ORIGINAL SUBMISSION

August 21, 2012

Food and Drug Administration
Center for Food Safety & Applied Nutrition
Office of Food Additive Safety (HFS-255)
5100 Paint Branch Parkway
College Park, MD 20740-3835

Attention: Dr. Mary D. Ditto

Re: GRAS Notification - ChitoClear® Shrimp-Derived Chitosan

Dear Dr. Ditto:

On behalf of Iceland-based Primex, ehf, we are submitting for FDA review the accompanying GRAS notification for ChitoClear® which is chitosan that is derived from shrimp. The attached documentation contains the specific information that addresses the safe human food uses for the subject notified substance. Four copies of the subject notification are provided since meat and poultry product applications are anticipated for the notified substance which requires coordinated review with USDA.

If additional information or clarification is needed as you and your colleagues proceed with the review, please feel free to contact me *via* telephone or email.

We look forward to your feedback.

Sincerely,

(b) (6)

Robert S. McQuate, Ph.D.
CEO & Co-Founder
GRAS Associates, LLC
20482 Jacklight Lane
Bend, OR 97702-3074
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Enclosure: GRAS Notification for ChitoClear® Shrimp-Derived Chitosan (4 copies)

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Division of
Biotechnology and
GRAS Notice Review

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Enclosure: GRAS Notification for ChitoClear® Shrimp-Derived Chitosan (4 copies)

GRAS ASSESSMENT

ChitoClear Shrimp-Derived Chitosan

Food Usage Conditions for General Recognition of Safety

for

Primex, ehf

Siglufjordur, ICELAND

Evaluation by

Richard C. Kraska, Ph.D., DABT
Robert S. McQuate, Ph.D.
Madhusudan G. Soni, Ph.D., FACN, FATS

August 20, 2012

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I. GRAS EXEMPTION CLAIM

A. Claim of Exemption from the Requirement for Premarket Approval Pursuant to Proposed 21 CFR 170.36(c)(1)[1]

#

In accordance with Section 201(s) of the Federal Food, Drug, and Cosmetic Act, Primex, ehf has determined that its shrimp-derived chitosan, meeting the specifications described below for the branded ChitoClear® products, is Generally Recognized As Safe (GRAS). Primex has made this GRAS determination based on scientific procedures in concert with an appropriately convened panel of experts who are qualified by scientific training and experience. The evaluation accurately reflects the intended food uses of the shrimp-derived chitosan.

Signed:

(b) (6)

August 20, 2012

Robert S. McQuate, Ph.D.
GRAS Associates, LLC
20482 Jacklight Lane
Bend, OR 97702-3074

B. Name Address of Notifier

Primex, ehf
Oskarsgata 7
580 Siglufjordur
ICELAND

As the notifier, Primex, ehf, Siglufjordur, Iceland, ("Primex") accepts responsibility for the GRAS determination that has been made for ChitoClear® shrimp-derived chitosan products as direct additives serving multiple food use purposes as described in the subject notification; consequently, ChitoClear® shrimp-derived chitosan, meeting the conditions described herein is exempt from premarket approval requirements for food ingredients.

C. Common Name Identity of Notified Substance

The common name of notified substance is shrimp-derived chitosan and shrimp chitosan. See also Section III.

D. Conditions of Intended Use in Food

Shrimp-derived chitosan, in the form of selected ChitoClear® products, is intended to be added to designated foods---including meat and poultry products---as a direct additive to serve one or more

[1] See 62 FR 18938 (17 April 1997); http://www.gpo.gov/fdsys/pkg/FR-1997-04-17/pdf/97-9706.pdf

of the designated food uses shown below. In order to classify the various effects ingredients may have in food, FDA has published a list of 32 physical or technical effects for which ingredients may be added to food. These are codified at 21 CFR 170.3(o)(1-32). The intended applications for shrimp-derived chitosan are covered under one or more of the following designations listed within 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."

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

(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.

E. Basis for GRAS Determination

Pursuant to 21 CFR 170.30, shrimp-derived chitosan---as ChitoClear® products---has been determined to be GRAS on the basis of scientific procedures as discussed in the detailed description provided below.

F. Availability of Information

The data and information that serve as the basis for this GRAS notification will be sent to the US Food and Drug Administration (FDA) upon request or will be available for review and copying at reasonable times at the offices of GRAS Associates, LLC, located at 20482 Jacklight Lane, Bend, OR 97702-3074.

II. INTRODUCTION

A. Objective

At the request of Primex, ehf, GRAS Associates, LLC ("GA") has undertaken an independent safety evaluation of ChitoClear® products for use in foods. The purpose of the evaluation is to ascertain whether or not the intended food uses of ChitoClear® can be considered to be Generally Recognized As Safe (GRAS) when used as a direct food ingredient for one or more food use purposes in selected food categories.

B. Foreword

Primex is a manufacturer and supplier of shrimp-derived chitosan ingredients for the food and dietary supplement industries. Primex provided GA with substantial background information to enable the GRAS assessment for ChitoClear® products to be undertaken. Determining how much ChitoClear® can be safely consumed, i.e., the exposure or use levels, is critical in the determination of safe dietary levels of ChitoClear® when consumed as a food ingredient.

The safety/toxicity studies, consumption/exposure information, and other related documentation was augmented with an independent search of the scientific and regulatory literature through June 2012. The GRAS assessment for intended ChitoClear® uses was based primarily on the composite safety information---that is, it is based on scientific procedures. The composite safety/toxicity studies in concert with exposure information ultimately provide the specific scientific foundation for the GRAS determination. Those references that were deemed pertinent to the objective at hand are listed in Section VIII.

C. FDA Regulatory Framework

Ingredients for use in foods must undergo premarket approval by FDA as food additives or, alternatively, the ingredients to be incorporated into foods must be determined to be generally recognized as safe (GRAS). The authority to make GRAS determinations is not restricted to FDA. In fact, GRAS determinations may be provided by experts who are qualified by scientific training and experience to evaluate the safety of food and food ingredients under the intended conditions of use.

In 1997, FDA altered the GRAS determination process by eliminating the formal GRAS petitioning process and replacing the petitioning process with a notification procedure. While outlining the necessary content to be considered in making a GRAS determination, FDA encouraged that such determinations should be provided to FDA in the form of a notification. However, notifying FDA of such determinations is voluntary.

D. Regulatory Background for Chitosan

Chitosan is allowed for dietary use in several countries. Chitosan is approved as a natural food additive for general food use in Japan and Korea (Japan Food Chemical Research Foundation, 2011; KFDA, 2011). Chitosan has been the subject of a notification in the European Union as a

novel food for use in supplements (EU, 2007), and the European Food Safety Authority (EFSA) Panel on Dietetic Products, Nutrition and Allergies has permitted the use of a health claim---maintenance of normal blood LDL-cholesterol concentrations---for chitosan (EFSA, 2011).

In 2001 and 2005, Primex submitted GRAS notices (GRN 73 and GRN 170) on shrimp-derived chitosan to FDA (FDA, 2001, 2005). Based on discussions with the agency, Primex requested FDA to cease the evaluation of both submissions. Among the primary concerns noted by the agency from the two review meetings were:

- The unpublished status of a key clinical study (Tapola et al., 2008) on chitosan;
- Scientific concerns stemming from three publications on chitosan by Deuchi and co-workers (Deuchi et al., 1994; 1995a, 1995b);
- The commitment by the National Toxicology Program (2000) to undertake a toxicology study on chitosan; and
- Doubts about consensus existing within the scientific community on the safety conclusion.

Since the withdrawal of the second chitosan GRAS notification (GRN 170), significant new information has appeared in the published literature on chitosan. The recently published safety studies and other information as discussed in this safety evaluation support a GRAS determination since these studies adequately resolve the scientific questions and concerns that were previously raised. More detailed responses to these concerns are found elsewhere in this notification.

Based on the information available from FDA's GRAS Notice Inventory[2] website, on August 08, 2011, FDA filed a GRAS notice on chitosan derived from *Aspergillus niger* (GRN 397) for use as a secondary direct food ingredient (FDA, 2011). On December 19, 2011, this notice received a "no questions" letter from FDA. Although the source organism (*A. niger*) for chitosan in the FDA accepted GRAS notice is different, the shrimp-derived chitosan---the subject of the present GRAS determination---is substantially equivalent chemically and structurally, which also includes its CAS number.

[2] See: http://www.accessdata.fda.gov/scripts/fcn/fcnNavigation.cfm?rpt=grasListing&displayAll=true.

III. INGREDIENT IDENTITY, CHEMICAL CHARACTERIZATION, MANUFACTURING PROCESS PURITY

A. Description of Shrimp-Derived Chitosan

Chitosan, a poly-glucosamine, is a natural substance found in the cell walls of fungi, and in soil, and sediments (Gooday, 1990; Shimahara et al., 1989). 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 prepared by deacetylating chitin from the exoskeletons of a variety of crustaceans. Chitin is the second most abundant natural biopolymer after cellulose (Shahidi et al., 1999). The structure of chitin is similar to that of cellulose, and chitosan has long been considered a source of dietary fiber since it cannot be completely broken down in the upper human digestive tract (Ormrod, 1998). Three functional groups of chitosan, amino groups as well as both primary and secondary hydroxyl groups, are believed to contribute to the unique properties of the polymer which provide diverse functionality in foods (Shahidi et al., 1999).

As defined in European Pharmacopoeia (2004) monograph, chitosan hydrochloride is the chloride salt of an unbranched binary heteropolysaccharide consisting of the two units, N-acetyl-D-glucosamine and D-glucosamine, obtained by partial deacetylation of chitin normally leading to a degree of deacetylation of 70 to 95%. Chitin is extracted from the shells of shrimp and crab. Primex has developed a unique process to manufacture chitosan from the arctic shrimp, *Pandalus borealis*. A consistent source of raw material and a well-designed manufacturing practice have made it possible to use chitosan more widely as a food ingredient.

B. Chemical Identity of Shrimp-Derived Chitosan

1. Common or Usual Name

Shrimp-derived chitosan and shrimp chitosan.

2. Chemical Names (IUPAC or Chemical Abstracts Name)

Poliglusam

3. CAS Registry Number

9012-76-4

4. Synonyms Common Names

a) Polyglucosamine
b) Poly-[1→4]-1-β-D-glucosamine
c) Poly 2-Amino-2-deoxy-D-Glucopyranose
d) Binary heteropolymer of β-[1→4]-2-Amino-2-deoxy-D-Glucopyranose and β-[1→4]-2-deoxy-2-acetamido-D-glucopyranose]

5. Trade Names

a) ChitoClear® (marketed as ChitoClear® FG and FG95)
b) Primex Chitosan
c) Deacetylated Chitin

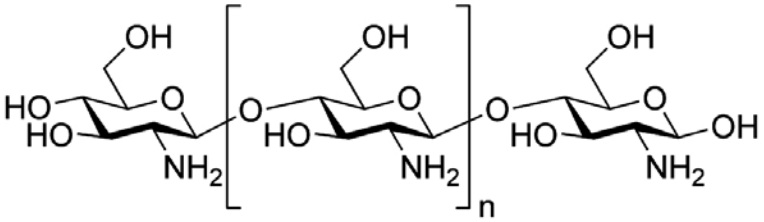
6. Chemical Formula

Chitosan is a polysaccharide composed of GlcN and GlcNAc linked with β-[1→4]-glycosidic linkages, where the molecular subunits are as noted below.

GlcN = D-Glucosamine = 2-Amino-2-deoxy-D-Glucopyranose and
GlcNAc = N-Acetyl-D-glucosamine = 2-deoxy-2-acetamido-D-glucopyranose

Empirical Formula: $(GlcN)_n + (GNAC)_m$ or $(C_6H_{11}N_10_4)_n + (C_8H_{13}\ N_10_5)_m$

7. Structural Formula



8. Molecular Weight (scientific literature)

Native Form (typical): 1 million Daltons (Wu et al., 1976)

9. Composition of Primex Shrimp-Derived Chitosan

The Primex chitosan products are binary heteropolymers of β-[1→4]-2-Amino-2-deoxy—D-Glucopyranose and β-[1→4]-2-deoxy-2-acetamido-D-Glucophyranose produced by the deacetylation of chitin, a structural element in the exoskeleton of crustaceans. 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 which is 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 either soluble or insoluble in 0.1 M acetic acid, respectively, or by degree of deacetylation, where a substance with >20 % deacetylation would be defined as chitosan (Shahidi et al., 1998; Shahidi et al., 1999).

C. Manufacturing Process

1. Scientific Literature of Chitosan Production

Chitosan is a nitrogen-containing polycationic polysaccharide prepared by deacetylation of chitin from various biological sources. Common plentiful sources of chitin are crustacean shells, especially shrimp, crabs, and krill. After deproteinization of crustacean shells, most processes as described in the literature are followed by an acid or enzymatic hydrolysis of chitin and then a deamination with sodium hydroxide solution (Shahidi et al., 1999). Several investigators have concluded that the chemical, biological, and physical properties of chitosan depend on the degree of deacetylation and the molecular size of the polymer (Wu et al., 1976; Hwang and Damodaran, 1995).

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 Program Board, 2000). The 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. More recently, the global market for chitosan was estimated as 13.7 thousand tons for the year 2010 with the Asia-Pacific region using an estimated 7.8 thousand metric tons and the US market consuming 3.6 thousand metric tons. Water treatment is reported to represent the largest end-use application segment for chitosan (Cosgrove, 2010).

2. Primex Manufacturing Process for Shrimp-Derived Chitosan

Primex has developed a novel production process for chitosan based on chitin from wild caught 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 and a peeling plant in Saudarkrokur that is some 60 miles from the Primex chitosan plant. 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 have allowed the peeling industry to supply the shell raw material to Primex in full accordance with HACCP standards, thereby ensuring high quality raw material.

The adjacent shrimp peeling plant in Siglufjördur is processing freshly caught unfrozen shrimp and the collection of raw material occurs directly *via* pipelines to the Primex processing plant. The shrimp raw material processed in the peeling plant in Saudarkrókur is stored frozen immediately upon harvest at a temperature of less than -18°C on board the shrimp fishing vessels. The shrimp are stored frozen until they are thawed immediately prior to the peeling process. Once the shrimp are peeled, the shells are immediately sent to the Primex chitosan manufacturing plant for processing.

The Primex production processes are optimized with the aid of specialized production equipment. Primex personnel design all process equipment, and the entire process is fully automated from the receipt of the shrimp shells at the 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. Shrimp-derived chitosan produced by this process is now being sold as a dietary supplement in more than 50 countries worldwide, including the European Union, the US, and Japan.

The following is a basic description of the production process for Primex chitosan. Figure 1 shows the flow diagram for the manufacturing process for the various forms of ChitoClear® products.

Upon receipt, Primex quality control personnel perform a visual inspection of the raw material. Rejected material is discarded. Suitable shells are transferred to the automated processing equipment.

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.

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.

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 0.5%. This demineralized chitin is washed with heated and pH-adjusted tap water to remove soluble fat and color.

The chitin is added to a solution of NaOH of appropriately high concentration in order to remove the acetyl groups from the chitin. Analyses of the degree of deacetylation are conducted in the Primex laboratory.

After the process of deacetylation, the mixture is washed and filtered through a mechanical filtering system.

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

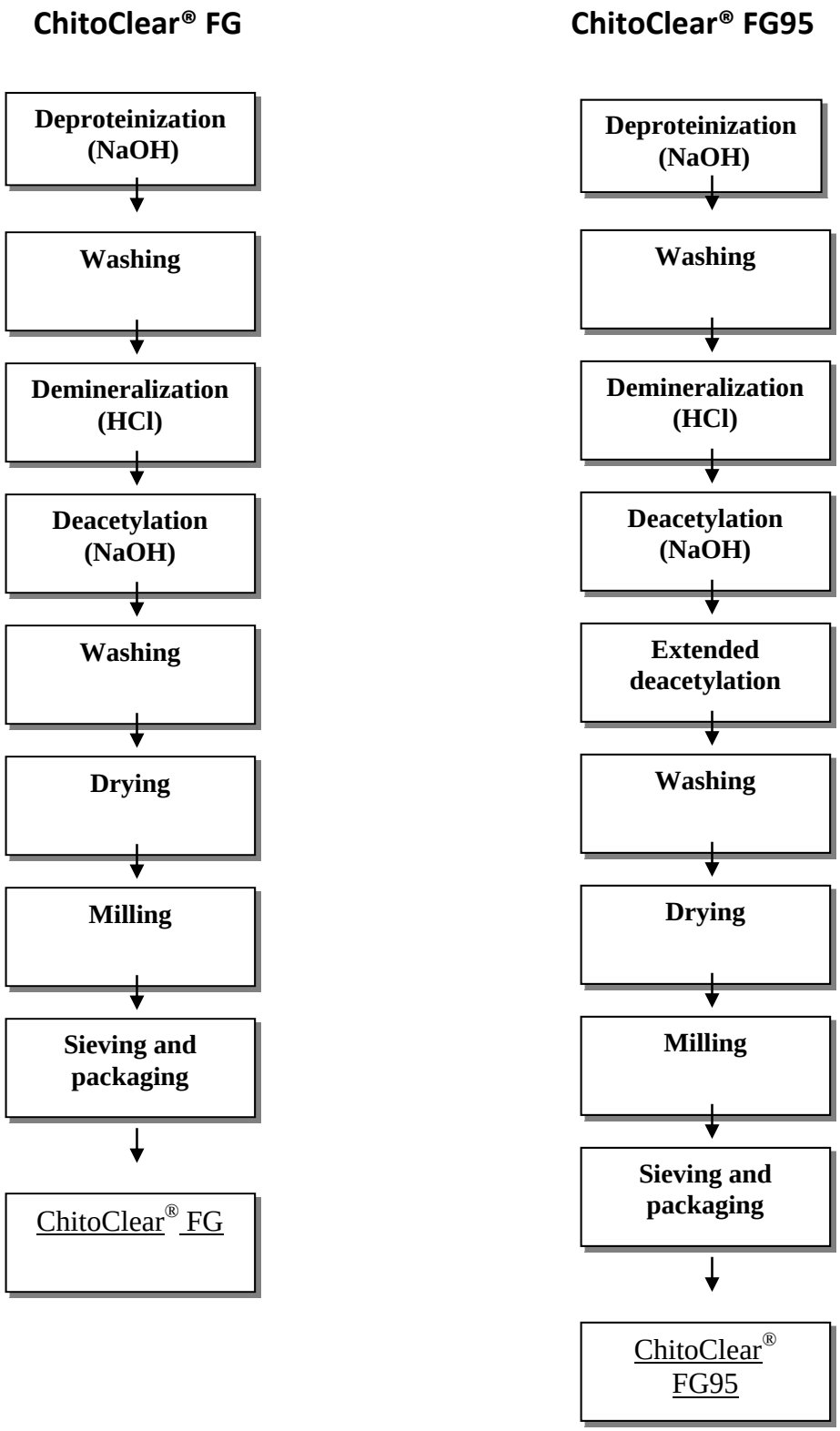
1) The chitosan wet flakes are dried in a Turbo drier until the desired moisture level has been achieved. The moisture content of the chitosan is reduced to less than 10% before drying is completed.

