S. = difference not significant

000234

2. Fate of Chitosan in the Gastrointestinal Tract

Chitosan has been characterized as a polymer of glucosamine, which meets most of the criteria of dietary fibers with hypocholesterolemic potential [Fukada, *et al.,* 1991 Vol 4 Tab 38 and Sugano, *et al.,* 1988 Vol 9 Tab 156]. These criteria include nondigestibility in the upper gastrointestinal tract, high viscosity, a polymeric nature, and high water-binding capacity, which becomes reduced in the lower gastrointestinal tract. Sugano, *et al.,* 1988, stated that chitosan might not be hazardous to the morphology and functioning of the intestine because of its fiber-like behavior and its low toxicity [Sugano, *et al.,* 1988 Vol 9 Tab 156].

The metabolic fate of chitosan in the digestive tract of man has been tracked through radiolabelled preparations [Macleod, *et al.,* 1999 Vol 6 Tab 97]. A study was carried out to assess the potential of pectin:chitosan:hydroxypropyl methylcellulose [HPMC] [P:C:H] films for colonic drug delivery. Radiolabelled [^{99m}Tc] tablets were coated with a 3:1:1. P:C:H film and administered to four healthy male human volunteers. The gastro-intestinal transit of the tablets was assessed by gamma scintigraphy. The results showed that in all cases, the tablets were able to pass through the stomach and small intestine intact. Degradation of the tablets began once they had reached the colon, due to digestion of the coat by colonic bacteria. The study highlighted the potential of this coating system for colonic drug delivery, and demonstrated that chitosan is not degraded until it reaches the colon [Macleod, *et al.,* 1999 Vol 6 Tab 97].

The amount of radioactivity released in relation to tablet position was measured in one healthy male volunteer. Results showed that all the radioactivity remained (105.7%) through the small intestine (Table 32 below). However, only 38.0% of the radioactivity remained at the end of the ascending colon at 335 minutes post ingestion. After 405

minutes in the digestive tract, only 11.3% remained in the transverse colon [Macleod, *et al.,* 1999 Vol 6 Tab 97].

Table 32
Tablet Position and Radioactivity Released with Time After Administration to One Volunteer

Time after tablet administration (min)	Estimated radioactivity remaining (%) in tablet	Estimated tablet position
2	100.0	Stomach
15	101.2	Stomach
30	102.7	Stomach
60	105.0	Small intestine
120	103.3	Small intestine
180	105.7	Small intestine
260	90.7	Ileo caecal junction
275	66.4	Ascending colon
290	63.5	Ascending colon
305	49.0	Ascending colon
335	38.0	Ascending colon
365	16.7	Spread into transverse colon
405	11.3	Spread into transverse colon

The hydrolysis of chitosan has been studied, and the degradation of natural polymers, such as chitosan and chitin proceeds via enzymatic degradation [Domard, *et al.*, 1989 Vol 12 Tab 255 and Lee *et al.*, 1995 Vol 6 Tab 91]. The mechanism of enzymatic attack on a polymer is usually specific with regard to the coupling of the enzyme to the polymer, and the site of the initial degradation. Both chitin and chitosan have been known to be digestible by chitinase and lysosyme in accordance with the quantity of N-acetyl groups and their distribution within the backbone of the molecule [Lee, *et al.*, 1995 Vol 6 Tab 91, Aiba, 1993 Vol 12 Tab 227, Aiba, 1992 Vol 12 Tab 228, and Sashiwa, *et al.*, 1990 Vol 15 Tab 334].

Muzzarelli, 1997, has provided evidence for the degradation of chitosan in humans via non-specific chitinase and lysosymal enzyme systems [Muzzarelli, 1997 Vol 7 Tab 104]. The highest lysozyme

susceptibility was shown by a degree of deacetylation of approximately 70% [Sashiwa, *et al.*, 1990 Vol 15 Tab 334]. The authors indicated that chitosans with degrees of deacetylation of approximately 70% have also been reported to have the highest immunoadjuvant activity through mouse peritoneal macrophage activation [Sashiwa, *et al.*, 1990 Vol 15 Tab 334]. Other authors have demonstrated the existence of bacterial chitosanases in nature [Gooday, 1990 Vol 4 Tab 45]. Enzymatic methods have been developed for determining the degree of deacetylation of chitosan [Nanjo, *et al.*, 1991, Vol 14 Tab 312].

Chitosan is degraded into chitooligosaccharides, and then finally into glucosamine [Domard, 1989, Vol 12 Tab 255, Aiba, 1993 Vol 12 Tab 227, and Aiba, 1992 Vol 12 Tab 228]. Glucosamine (2-amino-2-deoxy- alpha-D-glucose) is one of the two hexosamine sugars (6 carbon amino sugars) common in animal cells (the other being galactosamine). Structurally, glucosamine is modified glucose with an NH3 group replacing the OH group found on carbon two (C-2). G6-P is an aminomonosaccharide (amino sugar) produced in the body by the combination of glutamine with fructose, through the enzymatic action of glucosamine synthetase [Kelly, 1998 Vol 14 Tab 291].

Glucosamine is also formed in the human body as glucosamine 6-phosphate (G6-P) [Kelly, 1998 Vol 14 Tab 291]. It is the most fundamental building block required for the biosynthesis of certain classes of compounds, such as glycolipids, glycoproteins, glycosaminoglycans (formerly called mucopolysaccharides), hyaluronate and proteoglycans, which require amino sugars. Because it is a component of all these compounds, it is an essential component of cell membranes and cell surface proteins as well as interstitial structural molecules that hold cells together. Kelly, 1998 states that directly or indirectly, glucosamine plays a role in the formation of articular surfaces, tendons, ligaments, synovial fluid, skin, bone, nails, heart valves, blood vessels, and mucus secretions

of the digestive, respiratory, and urinary tracts [Kelly, 1998 Vol 13 Tab 276].

The synthesis of G6-P begins with the structural rearrangement of glucose 6-phosphate to fructose 6-phosphate to facilitate interaction with the amino acid glutamine. The enzyme glucosamine synthetase facilitates the transfer of an amide group (NH3) from glutamine to fructose 6-phosphate. The enzyme simultaneously isomerizes (creates an intramolecular rearrangement of a compound without any net change of the components of the compound) this compound to form G6-P. The resulting G6-P molecule is the precursor to all hexosamines and hexosamine derivatives. This first biotransformation of glutamine and fructose 6-phosphate to G6-P is considered the rate-limiting step in amino sugar biosynthesis, and is an essential step in the glycosylation of all proteins [Kelly, 1998 Vol 13 Tab 276].

Setnikar, *et al.*,1984 studied the fate of glucosamine when taken orally [Setnikar *et al.*,1984 Vol 15 Tab 339]. Using a rat model, blood levels, tissue distribution and excretion patterns of radioactivity were studied in after administration of [^{14}C] glucosamine sulphate by either the intravenous or oral route. After intravenous administration, plasma radioactivity declined in the first 30 min, then increased, reaching a peak at the 2nd hour, and disappeared, with a half-life of 28 hours. The radioactivity was reported to have diffused rapidly in the tissues. The liver and kidney levels were found to be higher than those in plasma. There was early incorporation of radioactivity in the skeletal tissues (cartilage and bone). About 50% of the administered radioactivity was excreted with expired CO_2 and about 35% was excreted in the urine. Fecal excretion was small (2% of the administered dose). After oral administration, radioactivity was quickly found in plasma, where it reached a peak at 4 hours. Plasma levels then declined slowly, with biphasic kinetics. The tissue distribution, including uptake in the skeleton, were similar to those seen with intravenous administration. Fecal excretion was minor,

demonstrating that glucosamine was nearly completely bioavailable when administered orally. The large excretion (82%) with expired CO_2 indicated that glucosamine is to a large extent broken down to smaller fragments [Setnikar *et al.,*1984 Vol 15 Tab 339].

Two later studies by Setnikar, *et al.,* (1986 and 1993) investigated the metabolism of glucosamine or glucosamine sulphate in dogs and man. The pharmacokinetics, organ distribution, metabolism and excretion of glucosamine were studied in the dog by administering uniformly labeled [^{14}C]-glucosamine (sulfate), either i.v. or orally, in single doses. Immediately after i.v. administration, the radioactivity in plasma resulting from the labeled glucosamine, freely diffused into organs and tissues. The investigators found that the radioactivity disappeared quickly from plasma with an initial half life of 13 minutes, and a terminal half life of 118 min. After 30-60 min the radioactivity in the plasma was no longer due to glucosamine, but was incorporated into alpha-and beta globulins. The protein-incorporated radioactivity was detected within 20-30 min after i.v. administration. The signal reached a peak after 8 h and then slowly diminished, with a half life of 2.9 days. Of the administered radioactivity, more than 34% was excreted in the urine, mainly as glucosamine, and 1.7% was excreted in the feces. Radioactivity was excreted also as [^{14}C]-CO2 in the expired air. The radioactivity, after i.v. administration, diffused rapidly from blood into the body. Some organs showed an active uptake of radioactivity, e.g. the liver and the kidney. The data also showed that other tissues, such as the articular cartilage, had an active uptake. The authors stated that In most other organs, the radioactivity found could be explained by passive diffusion from plasma. Following oral administration of a single dose of [^{14}C]-glucosamine, the radioactivity was quickly and nearly completely absorbed from the gastrointestinal tract. The pattern of disappearance, metabolic transformation, tissue distribution and excretion of the radioactivity were consistent with those found after i.v. administration [Setnikar, *et al.,* 1986 Vol 15 Tab 340].

The pharmacokinetics of glucosamine sulfate (CAS 29031-19-4) was investigated by the same laboratory in 6 healthy male volunteers (2 per administration route) using ^{14}C uniformly labeled glucosamine sulfate and administering it in single doses by either an intravenous (i.v.), an intramuscular (i.m.) or an oral route. The results showed that after i.v. administration, the radioactivity due to glucosamine could be detected in plasma and was rapidly eliminated, with an initial half life of 0.28 hours. 1-2 hours after administration, the radioactivity due to glucosamine disappeared almost completely, and was replaced by radioactivity originating from plasma proteins, in which glucosamine or its metabolites were incorporated. This radioactivity reached a peak after 8-10 hours and then declined with a half life of 70 hours. About 28% of the administered radioactivity was recovered in the urine 120 hours following the administration, and less than 1% was recovered in the feces. After i.m. administration similar pharmacokinetic patterns were observed. After oral administration a proportion close to 90% of glucosamine sulfate was absorbed. Free glucosamine was not detectable in plasma. The radioactivity incorporated in the plasma proteins followed pharmacokinetic patterns, which were similar to those after i.v. or i.m. administration, but the concentration in plasma was about 5 times less than after parenteral administration. The counts after oral administration were 26% of that after i.v., or i.m. administration. The authors hypothesized that the lower levels of plasma radioactivity after oral administration were probably due to a first pass effect in the liver, which metabolizes a notable proportion of glucosamine into smaller molecules and ultimately into CO2, water and urea [Setnikar, *et al.,* 1993 Vol 15 Tab 343].