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 level and particle size are achieved. The moisture content of the chitosan is reduced to less than 10% before drying is completed.

Potassium hypochlorite is used for decoloration (bleaching) of the chitin flakes. In this process the hypochlorite is only a technical aid for removing the red color in the product and does not leave harmful residues. Subsequently, the product is washed and dried, followed by deacetylation. No traces of the hypochlorite are expected in the products.

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 completed.

Figure 1. Flow Chart for the Manufacturing Process of Shrimp-Derived Chitosan



The sequence of production steps specific for each of the Primex chitosan products is depicted in Table 1 and Figure 1. Specific market categories for the various products are shown below in Table 2.

Table 1. Specific Production Steps for ChitoClear Products

PRODUCT	PRODUCTION STEPS
ChitoClear® FG	Deproteinization, demineralization, deacetylation, drying/milling, sieving/packaging
ChitoClear® FG95	Deproteinization, demineralization, deacetylation, extended deacetylation, drying/milling, sieving/packaging

Table 2. ChitoClear Products Market Properties

PRODUCT	PROPERTIES	MARKET
ChitoClear® FG	Highly deacetylated; Off – white powder/flakes	General use in foods; dietary supplements
ChitoClear® FG95	Very highly deacetylated; Off –white powder/flakes	Dietary supplements; foods in general especially as dietary fiber

3. Quality Control of Manufacturing Process

The plant where shrimp-derived chitosan is produced is a HACCP-designed facility. The HACCP plan was specifically designed for the entire shrimp-derived chitosan products manufacturing processes. Primex has standardized product recall, internal quality audit, cleaning and sanitation, finished product label control, allergen policy, and microbiological and environmental programs in place. In addition, Standard Operating Procedures for all equipment---which include regular maintenance and cleaning requirements---have been instituted. Primex has received a Quality Control Certificate from the Iceland Office for Health and Environment 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."

The Primex shrimp-derived chitosan plant has a certified Hazards Analysis Critical Control Point Program (HACCP) and ISO 22000 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.

The critical control points of the Primex production process are listed below.

a) Receipt of raw material: Visual control of shell and check for odor.

b) Receipt of production chemicals: Visual control of package.

c) Pressing: Water pressed from the raw material prior to each processing step.

d) Deproteinization: pH level, temperature, time of stirring, NaOH content, dry matter.

e) Demineralization: pH level, temperature, time of stirring, acid content.

f) Deacetylation: NaOH content, temperature, time of stirring, dry matter.

g) Washing after each processing step: pH level, temperature.

h) Drying: Dry matter content after drying.

i) Packaging and storage: contaminants, temperature, humidity.

4. Quality Control of Finished Product

The safety and quality of the final product is ensured in a number of ways. The raw materials used in the manufacture of ChitoClear® products are food grade quality and come with a Certificate of Analysis from the supplier. As outlined in Section III.C.3., a variety of critical control points are used to ensure the wholesomeness of the product. The final product is tested, and certificates of analysis are prepared which adhere to the specifications contained in Table 3. Primex offers products with a variety of viscosity specifications to meet customer-specific needs within the range shown in Table 3. In addition, Primex reports that it has the programs in place to execute a limited or general recall in the event that its quality control system is breached.

D. Product Specifications

1. General Physical Chemical Properties

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. Its raw material, chitin, is the (1→4)-β-linked homopolymer of N-acetyl-D-glucosamine. The individual polymer chains of chitin appear to be helices since each sugar unit is inverted in comparison to adjacent sugar units (Gooday, 1990). This configuration may account for the great stability and unique characteristics of chitin and chitosan.

Chitosans also 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 chitosans and therefore have considerable influence on their biological activities (Illum, 1998; Shahidi et al., 1999).

2. Physical Chemical Specifications

Primex 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 0.5% ash, and negligible amounts of protein and fat. The percent deacetylation of Primex chitosan is greater than 75%, and the turbidity rating is ≤ 30 NTUs. The product is further characterized by a solubility of ≥ 99% in 1.0% acetic acid. Lead levels are very low (< 0.1 ppm), and microbial limits have been set to meet food grade specifications. The physical properties and specifications for ChitoClear® products are compiled in Table 3.

Table 3. Final Product Specifications for ChitoClear® FG □ ChitoClear® FG95[a]

PARAMETERS	CHITOCLEAR® FOOD GRADE	CHITOCLEAR® FOOD GRADE 95
Dry Matter	≥ 90%	≥ 90%
Ash	≤ 0.5%	≤ 0.5%
Degree of Deacetylation	75.0% - 95.0%	≥ 95.0%
Solubility	≥ 99.0%	≥ 99.0%
Turbidity	≤ 30 NTU	≤ 30 NTU
Protein	≤ 0.02 g/100g	≤ 0.02 g/100g
Viscosity	25 - 5000 mPa·s (cP)[b]	25 - 5000 mPa·s (cP)[b]
Micromilled Powder	99% through 18-200 mesh sieve [b]	99% through 18-200 mesh sieve [b]
Appearance	White to off white powder	White to off white powder
HEAVY METALS		
Arsenic	< 0.100 ppm	< 0.100 ppm
Cadmium	< 0.050 ppm	< 0.050 ppm
Lead	< 0.100 ppm	< 0.100 ppm
Mercury	< 0.005 ppm	< 0.005 ppm
MICROBIOLOGICAL		
Total Plate Count	< 300 cfu/g	< 300 cfu/g
Escherichia coli	Absent	Absent
Staph.	Absent	Absent
Salmonella	Absent	Absent
Listeria	Absent	Absent
Pseudomonas	Absent	Absent
Mold and Yeast	< 50 cfu/g	< 50 cfu/g

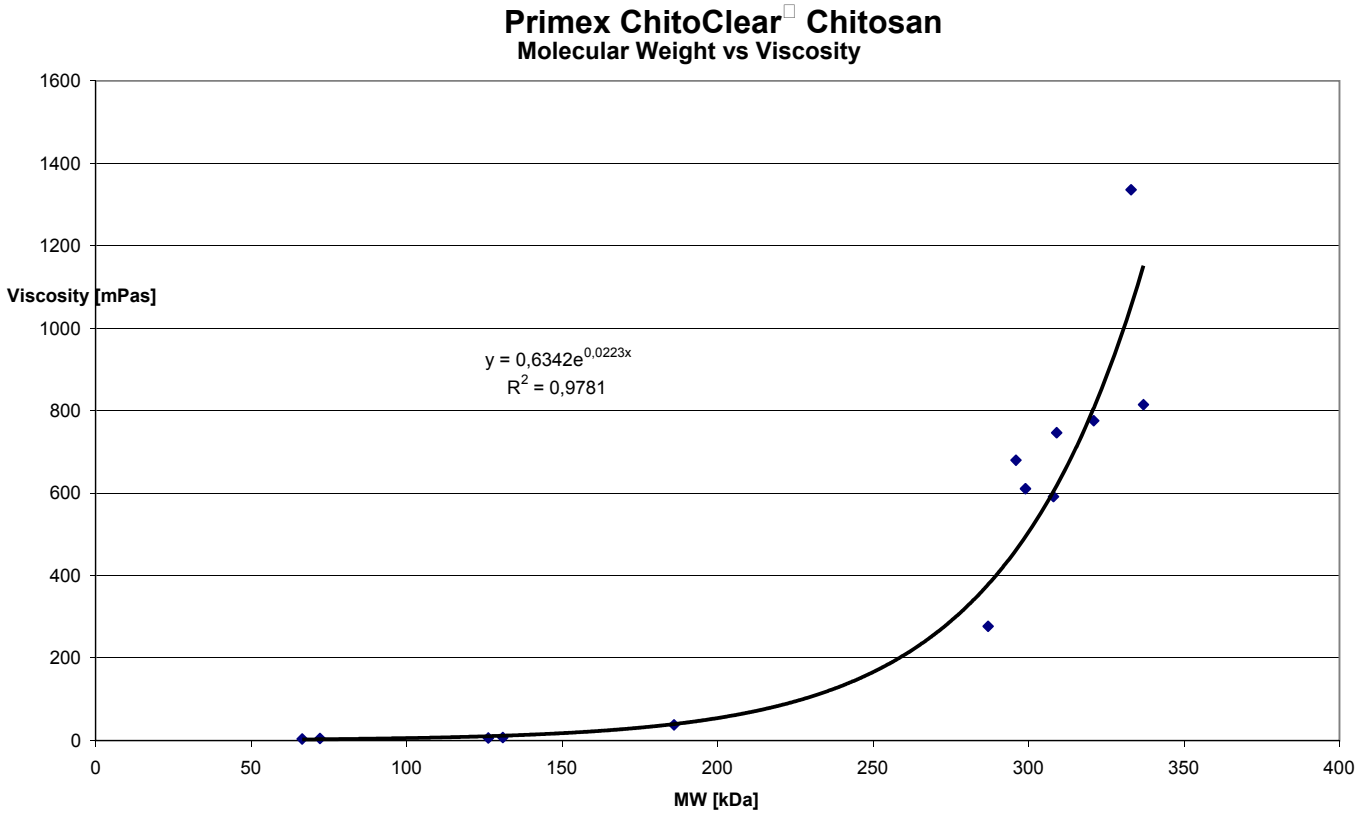
[a] Product: Off-white chitosan made from fresh shrimp shells, *Pandalus borealis.* [b] According to customer needs.

The product occurs in several food grade forms characterized by their degree of deacetylation (>75% –> 95%) and polymerization. ChitoClear® FG is a food grade product defined as having > 75% deacetylation. ChitoClear® FG95 has undergone extended deacetylation to yield a product with a degree of deacetylation >95%. 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. The viscosity of the product is directly related to the molecular weight of the chitosan. The results of the study done by Primex to show the relation of viscosity to molecular weight are depicted in

Figure 2. Based on this study, with a minimum product viscosity of 25 mPas, the minimum molecular weight of chitosan products is 165 kDa. Further experimental details for this study are given in Appendix A.

The product specifications for ChitoClear® FG and ChitoClear® FG95 are also reported in Table 3. Certificates of Analysis for several production batches of ChitoClear® FG and ChitoClear® FG95 are provided in Appendix B and Appendix C, respectively.

Figure 2. Primex ChitoClear Chitosan Molecular Weight Versus Viscosity



3. Stability Characteristics of Chitosan

The basic structure of chitosan and chitin is very similar to cellulose. Both polymers have β-1,4-links between the glucose molecules, and the only difference is that the hydroxyl groups in cellulose at the 2-position in chitin and chitosan are replaced with N-acetyl and amine groups, respectively.

In the isolation process of chitin and chitosan, the shrimp shell undergoes quite harsh chemical treatment in several steps with hydrochloric acid and sodium hydroxide at pH below 2 and above 12, respectively, and at temperatures over 100°C for more than one hour. In this process, all other parts of the shell---such as protein and fat, as well as minerals---are dissolved and removed while the chitin and chitosan remain relatively intact.

As a part of the diet, chitosan is a dietary fiber that is not broken down during the passage through the stomach and the intestine where it has to endure low pH and bile acids, along with the microorganisms and the enzymes in the digestive tract. All this indicates that chitosan is a very

stable polysaccharide in general and is therefore expected to be a stable ingredient in food products of various types.

Chitosan is only soluble in weak acids and is insoluble at pH levels above 5-6. Chitosan can therefore be used as an ingredient in food products either as a solid ingredient or as a chitosan solution in food solutions with pH <5.

a. Stability of Solid Chitosan

As a raw material in the solid state, chitosan is a very stable polymer. Primex has done some stability test on the solid ChitoClear product (see Appendix D-1). Four quality parameters of ChitoClear have been tested for changes upon storage for proximately three years. The parameters studied are dry matter content (DMC), viscosity (MW), degree of deacetylation, color and microbial content which includes total plate count, yeast and mold.

The results from the tests show that all samples have a little higher moisture content than when first measured. The difference is more pronounced as the samples get older. After two months the moisture content had increased slightly or between 0.2 to 0.5%, but all samples where within specification (DMC > 90%). After 34 months the DMC had decreased between 1.5% and 3.2%. Two batches are just out of the specification for ChitoClear after 34 months or 0.1 - 0.2% below the 90%. In this regard, it is important to note that Primex studies have shown that from a microbiological point of view, the ChitoClear Chitosan is stable at DMC levels as low as 85%.

For the viscosity, which is also an indicator of molecular weight, the stability tests indicate that there are no obvious changes. All batches are within the general specification of ChitoClear after 34 months.

Measurements of the changes in degree of deacetylation reveal that, after 34 months, all samples are within specifications. The results show that the degree of deacetylation is increasing a little with study time.

Total Plate Count microbiological tests illustrate that most batches exhibit no growth of colonies. Compared to the QC results of the same batch from nearly three years ago, we see that the chitosan environment is hostile to bacteria, further demonstrating chitosan's antimicrobial capacity.

Yeast and mold measurements show similar results; after 34 months the microbial numbers had decreased considerably during the storage time.

b. Stability of Chitosan in Various Acid Solutions

Primex conducted stability tests on chitosan solutions in three different acid solutions, i.e., acetic acid, lactic acid and glycolic acid (See Appendix D-2).

In these tests 4% chitosan solutions were tested in 1% and 2% acid solutions. These two different solutions for all batches were kept at two different incubation temperatures---at 25°C and 50°C. Samples from all solutions were collected weekly at 7 day intervals or at 0, 7, 14, 21 and 29 days of incubation. Product stability was assessed by measuring changes in viscosity and absorption spectra, as well as by visual evaluation of the solution color and formation of precipitates.

Most changes in the parameters examined were in viscosity and with the browning index and color. Clearness of solutions, turbidity and precipitate formation did not change during the incubation time at any acid concentration or temperature. Analyses of different batches showed the same pattern of changes in viscosity and browning index.

The reduction in viscosity was highest at 50°C in 2% acetic acid, followed by 50°C in 1% acetic acid. The reduction was much lower at 25°C with the lowest observed in 1% acetic acid at 25°C. More browning occurred at 50°C than 25°C, and in both cases the 1% acetic acid solutions exhibited less browning than with the 2% acetic acid. The relatively diminished browning effect is interesting since acetic acid is known to cause browning faster than most other organic acids.

With lactic acid, the only changes observed were in viscosity, with the browning index, and in color. Clearness of solutions, turbidity and precipitate formation did not change during the incubation time at any acid concentration or temperature. After 4 weeks there was relatively little difference in viscosity of the chitosan stored at 25°C for the 1% and 2% lactic acid solutions while when stored at 50°C some decrease in viscosity was observed. Similar results were found regarding the browning index with minor changes noted at 25°C; after 4 weeks at 50°C it had increased.

Eight batches of milled chitosan were used to test for stability in glycolic acid. The results show that, as with lactic acid, the main changes were in viscosity, browning index and color. For the 4% chitosan in 1% glycolic acid at 25°C, an increase in viscosity was observed in two batches in the first 7 days (see Fig. 1 in Appendix D-2), followed by a reduction back to around the original values after two weeks. The results show a similar trend as for the lactic acid and the acetic acid regarding changes in viscosity that are much lower at 25 °C than at 50°C.

In summary ChitoClear is very stable in solid state up to at least three years. Only minor changes are observed regarding the critical parameters, i.e., dry matter content, viscosity (MW), degree of deacetylation, color and microbial content. In acid solutions the chitosan shows little change at 25°C during four weeks in the measured designated parameters of viscosity, browning index and color while at 50°C, it was clearly evident that more browning, changes in color and viscosity had occurred.

The results from the testing of the stability of ChitoClear in solid state and in weak acid solutions indicate that ChitoClear chitosan is a very stable polysaccharide at temperatures up to at least 25°C and is, therefore, expected to be a stable ingredient in food products of various types.

4. Potential Allergenicity Considerations

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. As noted in Section V.D.2., Primex has confirmed through human clinical testing that its chitosan product is not allergenic. In adopting a conservative position in the interests of safety, Primex set a protein specification of less than 0.02% for its food grade products. Since chitosan naturally contains nitrogen, the use of the familiar Kjehldahl method for protein content determination is likely to provide erroneous results since this method relies on the estimation of nitrogen content. In order to establish what level of protein may be present in any of its lots of chitosan, special testing methods have been adapted by Primex that rely on amino acid estimation. The actual amino acid content of ChitoClear® chitosan is presented in Table 4. Total protein was determined by summing

the content of amino acids. These analyses confirmed that protein levels in the products are extremely low (<0.02%). The method used is included as Appendix E.

Table 4. Typical Protein Analysis of ChitoClear

LOT #	DATE SUBMITTED FOR ANALYSIS	PROTEIN % (W/W)
TM 3502	October 29, 2009	Below detection limit
TM 3519	December 1, 2009	0.005
TM 3548	February 15, 2010	0.005
TM 3550	February 15, 2010	0.005
TM 3636	August 2, 2010	0.005
TM 3695	November 22, 2010	0.005
TM 3696	November 22, 2010	0.005
TM 3711	March 28, 2011	Below detection limit
TM 3723	May 19, 2011	Below detection limit
TM 3724	May 19, 2011	Below detection limit

IV. INTENDED USE ANTICIPATED DIETARY EXPOSURE

A. Intended Uses

Chitosan has been identified as being biocompatible, non-antigenic, non-toxic, and biofunctional (Vidanarachchi and Kurukulasuriya, 2011). 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 applications.