In another study Setnikar *et al.,*1991, found that oral daily doses of glucosamine sulphate of 50-800 mg/kg were effective in assisting with symptoms of arthritis. The authors stated that the potency of glucosamine sulphate in comparison of that of the indomethacin used as a reference substance was found 50-300 times lower. However, since the toxicity of indomethacin in chronic toxicity experiments has

been reported to be 1000-4000 times larger, the therapeutic margin with regard to prolonged treatments of inflammatory disorders results in a margin of safety, which is 10-30 times more favorable for glucosamine sulphate than for indomethacin [Setnikar *et al.*,1991 Vol 15 Tab 341].

3. Chitosan and other Foods as Sources of Glucosamine

According to Muzzarelli, chitosan provides a sustained-release form of glucosamine. Oral ingestion (1g/day) of N-acetylglucosamine or chitosan increases the serum concentration of glucosamine [Muzzarelli, 2000 Vol 7 Tab 105]. With ingestion of chitosan, the serum N-acetylglucosamine concentration remains elevated for at least 48 hours, although glucosamine itself is normally cleared rapidly from the blood stream [Muzzarelli, 2000 Vol 7 Tab 105]. Blood glucose levels are not altered with supplemental N-acetylglucosamine. Setnikar *et al.*, 1993, indicated that these results confirmed the efficacy of orally administered chitosan for the improvement of joint function in human subjects [Muzzarelli, 2000 Vol 7 Tab 105].

Experimental evidence from various laboratories indicates that as with other polysaccharides, a small portion of orally administered chitosan is digested in the upper gastrointestinal tract [Muzzarelli, 2000 Vol 7 Tab 104 and Varum, *et al.*, 1997 Vol 11 Tab 213]. It is hypothesized that the digestion is due to the action of hydrochloric acid in the stomach, and the activities of nonspecific enzymes present in saliva and gastric juice. In the large intestine, enzymes from microorganisms digest the main portion of chitosan into glucosamine units [Muzzarelli, 2000 Vol 7 Tab 105].

Other foods have also been reported to provide a source of glucosamine. Tempe, a traditional Indonesian food, is prepared with cooked soya beans bound into a solid growth of *Rhizopus oligosporus*. *Rhizopus* species have been reported to secrete

chitosan, and continued fermentation of chitosan produces glucosamine in the food product. The glucosamine content of Tempe is 51 - 107 g/kg (dry biomass). This example illustrates the fact that polymeric forms of glucosamine (chitosans) have been present in the human diet for generations in various foods [Muzzarelli, 2000 Vol 7 Tab 105 and Shimahara, *et al.,* 1989, Vol 9 Tab 154].

The pharmacokinetics of glucosamine sulfate have been investigated after intravenous, intramuscular and oral administration in dogs, rats and humans. After intravenous administration of radio labeled glucosamine in dogs, investigators found that the substance was cleared from plasma in less than 2 hours and appeared to be incorporated into plasma proteins within a maximum period of 8 - 10 hours after injection. Nearly one third of the injected glucosamine could be recovered from the urine after 120 hours, and 1% was excreted in the faeces [Muzzarelli, 2000 Vol 7 Tab 105].

In human volunteers the data was similar. Upon oral administration, the concentrations of glucosamine were five times lower than after intravenous administration, probably because the glucosamine was removed in the liver, where it is metabolized to carbon dioxide, water and urea [Muzzarelli, 2000 Vol 7 Tab 105]. Glucosamine is known to diffuse very rapidly in most tissues and organs, even after oral administration, accumulating in the articular tissue and in the bone [Muzzarelli, 2000 Vol 7 Tab 105].

The clinical efficacy and safety of orally administered glucosamine has been surveyed in small animals. Over three thousand practitioners were asked to report on the effects of glucosamine treatment of degenerative joint diseases in dogs. Over 90% (96.8%) reported that dogs older than 5 years were the most responsive to treatment. The response to supplementation was estimated to occur within 4 weeks (83%). The glucosamine product was rated good or excellent in improving mobility and alleviating pain in the animals. No practitioner reported a worsening of pain or mobility [Muzzarelli, 2000

Vol 7 Tab 105].

Although the anti-inflammatory activity of glucosamine is 50 - 300 times lower than that of drugs, such as indomethacin, the toxity of the latter is 1000 - 4000 times greater, and therefore, the therapeutic margin favors dietary glucosamine. Its anti-inflammatory action appears to be related to mechanisms substantially different from those of non-steroidal drugs, which act primarily through inhibition of cyclooxygenase. Glucosamine is not an inhibitor of cyclooxygenase, and therefore, its effects are prostaglandin independent [Muzzarelli, 2000 Vol 7 Tab 105].

Glucosamine stimulates proteoglycan biosynthesis by providing nutritional support, and the newly synthesized proteoglycans may stabilize cell membranes, resulting in an anti-inflammatory effect. Glucosamine also reduces the generation of superoxide radicals by macrophages and inhibits lysosomal enzymes [Muzzarelli, 2000 Vol 7 Tab 105]. Deal and Moskovitz, 1999, as reported by Muzzarelli list the numerous clinical trials (> 20), where glucosamine has been tested on over 3000 subjects. One of the most recent clinical trials was conducted by Laffler *et al.*, 1999, on 34 males from the U.S. Navy diving and special warfare community, who presented chronic pain and radiographic evidence of knee or low back diseases. Knee osteoarthritis symptoms were relieved as demonstrated by various assessments including a physical examination score (-43.3%) [Muzzarelli, 2000 Vol 7 Tab 105].

Thus it was concluded that the supplementation relieved symptoms of knee osteoarthritis, while a larger set of data was needed before comment could be made on spinal diseases. Houpt *et al.*, 1999 who investigated the efficacy of glucosamine on pain and disability, concluded that glucosamine hydrochloride benefits some subjects with knee osteoarthritis [Muzzarelli, 2000 Vol 7 Tab 105]. Muzzarelli, 2000 concluded that, in general, the studies discussed above demonstrated a favorable response by the body to glucosamine,

which is the ultimate breakdown product of chitosan [Muzzarelli, 2000 Vol 7 Tab 105].

4. Chitosan Degradation

The initial degradation rates [r] in human serum of three chitosans characterized by varying fractions of N-acetylated units (F_A) were determined by measuring the decrease in viscosity as a function of time. The F_A of the three chitosans was 0.42, 0.5 1, and 0.60 respectively. A strong increase in r with increasing F_A of the chitosans was observed. The degradation rate increased proportionally to $F_A^{4.5}$. With increasing concentrations of lysozyme added to the reaction mixtures of chitosan and serum, the relative increase in degradation rate of chitosans with increasing F_A was almost the same as that without lysozyme added. Addition of the chitinase inhibitor allosamidin [50 μM] did not inhibit the degradation rate of chitosan [F_A = 0.60] by human serum. The results suggested that chitosans are actually mainly depolymerized by lysozyme in human serum, and not by other enzymes or other depolymerization mechanisms [Varum, *et al.*, 1997 Vol 11 Tab 213].

Chitin was deacetylated to various extents with NaOH to obtain partially and thoroughly deacetylated chitins. The specimens used in this study were deacetylated by 0 [chitin], 68.8, 73.3, 84.0, 90.1 and 100 mol% [chitosan]. Films with a thickness of 150 microns were prepared from these specimens by the solution casting method. The equilibrated water contents of the films were 52.4 [chitin], 73.8 [68.8 mol%], 64.2 [73.3 mol%], 61.8 [84.0 mol%], 57.8 [90.1mol%] and 49.7 wt% [chitosan], while the tensile strengths of the water swollen films were 244 [chitin], 197 [68.8 mol%], 232 [73.3 mol%], 320 [84.0 mol%], 293 [90.1 mol%] and 433 g mm-2 [chitosan]. The maximum water content and the minimum tensile strength observed for a specimen deacetylated between 0 and 68.8 mol% may be ascribed to the lowered crystallinity by deacetylation of chitin, since both chitin and chitosan are crystalline polymers. Unlike their physical

properties, *in vitro* and *in vivo* degradations of these films occurred less rapidly without passing a maximum or minimum, as their degree of deacetylation became higher. The *in vitro* degradation was carried out by immersing the films in buffered aqueous solution of pH 7 containing lysozyme at 37° C, while the *in vivo* degradation was studied by subcutaneously implanting the films in the back of rats. It was found that the rate of *in vivo* biodegradation was very high for chitin and 68.8 mol% deacetylated chitin, compared with that for the 73.3 mol% deacetylated chitin. The films, which were more than 73.3 mol% deacetylated, showed slower biodegradation. Interestingly, the tissue reaction towards highly deacetylated derivatives including chitosan was very mild, although they had cationic primary amines in the molecule [Tomihata, *et al.*, 1997 Vol 9 Tab 163].

5. Exposure

The mean and the 90th percentile of estimated exposure to Primex ChitoClear™ chitosan (approximately 2.0 and 4.0 grams respectively) remains lower than the recommended levels of intake for dietary fiber (25-30 grams per day) in the United States [Enns, *et al.,* 1997 Vol 4 Tab 36]. Additionally, dietary fiber intake in the US (averaged for men and women) has been estimated by USDA at 16.1 grams per day [Enns, *et al.,* 1997 Vol 4 Tab 36]. This value still represents a deficit in relation to levels established for good health.

Primex ehf is aware that certain individuals may consume chitosan as a dietary supplement. The recommended dose of chitosan as a dietary supplement is 1.5 to 3.0 grams per day. This dosage has been recommended based on the results of clinical trials with human volunteers [Colombo, *et al.*, 1996, Vol 3 Tab 24]. It is possible that consumption of chitosan may provide health benefits from additional dietary fiber intake. Further, no untoward effects have been reported in multiple studies involving chitosan consumption by human volunteers. Therefore, Primex ehf submits that the exposure to chitosan represented by the estimate provided in Section III above,

will pose no threat to the public health.

In reality, exposure to Primex chitosan is expected to be limited by:

1. Cost - only certain products within a given food subcategory will be of high enough value to support an ingredient costing US$ 20 - 40 dollars per kilogram.
2. Organoleptic considerations – use of chitosan may not provide the desired organoleptic targets for all
3. Functional properties - certain functional properties may not be appropriate for a given consumer product.

6. Technical Effects

Applications for chitosan include general use in foods as a multiple-use direct additive. The manufacturer expects that the ingredient will be used under the conditions of current Good Manufacturing Practice.