Use of chitosan in the food industry provides several benefits, and this has been well reviewed in the scientific literature. Chitosan is useful in foods not only as a source of dietary fiber, but also because of its functionality to affect protein aggregation, emulsification capacity, film-forming ability, clarifying ability, and fatty acid absorption capability (Knorr, 1991; Shahidi et al., 1999). A more detailed description of the possible uses of chitosan is given in these references. In addition, the antimicrobial and antioxidative action of chitosan in food has been reviewed by Friedman and Juneja (2010). This review summarizes antimicrobial action in noodles, rice cakes, eggs, hummus, pasta, cheese, milk, yogurt and various fruit products with extension of shelf life shown in all cases. Another review describes uses of chitosan-based biofilms with protective antimicrobial activity on various foods (Dutta et al., 2012). Summaries of reports showing the usefulness and acceptability for meat products are provided in Appendix F.

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 rather 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; Rodriguez, et al., 2000). Excessive chitosan usage may produce aggregation of ingredients, depending on the synergies involved and the pH of the formulation (Poole, 1988). Further, Rodriguez et al. (2000) stated that the affinity between chitosan and water decreased with increasing pH. Therefore, use of chitosan is expected to be limited by its physicochemical properties. Additionally, chitosan imparts an unpleasant taste to food at higher concentrations.

B. Anticipated Dietary Exposure

Based on the intended food uses of ChitoClear® products, a realistic estimate for dietary intake for shrimp-derived chitosan was calculated using the FDA's guidance document "Estimating Exposure to Direct Food Additives and Chemical Contaminants in the Diet"[3] and USDA's Continuing Survey of Food Intakes by Individuals (Enns, et al., 1997).

In order to estimate the probable human exposure to shrimp-derived chitosan on a continual daily basis, Primex has relied upon (1) examples of commercial formulations from Asia and Europe, (2) potential shrimp-derived chitosan use from recent published studies, (3) the patent literature, and (4) the results of its own food technology research. Use of the material was estimated for the 94 NAS food categories on a working Table entitled "GRAS Food Additive Categories and Sub-Categories" (data not shown). Use levels as a percent of the final food products were then

[3] http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/FoodIngredientsandPackaging/ucm074725.htm.

estimated across food sub-categories to yield averages for the major food groups. The CSFII food categories, along with shrimp-derived chitosan use levels, food sub-category, mean intake, and shrimp-derived chitosan intake are shown in Table 5. This table presents mean intake data for adults 20 years and over by considering eaters only.

The results of these calculations and potential exposures from other sources are summarized at the bottom of Table 5. The estimated daily mean exposure derived from Table 5 yielded 1.69 grams of shrimp-derived chitosan per day. The 90th percentile is then estimated to be 3.37 grams per day. While the percentage of use assigned to each food sub-category was viewed to be realistic, this exposure estimate is considered to be conservative because 100% market penetration was assumed, and shrimp-derived chitosan was assumed to be included in all products in a given sub-category.

The calculation in Table 5 assumes 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 sub-category. Further, use within a sub-category 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.

C. Other Information on Human Exposure to Chitosan

Chitosan derived from shellfish has a long history of safe use in the food supply. Presently, chitosan is approved as a natural food additive for general food use in Japan and Korea (Japan Food Chemical Research Foundation, 2011; KFDA, 2011). In other countries such as the US, European Union, and other countries, chitosan is widely used as a dietary supplement. The dietary supplement products typically recommend consumption of 1 to 3 g chitosan/person/day.

Table 5. Estimated Intake of Shrimp-Derived Chitosan in Selected Food Categories[a]

Major Food Codes	Food Category	Chitosan Solids Use Level (%)	Food Intake (g)	Chitosan Solids Intake/day (g)	Major Food Application
Baked goods, baking mixes					
51	Yeast Breads & Rolls	0.0256	50	0.0128	Functional food ingredient, stabilizer, natural preservative, natural dietary fiber
52, 55	Quick breads, pancakes, etc.	0.04	20	0.008	Functional food ingredient, stabilizer, natural preservative, natural dietary fiber
53	Cakes, cookies, pastries, pies[b]	0.04	38	0.0152	Functional food ingredient, stabilizer, natural preservative, natural dietary fiber
58	Mixtures mainly grain	0.153	107	0.16371	Functional food ingredient, natural dietary fiber
Breakfast Cereals					
571 - 574, 578	Ready-to-eat cereals	0.032	17	0.00544	Functional food ingredient, natural dietary fiber
Grain Products & Pastas					
562	Rice	0.0256	19	0.004864	Functional food ingredient, natural preservative, natural dietary fiber
561	Pasta	0.0256	21	0.005376	Functional food ingredient, natural preservative, natural dietary fiber
54	Snack foods (crackers, chips)	0.75	12	0.09	Functional food ingredient, natural dietary fiber
Total Vegetables					
	Fresh vegetables[c]	0.21	45	0.0945	Natural preservative, moisture retention
	Processed vegetables, juices	0.21	14	0.0294	Natural preservative, functional food ingredient, clearing agent
	Dark Green Vegetables	0.21	13	0.0273	Natural preservative, moisture retention
Total Fruits					
612	Citrus juices[d]	0.46	17.7	0.08142	Natural preservative, functional food ingredient, clearing agent, natural dietary fiber
	Fruits & mixtures	0.46	19.5	0.09	Natural preservative, moisture retention, natural dietary fiber
Milk Products					
114	Yogurt	0.77	8	0.0616	Natural preservative, functional food ingredient, stabilizing agent, natural dietary fiber

131,132, 133, 134	Milk Desserts[e]	0.77	24	0.1848	Natural preservative, dietary functional ingredient, stabilizer, natural dietary fiber
140-147	Cheese	0.56	8	0.0448	Natural preservative, dietary functional ingredient, stabilizer, moisture retention
116, 118	Milk-Based Beverages	0.77	34	0.1818	Natural preservative, functional food ingredient, stabilizer, natural dietary fiber
MEAT PRODUCTS					
252	Sausages, Processed Meats	0.45	21	0.0945	Natural preservative, dietary functional ingredient, emulsifier
	Fresh Meat	0.25	34	0.085	Natural preservative, moisture retention
	Poultry	0.25	20	0.05	Natural preservative, moisture retention
	Fish	0.25	11	0.0275	Natural preservative, moisture retention
EGGS					
321,323,324, 33,34	Egg Products[f]	0.213	9	0.01917	Natural preservative, dietary functional ingredient, emulsifier
LEGUMES					
	Legumes[g]	2.2	1.8	0.0396	Natural preservative, functional food ingredient, stabilizer
FATS & OILS					
832	Salad Dressings	0.22	8	0.0176	Natural preservative, functional food ingredient, emulsifying agent, stabilizer
TOTAL SUGARS & SWEETS					
917.918	Candy (Soft & Hard)	0.5	7	0.035	
911 - 916	Other Sugar Products[h]	0.25	15	0.0375	Natural preservative, functional food ingredient, stabilizer
BEVERAGES NON-ALCOHOLIC					
925	Low Cal. Fruit Drinks & Ades	1	18	0.18	Natural preservative, functional food ingredient, natural dietary fiber, clearing agent
	Total Mean Intake (grams)			1.69	
	90th Percentile			3.37	
	Potential Dietary Supplement Use			1.5-3.0	

[a] Enns, et al., 1997. [b] Includes dietetic bars. [c] Includes tomatoes and lettuce products. [d] Includes citrus juices with pulp. [e] Includes ice creams etc. [f] Includes processed egg products. [g] Includes tofu and similar products. [h] Includes jams, jellies, etc.

V. REVIEW OF SAFETY DATA

There have been several published review articles (Ylitalo, et al., 2002; Shields, et al., 2003; Mhurchu, et al., 2005; Jull, et al., 2008) which discuss possible benefits and safety of oral consumtion of chitosan at dosages as high as 6 grams/person/day. In addition, there have been two reviews which discuss the safety of chitosan for use as an excipient in pharmaceuticals (Illum, 1998; Baldrick, 2010). The most relevant studies related to the safety of chitosan are reviewed in this section.

A. Metabolism of Chitosan

The metabolism of chitosan has been characterized in a number of *in vivo* and *in vitro* studies. As described in a review article by Baldrick (2010), ^{14}C-chitosan in rats is digested into low molecular weight substances within the gastrointestinal tract, and these substances are then distributed in tissues (Nishimura et al., 1997).

Chitosan is degraded into chitooligosaccharides and then finally into glucosamine (Domard and Cartier, 1989; Aiba, 1993; Aiba, 1992). Chitosan have been shown to be digested in the gastrointestinal tracts of rabbits (Hirano et al., 1990; Lee et al., 2004). Chitosan is digested by enzymes found in intestinal mucosa and microflora of animals and is then converted to N-acetylglucosamine.

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; Sugano, et al., 1988). 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.

In an *in vitro* study, Varum et al. (1997) reported that degradation of chitosan with egg white and human lysozome occurs primarily *via* enzymatic depolymerization. In a study by Hirano et al. (1991), serum lysozyme levels were elevated in rabbits following intravenous administration of chitosan oligosaccharides. Furthermore, it has been suggested that degradation of chitosan depends on the degree of polymerization. With a higher degree of polymerization, less degradation occurs (Tomihata and Ikada, 1997; Ren et al., 2005; Freier et al., 2005). In a 12-week study, subcutaneous implantation of chitosan films in rats revealed that as the degree of deacetylation increased, degradation occurred less rapidly (Tomihata and Ikada, 1997). The degradation of chitosan produces chitosan oligosaccharides of variable lengths.

The absorption of chitosan from the gastrointestinal tract decreases with increasing molecular weight. Chae et al. (2005) investigated absorption of fluorescein isothiocyanate-labeled chitosans with varying molecular weights and degrees of deacetylation in an *in vitro* study in Caco-2 cells and also in an *in vivo* study following oral administration to rats at a dose of 20 mg/kg bw/day. In both *in vitro* and *in vivo* experiments, absorption profiles were observed to increase more than 23 and 25 times with low molecular weight (3.8 kDa) compared to high molecular weight (230 kDa) chitosan. Plasma C_{max} and AUC values ranged from <0.5 up to 20.23 µg/mL and 0.97 up to 24.13 µg/mL/h, respectively. In the epithelium of villi in duodenum and jejunum, dense fluorescence intensities were observed with the 3.8 kDa chitosan, and intensities decreased with increasing chitosan molecular weight with no sign of absorption observed with the 230 kDa form. Qin et al. (2006) reported that the chitooligomers that are formed by depolymerization of chitosan have good intestinal absorption.

In another study by Zeng et al. (2008a), fluorescein isothiocyanate-labeled chitosan preparations were gavage-administered to female mice at single oral doses of 500 mg/kg bw/day. In this study, four preparations of chitosans [chitosan oligomer (MW=0.99 kDa, 85.9% deacetylated); middle molecular weight chitosan (M-chitosan, MW=32.7 kDa, 85.0% deacetylated), water-soluble chitosan (MW=39.1 kDa, 52.6% deacetylated); and high molecular weight chitosan (H-chitosan, MW=760 kDa, 85.5% deacetylated)] were used. Blood samples were collected at 30, 60, 120, and 240 minutes after the administration for pharmacokinetic analyses. The extent of chitosan absorption was found to be inversely related to its molecular weight for three of the samples, with absorption occurring in the following order: H-chitosan < M-chitosan < chitosan oligosaccharide. Water-soluble chitosan with its lower degree of deacetylation, however, had the greatest amount absorbed compared to all of the compounds tested despite its moderate molecular weight. Labeled material was found in all organs examined including liver, kidney, spleen, thymus, heart and lung from the 4 samples in the same relative order as was found in plasma. In this study and the Chae et al. (2005) study noted above, no control groups were included, and non-specific binding of the label may have occurred.

The hydrolysis of chitosan has been studied, and the degradation of natural polymers, such as chitosan and chitin proceeds *via* enzymatic degradation (Domard and Cartier, 1989; Lee et al., 1995). 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; Aiba, 1993; Aiba, 1992; Sashiwa, et al., 1990).

Muzzarelli (1997) has provided evidence for the degradation of chitosan in humans *via* non-specific chitinase and lysosymal enzyme systems (Muzzarelli, 1997). The highest lysozyme susceptibility was shown by a degree of deacetylation of approximately 70% (Sashiwa, et al., 1990).

It has been suggested that chitosan is highly biodegradable with no bioaccumulation or retention in the body (Baldrick, 2010). Lee et al. (2004) reported that oral administration of ^{14}C-labeled N-acetylglucosamine (copolymer of chitosan) to rats resulted in excretion of 4%, 17% and 54%, in the urine, feces and exhaled air, respectively, during first week following the dosing. The other 25% was distributed into connective tissue or cartilage.

In summary, the collective studies on chitosan metabolism suggest that systemic exposure to parent chitosan following its oral exposure appears to be limited (due to digestion in the gastrointestinal tract). Any amount that is absorbed is likely to undergo enzymatic degradation to its copolymers glucosamine and N-acetylglucosamine which are excreted or used in the amino sugar pool.

B. Toxicology

A number of published toxicology studies found in the literature were reviewed by Illum (1998) and Baldrick (2010). Additionally, several safety-related studies of chitosan are summarized in a recent FDA (2011) GRAS notification. The most relevant and important studies on chitosan toxicity are summarized below with additional studies summarized in Table 7.

In an extensive review article, Baldrick (2010) critically reviewed and summarized the safety-related information of chitosan as a pharmaceutical excipient. In this review, several preclinical and clinical studies of chitosan have been summarized. The oral LD_{50} of chitosan has been evaluated in a number of studies with reported results of >1500 mg/kg bw/day in rats and >16,000 mg/kg bw/day in the mouse (Baldrick, 2010). It was stated that no toxicity was noted in rats receiving 2000 mg/kg bw/day by gavage or at levels up to 5% (equivalent to approximately 3000 mg/kg bw/day) in the diet for up to 3 months. The Baldrick (2010) review also states that there are a number of published studies which examined the effects of feeding chitosan in the diet of rats at high g/kg levels, and the results of these studies revealed that chitosan is reasonably well tolerated. It was also noted that data on modern genotoxicity testing studies are limited for chitosan. A few studies were quoted for chitooligomers, but no evidence of genotoxicity was noted. The review also states that no data on reproductive toxicity or the carcinogenic potential of chitosan were found and that there were no immunotoxicity findings from oral rodent studies with chitosan.

Kim et al., (2001) completed a subacute study to evaluate the toxicity of chitosan oligosaccharide in rats. Male and female Sprague-Dawley rats received 0, 500, 1,000 or 2,000 mg/kg bw/day chitosan by oral gavage for 28 days. No significant differences were observed between groups for food intake, body weight, clinical signs, or urinalysis parameters. Furthermore, no significant differences between groups in hematological parameters were reported, except for a significantly increased mean leukocyte concentration in males receiving the 2,000 mg dosage of chitosan that was considered not to be treatment related or toxicologically relevant. A significant decrease in albumin and total blood protein observed in male rats administered the 500 mg dosage of chitosan was also not considered to be toxicologically relevant. There were no significant differences in organ weights or histopatholgical findings between groups. The authors concluded that the no-observed-adverse-effect level (NOAEL) was 2,000 mg/kg bw/day in rats.

Zeng et al. (2008b) conducted a 90-day toxicity study in mice using four chitosan preparations with different molecular weights---high molecular weight chitosan (H-chitosan MW=760 kDa), middle molecular weight chitosan (M-chitosan MW=32.7 kDa), water-soluble chitosan (MW=39.1 kDa) and chitosan oligomer (MW=0.99 kDa). These four samples were the same used in a previous study measuring the absorption of chitosan from the GI tract, as well as tissue distribution (Zeng et al., 2008a). Animal diets were supplemented with 1.05% chitosan per day, which is equal to an intake of approximately 500 mg/kg bw/day. No mortality occurred during the study, and there were no body weight differences between groups. In the H-chitosan, M-chitosan and chitosan oligomer treatment groups, the relative organ weights of the heart, liver, spleen, thymus and kidney were not changed. However, in the water-soluble group, the relative thymus weight was significantly increased by 46% compared to the controls. There were no differences in histopathological findings or gross evaluation of the organs. The animals in the M-chitosan group had significantly higher iron levels in the liver and spleen; significantly higher zinc levels in the liver, spleen and heart; and significantly higher copper levels in the liver. The authors concluded that there may be a greater accumulation of M-chitosan in the liver and other organs that exert metal-chelating properties. The investigators concluded that dietary ingestion of chitosan did not cause any adverse effects, did not depress the levels of Fe, Zn and Cu absorption in mice, and, moreover, may improve absorption of these trace metals.

Choi et al. (2012) conducted a study to determine the reproductive effects of chitosan. Female B6C3F1 obese mice (15/group) were induced to ovulate and were orally administered water soluble chitosan at doses of 480 mg/kg bw/day for 4 days. The oocyte production and fertilization

rates were not affected by chitosan treatment. Chitosan treatment increased the number of ovulated oocytes as well as *in vivo* and *in vitro* fertilization rates compared to controls. The authors concluded that there were no negative effects of chitosan treatment on reproduction in mice and that chitosan may improve the ovary function in obese mice.

Yoon et al. (2005) studied 4 generations of ICR mice that ingested chitosan oligosaccharides in drinking water for up to 180 days. No adverse effects were reported in all generations. In the parental generation, male and female mice were given drinking water containing 0.1% chitosan oligosaccharide for 30 days. The F_1, F_2 and F_3 generations were given drinking water containing 0, 0.01, 0.1, or 1% chitosan oligosaccharide (approximately 0, 0.1, 1, or 10 mg chitosan oligosaccharide/kg bw/day) for up to 180 days. Bone marrow from the femur of each mouse was used to test for chromosomal aberrations, which showed no significant differences between any of the treated groups compared to the control group. No adverse effects on safety parameters were observed in this study.