In order to classify the various effects ingredients may have in food, FDA has published a list of 32 physical or technical functional effects for which direct food ingredients may be added to food. These are codified at 21 CFR §170.3 (o) (1-32). Technical effects for chitosan are covered under several of the following terms as listed under 21 CFR §170.3 (o).

(2) "Antimicrobial agents": Substances used to preserve food by preventing the growth of microorganisms and subsequent spoilage, including fungistats, mold and rope inhibitors, and the effects listed by the National Academy of Sciences/National Research Council under "preservatives."

(3) "Antioxidants": Substances used to preserve food by retarding deterioration, rancidity, or discoloration due to oxidation.

(5) "Curing and pickling agents": Substances imparting a unique flavor and/or color to a food, usually producing an increase in shelf life stability.

Section IV Detailed Summary of the Basis for the Determination that Primex Chitosan is GRAS

(6) "Dough strengtheners": Substances used to modify starch and gluten, thereby producing a more stable dough, including the applicable effects listed by the National Academy of Sciences/National Research Council under "dough conditioner."

(8) "Emulsifiers and emulsifier salts": Substances which modify surface tension in the component phase of an emulsion to establish a uniform dispersion or emulsion.

(14) "Formulation aides": Substances used to promote or produce a desired physical state or texture in food, including carriers, binders, fillers, plasticizers, film-formers, and tableting aids, etc.

(16) "Humectants": Hygroscopic substances included in food to promote retention of moisture, including moisture-retention agents and antidusting agents.

(20) "Nutrient supplements": Substances which are necessary for the body's nutritional and metabolic processes.

(24) "Processing aids": Substances used as manufacturing aids to enhance the appeal or utility of a food or food component, including clarifying agents, clouding agents, catalysts, flocculants, filter aids, and crystallization inhibitors, etc.

(28) "Stabilizers and thickeners": Substances used to produce viscous solutions or dispersions, to impart body, improve consistency, or stabilize emulsions, including suspending and bodying agents, setting agents, jellying agents, and bulking agents, etc.

(29) "Surface-active agents": substances used to modify surface properties of liquid food components for a variety of effects, other than emulsifiers, but including solubilizing agents, dispersants, detergents, wetting agents, rehydration enhancers, whipping agents, foaming agents, and defoaming agents, etc.

(31) "Synergists": Substances used to act or react with another food ingredient to produce a total effect different or greater than the sum of the effects produced by the individual ingredients.

(32)"Texturizers": Substances which affect the appearance or feel of the food.

7. Contaminants and Manufacturing

Product Purity

Primex ehf has performed a number of tests on its products to assure the purity of the product. Analyses for contaminants are shown in Table 33 below. The analyses demonstrated that the product was free of the contaminants of most concern [Primex Ingredients, 2000 Vol 7 Tabs 120 and 123]. Likewise, Table 34 below demonstrates that Primex chitosan is much lower in dioxins than other common foodstuffs, and lower than other raw materials from which other chitosan products are made (crabs) [Primex Ingredients Dioxin Testing, 2000 Vol 7 Tab 120]. Table 33 below demonstrates that the shrimp used as raw material in the Primex process are free of the White Spotted Virus and the Yellow Head Virus (WSSV and YHV) known to infect shrimp [University of Arizona Virus Scan, 2000 Vol 3 Tab 1].

This information in combination with the data presented in Section I demonstrates that Primex chitosan is free of both organic and *inorganic contaminants (heavy metals)*. Therefore, based on its chemistry, chitosan, as manufactured by Primex may be considered a purified source of polysaccharides.

Section IV Detailed Summary of the Basis for the Determination that Primex Chitosan is GRAS

Table 33

Contaminant Analysis

(Translated from the original Norwegian by the Laboratory)

NORWEGIAN INSTITUTE FOR WATER RESEARCH P.O. Box 173 Kjelsaas, 0411 OSLO	TEST REPORT
Name	PRIMEX
Contract number	99019-01
Date of sample reception	25.01.99
Laboratory Code	137 1-2
Sample Type	*Biological matter (chitosan)*
Concentrations in	Micrograms/kg wet weight (pp., w.w.)
Analytical method	H2-4
Date of analysis	12.03.99
Analyst	Lasse Berglind

1: Primex Chitosan batch no. 355
2: Primex Chitosan batch no. 355

Parameter/sample no.	1	2
Naphthalene	<0,5	<0,5
2-Methyl-naphthalene	<0,5	<0,5
1-Methyl-naphthalene	<0,5	0.9
Biphenyl	2.7	4.6
2,6-Dimethylnaphthalene	<0,5	<0,5
Acenaphthalene	<0,5	<0,5
Acenaphthene	0.7	1.3
2,3,5-Trimethylnaphthalene	<0,5	<0,5
Fluorene	1.6	1.3
Phenanthrene	3.9	2.9
Anthracene	<0,5	<0,5
1-Methylphenanthrene	1.1	<0,5
Fluoranthene	3.1	3.6
Pyrene	1.9	2.7
Benzo(a)anthracene*	<0,5	0.8
Chrysene/triphenylene	0.9	0.9
Benzo(b,j,k)fluoranthene*	1.2	1.2
Benzo(e)pyrene	<0,5	<0,5
Benzo(a)pyrene*	<0,5	<0,5
Perylene	<0,5	<0,5
Indeno (1,2,3-cd) pyrene*	<0,5	<0,5
Dibenzo (a, c/a, h) anthracene* 1)	<0,5	<0,5
Benzo (ghi) perylene	0.6	0.5
Total PAH	17.7	20.7
Sum carcinogenic PAH*	1.2	2
% Carcinogenic PAH	6.8	9.7
% Dry substance	91.9	92.2

Section IV Detailed Summary of the Basis for the Determination that Primex Chitosan is GRAS

Table33 (continued)

*Potentially carcinogenic according to Int. Agency for Research on Cancer (1987)
1) Only the dibenzo (a, h)-isomer

This test report may only be copied completely and without changes.
The test result is only valid for the sample, which has been tested.

Table 34
Dioxin Analysis

Sample	No.	Concentration (pg TE/g ww)[1] Average	Concentration (pg TE/g ww)[1] min-max	Fat (%)	Concentration (pg TE/g fw)[2]
Cod Fillet	2	0.1	0.03-0.2	0.3	33
Mackerel	2	1.5	0.7-2.2	16	4.4-14
Herring	6	2.3	1.8-2.8	14	16
Farmed Salmon	4	0.7	0.5-1.1	3	23
Cod Liver	2	7.7	6.8-8.5	58	13
Cod liver Oil	4	5.8	0.6-16	100	5.8
Crab	3	29	17-44	20	145
Margarine	4	1	0.9-1.1	81	1.2
Butter	3	1.2	0.9-1.4	81	1.5
Lambs fat	3	1.3	0.5-2.6	80	1.6
Egg	3	0.2	0.1-0.2	11	1.8
Milk	8				0.3-0.9
Milk (near industries)	5				1.3-3.1
Primex Ingredients for PCDD/PCDF and non-ortho PCB[3]	1	0.07			

[1] TE =2378-TCDD toxicity equivalents according to the international model (NATO/CCMS, 1989); pg TE/g ww = a measure of the toxicity equivalents per wet weight.
[2] pg TE/g fw = a measure of the toxicity equivalents per fat weight
[3] Measured as polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans in pg i – TE/g

Table 35
Virus Analysis

UAZ #	Sample ID	WSSV	YHV
00-131A/1	Shrimp shells	Not detected	Not detected
00-131A/2	Shrimp shells	Not detected	Not detected
00-131B/1	ChitoClear: TM661	Not detected	Not detected
00-131B/2	ChitoClear: TM761	Not detected	Not detected
00-131B/3	ChitoClear: TM93	Not detected	Not detected

G. Animal Safety and Toxicology Studies

Table 36 below summarizes animal studies, in which the toxicological effects of chitosan have been analyzed. Several of the studies mentioned desirable attributes of chitosan, when delivered as part of a diet.

Muzzarelli, 1997 stated that chitosan is a non-toxic natural polysaccharide of β-1,4-linked glucosamine residues that has been produced industrially by deacetylation of chitin obtained from crab and prawn shells [Muzzarelli, 1997 Vol 7 Tab 104]. Because of chitosan's well-recognized physiological adaptability, it is used as a food additive and in biomedicinal materials, etc. Also, because chitosan is not hydrolyzed by the digestive enzymes of man, and its chemical structure is similar to that of cellulose, it can be used as a new source of dietary fiber. However, because chitosan contains one amino group per residue, the author felt that its cationic character might confer properties quite different from those of other dietary fibers, and that most of its chemical functions were attributable to the amino group. Among them, the most noticeable dietary consideration is its adsorption of acidic metabolites such as uric acid and bile acid [Jing, *et al.,* 1997 Vol 5 Tab 67]. Its hypocholesterolemic effect on healthy man has also been reported [Sugano *et al.*, 1988 Vol 9 Tab 156, Maezaki *et al.*, 1993 Vol 6 Tab 98, and Jing, *et al.,* 1997 Vol 5 Tab 67].

Section IV Detailed Summary of the Basis for the Determination that Primex Chitosan is GRAS

Table 36: Toxicity Assessments of Chitosan in Animals

Species	Dosage of Chitosan	Duration	Effect	Reference
Mice	5 mg subcutaneous injection; Oral administration of 5% chitosan in a casein-based diet	Every two weeks for 12 weeks; Oral administration for 12 weeks	Injections did not evoke general cellular abnormalities; With oral administration, mouse body weights decreased, and probiotic bacterial populations decreased	Tanaka, *et al.*, 1997
Mice	1 mCi/kg body weight of ^{166}Ho-Chit (DW-166 HC)	Single Dose	Slightly decreased kidney weights, without histological alterations	Lee *et al.*, 1998
Sprague-Dawley Rats	5% of diet	21 days	No Mucosal change in large bowel; No untoward effects; Chitosan did not alter hemoglobin concentration, and showed least change in serum iron (-1.8%) among oat gum, cholestyramine, and Chitosan	Jennings, *et al.*., 1988
Rats	5-7.5% of the Diet, with 1% Cholesterol and 0.2% Cholic Acid	1, 3, or 6 weeks	No change in liver weight or appearance	LeHoux, *et al.*, 1993
One-Day-Old Broiler-Chicks	30 g/kg	21 Days	Reduced Short Chain Fatty Acids shows Antimicrobial Nature of Chitosan	Razdan, *et al.*, 1994
One-Day-Old Broiler-Chicks	30 g/kg	21 Days	Chitosan reduced body weights and Feed Intakes	Raman, *et al.*, 1997

Section IV Detailed Summary of the Basis for the Determination that Primex Chitosan is GRAS

Table 36: Toxicity Assessments of Chitosan in Animals (*Continued,*)

Species	Dosage of Chitosan	Duration	Effect	Reference
Rats	0.5, 2, 5 and 10 % of Diet	81 Days	No Constipation or diarrhea; 0.5% increased HDL in cholesterol-free diets; 2% & 5% did not influence food intake or growth; Finer particles at 2% restrained growth	Sugano, *et al.*, 1980
Oral Toxicity in Rats and Mice	Chitosan	Being Translated	LD_{50} was reported to be 16g/kg	Illum, 1998, reporting on Arai, *et al.*, 1968
Rats	5% Diet		Urinary Excretion of Calcium Increased	Wada, *et al.*, 1997
Mice	Chitosan Microspheres	60 Days	Minimized drug toxicity	Jameela, *et al.*, 1996
Mice	300 to 1,500 mg/kg	8 Days	Prevented the side effects and toxicity of a cancer-inhibiting drug.	Kimura, *et al.*, 1999
Mice Rabbits Guinea Pigs	72 hours Eye Irritation Tests Skin Irritation Tests	-	No Undesirable Toxic Effects	Rao, *et al.*, 1997
Rabbits		-	No irritability to Keratinocytes	Augustin, *et al.*, 1995
Sprague-Dawley Rats	5% of Diet with High Fat	2 Weeks	Decrease in Mineral Absorption and Bone Mineral Content; Additional Calcium Required; Decrease in serum Vitamin E Levels	Deuchi, *et al.*, 1995
NIH Rats	1.8 g/kg/day	90 Days	No Toxic Effects	Ling, *et al.*, 1999

Of particular interest is an animal study by Ling, *et al.*, 1999 in which the investigators measured both acute and chronic toxicity in NIH rats.