The most rigorous toxicology study conducted was by the National Toxicology Program (NTP). This study was initiated as a specialized mechanistic study designed to determine the potential for vitamin E depletion and osteoporosis from the ingestion of chitosan (NTP, 2008). While it is unclear whether the mechanistic endpoints were examined, NTP has released much of the data on the toxicology study. The 26-week feeding study in male and female Sprague-Dawley rats used diets containing 0, 1, 3 and 9% chitosan.[4] On a body weight basis the resulting intake of chitosan will be approximately 0, 500, 1500 and 4500 g/kg bw/day, respectively. The study protocol details, along with raw data summary tables, can be found in Appendix G. The study was completed in 2008 but only raw data results are currently available for some of the endpoints---body weights, survival data, clinical observations and non-neoplastic lesions. No statistical evaluation has been released on the data by NTP, but a review indicates that the high dose group shows a lowered body weight gain in both sexes. The incidence rates of the non-neoplastic lesions did not reveal any dose related effects. Based on a discussion with NTP staff member, Dr. Scott Masten, and given the lack of any toxicological or nutritional findings in this long-term study at very high doses, NTP will likely cease its investigation into chitosan. The histopathology is currently in peer review, and that process has yet to be completed. Based upon the review of available data, the NOAEL for chitosan appears to be 3% (1500 mg/kg bw/day) in the diet under the conditions of this study. The NTP has not published its final report, but the results of the study are publicly available.

In a recent study, Omara et al. (2012) investigated the effect of chitosan given orally to male and female mice at dose levels of 150 and 300 mg/kg bw/day for 35 days. Chitosan treatment caused significant elevation in transaminases (ALT, AST) and alkaline phosphatase (ALP) in liver and in serum urea and creatinine in a dose dependent manner. Lipid profile parameters significantly decreased, while a significant increase in glycolytic enzymes activities in all treatment groups was noted. Female mice in the 300 mg/kg bw/day revealed significant reduction in lipid profile parameters compared to male groups receiving the same dose. Phosphofructokinase and lactate dehydrogenase activities were significantly enhanced, while glucose phosphate isomerase and hexokinase were significantly elevated in the higher dose females compared to males. Histopathological study of liver and kidney tissues showed moderate to severe histopathological changes dependent on the dose and gender. Image analysis resulted in significant depletion in glycogen and protein contents, more so in females than males. The investigators concluded that

[4] NTP study results: http://ntp_apps.niehs.nih.gov/ntp_tox/index.cfm?fuseaction=ntpsearch.searchresults&searchterm=9012-76-4.

female mice were more susceptible to the toxic effect of chitosan than males when administered with the higher dose for a long period. The results of this study are difficult to interpret; as in the above-described NTP study, no adverse effects were noted at levels up to 4500 mg/kg bw/day for 6 months. In the Omara et al. study, product specifications such as molecular weight, degree of polymerization/deacetylation, etc., were not provided. Additional discussion and relevance of this study to the present GRAS assessment can be found in Section VI.

C. Other Biological Studies on Chitosan

Of the many biological studies conducted where chitosan was administered orally, several are important for the safety review, and they are summarized in the following paragraphs of this Section. Other supporting studies are summarized in Appendix H.

In three separate short-term studies in rats, Deuchi et al. (1994; 1995a; 1995b) investigated the effects of chitosan on fat excretion in feces and on mineral and fat-soluble vitamin status. In these studies, various dietary fibers were examined for their effects on fat digestibility and fecal fat excretion in rats fed a high fat diet. Rats were fed high fat diets along with various levels of dietary fiber, one of which was a high level of chitosan, for a total of two weeks. In the first study (Deuchi et al., 1994), the effects of 22 different dietary fibers were compared at a level of 5% in the diet. These were compared to the control diet which contained 5% cellulose. Only two fiber sources, chitosan and propylene glycol alginate, resulted in less than 90% fat absorption. Only three fiber sources resulted in an increase in fecal fat---Kapok, propylene glycol alginate and chitosan. The effect of chitosan and other fibers on dry weight of feces, lipid in feces and apparent fat digestibility excerpted from the Deuchi et al. (1994) study are summarized in Table 6. No adverse effects were reported following chitosan administration.

Table 6. Effect Chitosan Other Fibers on Dry Weight of Feces, Lipid in Feces Apparent Fat Digestibility

Fiber Source	Dry weight of feces (g 3 days)	Total lipid in feces (mg 3 days	Apparent fat digestibility ( )
Cellulose (control)	4.86	578.8	94.9
Kapok	7.55	924.0	91.7
PGA	5.75	1641	83.4
Chitosan	8.95	5380	50.8

In the second study, Deuchi, et al. (1995a) investigated the effect of various chitosan preparations for their effect on fecal fat excretion when rats were fed a high fat diet. The chitosan preparations varied, either with a comparable degree of deacetylation but different viscosity or with similar viscosity but varying degrees of deacetylation. The study reported that increasing viscosity of the chitosan and increasing the degree of deacetylation tended to decrease the apparent fat digestibility. No adverse effects were reported following chitosan administration.

In the third study by Deuchi et al. (1995b), the effects of continuous and massive intakes of chitosan with sodium ascorbate (AsN) on mineral and fat-soluble vitamin status were evaluated in male Sprague-Dawley rats fed a high-fat diet. Four experiments were included in this reference.

In the first experiment, rats were divided into four groups; cellulose without AsN, cellulose with AsN, chitosan without AsN and chitosan with AsN. The study continued for 13 days with food intake recorded daily and body weights recorded every 3 or 4 days. Supplemental AsN did not affect body weight gain or food efficiency ratios, however, these parameters and fat digestibility were significantly lower in the chitosan exposed groups compared to controls. The addition of AsN caused a significant decrease in the fat digestibility in the groups exposed to chitosan but not in the cellulose exposed groups. Decreases in calcium, magnesium and iron absorptions were noted in the chitosan groups, but these were improved with AsN supplementation. The mineral content of the bone was significantly lower in the chitosan groups and was significantly increased with AsN supplementation.

In the second experiment, rats were divided into three groups; chitosan with AsN, chitosan with AsN enriched with calcium and chitosan with AsN enriched with magnesium. Feces were collected and analyzed in the last three days of the 14-day test period. Enrichment of the chitosan diet with either calcium or magnesium significantly increased the body weight gain but not the food efficiency ratio or fat digestibility.

In the third experiment, rats were divided into four groups: cellulose with AsN and minerals; chitosan with AsN and minerals; chitosan with AsN, minerals and fat-soluble vitamins; and, glucosamine with AsN, minerals and fat-soluble vitamins. Feces were collected and analyzed in the final three days of the 14-day test period. Body weight gain was significantly lower in the chitosan groups as compared to the cellulose and glucosamine groups, even with supplementation of AsN, fat-soluble vitamins and minerals. The food efficiency ratio and fat digestibility was significantly lower in the chitosan fed groups when compared to the cellulose and glucosamine groups. The retinol and retinyl palmitate levels were significantly lower in the livers of the chitosan groups when compared to the cellulose and glucosamine groups. Serum retinol levels were not significantly different between groups. The liver vitamin A levels did not improve in the chitosan group with fat-soluble vitamin supplementation. There was a large decrease in the serum vitamin E levels in the chitosan groups compared to the cellulose or glucosamine groups, and the liver vitamin E levels were also lower in the chitosan groups. The serum levels of triglyceride and cholesterol tended to be lower in the chitosan groups compared to the cellulose and glucosamine groups.

In the final experiment, rats were divided into four groups with the same feeding conditions as in the third experiment for 14 days. Feces were collected for the last 3 days to determine the digestibility of chitosan and glucosamine. On day 15, animals were euthanized following a 24-hour fast, and blood was collected for analysis of the plasma vitamin K levels. Prothrombin time and activated partial thromboplastin time were used to indirectly evaluate the plasma vitamin K levels. The vitamin K levels were not markedly affected by chitosan consumption.

The Deuchi et al. (1995b) study showed that chitosan administration resulted in calcium loss from bone, depletion of vitamin A and E in plasma and liver, and a slight increase in vitamin K plasma levels. A complex experimental design was used to look at the effects of vitamin and mineral supplementation. It was also shown that glucosamine, the major metabolite of chitosan, did not mimic the effects of chitosan. Deuchi et al. (1995b) did not proffer a plausible theory for the

mechanism of the complex effects observed. Although these investigators cited a previous study in which feeding of chitosan at 5% in the diet of rats did not affect calcium levels, no reasons for differential findings were provided. The investigators agreed that in their studies the intake of chitosan was massive. No effects at lower doses on any of the parameters were observed in any of the studies by these investigators.

Contrary to the observation from the Deuchi et al., studies, Fukada et al. (1991) did not find any significant differences in growth, food intake, liver weight, and dried fecal weight between control and chitosan-fed (2 or 5%) male Wistar rats after 21 days on a low cholesterol diet. In this study, Fukada et al. (1991) determined the effect of chitosan on intestinal bile acids in male Wistar rats. Animals were divided into three groups; control diet supplemented with 0.5% cellulose and 4.5% sucrose, control diet supplemented with 0.5% cellulose, 2.5% sucrose and 2% chitosan and control diet supplemented with 5% chitosan. The rats were fed *ad libitum* for 21 days and food intake was measured daily while body weights were measured every second day. Feces were collected for 2 days prior to termination and the blended, lyophilized samples were used for extraction and identification of bile acids. On the day of euthanasia, blood was collected and the intestines excised. The small intestine was divided into the duodenum, jejunum and ileum, and the large intestine was divided into the cecum and colon. The intestinal contents from each segment were evaluated for pH and bile acid analysis. No significant differences in growth, feed consumption, liver weight and dried fecal weight were noted among the groups. Serum cholesterol was lower in the 5% chitosan-fed group compared to controls. There were no significant differences between the control and chitosan groups in fecal excretion of neutral sterols and bile acids. While there was no increase in the excretion of fecal bile acids, there was a change in the fecal bile acid composition with chitosan consumption. Lithocholic acid and deoxycholic acid increased while 5-emimeric 3α-hydroxy-6-keto-cholanoic acid(s) decreased. The conversion of primary bile acids to secondary bile acids in the large intestine was affected by the increased pH secondary to chitosan feeding. The concentration of α, β and ω-muricholic acids and lithocholic acid increased with chitosan feeding and the levels of hyodeoxycholic acid (and its 6β-isomer), monohydroxy-monoketo-cholanoic acids and 3α, 6ξ, 7ξ-trihydroxy-cholanoic acid decreased. The effects on bile acids observed at the 5% dietary level of chitosan were not observed at the 2% level.

D. Human Studies

Clinical studies on chitosan have been well reviewed (Baldrick, 2010; FDA 2011). Egras et al. (2011) reviewed several human studies which investigated the use of chitosan as a weight loss supplement and concluded that, even though chitosan is well-tolerated in humans, the data are still limited. Several important safety studies on chitosan use in humans are summarized below.

1. Clinical Trials Assessing Safety of Chitosan

In a double-blind, placebo-controlled, parallel trial, Jaffer and Samplis (2007) investigated safety and efficacy of low molecular weight chitosan. In this trial, chitosan was given at daily doses of 1.2, 1.6, and 2.4 g to subjects with low-to-moderate hypercholesterolemia for 12 weeks. Of the 105 subjects that fulfilled the study criteria, 95 completed the study. Safety was assessed by the incidence of adverse events and clinically important changes in laboratory results, including serum 25-hydroxyvitamin D (25(OH)D). There were 29 predominantly mild adverse events reported by 24 (23%) patients---most frequently constipation (3%) and diarrhea (3%). The overall incidence of adverse events was similar among the study groups. No significant changes in serum 25-

hydroxyvitamin D (25(OH)D) levels at the end of 12 weeks were noted between treatment and placebo groups. Although no details of the safety-related blood parameters were reported, the article mentions that "blood was drawn for lipid profile and safety parameter testing." The results of this study suggest that ingestion of chitosan at a dose level up to 2.4 g/day for 12 weeks is safe.

In another, randomized, double-blinded, placebo-controlled dietary intervention, Kaats et al. (2006) examined the safety and efficacy of a chitosan. Participants were assigned to a treatment group, a placebo group or a control group. In this study a total of 150 overweight adults were enrolled; 134 (89.3%) completed the study; 111 (82.8%) were women who were similarly distributed among the three groups. The treatment group received 3000 mg (six 500 mg capsules) chitosan/day, and both treatment and placebo groups wore pedometers during their waking hours and recorded daily step totals. The control group followed weight loss programs of their choice and took the same baseline and ending tests. Subjects underwent Dual Energy X-ray Absorptiometry tests, fasting blood analysis (43-chemistry parameters), and self-reported daily activity levels and caloric intakes. The results of this study provide evidence for the efficacy and safety of a chitosan compound. There were no self-reported significant adverse side effects or adverse changes in blood chemistry parameters.

In 2005, Cochrane Reviews published a review of chitosan use for obese and overweight individuals (Jull et al., 2005). Cochrane Reviews are systematic reviews of primary research in human health care and health policy, and they are internationally recognized as the highest standard in evidence-based health care. Fifteen trials including a total of 1219 participants met the stringent inclusion criteria. No trial to date has measured the effect of chitosan on mortality or morbidity. Analyses indicated that chitosan preparations result in a significantly greater weight loss, decrease in total cholesterol and a decrease in systolic and diastolic blood pressure compared with placebo. There were no clear differences between intervention and control groups in terms of frequency of adverse events or in fecal fat excretion.

Tapola et al. (2008) published an important study to assess the effect of two different doses of chitosan on serum fat-soluble vitamin concentrations, cholesterol concentrations, and other safety parameters. The chitosan used in this study was ChitoClear® FG95, the subject of the present GRAS assessment. A total of 65 men and women consumed 0, 4.5, 6.75 g per day of chitosan or 6.75 g per day glucomannan for eight weeks in a parallel, placebo-controlled, single-blind study. The number of participants in each of the groups was 14 (5M/9F), 15 (7M/8F), 12 (7M/5M), and 15 (8M/7F), respectively. The ages of the subjects ranged from 18 to 55 years. Altogether, 56 participants completed the study. No differences were detected among the treatments in serum vitamins (vitamin A, vitamin E, 25-hydroxyvitamin D), carotenes (α- and β-carotene), clinical chemistry or hematology measurements. There were no significant differences in clinical chemistry

Table 7. Additional Toxicology Studies on Chitosan

Animal Species & Number of Animals	Route of Adminis-tration	Study Duration	Dose	Test Sample & Source	Results	Reference
Female Long-Evans rats; 5/group	Oral	56 days	0 or 2% of diet	Chitosan from shrimp shells	No significant changes in body weight or food consumption. No differences in liver lipids, or plasma palmitic & steric acid levels. Treatment animals had increased	Hossain et al., 2007

					oleic, linoleic, & docosapentaenoic acid, decreased arachiodonic acid & decreased plasma cholesterol level.	
Female, Ovariectomized Sprague-Dawley rats; 8/group	Oral	42 days	0 or 2% of diet	Chito-oligosacchar-ides	No significant differences in weight gain, food intake, or serum calcium. Chitooligosaccharide increased bone marrow density in femur.	Jung et al., 2006
Male Sprague-Dawley rats; 8/group	Oral	28 days	0, 2%, or 5% of diet	Chitosan from crab shell	No differences in body weight or food intake. Chitosan-treated rats had significantly lower plasma total cholesterol & LDL-cholesterol. No other adverse effects reported.	Moon et al., 2007
Male Sprague-Dawley Rats; 7/group	Oral	28 days	0 or 5% of diet	Chitosan from shrimp shells	Treatment group had significantly lower liver weight (absolute & relative), lower levels of liver metabolizing enzymes, & lower final body weight. No other adverse effects reported.	Yao et al., 2010
Sprague-Dawley rat; 6/group	Oral	28 days	0, 5% (high viscosity chitosan), 5% (low viscosity chitosan) of diet	Chitosan from shrimp shells	No changes in body weight, plasma lipid values. Chitosan treatment resulted in decreased liver weight, total liver lipids, plasma cholesterol, VLDL, LDL and HDL cholesterol. In high-viscosity group, higher liver lipid peroxide. No other adverse effects reported.	Chiang et al., 2000
Sprague-Dawley rat; 6-7/group	Oral	28 days	0, 2% or 5% of diet	Chitosan from crab shells	No significant changes in body weight or food intake. Chitosan group had lower liver weight & liver cholesterol. No adverse effects reported.	Sugano et al., 1988
Male Sprague-Dawley rats; 8-9/group	Oral	18 days	0, 10% (during week 1 of study) or 7.5% (for the rest of the study)	Chitosan (source not reported)	Chitosan group had reduced food intake & slower rate of growth. Lower level of liver cholesterol & higher fat excretion observed in chitosan group. No other adverse effects reported.	Gallaher et al., 2000
Male C57bl6/J mice; 8/group	Oral	10 weeks	0 (high-fat diet) or 5% of a high fat diet	Chitosan from exoskeleton fungi	In chitosan group, body weight gain decreased & feed efficiency lower. Fat staining of tissue showed lipid accumulation reduced in liver & muscle in chitosan group. No differences in liver weight, insulin resistance index, & glucose tolerance. Chitosan group had decreased serum triglycerides & cholesterol. No other adverse effects reported.	Neyrinck et al, 2009
Swiss Webster male and female mice; 29-30/group	Oral	70 days	0 or 15,000 mg/kg bw/day	Source not reported	After 10 week, chitosan-treated mice had a reduced body weight, an increased small intestine length, & increased liver weight & mass. No other adverse effects reported.	Kimura et al., (2004)
Female ICR mice; 13/group	Oral	63 days	0, 4,500, 10,500, 22,500	Chitin-chitosan	Chitin-chitosan treated mice had significantly reduced body weight & serum triacylglycerol levels. Liver	Han et al., 1999

			mg/kg bw/day		weight reduced in groups receiving 7 or 15% chitosan. No adverse effects reported.	
Female CF1 mice; 12/group	Oral	42 days	0, 3000 HMWC, 3000 mg/kg bw /day LMWC	High molecular weight chitosan (HMWC) and Low molecular weight chitosan (LMWC); source not reported	Chitosan group had lowered body weight, but HMWC not statistically significant. No adverse effects reported.	Torzsas et al., 1996
Male and female BALB/c mice	Oral	28 days	0, 750, 7,500 mg/kg bw/day	Chitosan from crab shells	At end of study, mice receiving 5% chitosan had significant reduction in body weight & average food consumption. In 5% group, the facultative anaerobes & lactobacillus concentrations were statistically less than controls & anaerobe colonies higher. No adverse effects reported.	Tanaka et al., 1997
Male broiler chicken; 64/group	Oral	42 days	0, 5%, 10%, 15%	Chitooligosaccharides	In highest dose group, lower feed consumption noted in first few weeks of study. Illeal digestibility for calcium & phosphorus increased with increasing chitooligosachharide concentration. No adverse effects reported.	Huang et al., 2005
Male broiler chicken; 42/group	Oral	42 days	0, 0.02, 0.05, 0.1, 0.3, 0.5 % of diet	Chitosan, source not reported	Average bw gain increased during weeks 3 to 6 in 0.05 & 0.1% groups. No changes in average daily feed, but feed conversion efficiency increased in 0.05 & 0.1% groups.	Shi et al., 2005

(plasma creatinine, plasma urate, plasma gamma-glutamyl transferase, plasma calcium, serum ferritin) or routine hematology (blood counts) measurements among the study groups. However, the hematocrit increased in the glucomannan group, and plasma calcium concentration decreased in every study group. The increase in hematocrit was statistically but not clinically significant. Four subjects in the chitosan 6.75 dosage group and one subject in the glucomannan group discontinued the study due to transient adverse events. Common gastrointestinal symptoms (loose feces, constipation, abdominal pain, repeated flatulence, abdominal bloating and abdominal rumbling) were reported in each group. There was a significant association between the study groups and the incidence of constipation, heartburn and nausea during the first 4-week period and between the study groups and nausea during the second 4-week period. The incidence of constipation seemed to be greatest in the chitosan 4.5 dosage group during the first four weeks of ingestion of chitosan. The incidence of heartburn and nausea seemed to be greatest in both chitosan groups. However, there were no significant differences between the groups after performing pair-wise comparisons. The changes in the total and LDL-cholesterol concentrations among the study groups were not statistically significant. The authors concluded that the consumption of chitosan tablets was found to be safe, but there was no significant effect on cholesterol concentration. Chitosan administration did not show any effect on vitamin status in humans. The investigators concluded that the consumption of chitosan was safe.

In an extensive review article, Baldrick (2010) concluded that chitosan exhibits low oral toxicity, and the available literature shows prior human oral exposure to chitosan *via* dietary supplements

and food additive, medical devices, and cosmetic applications. Although the focus of this review was related to the use of chitosan as an excipient, several studies following oral administration of chitosan were discussed. The reviewer stated that human exposure of gram levels of chitosan have occurred from oral dietary supplementation intake, and no adverse findings appear to have been manifested. An observed safe level of oral intake for the chitosan constituent, glucosamine, which is used in health supplements, of up to 2000 mg/day has been established for human use.

Choi et al. (2012) investigated oral chitooligosaccharides reduction of plasma lipids in smokers and non-smokers. Healthy male smokers (n=11) and non-smokers (n=8) consumed 500 mg of chitooligosaccharides in water two times per day before a meal for six weeks.

Chitooligosaccharides were prepared from crab shell chitosan. At the end of the study, there was a significant decrease in total cholesterol and low-density lipoprotein (LDL) cholesterol levels in both smokers and non-smokers. There were no significant differences in body weight or BMI after 6 weeks. No treatment related adverse effects were noted in any of the volunteers.

The summaries of several additional clinical studies are provided in Table 8. These studies were excluded from the above summaries because they were efficacy studies and did not provide rigorous safety endpoints.

2. Clinical Trials to Assess Allergen Concerns of Chitosan

Primex sponsored a study to investigate whether its commercial chitosan, ChitoClear®, 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. The test material was ChitoClear® FG95 Lot TM762 as manufactured by Primex. 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 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 noncommercial chitosan. 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.

Table 8. Additional Clinical Studies on Chitosan

Information on Subjects	Route of Administration	Study Duration	Dose	Test Sample & Source	Results	Reference
30 overweight, hyperlipemic subjects, under physical activity training	Oral	4 months	0 or 2 g/day	Low molecular weight chitosan, polyglucosamine	Significant reduction in body weight, waist circumference, LDL cholesterol & triacylglycerol than placebo. HDL increased more than placebo control. No adverse effects reported.	Cornelli et al., 2008
18 subjects with type	Oral	12 weeks	0 or 1.8	Chitosan,	In chitosan treated group, no	Ausar et al.,

2 diabetes			g/day	source not specified	significant differences in cholesterol or triglyceride concentration. Increase in HDL-cholesterol & reduced LDL-cholesterol. Mild digestive discomfort in subjects reported.	2003
68 obese subjects	Oral	12 weeks	0 or 3 g/day	Asorbitol, a salt of chitosan from shellfish	No significant changes in weight, body composition, blood composition, blood pressure, lipid profile or fasting insulin levels. No adverse effects reported.	Ho et al., 2001
12 obese subjects without diabetes	Oral	3 months	0 or 2.25 g/day	Chitosan (Vitamin World, 750 mg tablet)	No significant changes in serum glucose or lipid profile. Significant decrease in triglycerides in chitosan-treated group. Significantly increase insulin sensitivity. No adverse effects reported.	Hernandez, Gonzalez et al., 2010
134 overweight subjects	Oral	60 days	0 or 3 g/day	Chitosan, source not specified	Significantly reduced weight in chitosan treated group. No changes in cholesterol, HDL, LDL or bone density.	Kaats et al., 2006
56 subjects with mild hypercholesterolemia	Oral	55 days	0, 4.5, 6.75 g/day	Chitosan from shellfish	No changes in body weight, hematology, serum biochemistry or plasma lipids. Adverse effects included heartburn, constipation & nausea. Some subjects (in both groups) reported adverse skin symptoms.	Tapola et al., 2008
95 subjects with mild/moderate hypercholesterolemia	Oral	8 weeks	0 or 1.2 g/day	Chitosan, source not specified	No significant differences in body weight, BMI, blood pressure or food consumption. Chitosan treated group had significant reduction in total cholesterol at 8 weeks. No significant differences in HDL cholesterol or triglyceride levels. No adverse effects reported.	Bokura and Kobayashi, 2003
10 healthy subjects	Oral	4 weeks	0 or 0.54 g/day	Water-soluble chitosan	No significant differences in blood pressure, BMI, total cholesterol, phosphorus or calcium. The levels of plasma glucose decreased & atherogenic index decreased after 2 weeks in chitosan group. Concentration of HDL cholesterol increased in chitosan group, and no difference in LDL-cholesterol. Increased total plasma antioxidant activity in chitosan group. No adverse	Anraku et al., 2009

					effects reported.	
30 overweight subjects	Oral	28 days	0 or 2 g/day	Chitosan, source not specified	No significant changes in serum cholesterol or triglycerides, or body mass index. No change in vitamin A, D, E, β-carotene. Nine subjects in chitosan group reported constipation.	Pittler et al., 1999
8 healthy males	Oral	14 days	Week 1: 0 g/day Week 2: 3 g/day Week 3: 6 g/day Week 4: 0 g/day	Chitosan from sea crab shells	Decreased in lecithinase-negative clostridia, which may improve intestinal environment. Decreased fecal ammonia. No adverse effects reported.	Terada et al., 1995
8 healthy males	Oral	14 days	0, 1, 3, 6 g/day	Chitosan, source not reported	No change found in intake of energy, protein, fat or cholesterol. In chitosan treated subjects, decrease in total serum cholesterol & increase in HDL-cholesterol was noted. No changes in bile acid excretion, & the amount of bile acid excreted as lithocholic acid significantly decreased. Excreted amount of coprostanol (metabolite of cholesterol) significantly lower.	Maezaki et al., 1993
6 healthy adults	Oral	3 days during week two & 3 days during week three	1 g/day during week one & 3 g/day during week 2	Chitosan (FH 80, Koyo Chemicals, Japan)	No appreciable change in laboratory values. Headache & fatigue only reported subjective adverse events but were not related to chitosan intake---were reported during week one when chitosan was not consumed	Kohda et al., 2012

The protocol was designed such that subjects who reacted positively to chitosan in the first prick-test would 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, 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.

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 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 to be 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. 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 reacted positively to chitosan, including those found to be allergic to fish or shrimp:

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

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., 2001).

E. EFSA Assessment

Following a request from the European Commission, the European Food Safety Authority (EFSA, 2011) provided a scientific opinion on a list of health claims for chitosan. This opinion addressed the scientific substantiation of health claims in relation to chitosan and reduction in body weight, maintenance of normal blood LDL cholesterol concentrations, reduction of intestinal transit time and reduction of inflammation. Among the different claims, the EFSA Panel concluded that a cause and effect relationship has been established between the consumption of chitosan and maintenance of normal blood LDL-cholesterol concentrations based on a meta-analysis of several clinical studies. The Panel noted that, in order to obtain the claimed effect, 3 g of chitosan should be consumed daily. The EFSA Panel did not endorse any of the other suggested claims for chitosan. Although the Panel did not review the safety, this recommendation supports the notion that chitosan is safe at dose levels up to 3 g/day.

F. Cytotoxicity Hemocompatability of Chitosan

In the 2010 Baldrick review on the safety of chitosan, several studies about the potential cytotoxicity and hemocompatablity of chitosan were reviewed. *In vitro* studies with blood showed evidence of coagulation, thrombus formation and platelet aggregation and adhesion (Lee et al., 1995). Other cell culture studies have shown that chitosan polymers can be cytotoxic at

concentrations in the µg/mL to mg/mL range with an IC_{50} of 0.21 mg/mL for chitosan hydrochloride. The level of cytotoxicity depended on the salt form used and the molecular weight of the polymer (Carreno-Gomez and Duncan, 1997; Chae et al., 2005; Opanasopit et al., 2007). One study failed to show cytotoxicity using cultures of human fibroblasts, endothelial cells and smooth muscle cells (Ishihara et al., 2002). Carreno-Gomez and Duncan (1997) demonstrated that high molecular weight chitosans have the potential to induce cellular toxicity when used as polymeric carries for intravenous administration. *In vitro* cytotoxic effects of various chitosans in Caco-2 cells were studied by Chae et al. (2005) with little or no cytotoxicity at 1 mg/mL, while EC_{50} values for cell viability in the 3.2 to >20 mg/mL range were seen. The authors suggested that different binding affinities of the chitosans on cellular membranes result in different degrees of cytotoxicity.

G. Glucosamine Toxicology

In addressing the metabolism of chitosan in Section V.A, it was noted that glucosamine is a probable metabolite of chitosan. Glucosamine, 2-amino-2-deoxy-D-glucose, is an amino monosaccharide that is an essential component of mucopolysaccharides and chitin. Glucosamine is a component of connective tissue in all mammals. It is readily synthesized in the body from glucose and forms mucopolysachharides, which are large complexes of carbohydrate. Because of the high concentration in joint tissues, glucosamine supplements are widely used to relieve symptoms of osteoarthritis. Three forms of glucosamine are commonly available as supplements: glucosamine hydrocholoride, glucosamine sulfate, and N-acetyl-glucosamine. These compounds are derived from chitin. The effects of glucosamine on glucose metabolism have been well-studied because pharmacologic concentrations of glucosamine affect insulin action and secretion. Altered glucose metabolism has been observed with parenteral administration of large doses of glucosamine in animals and with high concentrations in *in vitro* studies. However, oral administration of large doses of glucosamine in animals has no documented effect on glucose metabolism (Anderson et al., 2005).

A critical review by Anderson et al. (2005) summarized extensive research on glucosamine administration in humans and animals. Acute oral administration of glucosamine in laboratory animals at large doses (5,000–15,000 mg/kg bw/day) is well-tolerated without documented toxicity. The oral LD_{50} for glucosamine in rats and mice was found to be 5,000 mg/kg bw/day in all studies.

In subchronic and chronic toxicity studies conducted by the oral route, glucosamine was well-tolerated in rats, mice, rabbits and dogs at doses of approximately 159-8,000 mg/kg bw/day for 12-365 days. In most comprehensive studies, the NOAEL for rats was determined to be 2700 mg/kg bw/day (365 days) and is 2149 mg/kg bw/day in dogs (183 days) (Anderson et al., 2005).

In a comprehensive review by Anderson et al. (2005), results from 32 clinical trials were reviewed to examine possible toxic effects of glucosamine in humans. These studies included 3063 subjects for an average of 17 weeks. Sixteen of these studies demonstrated that glucosamine administration for up to 37 weeks did not adversely affect glucose metabolism. In other studies, healthy subjects had no adverse effects from an infusion of 9.7 g and only one subject developed a headache after an infusion of 30.5 g. In 13 clinical trials reporting safety parameters, there were no adverse effects of glucosamine on blood chemistry, hematologic markers, urinalysis, occult blood in feces, or cardiovascular parameters. Thus, in numerous randomized, double-blind, placebo-controlled human studies with doses up to 2,000 mg/day, conducted in both normal subjects and in individuals suffering from type 2 diabetes, no evidence of glucosamine toxicity,

intolerance, or adverse effects on glycemic control was noted. Adverse effects reported by individuals receiving glucosamine have not differed in incidence, character, or severity from those experienced by controls receiving placebos. The authors concluded that glucosamine is safe under current conditions of use as a dietary supplement based on many animal toxicology studies, as well as clinical studies in humans.

VI. DISCUSSION

A. GRAS Criteria

FDA defines "safe" or "safety" as it applies to food ingredients as:

> "…reasonable certainty in the minds of competent scientists that the substance is not harmful under the intended conditions of use. It is impossible in the present state of scientific knowledge to establish with complete certainty the absolute harmlessness of the use of any substance."[5]

Amplification is provided in that the determination of safety is to include probable consumption of the substance in question, the cumulative effect of the substance and appropriate safety factors. It is FDA's operational definition of safety that serves as the framework against which this evaluation is provided.

Furthermore, in discussing GRAS criteria, FDA notes that:

> "…General recognition of safety requires common knowledge about the substance throughout the scientific community knowledgeable about the safety of substances directly or indirectly added to food."
>
> "General recognition of safety through experience based on common use in food prior to January 1, 1958, shall be based solely on food use of the substance prior to January 1, 1958, and shall ordinarily be based upon generally available data and information."[6]

FDA discusses in more detail what is meant by the requirement of general knowledge and acceptance of pertinent information within the scientific community, i.e., the so-called "common knowledge element," in terms of the two following component elements:[7]

- Data and information relied upon to establish safety must be generally available, and this is most commonly established by utilizing published, peer-reviewed scientific journals; and

- There must be a basis to conclude that there is consensus (but not unanimity) among qualified scientists about the safety of the substance for its intended use, and this is established by relying upon secondary scientific literature such as published review articles, textbooks, or compendia, or by obtaining opinions of expert panels or opinions from authoritative bodies, such as JECFA and the National Academy of Sciences.

The apparent imprecision of the terms "appreciable," "at the time," and "reasonable certainty" demonstrates that the FDA recognizes the impossibility of providing absolute safety in this or any other area (Lu, 1988; Renwick, 1990; Rulis and Levitt, 2009).

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[5] See 21 CFR 170.3(i).
[6] See 21 CFR 170.30(a).
[7] See Footnote 1.

As noted below, this safety assessment to ascertain GRAS status of chitosan for the defined food uses meets FDA criteria for reasonable certainty of no harm by considering both the technical and common knowledge elements.

B. Safety of ChitoClear® Shrimp-Derived Chitosan

The Expert Panel has reviewed the manufacturing process, specifications and composition of ChitoClear® products, as well as the scientific literature and patents pertaining to the use of shrimp-derived chitosan in foods. In addition, the safety studies addressing the safety of shrimp-derived chitosan were evaluated by the Panel. The Panel also reviewed the previous chitosan GRAS notices (GRNs 73 and170) and the safety-related issues raised by FDA during the review of these notifications. The Panel believes that composite safety studies---including the most recently published studies---adequately support a GRAS determination as described herein and that no additional safety concerns remain with the identified intended food uses.

Primex manufactures chitosan from a very pure and uniform source of raw materials under very controlled conditions in compliance with FDA Good Manufacturing Practices regulations. The chitosan products are high molecular weight and have a high degree of deacetylation (>75%). The food grade specifications show that all possible contaminants of concern, such as heavy metals, are present at levels viewed as acceptable for a food ingredient. Primex offers customized preparations of chitosan at various viscosity ranges to meet customer technical needs. The lowest viscosity offered is 25 mPA; this corresponds to a product with a minimum molecular weight of 150 kDa. The Expert Panel agrees that these properties and specifications define a family of products that are safe as demonstrated by many published safety studies.

Primarily, FDA's expressed concerns with the previous GRAS notifications were related to the unpublished status of a key clinical study, commitment by the National Toxicology Program to undertake a toxicology study, and scientific issues arising from three publications on chitosan by Deuchi and co-workers (Deuchi et al., 1994, 1995a, 1995b). However, much progress has been made on these issues over the last six years. Most notably, the availability of the NTP study, the publication of the Tapola et al. (2008) study, and the review by Anderson et al. (2005) on glucosamine has resolved FDA's previous questions about the safety of chitosan under the proposed food use conditions. Additionally, more recently FDA has reviewed a related GRAS notice on chitosan---GRN 397---and completed its evaluation with a favorable finding. The important new findings and conclusions can be summarized as follows:

1. The key human study on safety of shrimp-derived chitosan has been published (Tapola et al., 2008) in a peer-reviewed journal of international repute and has been available in the public domain for the past few years.