H. Other Regulatory Approvals

000253

Chitosan is approved as a food in Japan, and several products ranging from biscuits to fishcakes have been approved by the Japanese Ministry of Agriculture, Fisheries and Foods as "Foods for Specified Health Use"

(FOSHU) [FOSHU, 1999 Vol 17 Tab 372]. Foods bearing a FOSHU approval are considered to improve the diet and to help maintain or improve health, when consumed in the normal way. Japan is the world's leader in the manufacture of chitosan, accounting for 90% of the global chitosan market worth approximately 4 trillion yen (120 yen = 1 US dollar) [*Korea Times*, April 7, 1999 Vol 13 Tab 280]. Nearly 50% of the chitosan manufactured in Japan is used in purifying drinking and wastewater streams. The *Korea Times* reports that there are ten firms manufacturing chitosan in Japan, and that 60 package chitosan into various products [*Korea Times*, April 7, 1999 Vol 14 Tab 297].

Chitosan has also been approved for use in Korea, and appears in that country as a food for the improvement of the health of the digestive tract [Lian, *et al.*, 1998, Vol 14 Tab 300]. Five firms are reported to manufacture chitosan in Korea, and 70 companies make end products using chitosan [*Korea Times*, April 7, 1999 Vol 14 Tab 297].

Recently, the United States Environmental Protection Agency (EPA) has exempted poly-D-glucosamine (chitosan) from the requirement of a tolerance [Federal Register Vol 13 Tab 261]. Under the Federal Food, Drug, and Cosmetic Act, the biochemical growth regulator poly-D-glucosamine (chitosan) is exempt from the requirement of a tolerance for residues when used as a seed treatment in or on rice. The Agency stated that based on the nontoxic nature of the chemical, the EPA was also establishing an exemption from the requirement of a tolerance for residues of poly-D-glucosamine (chitosan) when used as a pesticide in the production of any raw agricultural commodity.

The Australian Complementary Medicines Evaluation Committee considered the use of chitosan under Section 7 of the *Therapeutic Goods Act 1989* in several meetings from June 1998 through September 1998. Under Section 7 the Australian government may declare certain goods to be therapeutic goods when used, advertised, or presented for supply in a particular way. Members of the committee considered data and information, both pro and

con, regarding the consumption of chitosan by Australians at doses of 1-2 grams per day. Recommendations to the committee included the following:

1. Chitosan is suitable for use as an active ingredient in Listable products without limits.
2. A warning should be required on the effect of chitosan on the absorption of other drugs (subject to advice from a chemist) and a statement advising that the product of seafood origin.
3. Seek another round of public comment on the proposal that fiber in capsule, tablet or pill form be declared to be therapeutic goods, under section 7 of the Act.

The reasons for these recommendations were as follows:

a. Chitosan does not appear to present a significant risk to consumers in the manner and dosage likely to be consumed.
b. Chitosan can already be consumed in foods and no evidence is available to date to suggest this poses any safety risk.
c. Sufferers of seafood allergy would be alerted to any dangers if they were aware that chitosan is derived from seafood. A warning statement is not necessary, provided that the product contains a statement "is derived from seafood."

At their final meeting on the topic, members concluded that therapeutic goods containing chitosan should contain dosage directions on the label so that consumers could avoid any interaction between chitosan and prescription drugs.

Conclusion

Section IV Detailed Summary of the Basis for the Determination that Primex Chitosan is GRAS

I. Summary

Chitosan is a natural material that has been consumed by man for thousands of years. The human body treats chitosan as a dietary fiber, fermenting the nondigestible material by means of indigenous bacteria in the colon. Breakdown products from chitosan include chitooligosaccharides and ultimately glucosamine, which is a normal constituent of the human body. Multiple animal and human studies conducted over a period of time by scientists in various parts of the world have confirmed that chitosan is safe for consumption, and elicits no toxicological effects.

Primex has responded to certain questions posed by CFSAN, regarding the potential effect of chitosan consumption on the nutritional status of humans. Primex found no data to demonstrate that chitosan caused significant fecal fat excretion in humans, when the material was consumed at levels of approximately five grams per day or less. Further the several studies conducted in humans, where mineral status was measured, showed no degradation in the mineral status of the subjects. On the basis of this information, Primex submits that consumption of chitosan at the 90th percentile, as estimated by the company (4.0 grams per day), elicits no adverse consequences to human nutrition.

Appendix I
Expert Opinions

UNIVERSITY OF ILLINOIS
AT URBANA-CHAMPAIGN

Department of Animal Sciences

College of Agricultural, Consumer
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132 Animal Sciences Laboratory
1207 West Gregory Drive
Urbana, IL 61801

May 2, 2005

Dr. Robert Martin
United States Food and Drug Administration
Center for Food Safety and Applied Nutrition
HFS 255
5100 Paint Branch Parkway
College Park, MD 20740

Dear Dr. Martin:

Primex ehf asked that I review the results of recently published human safety studies dealing with the use of their shrimp (*Pandalus borealis)*-derived chitosan and provide an opinion as to whether it should be regarded as GRAS in light of the company's earlier GRAS determination. I am happy to do so.

As you know, Primex sponsored two human studies to respond to queries from the GRAS review team in the Center for Food Safety and Applied Nutrition regarding whether chitosan might interfere with fat-soluble vitamin and mineral status of humans, and whether Primex chitosan might elicit hypersensitivity reactions in allergy-prone individuals. I have reviewed both of those studies, and additional ones, in some depth and offer the following opinions.

Regarding the issue of fat-soluble vitamin and mineral status, two concentrations of Primex chitosan (4.50 and 6.75 g/d) along with a negative control group (0 g/d) and a positive control group fed 6.75 g glucomannan/d were fed to 56 mildly hypercholesterolemic males and females. The experimental design was a randomized, single blind, placebo-controlled parallel study. Test ingredients were provided in tablets that were consumed with typical Finnish diets for 8 weeks.

There was no evidence that shrimp-derived chitosan affected serum concentrations of Vitamin A, alpha-carotene, beta-carotene, 25-hydroxy vitamin D, vitamin E, or plasma cholesterol. The concentrations of the fat-soluble vitamins did not change compared with the controls when normalized for cholesterol concentration. Calcium concentrations in all groups decreased slightly throughout the course of the study, but all concentrations remained within the normal range. A number of hematological measurements, as well as clinical chemistry metabolites (e.g., white blood cells, creatinine, ferritin), were measured

and all remained within the normal range for all treatments, with no differences being noted among treatments.

This study supports the claim of Primex ehf that their shrimp-derived chitosan does not lower human fat-soluble vitamin concentrations in blood, nor is serum calcium concentration lowered below what is considered to be normal. Other studies where as much as 5 g chitosan/d were fed to humans ingesting a high fat diet report no loss of fecal fat due to treatment (Gades and Stern. 2002. *International Journal of Obesity and Related Metabolic Disorders* 26:119-222). Just this year, these same researchers (Gades and Stern. 2005. *Journal of the American Dietetic Association* 105:72-77) expanded on their previous study to include both men and women as subjects and to have them follow customized diet plans where 2.5 g chitosan was provided each day. Their conclusion is worthy of a quote: "The authors now have evidence using three different commercial products (this study, the 2002 reference, and a 2003 reference [Gades and Stern. 2003. *Obesity Research* 11:683-688]) that the claims that chitosan is a fat-trapping weight-loss aid are not merely unsubstantiated, they are false". And if fecal fat excretion is not increased, there can be no increased loss of fat-soluble vitamins.

Regarding the matter of hypersensitivity reactions allegedly resulting from chitosan intake by allergy-prone individuals, Primex ehf sponsored a study entitled "Evaluation of Chitosan as a Possible Compound Causing Immediate Hypersensitivity Reactions". The study was conducted in Finland and used 221 allergy-prone individuals (average age, 43.5 years). Subjects were assigned to a single-blind, open test, experimental design. The investigators reported no positive reactions to chitosan. Eleven of their subjects reacted positively to fish/shrimp allergens but tested negative to chitosan. These same individuals analyzed negative for chitosan-specific IgE as well. Therefore, the probability for immediate hypersensitivity reactions to chitosan derived from shrimp is very small, adding to the current database on this topic dealing with safety in that no cross-reactivity with fish/shrimp allergens was noted. This outcome is logical given that, in the conversion of shrimp chitin to the chitosan produced by Primex ehf, harsh chemical are used that would denature allergens present in shrimp shell. Upon examination of Primex ehf's Critical Control Points associated with the manufacture of chitosan, along with data from the testing of numerous lots of final product produced by Primex ehf, it is apparent that the final product meets the company's published specifications, and that being the case, this chitosan would have very low allergenic potential.

In conclusion, my experience is that shrimp-derived chitosan exhibits properties similar to a number of dietary fiber sources. Thus, it should not result in problems when consumed by humans at concentrations ranging from 4.50 to 6.75 g/d. On both fronts (i.e, fat excretion via feces and demonstration of immediate hypersensitivity reactions), Primex ehf chitosan proved to have no effect. I have no hesitation in recommending that this product be given GRAS status.

Please let me know if I can provide additional information.

Sincerely.

George C. Fahey, Jr.
Professor
Animal Sciences and Nutritional Sciences

JOSEPH F. BORZELLECA

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03 May 2005

Ms. Lee B. Dexter
Lee B. Dexter & Associates
Technology Consultants
15704 Webberville Road
Austin, Texas 78724- 7489

Re: GRAS Notification for Primex Shrimp-Derived Chitosan
Review of Recent Studies

Dear Lee:
Greetings and best wishes.