2. The available results of the dose-response 26-week NTP (2008) study did not reveal any safety-related concern. It is not likely that NTP will pursue their investigation of chitosan any further because of the lack of any safety concerns from experimental data in their 6-month study in rats.

3. The Deuchi et al. (1994; 1995a; 1995b) studies were all done at a single dose of 5% of the rat diet. No dose-response was established for the effects of chitosan in these studies. An equivalent dose in humans is 75 g/person/day.

4. The available safety-related information on glucosamine by Anderson (2005), a metabolite of chitosan, suggests that glucosamine is unlikely to present any safety concerns at levels expected to be generated from the intended food uses of chitosan.

5. In addition to the safety study published by Tapola et al. (2008), Jaffer and Samplis (2007) as well as Kaats et al. (2006) published a safety-related study of chitosan in human subjects that did not raise any new safety concerns. Importantly, these studies support the safety of chitosan at the intended use levels. The results of the Jaffer and Samplis (2007) study suggest that ingestion of chitosan at dose levels up to 2.4 g/day for 12 weeks is safe, while the results from Kaats et al. (2006) support the safety of chitosan at 3.0 g/day for 60 days.

6. The available human studies suggest that chitosan is unlikely to inhibit vitamin and mineral absorption at levels resulting from its intended uses as a food ingredient. One study in mice indicates that chitosan may actually improve trace metal absorption (Zeng et al., 2008a).

7. In several clinical trials efficacy of shrimp-derived chitosan has been investigated. All of these trials reported minimal or no adverse effects with doses ranging from 2.5 g/day to over 5 g/day, well within levels expected to be generated from the intended food uses of chitosan.

8. In several comprehensive review articles (Ylitalo, et al., 2002; Shields, et al., 2003; Mhurchu, et al., 2005; Jull, et al., 2008), current knowledge about cholesterol-lowering, weight management and safety properties of chitosan at dosages as high as 6 g/day are discussed. Ylitalo, et al., and Shields et al., reported mild and transitory gastrointestinal disturbances in a small number of patients (Appendix H). Both groups also conclude that chitosan has been clinically well-tolerated, but individuals with a shellfish allergy should use extreme caution in taking chitosan supplements. Shields, et al. (2003) also remarked that chitosan products may be contaminated with heavy metals, such as lead, mercury, iron, and copper; therefore, chitosan should be avoided by children, pregnant patients, and nursing mothers. Shields et al. went on to state that no drug interactions involving chitosan have been reported, but caution may be warranted when administering chitosan concurrently with highly lipophilic drugs. They also had theoretical concerns about chitosan decreasing the absorption of fat-soluble vitamins, although they acknowledge that clinical trials have not shown this to be the case. Mhurchu et al. (2005) and Jull et al. (2008) reported that there was no clear difference between intervention and control groups in terms of frequency of adverse events. In the review article, Jull et al. also added that, despite some side effects, none of the reviewed trials showed changes in blood chemistry which would suggest any significant effects of chitosan on these parameters.

9. One author of a published review concluded that more study is needed to resolve the possible effects of chitosan on blood clotting (Baldrick, 2010). The Expert Panel has reviewed this opinion and notes that this concern relates to parenteral administration and that there is no evidence that orally administered chitosan affects blood clotting.

10. The EFSA (2011) Panel concluded that a cause and effect relationship exists between the consumption of chitosan and maintenance of normal blood LDL-cholesterol concentrations; in order to obtain the claimed effect, 3 g of chitosan should be consumed daily. This recommendation indicates that EFSA also believes that consumption of chitosan is safe at this level.

Perplexing results were observed in a recent study (Omara et al. 2012) where chitosan was administered orally to male and female mice at dose levels of 150 and 300 mg/kg bw/day for 35 days. Many effects were seen on enzyme levels and creatinine. Histopathology was also observed in the kidney and liver. Little detail was given on the test sample in the Omara study. Purity as well as basic specifications on the chitosan---such as molecular weight and degree of polymerization/deacetylation---were not provided. The corresponding author of this study (Dr. Omara) was contacted to seek additional sample specifications and study-related details. However, the author did not respond to queries and only provided the article. In the absence of additional details, the results of this study are difficult to interpret. In light of the results of other studies, most notably in the NTP study where no adverse effects were noted at levels up to 4500 mg/kg bw/day for 6 months in rats and a 3-month study in mice at 500 mg/kg bw/day, the effects noted by Omara et al. (2012) at dose levels of 150 and 300 mg/kg bw/day for 35 days seem to be due to differences in products or contamination. As noted above, Shields, et al. (2003) also remarked that chitosan products may be contaminated with heavy metals, such as lead, mercury, iron, and copper, if proper quality control measures are not employed. Therefore, the Panel questions the reliability of this study and speculates that the source of the material may have been contaminated.

Overall, the preponderance of evidence of safety of chitosan, including new studies---several of which address concerns previously raised by FDA in 2005 in the review of GRN 170---supports the position that the proposed food uses of shrimp-derived chitosan are GRAS. This finding requires that the subject chitosan has a molecular weight in excess of 150 kDa, that this food substance complies with adequate purity specifications as described in Table 3, and that the subject chitosan would be used as a food ingredient in the designated food categories which would result in an estimated daily intake of 1.69-3.37 g/person.

C. Common Knowledge Elements with GRAS Determination

The first common knowledge element for a GRAS determination is that data and information relied upon to establish safety must be generally available; this is most commonly established by utilizing published, peer-reviewed scientific journals for the safety assessment. Numerous clinical studies which support the safety assessment have been published in the scientific literature---most notably those by Jaffer and Samplis (2007), Tapola et al., (2008), Kaats et al., (2006) and Jull et al. (2005). The safety is also supported by several toxicology studies in laboratory animals, the more definitive of which was conducted by NTP (2008). While a report has not been issued on the NTP study, the data are readily available on the NTP website (also see Appendix G).

The second common knowledge element for a GRAS determination is that there must be consensus among qualified scientists about the safety of the substance with its intended use. The safety data on chitosan have been reviewed by regulatory authorities who have all concluded that chitosan is safe as a dietary supplement (Japan Food Chemical Research Foundation, 2011; KFDA, 2011; EFSA, 2011). In a review of the use of chitosan as a pharmaceutical excipient,

Baldrick (2010) seems to conclude that there is adequate information to support the safety of oral consumption of gram levels of chitosan. It should be noted that Baldrick did not review the NTP study which this Expert Panel has concluded is very important due to the lack of adverse effects found in rats at high doses with over a six month treatment period. Baldrick also notes that purity of chitosan is an important issue, and this aspect relates to the Omara study.

In addition, many competent researchers responsible for the data referred to in this evaluation that have expertise in the fields of agriculture, nutrition, toxicology and medicine view shrimp-derived chitosan as a viable food ingredient and source of dietary fiber. In GRN 397, it is stated that there is no information on the toxicity of chitosan preparations derived from *A. niger*, however, a number of animal, human, and *in vitro* studies relevant to the safety of shellfish chitosan, have been published and support the safety of *A. niger* derived chitosan.

In light of the collective scientific investigations into the safety of chitosan products, including international review and evaluation for ingestion purposes, the Panel concludes that consensus exists regarding the safety of the intended human food uses of ChitoClear® products.

VII. CONCLUSIONS[8]

The Expert Panel offers the following conclusion:

ChitoClear products that are produced in accordance with FDA Good Manufacturing Practices requirements and which meet the purity specifications as set forth in Section III.D of this notification, are generally recognized as safe when consumed at levels up to 3.37 grams per person per day in the food categories and at the designated food use levels found in Table 5.

This declaration is made in accordance with FDA's standard for food ingredient safety, i.e., reasonable certainty of no harm under the intended conditions of use.

(b) (6)

Richard C. Kraska, Ph.D., DABT

(b) (6)

Robert S. McQuate, Ph.D.

(b) (6)

Madhusudan G. Soni, Ph.D., FACN, FATS

August 20, 2012

[8] The detailed educational and professional credentials for two of the individuals serving on the Expert Panel can be found on the GRAS Associates website at www.gras-associates.com. Drs. Kraska and McQuate worked on GRAS and food additive safety issues within FDA's GRAS Review Branch earlier in their careers and subsequently continued working within this area in the private sector. Dr. Soni's curriculum vitae can be accessed at: http://www.soniassociates.net. All three panelists have extensive technical backgrounds in the evaluation of food ingredient safety. Each individual previously served on multiple GRAS Expert Panels. Dr. Kraska served as Chair of the Panel.

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APPENDICES

Appendix A: **Analysis Report Determination of Molecular Weight Distribution**

Appendix B: **Certificates of Analysis for Food Grade ChitoClear**

Appendix C: **Certificates of Analysis Food Grade 95 ChitoClear**

Appendix D-1: **Stability Testing on Reference Samples of ChitoClear**

Appendix D-2: **Stability Tests Performed on Chitosan Solutions in Various Acids**

Appendix E: **Amino Acid Analysis of Proteins Peptides**

Appendix F-1: **Summary of Research of Suitability of Chitosan in Meat Products**

Appendix F-2: **Summary of Scientific Literature on Acceptability of Chitosan in Meat Products**

Appendix G: **Summary of NTP 26-Week Study with Chitosan in Rats**

Appendix H: **Additional Biological Effects Studies**

APPENDIX A □ PART 1

Polymer Standards Service GmbH · Postfach 3368 · D-55023 Mainz

PRIMEX
Sigridur Vigfusdottir

Oskarsgata 7

580 Siglufjordur

Iceland

Mainz, 2005-11-03

Analysis Report

Analysis Order: GPC-Analysis of 15 Chitosane samples. Determination of the molecular weight distribution (MWD). Calibration with pullulane standards

Batch no: TM2382- TM2396

Analysis Conditions:

Eluent:	0.1 M aqueous NaCl / 0.1 Vol.% Trifluoroacetic-acid (TFAc)
Columns:	PSS - Novema, 10 µm, linear, ID 8.0 mm x 300 mm PSS - Novema, 10 µm, 3000Å, ID 8.0 mm x 300 mm
Pump:	TSP P100
Flow:	1.0 ml/min
Sample Concentration:	about 2.0 g/l
Injector:	TSP AS 3000
Injectionsvolume:	20 µl
Temperature:	23 °C
Detector:	Shodex RI 71
Calculation:	PSS - WinGPC Version 7.20

In der Dalheimer Wiese 5, D-55120 Mainz
Telefon: +49-6131-96239-0; Telefax::+49-6131-96239-11
Ust.-Id.Nr.: DE 149 059 445; E-Mail: info@polymer.de
Homepage: www.polymer.de

Geschäftsführer: Dipl. Chem. Friedhelm Gores, Dipl. Chem. Peter Kilz
Joachim Kilz, Ralf Leinweber; HRB 3232, Reg. Amtsgericht Mainz
Bankverbindung: Mainzer Volksbank, BLZ 55190000, Kto.-Nr. 248629016
Deutsche Bank Mainz, BLZ 550 700 40, Kto.-Nr. 0 322 644

Sample Preparation

The sample was weighted and dissolved in 0.1 M aqueous NaCl / 0.1 Vol.% Trifluoroaceti-cacid at room temperature overnight. The concentration of the sample solution was about 2.0 g/ml.
The sample solution was filtrated through a 1.0 µm filter (Schleicher & Schuell) and 20 µl of the solution was injected by an autosampler.

Calibration and Calculation

A couple of pullulane standards with different molecular weights were measured fisrt in order to get a calibration curve.

The calculation of the average molecular weights and the molecular weight distribution of the samples was done by the so called slice by slice method based on the pullulane calibration..

The results are given on the attached plots.

Please note: The calculated molecular weights are not absolute values but pullulane equivalente molecular weights.

If you have any questions concerning the measurement and calculations, please don´t hesitate to contact me by e-mail: fgores@polymer.de

Best regards
PSS POLYMER STANDARDS SERVICE GMBH

(b) (6)

F. Gores

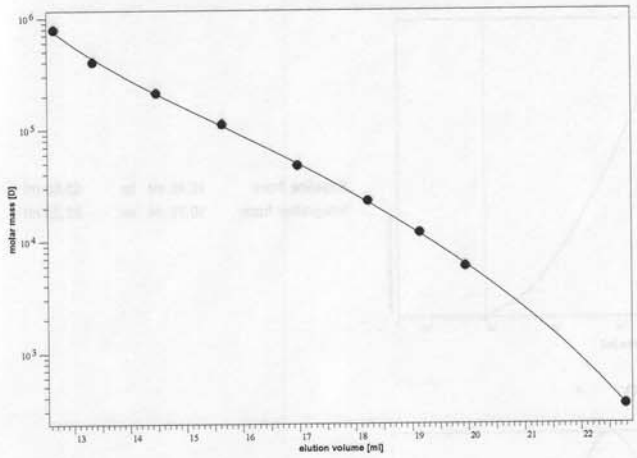
attachment:

- calibration curve and calibration data
- plot of the elugram and molecular weight distribution (MWD)
- overlay of the MWD

APPENDIX A □ PART 2

WINGPC Calibration

Comment: Pullulane in 0.1 M NaCl / 0.1 % TFAc
Filename: W:\GPC_DAT\Cal__05\Cal_pg07\pull_1101.CAL



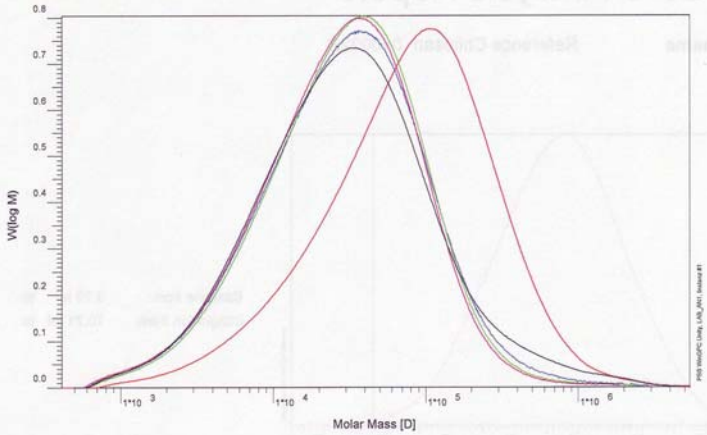
Calibration Parameters:

Columns:	Novema B: 10µm linear,3000Å
Mark-Houwink a:	0,000
Mark-Houwink k:	1,0000
Int. Standard:	Systeme (Salt)
Vp int. Standard:	23,00 ml
Fit:	PSS Poly 5
Operator:	F. Gores

Elutionvolume [ml]	Molar Mass Mp [D]	Sample Name	Slope	Deviation [%]
12,6667	788000	pull P800	-0,3850	-4,3853
13,3533	404000	pull P400	-0,3152	7,6664
14,4783	212000	pull P200	-0,2576	-1,0407
15,6450	112000	pull P100	-0,2463	-4,0181
16,9750	47300	pull P50	-0,2648	4,4975
18,2250	22800	pull P20	-0,2960	-3,0753
19,1383	11800	pull P10	-0,3241	-2,3460
19,9467	5900	pull P5	-0,3546	3,9603
22,7733	342	dxtp2	-0,5780	-0,5394

APPENDIX A □ PART 3

WINGPC Overlay MWD



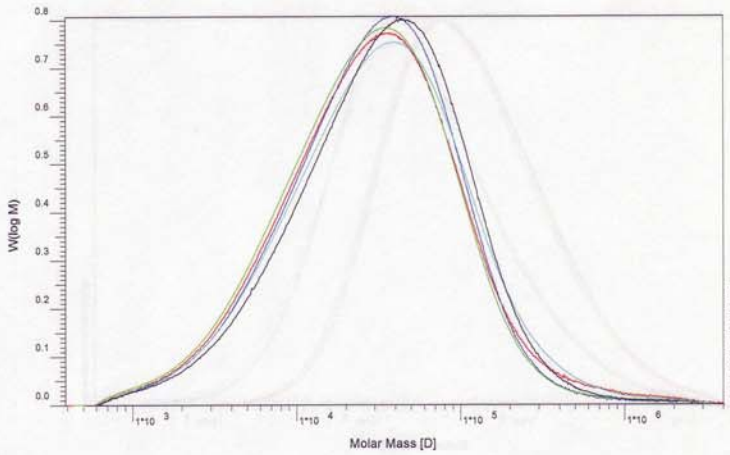
Curve: 1 Shodex RI 71, TM2382 051121
Curve: 2 Shodex RI 71, TM2383 051122
Curve: 3 Shodex RI 71, TM2384 051123
Curve: 4 Shodex RI 71, TM2385 051124
Curve: 5 Shodex RI 71, TM2386 051125

Curve No.	Mn [D]	Mw [D]	Mz [D]	D (Mw/Mn)	Vp (ml)	Mp [D]	Area (ml*\
1	28500	154000	520000	5,41	15,73	103000	0,010
2	13300	61980	317000	4,66	17,68	31800	0,010
3	13900	56980	251000	4,11	17,55	34600	0,010
4	12800	52200	195000	4,08	17,65	32400	0,010
5	13100	81380	606000	6,19	17,88	27800	0,010

Methodeninformation

Project:	W:\GPC_DAT\serv5_04_sax.LDX	Injection:	Mittwoch 02/11/05 0:
Calibration:	pull_1101.CAL	Calibration Curve-fit:	PSS Poly 5
Int. Standard:	Systeme (Salt)		
Int. Standard - K:	23,00 ml	Int. Standard - M:	22,99 ml
Sample concentration:	2,00 g/l	Inject-Vol.:	20 µl
Eluent:	0.1M NaCl /0,1% TFAc	Flow-Rate:	1,00 ml/min
Columns:	Novema B: 10µm linear,3000Å	Temperature:	23,0 °C
GPC-Instrument:	PG07	Operateur:	F. Gores