I have critically evaluated the two studies sponsored by Primex ehf of Iceland, "Evaluation of Chitosan as a Possible Compound Causing Immediate Hypersensitivity Reactions" and "Effect of Chitosan on Serum Fat-Soluble Vitamins and Plasma Cholesterol Concentrations in Mildly Hypercholesterolemic Subjects" in response to a request by the management of Primex ehf to review the results of these recent human safety studies and to provide an opinion stating whether or not these studies support or negate the company's earlier GRAS determination. I understand that these studies were conducted in response to two questions raised by CFSAN, a nutritional one involving a potential effect on the bioavailability of fat-soluble vitamins and minerals and the other, potential allergenicity. The GRAS Panel, of which I was a member, concluded that shrimp-derived chitosan (the Primex product) was non-toxic to humans and to the experimental animals studied. We further unanimously concluded that this product was GRAS for its intended use. We considered the issue of potential allergenicity in our deliberations and concluded that the manufacturing process was severe enough to denature any allergens present and the product would not be allergenic. Several other studies deemed appropriate were evaluated

The company indicated that the allergenicity study they sponsored was the first human clinical study of this type to be performed on a chitosan product (I have not verified this but none were identified in the literature search you conducted for the Expert Panel). This study was conducted at the Allergy Unit of the Department of Dermatology, Kuopio University Hospital in Finland and the report is dated 2002. In this study, 221 allergy prone subjects with a mean age of 43.5 ± 13.7 years were prick tested in a single blind open test design. Although the protocol allowed for an oral challenge for any positive reactors, no positive reactions for chitosan occurred. Further, 11 subjects showed positive reactions to fish or shrimp allergens and these were also negative in the chitosan prick-tests. These subjects were analyzed for chitosan-specific IgE, and none was found in the serum. The principal investigator concluded that the probability for immediate hypersensitivity reactions to chitosan derived from arctic shrimp is very small. This is probably because the harsh chemical treatment involved in converting the shrimp chitin to the Primex chitosan would denature any existing allergens present in the original shrimp shell. The product

tested met the specifications established by Primex. Therefore, Primex chitosan that meets the product specifications would have a very low order of potential allergenicity. The results of this study add an important element to the safety profile of shrimp-derived chitosan, since no cross-reactivity with fish or shrimp allergens was detected.

The primary objective of the nutritional study sponsored by Primex was to determine the effect of two different doses of Primex chitosan (4.5 g and 6.75 g) on serum concentrations of fat-soluble vitamins (vitamin A, α-carotene, β-carotene, vitamin E and vitamin D), and plasma cholesterol concentrations as compared with a placebo and a positive fiber control (glucomannan) in mildly hypercholesterolemic subjects. The study was designed as a randomized, single blind, placebo controlled parallel trial. Seventy-nine (79) subjects were screened for the study, and fifty-six (56) mildly hypercholesterolemic male and female subjects completed the trial. After a two-week run-in period the subjects were randomly assigned to one of four groups, 0, 4.5 g/day or 6.75 g /day of chitosan or 6.75 g/day of glucomannan in the form of tablets. The tablets were taken with main meals for eight weeks.

There was no evidence to suggest that shrimp-derived chitosan, when taken as a supplement to the habitual Finnish diet, effected serum concentrations of vitamin A, α-carotene, β-carotene, vitamin E, 25-hydroxyvitamin D or plasma cholesterol concentrations. Levels of the fat-soluble vitamins did not change in comparison to the controls, when normalized for cholesterol level. Calcium levels in each group, including the placebo, dropped slightly over the duration of the study, but all levels remained within the normal range. Other hematological and clinical chemistry parameters remained within the normal range for both chitosan groups.

This study supports the position of the Expert Panel and of Primex that Primex chitosan does not lower human fat-soluble vitamin levels, nor does it lower serum calcium levels below the normal range. This conclusion is also consistent with recently published studies which demonstrate that the consumption of a similar chitosan product at five gram per day, does not result in significant loss of fecal fat, even when the human subjects consumed a high fat diet (Gades, *et al.,* 2002 and 2003, and Guerciolini, *et al.,* 2001). If no significant fecal fat is lost, then there will be no significant loss of fat-soluble vitamins as well.

From a critical evaluation of the above information and from the knowledge that shrimp-derived chitosan has physiological effects similar to any number of other similar viscous fiber sources (Cummings, 2001), it may be concluded that the daily consumption of 4.5-6.7 grams of Primex chitosan should not pose a threat to human nutritional status. Further, data from the studies sponsored by Primex (summarized above), as well as data from other recently published studies covering the effect of chitosan on fecal fat levels, support the conclusion that Primex chitosan derived from shrimp can be considered Generally Recognized as Safe for its intended use.

Fondest personal regards.

Cordially,

Joseph F. Borzelleca

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Microbiology
Food Technology
Regulations
(NFPA Retired)

April 27, 2005

Dr. Robert Martin
US Food and Drug Administration
Center for Food Safety and Applied Nutrition
HFS 255
5100 Paint Branch Parkway
College Park, Maryland 20740

Dear Dr. Martin:

Re: GRAS Notification for Primex Shrimp-Derived Chitosan, Review of Recent Studies

At the request of Primex ehf of Iceland, the manufacturer of shrimp-derived chitosan I have reviewed the results of two new studies sponsored by the company, and I am prepared to provide an opinion stating whether or not these studies support or negate the company's earlier GRAS determination.

The two human clinical studies sponsored by Primex were entitled "Evaluation of Chitosan as a Possible Compound Causing Immediate Hypersensitivity Reactions" and "Effect of Chitosan on Serum Fat-Soluble Vitamins and Plasma Cholesterol Concentrations in Mildly Hypercholesterolemic Subjects".

In my opinion the studies are excellent and clearly show that Primex Chitosan did not cause immediate hypersensitivity reactions in a large sample of subjects undergoing skin-prick tests. Neither was there any cross-reactivity between chitosan and shrimp or fish. The second study was designed to test the effect of chitosan on human mineral and serum fat-soluble vitamins in mildly hypercholesterolemic subjects. Both studies appear to be well designed. The statistical difference in the test samples and the placebos was not significant and calcium levels remained within normal limits.

The few skin symptoms mentioned in the allergenicity test do not appear to be directly associated with the amount of chitosan consumed, and chitosan did not initiate allergic symptoms. After reviewing these studies in which a relatively large dose of shrimp-derived chitosan was consumed, I still see no reason why Primex chitosan should not be considered GRAS. Further, after studying the large volume of work available on Primex Chitosan, I believe that it is safe to consume in a diet at the exposure estimate for the 90th percentile of approximately 4 grams per day.

Sincerely,

Cleve Denny, Expert Panel member

Primex Chitosan Expert Panel Opinion

Introduction

An expert panel qualified by scientific training and experience to evaluate the safety of food and food ingredients was assembled to conduct an independent critical evaluation of the safety of Primex chitosan as a food ingredient when produced and used in accordance with current Good Manufacturing Practices and meeting the specifications described herein. The panel members included:

Dr. Joseph F. Borzelleca	Pharmacologist and Toxicologist, Chairman
Dr. Bert N. LaDu, Jr.	Pharmacologist
Mr. Cleve Denny	Microbiologist
Dr. Paul A. Sandford	Chemist, Applications Consultant
Dr. Olav Smidsrød	Chitosan and Polysaccharide Chemist

The qualifications of the members of the Scientific Expert Review Panel (the Panel) are provided in their curricula vitae, which are on file at the offices of the Norwegian Trade Council, 800 Third Avenue, 23rd Floor, New York, New York, 10022 USA. The Panel's qualifications in their respective scientific fields meet the requirements set forth in the Federal Food, Drug, and Cosmetic Act's definition of generally recognized as safe (GRAS) substances (§ 201(s)) and 21 CFR 170.30(a) "Eligibility for classification as generally recognized as safe (GRAS)".

The Panel was charged by Primex Ingredients EHF to critically evaluate the information and data regarding chitosan in general and Primex chitosan specifically. The Panel would then render an opinion, based on scientific principles, on the generally recognized as safe (GRAS) status of Primex chitosan. The opinion of the Panel is based on the general use of Primex chitosan in food when manufactured in accordance with U.S. current Good Manufacturing Practices (21 CFR Part 110) and meeting the specifications provided in the report.

Independently, members of the Panel critically evaluated the results of a literature review on chitosan, information provided by Primex Ingredients EHF, and other pertinent materials. The Panel conferred by telephone and then met in New York City on June 8, 2000 with representatives of Primex Ingredients EHF, who reviewed the pertinent safety and functionality information associated with Primex chitosan. This information was compiled into a twelve-volume report, which supported the eligibility of Primex chitosan as a GRAS ingredient in accordance with 21 CFR 170.30. The information provided for the Panel's review was presented in the form stipulated in 21 CFR 170.35.

Conclusion

Based on the Panel's independent and collective critical evaluation of all the information summarized above, it was determined that Primex chitosan can be considered generally recognized as safe (GRAS), when produced and used in accordance with cGMP as a food in the various major food categories as listed by the United States Department of Agriculture in the *Continuing Survey of Food Intake by Individuals* (CSFII), and when meeting the specifications described herein.

Signatures

Dr. Joseph F. Borzelleca, Chairm

Date 16 November 2000

Cleve Denny

Date 16 November 2000

Dr. Bert N. La Du, Jr.

Date Nov. 21, 2000

Dr. Paul A. Sandford

Date 11-15-00

Dr. Olav Smidsrød

Date 14/11-00

In reaching this conclusion, the Panel relied on the following information:
(References refer to those cited in the 12-volume GRAS Report)

Chemical Identity and Manufacturing

- Chitosan is a polysaccharide, composed mainly of glucosamine, 2-amino-2-deoxy-β-D-glucose [Shahidi, *et al.,* 1999 Vol 8 Tab 145].

- Chitosan occurs widely in nature in the cell walls of fungi, and as a breakdown product of chitin [Shahidi, *et al.,* 1999 Vol 8 Tab 145].

- Commercially, chitosan is prepared by deacetylating chitin, which is the second most abundant natural polymer on earth [Shahidi, *et al.,* 1999 Vol 8 Tab 145].

- Chitosan has three functional groups, an amino group, as well as both primary and secondary hydroxyl groups, which are the basis for its functionality in foods [Shahidi, *et al.,* 1999 Vol 8 Tab 145].

- Primex Ingredients EHF (Primex) of Avaldsnes, Norway has developed a unique process to manufacture chitosan from the arctic shrimp, *Pandalus borealis.*

- The Primex Ingredients EHF chitosan plant has a certified Hazards Analysis Critical Control Point Program (HACCP) in place to assure product quality. [Primex Ingredients Analytical Methods Vol 7 Tab 118].