WINGPC Overlay MWD



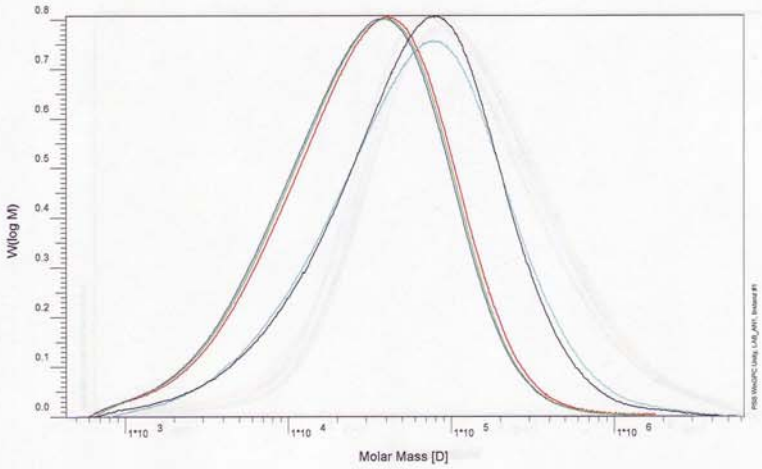
- Curve: 1 Shodex RI 71, TM2387 051126
- Curve: 2 Shodex RI 71, TM2388 051127
- Curve: 3 Shodex RI 71, TM2389 051128
- Curve: 4 Shodex RI 71, TM2390 051129
- Curve: 5 Shodex RI 71, TM2391 051130

Curve No.	Mn [D]	Mw [D]	Mz [D]	D (Mw/Mn)	Vp (ml)	Mp [D]	Area (ml*\
1	13000	67900	488000	5,22	17,73	30700	0,010
2	13600	54310	212000	4,00	17,60	33500	0,010
3	12300	54400	297000	4,42	17,79	29600	0,010
4	13700	70980	413000	5,20	17,61	33200	0,000
5	14800	64650	296000	4,37	17,45	36800	0,010

Methodeninformation

Project:	W:\GPC_DAT\serv5_04_sax.LDX	Injection:	Mittwoch 02/11/05 0
Calibration:	pull_1101.CAL	Calibration Curve-fit:	PSS Poly 5
Int. Standard:	Systeme (Salt)		
Int. Standard - K:	23,00 ml	Int. Standard - M:	22,93 ml
Sample concentration:	2,00 g/l	Inject-Vol.:	20 µl
Eluent:	0.1M NaCl /0,1% TFAc	Flow-Rate:	1,00 ml/min
Columns:	Novema B: 10µm linear,3000Å	Temperature:	23,0 °C
GPC-Instrument:	PG07	Operateur:	F. Gores

WINGPC Overlay MWD



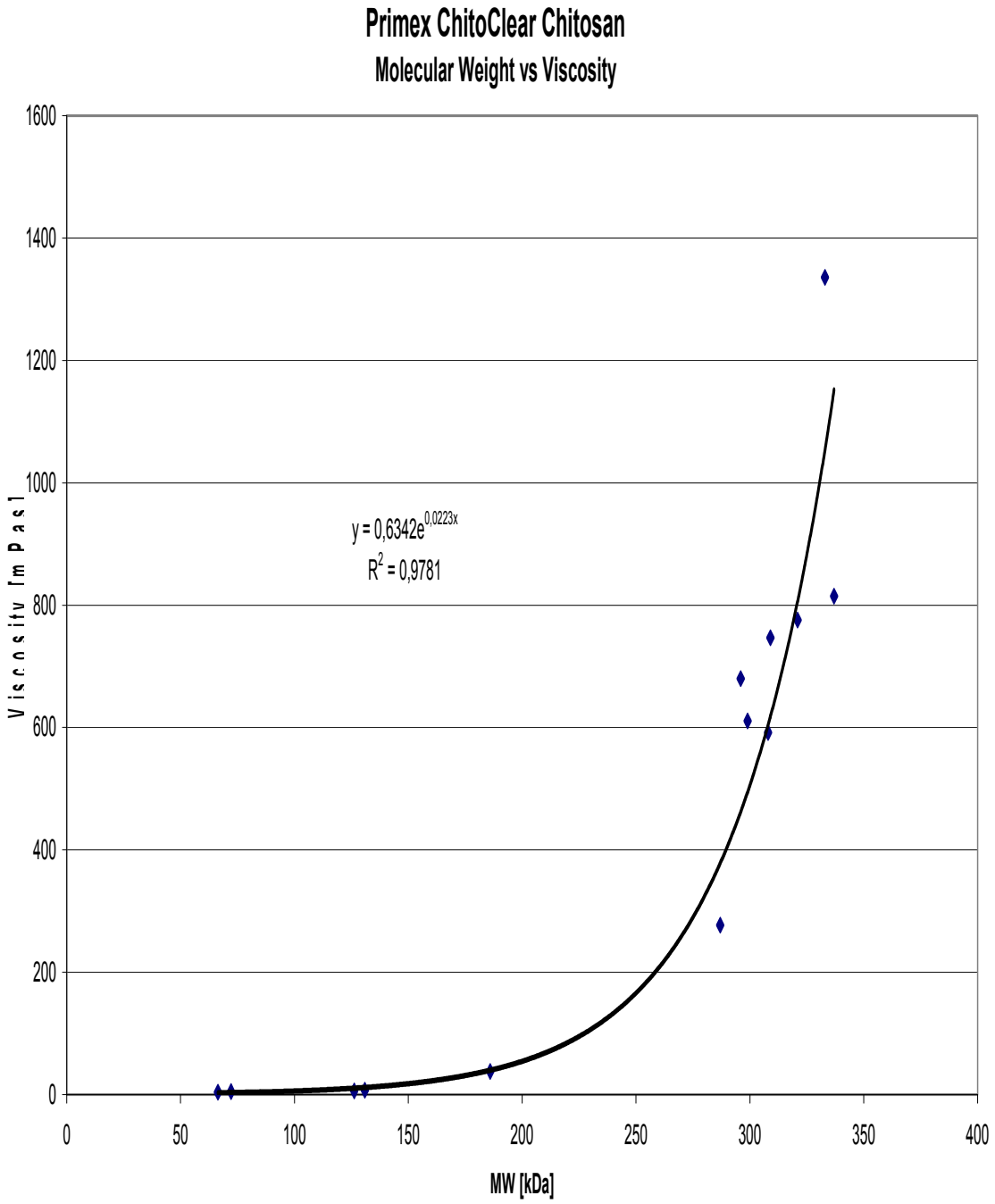
Curve: 1 Shodex RI 71, TM2392 051131
Curve: 2 Shodex RI 71, TM2393 051132
Curve: 3 Shodex RI 71, TM2394 051133
Curve: 4 Shodex RI 71, TM2395 051134
Curve: 5 Shodex RI 71, TM2396 051135

Curve No.	Mn [D]	Mw [D]	Mz [D]	D (Mw/Mn)	Vp (ml)	Mp [D]	Area (ml*\
1	13800	55600	183000	4,02	17,48	36100	0,010
2	13000	50830	153000	3,90	17,64	32500	0,000
3	13500	52230	162000	3,88	17,78	29700	0,010
4	24700	130900	591000	5,30	16,45	67800	0,010
5	23700	116600	445000	4,91	16,38	70300	0,010

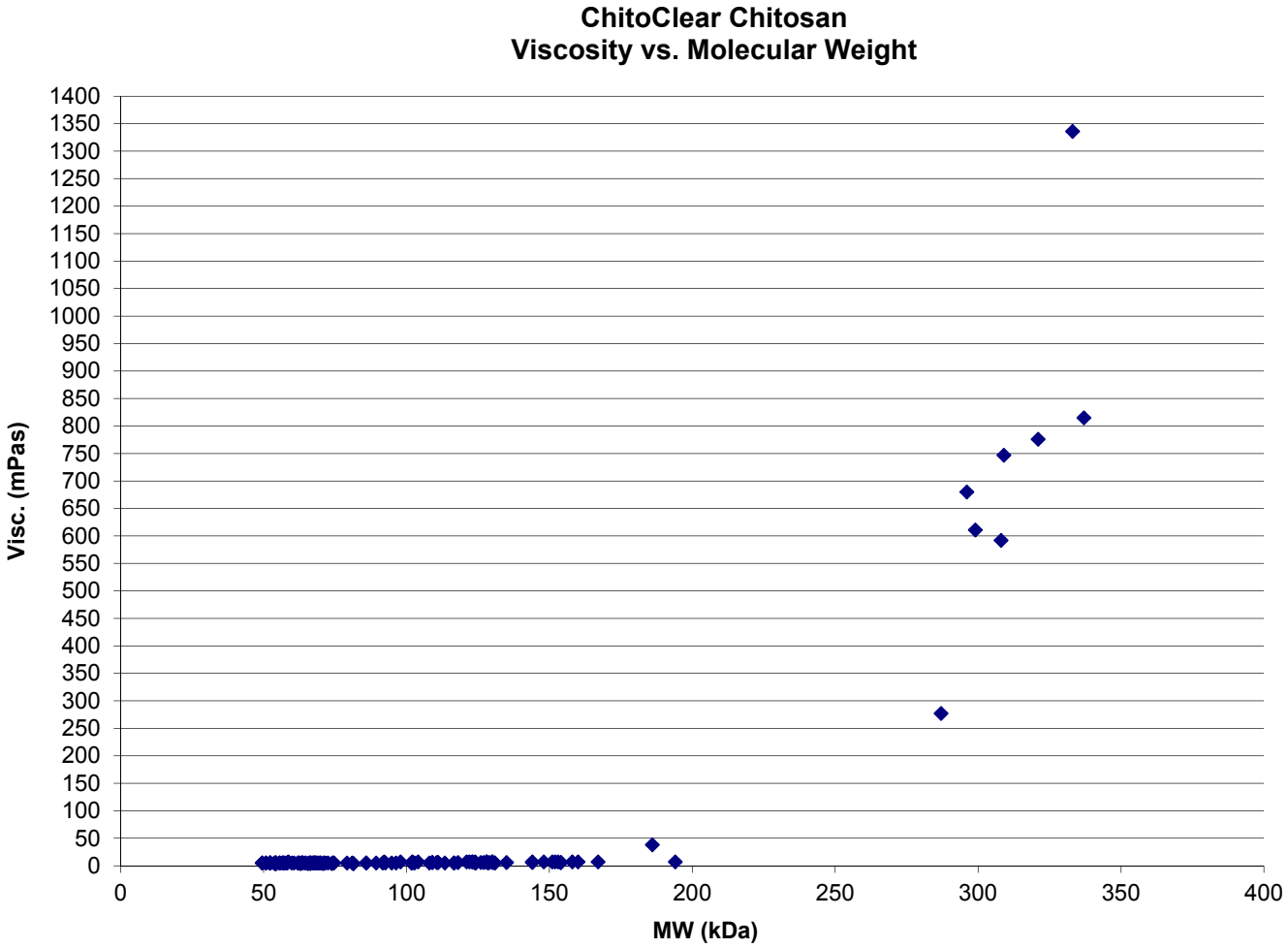
Methodeninformation

Project:	W:\GPC_DAT\serv5_04_sax.LDX	Injection:	Mittwoch 02/11/05 1
Calibration:	pull_1101.CAL	Calibration Curve-fit:	PSS Poly 5
Int. Standard:	Systeme (Salt)		
Int. Standard - K:	23,00 ml	Int. Standard - M:	22,89 ml
Sample concentration:	2,00 g/l	Inject-Vol.:	20 µl
Eluent:	0.1M NaCl /0,1% TFAc	Flow-Rate:	1,00 ml/min
Columns:	Novema B: 10µm linear,3000Å	Temperature:	23,0 °C
GPC-Instrument:	PG07	Operateur:	F. Gores

APPENDIX A □ PART 4



APPENDIX A PART 5



PSS POLYMER STANDARDS SERVICE GMBH

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Joachim Kilz, Ralf Leinweber; HRB 3232, Reg. Amtsgericht Mainz
Bankverbindung: Mainzer Volksbank, BLZ 55190000, Kto.-N r . 248629016
Deutsche Bank Mainz, BLZ 550 700 40, Kto.-Nr. 0 322 644

GPC-Analysis of ChitoClear Chitosan samples. Determination of the molecular weight distribution(MWD). Calibration with pullulane standards.

Sample Number	Mw [kDa]	Viscosity [mPas]
TM 1419	186	38
TM 1567	333	1336
TM 1625	58.6	7
TM 1690	287	277
TM 1847	337	815
TM 1923	296	680
TM 1961	321	776
TM2279	66.3	4
TM2386	81.38	4
TM2388	54.31	4
TM2077	96.3	5
TM2089	102	5
TM2091	89.4	5
TM2102	69.9	5
TM2106	79.2	5
TM2107	67.9	5
TM2108	59.8	5
TM2111	63.7	5
TM2123	124	5
TM2125	74.6	5
TM2126	57.8	5
TM2127	55.7	5
TM2128	53.9	5
TM2129	69.2	5
TM2131	92.6	5
TM2132	68.9	5
TM2138	58.3	5

Sample Number	Mw [kDa]	Viscosity [mPas]	
10 samples	66	4	average
87 samples	72	5	average
14 samples	126	6	average
23 samples	131	7	average
TM 1419	186	38	
TM 1690	287	277	
TM2642	308	592	
TM2823	299	611	
TM 1923	296	680	
TM2272	309	747	
TM 1961	321	776	
TM 1847	337	815	
TM 1567	333	1336	

TM2139	57.1	5
TM2140	66.5	5
TM2280	66.6	5
TM2286	94.8	5
TM2339	67.3	5
TM2340	68.2	5
TM2341	74.2	5
TM2342	72.5	5
TM2343	52.4	5
TM2344	49.5	5
TM2345	62.6	5
TM2346	69.8	5
TM2347	63.4	5
TM2348	56.6	5
TM2349	53.8	5
TM2351	54.2	5
TM2352	85.9	5
TM2383	61.98	5
TM2384	56.98	5
TM2385	52.2	5
TM2387	67.9	5
TM2389	54.4	5
TM2390	70.98	5
TM2391	64.65	5
TM2392	55.6	5
TM2393	50.83	5
TM2394	52.23	5
TM2395	130.9	5
TM2396	116.6	5
TM2074	111	6
TM2075	109	6
TM2076	109	6
TM2095	129	6
TM2124	126	6
TM2130	127	6
TM2281	130	6
TM2282	144	6
TM2284	135	6
TM2285	118	6
TM2338	111	6
TM2382	154	6
TM1702	122	7
TM2016	128	7
TM2017	104	7
TM2069	153	7
TM2070	130	7
TM2071	123	7
TM2078	151	7
TM2082	167	7
TM2083	128	7
TM2084	152	7
TM2085	144	7
TM2086	158	7

TM2090	102	7
TM2092	92.2	7
TM2093	130	7
TM2094	121	7
TM2101	97.9	7
TM2103	123	7
TM2283	148	7
TM2287	124	7
TM2288	160	7
TM2289	194	7
TM2338	110.6	6
TM2339	67.32	5
TM2340	68.25	5
TM2341	74.17	5
TM2342	71.6	5
TM2343	52.39	5
TM2344	49.51	5
TM2345	62.6	5
TM2346	69.81	5
TM2347	63.41	5
TM2348	56.6	5
TM2349	53.84	4
TM2351	54.16	5
TM2352	85.9	5
TM2382	154	6
TM2383	61.98	5
TM2384	56.98	5
TM2385	52.2	5
TM2386	81.38	4
TM2387	67.9	5
TM2388	54.31	4
TM2389	54.4	5
TM2390	70.98	5
TM2391	64.65	5
TM2392	55.6	5
TM2393	50.83	5
TM2394	52.23	5
TM2395	130.9	5
TM2396	116.6	5
TM2531	113.4	5
TM2532	81.03	5
TM2533	128.5	5
TM2534	91.65	5
TM2535	102.8	5
TM2536	124.2	5
TM2537	108	5
TM2538	73.78	4
TM2539	65.49	4
TM2540	62.89	4
TM2541	70.81	4
TM2542	101.7	5
TM2615	63.56	5

TM2616	65.91	5
TM2617	67.51	5
TM2618	66.11	5
TM2619	66.4	5
TM2620	68.23	5
TM2621	60.64	5
TM2622	63.53	5
TM2623	63.6	5
TM2272	309	747
TM2642	308	592
TM2823	299	611

Sample Number	Mw [kDa]	Viscosity [mPas]		
10	66	4		
87	72	5		
14	126	6		
23	131	7		
TM 1419	186	38	92%	collodial
TM 1690	287	277	99%	collodial
TM2642	308	592	95%	collodial
TM2823	299	611	80%	direct
TM 1923	296	680	87%	direct
TM2272	309	747	81%	direct
TM2696			82%	direct
TM 1961	321	776	81%	direct
TM 1847	337	815	80%	direct
TM 1567	333	1336	88%	collodial

APPENDIX B □ PART 1

primex

Certificate of analysis

Food Grade LV

Product name	ChitoClear® (Chitosan)	*Manufactured by:*
Source	*Pandalus borealis*	Primex ehf
Generic name	β(1→4) D-glucosamine / N-acetyl-D-glucosamine	Oskarsgata 7
Lot number	TM3472	580 Siglufjordur
Revision number	Rev. 03	Iceland
Date of manufacture	4.9.2009	Ph: +354 460 6900
Retest date	4.9.2012	Fax: +354 460 6909

PARAMETER	TEST METHOD	RESULTS	Comment
Dry Matter Content	CP-001	92 %	
Ash	CP-002	0,2 %	
Turbidity	CP-003	29 NTU	
Viscosity	CP-004 (1% chitosan)	9 cP (mPa·s)	
Solubility	CP-006	99,7 %	
Degree of Deacetylation	CP-010 (Colloidal titration)	95 %	
Protein	As total amino acids*	0,012 g/100g	
Sieve Analysis	CP-008	100% through 100 mesh	
Tap Density	CP-009	0,58 g/cc	
Appearance	CP-007	White powder	
Taste and odor	CP-007	No taste or smell	
Microbial:			*detection limit:*
Aerobic plate count	NMKL 86	<300 cfu/g	10 cfu/g
Yeast and mold	NMKL 98	<50 cfu/g	10 cfu/g
Coliform bacteria	ISO 4831	absent	10 cfu/g
E. coli	ISO 7251	absent	0,3 cfu/g
Salmonella	NMKL 71	absent	Neg.
Listeria	NMKL 136	absent	Neg.
Pseudomonas	ISO 16266	absent	10 cfu/g
Toxic Heavy Metals:			
Arsenic	SW-846 6020 ICP/MS	none detected	0,10 ppm
Cadmium	SW-846 6020 ICP/MS	none detected	0,05 ppm
Lead	SW-846 6020 ICP/MS	none detected	0,10 ppm
Mercury	SW-846 7471 CVAA	none detected	0,005 ppm

* Analysis performed at Uppsala Biomedical Center, Sweden

MBR – Master Batch Record
CP – Primex Standard Test Methods
NMKL - Nordic Committee on Food Analysis
ISO – International Organization for Standardization
ICP/MS – Inductively Coupled Plasma Mass Spectrometry
CVAA – Cold Vapor Atomic Absorption

(b) (6)

Reported By: _____
Name: Vala Arnadottir
Title: Laboratory Manager
Date: 27. 7. 2011.