- Final product specifications for the various Primex chitosan products have been provided.

- An analysis of five lots of Primex chitosan has demonstrated that the products can consistently meet their published specifications.

- Chemical and heavy metals analyses of Primex chitosan products have shown that the products contain no toxicants of concern. Primex chitosan contains less chemical and microbiological contaminants than many common food sources.

Use and Functionality

- Because of the high cost of producing chitosan, it has not been historically used as a food or food ingredient [Shahidi, *et al.,* 1999 Vol 8 Tab 145].

- Modern humans continue to consume small quantities of chitosan as part of a normal diet. Natural sources of chitosan in the diet include various food fungi, e.g. *Pleurotus ostreatus*, and certain invertebrate animals [Illum, 1998 Vol 5

Tab 61, Chobot, *et al.,* 1995 Vol 3 Tab 22, and Muzzarelli, 1997, Vol 7 Tab 104].

- Applications for chitosan include general use in foods as a multiple-use direct additive.

- Chitosans have been found to have many beneficial properties of use to the food industry, including fat absorption, protein aggregation, emulsification capability, film-forming ability, clarifying ability and antimicrobial activity [Knorr, 1991 Vol 6 Tab 76 and Shahidi *et al.,* 1999 Vol 8 Tab 145].

- Many food scientists have considered chitosan a source of dietary fiber [Furda, 2000, Vol 4 Tab 40, and Dreher, 1987 Vol 4 Tab 27].

Self-Limiting Use in Foods

- Studies by food scientists have shown that chitosan has been an effective emulsifier at percentages of use of less than 1.0% [Poole, 1988 Vol 11 Tab 225 and Rodriguez, *et al.,* 2000 Vol 17 Tab 376].

- Use of chitosan is expected to be limited by its physicochemical properties.

- Excessive chitosan usage may produce aggregation of ingredients, depending on the synergies involved and the pH of the formulation [Poole, 1988 Vol 11 Tab 225]. Poole found that use of greater than 0.8% chitosan yielded a dense aggregate in a whey protein isolate-corn oil emulsion [Poole, 1988 Vol 11 Tab 225].

Exposure

- Primex Ingredients EHF has provided an estimate of exposure to its ChitoClear™ chitosan products. Additional exposure to chitosan may result from its use as a source of dietary fiber in dietary supplement form [Enns, *et al.,* 1997 and Langhelle, 1999 Vol 17 Tab 375].

- The mean and the 90th percentile of estimated exposure to Primex ChitoClear™ chitosan assume that 100% of consumers are "eaters" of chitosan-containing products within a given food category, and that all foods within a given category contain chitosan.

- The mean and the 90th percentile of estimated exposure to Primex ChitoClear™ chitosan (7.97 and 15.94 grams respectively) remains lower than the recommended levels of intake for dietary fiber (25-30 grams per day) in the United States [Enns, *et al.,* 1997 Vol 4 Tab 36].

- The recommended dose of chitosan as a dietary supplement is 1.5 to 3.0

grams per day. This dosage has been recommended based on the results of clinical trials with human volunteers [Colombo, *et al.*, 1996, Vol 3 Tab 24].

Current Usage

- One company, Vanson Incorporated, Redmond, WA, manufactures chitosan in the United States.

- Chitosan is approved as a food in Japan, and several products ranging from biscuits to fishcakes have been approved by the Japanese Ministry of Agriculture, Fisheries and Foods as "Foods for Specified Health Use" (FOSHU) [FOSHU, 1999 Vol 17 Tab 372].

- Japan is the world's leader in the manufacture of chitosan, accounting for 90% of the global chitosan market, worth approximately 4 trillion yen (US$ 33.3 billion, where 120 yen = 1 US dollar) [*Korea Times*, April 7, 1999 Vol 13 Tab 280].

- The *Japan Times* of April 11, 2000 reported that sales of dietary chitosan in Japan totaled nearly US$1 billion per year [*Japan Times,* April 11, 2000 Vol 14 Tab 286].

- A 1999 US State Department Document entitled "Japan – Chitin and Chitosan Market – IMI990408" indicated that the current health food market for chitosan in Japan is slightly more than 200 metric tons per year.

- Ten firms are reported to manufacture chitosan in Japan, and 60 are reported to package chitosan into various products [*Korea Times*, April 7, 1999 Vol 14 Tab 297].

- Chitosan has also been approved for use in Korea, and appears in that country as a food for the improvement of the health of the digestive tract [Lian, *et al.*, 1998 Vol 14 Tab 300]

- Five firms are reported to manufacture chitosan in Korea, and 70 companies make end products using chitosan [*Korea Times*, April 7, 1999 Vol 14 Tab 297].

- The Australian Complementary Medicines Evaluation Committee considered the use of chitosan under Section 7 of the *Therapeutic Goods Act 1989* from June 1998 through September 1998. Members of the committee considered data and information, both pro and con, regarding the consumption of chitosan by Australians at doses of 1-2 grams per day, and concluded that Chitosan does not appear to present a significant risk to consumers in the manner and dosage likely to be consumed.

- Use of chitosan as a dietary supplement in the U.S. has been growing rapidly.

According to Information Resources, Inc., approximately US . has been sold by the three leading brands since February, 1999. This translates to approximately 240,000 kilograms of consumption, at an average wholesale price of U.S. . . Actual usage is now estimated to be 500,000 kilograms per year, since the *Natural Products Insider, 1999 Buyer's Guide* listed 72 suppliers/distributors of chitosan in the US.

- Recently, the United States Environmental Protection Agency (EPA) has exempted poly-D-glucosamine (chitosan) from the requirement of a tolerance [Federal Register Vol 13 Tab 261]. Chitosan is suitable for use as an active ingredient in listable products without limits.

Animal Studies

- Thirty-three animal safety and functionality studies in five species of animals were considered by the Panel.

- Both human and animal studies have shown that chitosan is well tolerated, and that its metabolism produces substances that are found in the human body and other dietary substances [Kelly, 1998 Vol 14 Tab 291].

- A contact hypersensitivity test with albino guinea pigs showed that none of the control and test animals were observed to have skin reactions after the challenge treatment with Primex chitosan at a concentration of 1.875 %.

Human Safety

- Ten published human studies conducted in various countries with volunteers consuming 1.0 – 6.0 grams of chitosan per day demonstrated that vitamin, mineral, and hemoglobin balances remained within normal limits.

- Adverse events accompanying the use of chitosan and a calorie-restricted diet included mild and transitory nausea, flatulence, or constipation in < 5% of the subjects. However, there was no statistically significant difference in the number or severity of adverse events between the treatment and control groups [Veneroni, *et al.,* 1996 Vol 11 Tab 214, and Sciutto, *et al.,* 1995 Vol 8 Tab 141].

Fate of Chitosan in the Gastrointestinal Tract

- Hirano indicated in 1989 that approximately 60 tons of chitosan per year went into the production of D-glucosamine [Hirano, 1990 Vol 5 Tab 54].

- The metabolic fate of chitosan in the digestive tract of man has been tracked through radiolabelled preparations [Macleod, *et al.,* 1999 Vol 6 Tab 97]. Studies demonstrated that chitosan is not degraded until it reaches the colon [Macleod, *et al.,* 1999 Vol 6 Tab 97].

- The hydrolysis of chitosan has been studied, and the degradation of natural polymers, such as chitosan and chitin proceeds via enzymatic degradation [Domard, *et al.*, 1989 Vol 12 Tab 255 and Lee *et al.*, 1995 Vol 6 Tab 91].

- Both chitin and chitosan have been known to be digestible by chitinase and lysosyme in accordance with the quantity of N-acetyl groups and their distribution within the backbone of the molecule [Lee, *et al.*, 1995 Vol 6 Tab 91, Aiba, 1993 Vol 12 Tab 227, Aiba, 1992 Vol 12 Tab 228, and Sashiwa, *et al.*, 1990 Vol 15 Tab 234].

- Muzzarelli, 1997, has provided evidence for the degradation of chitosan in humans via non-specific chitinase and lysosymal enzyme systems [Muzzarelli, 1997 Vol 7 Tab 104].

- Chitosan is degraded into chitooligosaccharides, and then finally into glucosamine [Domard, 1989, Vol 12 Tab 255, Aiba, 1993 Vol 12 Tab 227, and Aiba, 1992 Vol 12 Tab 228].

- Glucosamine, which is the final break-down product of chitosan is also formed in the human body as glucosamine 6-phosphate (G6-P) [Kelly, 1998 Vol 14 Tab 291].

Appendix II
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Primex Ingredients ASA Analytical Results for Amino Acids	119	7
Primex Ingredients ASA Analytical Results for Dioxin	120	7
Primex Ingredients ASA Analytical Results for Five Lots in Duplicate Raw Data	121	7
Primex Ingredients ASA Analytical Results for Minerals	122	7
Primex Ingredients ASA Analytical Results for Pesticides	123	7
Primex Ingredients ASA Certificates of Analyses	124	7
Primex Ingredients ASA Certificate of Free Sale	125	7

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	Tab	Vol
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Primex Ingredients ASA Production Process	127	8
Primex Ingredients ASA Environmental Permits	128	8
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Appendix III

Appendix III

Symptoms and Dropouts

In 2003 Primex ehf sponsored a study, which was conducted by Oy Foodfiles Ltd in Finland. The title of the study was "Effect of Chitosan on Serum Fat-Soluble Vitamins and Plasma Cholesterol Concentrations in Mildly Hypercholesterolemic Subjects" [Tapola, *et al.,* 2003 Vol 16 Tab 357]. This Appendix discusses the gastrointestinal symptoms reported to some extent by by all groups, including the placebo (Table 1).

Distressing gastrointestinal (GI) symptoms not related to identifiable pathology affect 15% to 30% of the United States population [Georges, *et al.,* 1994 Vol 13 Tab 270]. In a recent survey of 5430 households, Drossman *et al.* (1993) documented the presence of at least one functional bowel symptom in 69% of this study group. The symptoms reported included abdominal pain, distention, and changes in defecation patterns. These symptoms account for 100,000 hospital discharges each year and 30% to 70% of visits to gastroenterologists population [Georges, *et al.,* 1994 Vol 13 Tab 270].

In the study sponsored by Primex, common gastrointestinal symptoms were observed. However, there was no significant difference reported by the test subjects for their Physical Functioning, Vitality, or Bodily Pain at the completion of the trial as measured by the RAND-36 [Tapola, *et al.,* 2003 Vol 16 Tab 357]. There were no significant differences in the gastrointestinal symptoms reported among groups, except that the incidence of constipation, heartburn and nausea was greater during the first four weeks of the study in the chitosan groups in comparison to the placebo. In general the numbers of subjects reporting these symptoms declined during the last four weeks of the trial. Four and three subjects in the 4.5 and 6.75 gram chitosan groups, respectively, reported constipation in the last four weeks of the study, however, the severity of the constipation dropped in both groups from 2.5 to 1.5 and from 1.5 to 1.0 on a 4.0 point scale (See Table 2). Four and two subjects in the 4.5 and 6.75 gram chitosan groups, respectively, reported heartburn in the last four weeks of the study, however, the severity of the heartburn remained at a level of 1.0 in the

group consuming the 4.5g/day level of chitosan, while the two subjects who still experienced heartburn in the last four weeks of the study in the 6.75g/day group reported that their symptoms increased from 2.0 to 3.5 on a 4.0 point scale.

Subjects reporting nausea dropped from three to two in the 4.5g/day group during the last four weeks of the trial, and the severity of their symptoms dropped from 2.0 to 1.0 on a 4.0 scale. This data may indicate adaptation over time to additional fiber intake. Five subjects reported nausea in the 6.75g/day group during the first four weeks, with a median nausea level of 1.0. During the second four weeks, four subjects reported nausea with a median level of severity of 2.0.

There were no differences in defecation frequency among the groups (Table 3). However, two subjects in the Chitosan 4.5 g group reported blood in the feces and hemorrhoids, one subject reported dizziness, one subject sweating by nights and one subject bulging veins. In the chitosan 6.75 g group one subject reported tender anus. In the glucomannan group one subject reported dizziness. However, there were no significant differences between the groups in pairwise comparisons [Tapola, *et al.,* 2003 Vol 16 Tab 357].

Appendix III Symptoms and Dropouts of the Primex Sponsored Fat soluble Vitamin Study

Table 1. The number of the subjects reporting symptoms.

	Chitosan 4.5 g (n = 15)	Chitosan 6.75 g (n = 12)	Glucomannan 6.75g (n = 15)	Placebo (n = 14)	p-value[a]
Loose feces					
0-4 wk	5	3	5	4	NS
4-8 wk	2	0	4	4	NS
Repeated diarrhea					
0-4 wk	1	0	2	2	NS
4-8 wk	0	0	0	0	NS
Constipation					
0-4 wk	8	2	3	1	0.021
4-8 wk	4	3	1	0	NS
Abdominal pain					
0-4 wk	4	3	2	2	NS
4-8 wk	3	4	2	3	NS
Repeated flatulence					
0-4 wk	3	1	5	2	NS
4-8 wk	2	3	6	3	NS
Heartburn					
0-4 wk	4	5	2	0	0.044
4-8 wk	4	2	2	0	NS

Appendix III Symptoms and Dropouts of the Primex Sponsored Fat soluble Vitamin Study

Table 1 continued

	Chitosan 4.5 g (n = 15)	Chitosan 6.75 g (n = 12)	Glucomannan 6.75 g (n = 15)	Placebo (n = 14)	p-value[a]
Abdominal bloating					
0-4 wk	3	3	3	4	NS
4-8 wk	2	2	2	3	NS
Nausea					
0-4 wk	3	5	1	0	0.023
4-8 wk	2	4	0	0	0.016
Abdominal rumbling					
0-4 wk	1	1	3	1	NS
4-8 wk	2	0	2	2	NS
Abdominal crumbling					
0-4 wk	0	0	0	1	NS
4-8 wk	0	2	0	1	NS
Headache					
0-4 wk	2	0	2	0	NS
4-8 wk	0	0	0	0	NS
Fatigue					
0-4 wk	2	1	1	1	NS
4-8 wk	1	0	1	0	NS

[a] indicates the significance of the independence of the test group and reported symptoms analyzed with the Pearson chi-square test

Appendix III Symptoms and Dropouts of the Primex Sponsored Fat soluble Vitamin Study

Table 2. The severity of reported abdominal symptoms by degree (median)

1 (= the mildest) to 4 (= the strongest)

	Chitosan 4.5 g (n = 15)	Chitosan 6.75 g (n = 12)	Glucomannan 6.75 g (n = 15)	Placebo (n = 14)
Loose feces				
0-4 wk	1.0	1.0	1.5	1.5
4-8 wk	1.0	-	1.0	1.0
Repeated diarrhea				
0-4 wk	2.0	-	1.5	2.0
4-8 wk	-	-	-	-
Constipation				
0-4 wk	2.5	1.5	1.0	1.0
4-8 wk	1.5	1.0	3.0	1.0
Abdominal pain				
0-4 wk	2.0	2.0	1.0	2.0
4-8 wk	1.0	2.0	1.0	2.0
Repeated flatulence				
0-4 wk	2.0	1.0	2.0	2.5
4-8 wk	1.0	1.0	2.0	1.0
Heartburn				
0-4 wk	1.0	2.0	1.0	-
4-8 wk	1.0	3.5	1.5	-

Appendix III Symptoms and Dropouts of the Primex Sponsored Fat soluble Vitamin Study

Table 2 Continued

	Chitosan 4.5 g (n = 15)	Chitosan 6.75 g (n = 12)	Glucomannan (n = 15)	Placebo (n = 14)
Abdominal bloating				
0-4 wk	1.0	2.0	2.0	2.5
4-8 wk	1.0	2.5	2.0	2.0
Nausea				
0-4 wk	2.0	1.0	1.0	-
4-8 wk	1.0	2.0	-	-
Abdominal rumbling				
0-4 wk	1.0	2.0	1.0	3.0
4-8 wk	1.0	-	2.5	1.5
Abdominal crumbling				
0-4 wk	-	-	-	1.0
4-8 wk	-	2.0	-	1.0
Headache				
0-4 wk	3.0	-	1.5	-
4-8 wk	-	-	-	-
Fatigue				
0-4 wk	2.0	1.0	2.0	1.0
4-8 wk	2.0	-	1.0	-

Appendix III Symptoms and Dropouts of the Primex Sponsored Fat Soluble Vitamin Study

Table 3. Defecation frequency. Mean (SD)

	Chitosan 4.5 g (n = 15)	Chitosan 6.75 g (n = 12)	Glucomannan (n = 15)	Placebo (n = 14)	p-value[a]
Defecation frequency (times per day)					
habitual	1.1 (0.6)	1.0 (0.4)	1.1 (0.5)	1.2 (0.6)	NS
0-4 wk	1.2 (0.6)	1.2 (0.5)	1.2 (0.5)	1.2 (0.6)	NS
4-8 wk	1.3 (0.6)	1.3 (0.7)	1.1 (0.6)	1.3 (0.6)	NS
p-value[b]	NS	NS	NS	NS	

[a] The significance of the difference among the study groups analyzed with the Kruskal-Wallis test adjusted with the Bonferroni correction.

[b] The significance of the within-group difference analyzed with the Friedman test adjusted with the Bonferroni correction.

Study Dropouts

A total of 9 subjects withdrew from this trial, three males and 6 females. Two male subjects withdrew from the placebo group; one due to poor compliance, and the other due to a screening failure after randomization. One male dropped from the chitosan 4.5 gram group for unknown reasons.

Six females from the high fiber groups (6.75 gram per day glucomannan and chitosan groups) withdrew from the study. One, in the 6.75 gram per day glucomannan group was unable to consume the tablets. Five discontinued the study due to various gastrointestinal symptoms. Four subjects in the chitosan 6.75 g group and one subject in the glucomannan group discontinued the study due to symptom complaints (See Table 4 below).

Significantly, the women who discontinued the study were circa age 50, and therefore, peri-menopausal (See Table 4). Menopausal women are known to have a greater incidence of gastrointestinal symptoms than the population in general, and functional bowel distress (FBD) is present in a high percentage of the population. Subjects with these types of symptoms were not excluded from participating in this study. The women in the high fiber groups were consuming 23-24 grams of fiber per day in addition to 6.75 grams of either chitosan or glucomannan [Tapola, *et al.*, 2003 Vol 16 Tab 357, Georges, *et al.*, 1994 Vol 13

Tab 270]. Therefore, after the study began, their fiber intake approached 30 grams per day.

Georges, *et al.,* 1994, who studied dietary fiber and distressing gastrointestinal symptoms in 20 midlife women (mean age 52.7 years), noted a wide variability in GI symptom severity. This research group from Ohio State University found a significant negative relationship between dietary fiber intake and abdominal pain, awakening with abdominal pain, nausea, awakening with nausea, and awakening with rectal pain in American women consuming 5.4 to 13.0 gram of fiber per day (mean intake of dietary fiber for all subjects was 9.02g) [Georges, *et al.,* 1994 Vol 16 Tab 349]. These authors explained that American women are at particular risk for the development of distressing GI symptoms, and have demonstrated a 2:1 incidence of such symptoms in comparison to American males. In other recent studies of GI function and symptoms in menstruating women ages 20 to 43, approximately 30% of the women reported distressing GI symptoms compatible with functional bowel distress on a retrospective questionnaire.

Georges, *et al.,* 1994 stated that sudden increases in dietary fiber intake (such as would happen during a study) were likely to result in increased gastrointestinal symptoms. Table 5 below shows the correlation between menses and gastrointestinal symptoms in women 19-43 years of age [Jarrett, *et al.*, 1996 Vol 14 Tab 287]. Table 6 presents the range and severity of gastrointestinal symptoms in women ages 19-43 as studied by Jarrett, et al., 1996 age [Jarrett, *et al.*, 1996 Vol 14 Tab 287]. This data can be compared to that listed in Table 2.

Makkonen, *et al.,* 1993, who studied the endocrine and metabolic effects of 15 grams per day of guar gum in menopausal Finnish women, also noted that eight of 15 subjects in the guar gum group and four in the placebo group had gastrointestinal side-effects. The investigators stated that the incidence of the side-effects in the guar gum group was comparable to that found in previously published studies, and that the side-effects were mild [Makkonen, *et al.,* 1993 Vol 14 Tab 306]. The mean age of the Finnish women was 52.3±2.7 years.

Appendix III Symptoms and Dropouts of the Primex Sponsored Fat Soluble Vitamin Study

The discomfort of the 6 women in both the chitosan and glucomannan high fiber groups resolved after their fiber intake was reduced. The gastrointestinal symptoms picture of potential study subjects may need to be a screening criterion to consider before further studies are designed.

A few subjects reported skin symptoms during the study. In the chitosan 4.5 g group one subject reported dry skin, one subject oily skin and one subject itchy skin. In the chitosan 6.75 g group one subject reported dry skin in the palm of his hand, one subject rash and one subject itchy stomach skin. In the placebo group one subject reported pimples on the back. No one reported skin symptoms in the glucomannan group [Tapola, *et al.,* 2003 Vol 16 Tab 357]. Since this information does not appear to be related to any one treatment group, Primex is unable to draw the conclusion that skin symptoms were related to fiber intake.

The company has conducted an evaluation of chitosan as a possible stimulant of hypersensitivity in 221 human subjects, and as discussed elsewhere in this report, no allergenicity or other type of hypersensitivity was observed, even in those eleven subjects, who showed positive skin prick tests to fish or shrimp [Harvima, *et al.,* 2002 Vol 13 Tab 276]. Therefore, the company concludes that there is no reason to suspect that Primex Chitosan elicited the skin responses described above.

Appendix III Symptoms and Dropouts of the Primex Sponsored Fat Soluble Vitamin Study

Table 4
Subjects withdrawing from Study

Group	Chitosan 4.5 g	Chitosan 6.75 g	Glucomannan	Placebo
Subjects	1 Male	4 Females	2 Females	2 Males
Age	30	49 (4)	52 (2)	45 (7)
Reason	1: unknown reasons	4: Adverse Effects	1: Adverse Effects 1: Unable to eat Tablets	1: Poor Compliance 1: Screening Failure
Symptoms	-	1. Constipation	1. Abdominal pain, headache, feeling of nausea	-
	-	2. Feeling of Nausea, loss of appetite itching skin-palms and soles	-	-
		3. Hemorrhoids	-	-
	-	4. Burning sensation in the intestines, constipation, heartburn, hemorrhoids	-	-

Appendix III Symptoms and Dropouts of the Primex Sponsored Fat Soluble Vitamin Study

Table 5
Association of Gastrointestinal symptoms with Menses

	Cycle 1 M(SE)	Cycle 2 M(SE)	Cycle 3 M(SE)	Average M(SE)
Symptoms				
Stomach Pain	0.51 (0.13)	0.31(0.12)	0.36(0.10)	0.45 (0.11)
Nausea	0.40 (0.10)	0.22(0.10)	0.28(0.08)	0.32 (0.08)
Diarrhea	0.00 (0.12)	0.01(0.10)	0.16 (0.08)	0.10 (0.09)
Constipation	0.20 (0.11)	0.12(0.07)	-0.02(0.04)	0.13 (0.06)
Stool Characteristics				
Stool Consistency	0.02(0.13)	0.08(0.13)	0.18 (0.12)	0.10 (0.13)
Number of Stools	-0.33(0.34)	0.48(0.35)	0.48 (0.27)	0.36 (0.36)

[Jarrett, *et al.*, 1996 Vol 14 Tab 287]

Table 6. Mean Severity of distressing GI Symptoms Rated on a 0 to 4 scale on a Daily Basis		
	M	SD
Abdominal Pain	1.2	1.3
Abdominal Distension	1.2	1.2
Awakening with abdominal Pain	0.8	1.2
Nausea	0.4	0.8
Awakening with Nausea	0.3	0.7
Awakening with rectal pain	0.2	0.4

[Georges, *et al.*, 1994 Vol 13 Tab 270].

000318

Appendix IV

Appendix IV

Emulsification and Foaming Agent Properties of Chitosan

Chitosan has demonstrated an ability to improve foaming and emulsification systems in foods at relatively low percentages of use. Chitosan has been shown to produce a w/o/w double emulsion in one step without the addition of surfactants [Rodriguez *et al.*, 2000 Vol 12 Tab 237]. These technical effects are based on the basic physicochemical properties of the molecule, which function *in vivo* as well as in food systems. Rodriguez *et al.*, 2000 hypothesizes that the one-step formation of w/o/w double emulsions may be due to the heterogeneity of the molecule [Rodriguez *et al.*, 2000 Vol 12 Tab 237]. The varied technical effects of which chitosan is capable will allow it to be considered as an ingredient in a wide variety of food categories [Poole, 1988 Vol 11 Tab 225].

Poole determined that low viscosity chitosan behaves like basic proteins, that enhance the foaming of acidic proteins in the presence of lipids. In his laboratory, Poole, performed a number of experiments to optimize the effect of chitosan on foam formation and stability. Table 1 shows that at a constant chitosan to whey protein isolate (WPI) ratio of 1:10, a minimum concentration of whey protein and chitosan was necessary to enhance foaming. A minimum WPI concentration of 4% was required for foaming in the presence of 10% corn oil. However, a self-limiting level was reached at 8%, due to excessive precipitation, which prevented foaming [Poole, 1988 Vol 11 Tab 225].

Appendix IV

Table 1
Influence of WPI Concentration on Foaming with Corn Oil
(Chitosan: WPI = 1:10; corn oil = 10%; pH = 6.0; sucrose = 10%)

Conc. Of WPI (%)	Conc. Chitosan (%)	%Foam Expansion	%Foam Volume Stability	%Foam Liquid Stability	Foam Description
2.0	0.2	140	60	10	Poor
4.0	0.4	500	95	75	Fairly Stiff
6.0	0.6	550	100	90	Stiff
8.0	0.8	-	-	-	Dense
					Aggregate

The range of lipids used in food manufacture is extensive, according to Poole. Therefore, the ability of the chitosan-whey protein system to tolerate several lipids was investigated. The results are presented in Table 2. Foams described as "Good" were obtained with all the lipids tested with the exception of lecithin, a lipid that is known to be particularly detrimental to foaming [Poole, 1988 Vol 11 Tab 225]. Table 2 shows foaming scores for emulsions with and without low-viscosity chitosan added to whey protein isolate systems. The conclusion drawn from the experiment was that chitosan significantly improved foam properties with all the fats tested except lecithin.

Appendix IV

Table 2
Foaming properties of WPI/chitosan with different lipids
(WPI = 6%; chitosan = 0.7%; sucrose = 30 %; pH = 6.0; lipid = 20%)

Type of fat/oil	Chitosan	%Foam Expansion	%Foam Volume Stability	%Foam Liquid Stability	Description of foam
Corn oil	+	570	100	100	Very stiff
	-	130	55	0	Poor
Coconut oil	+	870	100	100	Stiff
	-	80	45	0	Poor
Butter oil	+	910	100	75	Fairly Stiff
	-	130	50	0	Poor
Cocoa butter	+	630	100	100	Fairly Stiff
	-	95	45	0	Poor
Lard	+	300	85	35	Poor
	-	90	45	0	Poor
Lecithin (0.1%)	+	170	70	20	Poor
	-	200	65	0	Poor
Lard (10%)	+	970	90	100	Very Stiff
	-	90	45	0	Poor

Likewise, various sugars and polysaccharides are used in foods to sweeten or alter texture. A representative range of these carbohydrates was tested in Poole's laboratory in a whey protein isolate foaming system. The carbohydrates tested comprised: sorbitol a non-cariogenic sugar alcohol; glucose syrup consisting of mono-, di-, and oligosaccharides derived by controlled hydrolysis of starch; polydextrose a reduced-calorie bulking agent comprising randomly polymerized glucose; methylcellulose an uncharged viscosity-enhancing food polysaccharide.

"Good" foams were obtained when 0.7% of a chitosan solution was added to the foaming system, with all the saccharides except methyl cellulose, which inhibited foaming (presumably because the high viscosity inhibited air incorporation) [Poole, 1988 Vol 11 Tab 225].

Appendix IV

Table 3 presents the effect of low-viscosity and hydrolyzed chitosans on the foaming of various proteins under similar conditions. "Good" foams were obtained with egg albumen, which, like whey protein, is a globular protein. However, hydrolysis of chitosan beyond 10% adversely effected foam-enhancement. Serum albumin (BSA), another acidic globular protein, also gave good results but no foam-enhancement was observed with caseinate and gelatin, which are extended random coil molecules. Both were precipitated excessively by chitosan. Therefore, it was determined that the conformation of the protein is an important factor to consider when adding chitosan to a foaming system.

Table 3
Effects of Low-Viscosity Chitosan and Hydrolyzed Chitosans
(DH 5, 10 and 15%) on the Foaming of Acidic Proteins
(protein = 6%; chitosan = 0.7%; sucrose = 30%; pH = 6.0)

Acidic protein	Chitosan	%Foam Expansion	%Foam Volume Stability	%Foam Liquid Stability	Description of foam
Egg albumen	LV	570	100	100	Very Stiff
Egg albumen	DH 5%	520	100	95	Moderately stiff
Egg albumen	DH 10%	500	95	75	Flowed
Egg albumen	DH 15%	235	80	35	Flowed
Caseinate	DH 5%	30	40	20	Very poor
Gelatin	DH 5%	-	-	-	No foam
WPI	DH 5%	720	100	100	Moderately stiff
BSA	DH 5%	710	95	75	Stiff

Appendix IV

The data presented above have confirmed the ability of chitosan to act as a foaming agent, and in addition they have established that minimum and self-limiting ranges exist for the performance of chitosan in these types of systems.

Submission End

AM

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TO: MR. RICHARD BONNETT, CFSAN
VIA FAX: (301) 436-2964

FROM: LEE B. DEXTER (b)(6)

PAGES: 2

DATE: OCTOBER 31, 2005

RE: LETTER WITHDRAWING GRAS NOTIFICATION NUMBER 000170

Dear Mr. Bonnett:

Please find attached a letter withdrawing the GRAS Notification for shrimp-derived chitosan. Primex ehf would like to withdraw without prejudice. The company would like to schedule a meeting with appropriate CFSAN personnel, so that Primex can determine if publication of unpublished studies will be sufficient to resolve any of the concerns the agency may have concerning the safety and intended use of this material, prior to submitting a new and revised document.

Please accept my thanks for calling me in advance on this issue.

000330

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October 31, 2005

Mr. Richard Bonnett
US Food and Drug Administration
Center for Food Safety and Applied Nutrition
5300 River Road, Room 2038
College Park, Maryland 20770
Via Fax: (301) 436-2964

Dear Mr. Bonnett:

Re: GRAS Notification for Primex Shrimp-Derived Chitosan
Withdrawal

Primex ehf, Siglufjordur, Iceland hereby notifies the Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety, that the company withdraws its GRAS Notification for shrimp-derived chitosan (GRN 000170) without prejudice. It is the intention of Primex ehf to resubmit a revised notice of a claim for premarket exemption based on a GRAS determination under proposed §170.36, following discussions with agency personnel.

In order to determine whether publication of studies sponsored by the company may assist in creating general recognition in the scientific community regarding the safety and intended uses of shrimp-derived chitosan, Primes ehf would like to schedule a meeting to discuss GRN 000170. Following the meeting, the company will prepare a revised Notification, which will incorporate any pertinent information contained in recent publications.

Primex ehf wishes to thank CFSAN officials for their cooperation and attention in reviewing GRN 000170. On the company's behalf, Steven Dillingham or I will telephone to make arrangements for a meeting time convenient to both the agency and Primex.

Sincerely,

(b)(6)

Lee B. Dexter
Technical Consultant

CC: Bjarte Langhelle, Primex ehf
Haukur Omarsson, Primex ehf
Steven Dillingham, Strategro, Inc.

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