Methods of analysis available upon request

NOTE: *Lack of colored logo indicates this document is a copy and is not controlled*

Primex ehf, Oskarsgata 7, 580 Siglufjordur, Iceland, Phone: +354 460 6900, Fax: +354 460 6909

primex

Certificate of analysis

Food Grade LV

Product name	ChitoClear® (Chitosan)	*Manufactured by:*
Source	*Pandalus borealis*	Primex ehf
Generic name	β(1→4) D-glucosamine / N-acetyl-D-glucosamine	Oskarsgata 7
Lot number	TM 3528	580 Siglufjordur
Revision number	Rev. 03	Iceland
Date of manufacture	9.12.2009	Ph: +354 460 6900
Retest date	9.12.2012	Fax: +354 460 6909

PARAMETER	TEST METHOD	RESULTS	Comment
Dry Matter Content	CP-001	94,3 %	
Ash	CP-002	0,1 %	
Turbidity	CP-003	28 NTU	
Viscosity	CP-004 (1% chitosan)	8 cP (mPa·s)	
Solubility	CP-006	99,8 %	
Degree of Deacetylation	CP-010 (Colloidal titration)	96 %	
Protein	As total amino acids*	< 0,02 g/100g	
Sieve Analysis	CP-008	100% through 100 mesh	
Tap Density	CP-009	0,59 g/cc	
Appearance	CP-007	White powder	
Taste and odor	CP-007	No taste or smell	
Microbial:			*detection limit:*
Aerobic plate count	NMKL 86	<300 cfu/g	10 cfu/g
Yeast and mold	NMKL 98	<50 cfu/g	10 cfu/g
Coliform bacteria	ISO 4831	absent	10 cfu/g
E. coli	ISO 7251	absent	0,3 cfu/g
Salmonella	NMKL 71	absent	Neg.
Listeria	NMKL 136	absent	Neg.
Pseudomonas	ISO 16266	absent	10 cfu/g
Toxic Heavy Metals:			
Arsenic	SW-846 6020 ICP/MS	none detected	0,10 ppm
Cadmium	SW-846 6020 ICP/MS	none detected	0,05 ppm
Lead	SW-846 6020 ICP/MS	none detected	0,10 ppm
Mercury	SW-846 7471 CVAA	none detected	0,005 ppm

* Analysis performed at Uppsala Biomedical Center, Sweden

MBR – Master Batch Record
CP – Primex Standard Test Methods
NMKL - Nordic Committee on Food Analysis
ISO – International Organization for Standardization
ICP/MS – Inductively Coupled Plasma Mass Spectrometry
CVAA – Cold Vapor Atomic Absorption

Reported By: (b) (6)
Name: Vala Arnadottir
Title: Laboratory Manager
Date: 27. 7. 2011.

Methods of analysis available upon request

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ISO 22000

CERTIFIED PRODUCT TÚN ICELAND

Primex ehf, Oskarsgata 7, 580 Siglufjordur, Iceland, Phone: +354 460 6900, Fax: +354 460 6909

primex

Certificate of analysis

Food Grade

Product name	ChitoClear® (Chitosan)	*Manufactured by:*
Source	*Pandalus borealis*	Primex ehf
Generic name	β(1→4) D-glucosamine / N-acetyl-D-glucosamine	Oskarsgata 7
Lot number	TM 3696	580 Siglufjordur
Revision number	Rev. 03	Iceland
Date of manufacture	11.11.2010	Ph: +354 460 6900
Retest date	11.11.2013	Fax: +354 460 6909

PARAMETER	TEST METHOD	RESULTS	Comment
Dry Matter Content	CP-001	97,6 %	
Ash	CP-002	0,2 %	
Turbidity	CP-003	23 NTU	
Viscosity	CP-004 (1% chitosan)	1227 cP (mPa·s)	
Solubility	CP-006	99,6 %	
Degree of Deacetylation	CP-010 (Colloidal titration)	91 %	
Protein	As total amino acids*	0,005 g/100g	
Sieve Analysis	CP-008	100% through 100 mesh	
Tap Density	CP-009	0,52 g/cc	
Appearance	CP-007	White powder	
Taste and odor	CP-007	No taste or smell	
Microbial:			*detection limit:*
Aerobic plate count	NMKL 86	<300 cfu/g	10 cfu/g
Yeast and mold	NMKL 98	<50 cfu/g	10 cfu/g
Coliform bacteria	ISO 4831	absent	10 cfu/g
E. coli	ISO 7251	absent	0.3 cfu/g
Salmonella	NMKL 71	absent	Neg.
Listeria	NMKL 136	absent	Neg.
Pseudomonas	ISO 16266	absent	10 cfu/g
Toxic Heavy Metals:			
Arsenic	SW-846 6020 ICP/MS	none detected	0.10 ppm
Cadmium	SW-846 6020 ICP/MS	none detected	0.05 ppm
Lead	SW-846 6020 ICP/MS	none detected	0.10 ppm
Mercury	SW-846 7471 CVAA	none detected	0.005 ppm

* Analysis performed at Uppsala Biomedical Center, Sweden

MBR – Master Batch Record
CP – Primex Standard Test Methods
NMKL - Nordic Committee on Food Analysis
ISO – International Organization for Standardization
ICP/MS – Inductively Coupled Plasma Mass Spectrometry
CVAA – Cold Vapor Atomic Absorption

(b) (6)

Reported By: _____
Name: Vala Arnadottir
Title: Laboratory Manager
Date: 27. 7. 2011.

Methods of analysis available upon request

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Primex ehf, Oskarsgata 7, 580 Siglufjordur, Iceland, Phone: +354 460 6900, Fax: +354 460 6909

primex

Certificate of analysis

Food Grade

Product name	ChitoClear® (Chitosan)	*Manufactured by:*
Source	*Pandalus borealis*	Primex ehf
Generic name	β(1→4) D-glucosamine / N-acetyl-D-glucosamine	Oskarsgata 7
Lot number	TM 3723	580 Siglufjordur
Revision number	Rev. 03	Iceland
Date of manufacture	16.5.2011	Ph: +354 460 6900
Retest date	16.5.2014	Fax: +354 460 6909

PARAMETER	TEST METHOD	RESULTS	Comment
Dry Matter Content	CP-001	95,8 %	
Ash	CP-002	0,4 %	
Turbidity	CP-003	27 NTU	
Viscosity	CP-004 (1% chitosan)	1228 cP (mPa·s)	
Solubility	CP-006	99,1 %	
Degree of Deacetylation	CP-010 (Colloidal titration)	91 %	
Protein	As total amino acids*	< 0,005 g/100g	
Sieve Analysis	CP-008	100% through 100 mesh	
Tap Density	CP-009	0,41 g/cc	
Appearance	CP-007	White powder	
Taste and odor	CP-007	No taste or smell	
Microbial:			*detection limit:*
Aerobic plate count	NMKL 86	<300 cfu/g	10 cfu/g
Yeast and mold	NMKL 98	<50 cfu/g	10 cfu/g
Coliform bacteria	ISO 4831	absent	10 cfu/g
E. coli	ISO 7251	absent	0,3 cfu/g
Salmonella	NMKL 71	absent	Neg.
Listeria	NMKL 136	absent	Neg.
Pseudomonas	ISO 16266	absent	10 cfu/g
Toxic Heavy Metals:			
Arsenic	SW-846 6020 ICP/MS	none detected	0,10 ppm
Cadmium	SW-846 6020 ICP/MS	none detected	0,05 ppm
Lead	SW-846 6020 ICP/MS	none detected	0,10 ppm
Mercury	SW-846 7471 CVAA	none detected	0,005 ppm

* Analysis performed at Uppsala Biomedical Center, Sweden

MBR – Master Batch Record
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NMKL - Nordic Committee on Food Analysis
ISO – International Organization for Standardization
ICP/MS – Inductively Coupled Plasma Mass Spectrometry
CVAA – Cold Vapor Atomic Absorption

(b) (6)

Reported By: _____
Name: Vala Arnadottir
Title: Laboratory Manager
Date: 25. 11. 2011.

Methods of analysis available upon request

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APPENDIX B □ PART 2

Certificate of analysis

Food Grade

Product name	ChitoClear® (Chitosan)	*Manufactured by:*
Source	*Pandalus borealis*	Primex ehf
Generic name	β(1→4) D-glucosamine / N-acetyl-D-glucosamine	Oskarsgata 7
Lot number	TM 3636	580 Siglufjordur
Revision number	Rev. 03	Iceland
Date of manufacture	14.7.2010	Ph: +354 460 6900
Retest date	14.7.2013	Fax: +354 460 6909

PARAMETER	TEST METHOD	RESULTS	Comment
Dry Matter Content	CP-001	96 %	
Ash	CP-002	0,1 %	
Turbidity	CP-003	17 NTU	
Viscosity	CP-004 (1% chitosan)	1912 cP (mPa·s)	
Solubility	CP-006	99,9 %	
Degree of Deacetylation	CP-010 (Colloidal titration)	91 %	
Protein	As total amino acids*	< 0,02 g/100g	
Sieve Analysis	CP-008	100% through 20 mesh	
Tap Density	CP-009	0,36 g/cc	
Appearance	CP-007	White powder	
Taste and odor	CP-007	No taste or smell	
Microbial:			*detection limit:*
Aerobic plate count	NMKL 86	<300 cfu/g	10 cfu/g
Yeast and mold	NMKL 98	<50 cfu/g	10 cfu/g
Coliform bacteria	ISO 4831	absent	10 cfu/g
E. coli	ISO 7251	absent	0,3 cfu/g
Salmonella	NMKL 71	absent	Neg.
Listeria	NMKL 136	absent	Neg.
Pseudomonas	ISO 16266	absent	10 cfu/g
Toxic Heavy Metals:			
Arsenic	SW-846 6020 ICP/MS	none detected	0,10 ppm
Cadmium	SW-846 6020 ICP/MS	none detected	0,05 ppm
Lead	SW-846 6020 ICP/MS	none detected	0,10 ppm
Mercury	SW-846 7471 CVAA	none detected	0,005 ppm

* Analysis performed at Uppsala Biomedical Center, Sweden

MBR – Master Batch Record
CP – Primex Standard Test Methods
NMKL - Nordic Committee on Food Analysis
ISO – International Organization for Standardization
ICP/MS – Inductively Coupled Plasma Mass Spectrometry
CVAA – Cold Vapor Atomic Absorption

(b) (6)

Reported By: _____
Name: Vala Arnadottir
Title: Laboratory Manager
Date: 19.12.2011

Methods of analysis available upon request

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APPENDIX B □ PART 3

Certificate of analysis

Food Grade

Product name	ChitoClear® (Chitosan)	*Manufactured by:*
Source	*Pandalus borealis*	Primex ehf
Generic name	β(1→4) D-glucosamine / N-acetyl-D-glucosamine	Oskarsgata 7
Lot number	TM 3695	580 Siglufjordur
Revision number	Rev. 03	Iceland
Date of manufacture	10.11.2010	Ph: +354 460 6900
Retest date	10.11.2013	Fax: +354 460 6909

PARAMETER	TEST METHOD	RESULTS	Comment
Dry Matter Content	CP-001	96,9 %	
Ash	CP-002	0,2 %	
Turbidity	CP-003	27 NTU	
Viscosity	CP-004 (1% chitosan)	1261 cP (mPa·s)	
Solubility	CP-006	99,2 %	
Degree of Deacetylation	CP-010 (Colloidal titration)	90 %	
Protein	As total amino acids*	0,005 g/100g	
Sieve Analysis	CP-008	100% through 20 mesh	
Tap Density	CP-009	0,38 g/cc	
Appearance	CP-007	White powder	
Taste and odor	CP-007	No taste or smell	
Microbial:			*detection limit:*
Aerobic plate count	NMKL 86	<300 cfu/g	10 cfu/g
Yeast and mold	NMKL 98	<50 cfu/g	10 cfu/g
Coliform bacteria	ISO 4831	absent	10 cfu/g
E. coli	ISO 7251	absent	0,3 cfu/g
Salmonella	NMKL 71	absent	Neg.
Listeria	NMKL 136	absent	Neg.
Pseudomonas	ISO 16266	absent	10 cfu/g
Toxic Heavy Metals:			
Arsenic	SW-846 6020 ICP/MS	none detected	0,10 ppm
Cadmium	SW-846 6020 ICP/MS	none detected	0,05 ppm
Lead	SW-846 6020 ICP/MS	none detected	0,10 ppm
Mercury	SW-846 7471 CVAA	none detected	0,005 ppm

* Analysis performed at Uppsala Biomedical Center, Sweden

MBR – Master Batch Record
CP – Primex Standard Test Methods
NMKL - Nordic Committee on Food Analysis
ISO – International Organization for Standardization
ICP/MS – Inductively Coupled Plasma Mass Spectrometry
CVAA – Cold Vapor Atomic Absorption

(b) (6)

Reported By:
Name: Vala Arnadottir
Title: Laboratory Manager
Date: 19.12.2011

Methods of analysis available upon request

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APPENDIX B □ PART 4

primex

Certificate of analysis

Food Grade

Product name	ChitoClear® (Chitosan)	*Manufactured by:*
Source	*Pandalus borealis*	Primex ehf
Generic name	β(1→4) D-glucosamine / N-acetyl-D-glucosamine	Oskarsgata 7
Lot number	TM 3743	580 Siglufjordur
Revision number	Rev. 03	Iceland
Date of manufacture	21.7.2011	Ph: +354 460 6900
Retest date	21.7.2014	Fax: +354 460 6909

PARAMETER	TEST METHOD	RESULTS	Comment
Dry Matter Content	CP-001	97 %	
Ash	CP-002	0,5 %	
Turbidity	CP-003	28 NTU	
Viscosity	CP-004 (1% chitosan)	1211 cP (mPa·s)	
Solubility	CP-006	99,1 %	
Degree of Deacetylation	CP-010 (Colloidal titration)	89 %	
Protein	As total amino acids*	< 0,02 g/100g	
Sieve Analysis	CP-008	100% through 100 mesh	
Tap Density	CP-009	0,52 g/cc	
Appearance	CP-007	White powder	
Taste and odor	CP-007	No taste or smell	
Microbial:			*detection limit:*
Aerobic plate count	NMKL 86	<300 cfu/g	10 cfu/g
Yeast and mold	NMKL 98	<50 cfu/g	10 cfu/g
Coliform bacteria	ISO 4831	absent	10 cfu/g
E. coli	ISO 7251	absent	0,3 cfu/g
Salmonella	NMKL 71	absent	Neg.
Listeria	NMKL 136	absent	Neg.
Pseudomonas	ISO 16266	absent	10 cfu/g
Toxic Heavy Metals:			
Arsenic	SW-846 6020 ICP/MS	none detected	0,10 ppm
Cadmium	SW-846 6020 ICP/MS	none detected	0,05 ppm
Lead	SW-846 6020 ICP/MS	none detected	0,10 ppm
Mercury	SW-846 7471 CVAA	none detected	0,005 ppm

* Analysis performed at Uppsala Biomedical Center, Sweden

MBR – Master Batch Record
CP – Primex Standard Test Methods
NMKL - Nordic Committee on Food Analysis
ISO – International Organization for Standardization
ICP/MS – Inductively Coupled Plasma Mass Spectrometry
CVAA – Cold Vapor Atomic Absorption

(b) (6)

Reported By: _____
Name: Vala Arnadottir
Title: Laboratory Manager
Date: 19.12.2011

Methods of analysis available upon request

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Primex ehf, Oskarsgata 7, 580 Siglufjordur, Iceland, Phone: +354 460 6900, Fax: +354 460 6909

APPENDIX C

primex

Certificate of analysis

Food Grade 95

Product name	ChitoClear® (Chitosan)	*Manufactured by:*
Source	*Pandalus borealis*	Primex ehf
Generic name	β(1→4) D-glucosamine / N-acetyl-D-glucosamine	Oskarsgata 7
Lot number	TM3375	580 Siglufjordur
Revision number	Rev. 03	Iceland
Date of manufacture	7.11.2008	Ph: +354 460 6900
Retest date	7.11.2011	Fax: +354 460 6909

PARAMETER	TEST METHOD	RESULTS	Comment
Dry Matter Content	CP-001	94,9 %	
Ash	CP-002	0,4 %	
Turbidity	CP-003	22 NTU	
Viscosity	CP-004 (1% chitosan)	103 cP (mPa·s)	
Solubility	CP-006	99,9 %	
Degree of Deacetylation	CP-010 (Colloidal titration)	95 %	
Protein	As total amino acids*	< 0,02 g/100g	
Sieve Analysis	CP-008	100% through 100 mesh	
Tap Density	CP-009	0,52 g/cc	
Appearance	CP-007	White powder	
Taste and odor	CP-007	No taste or smell	
Microbial:			*detection limit:*
Aerobic plate count	NMKL 86	<300 cfu/g	10 cfu/g
Yeast and mold	NMKL 98	<50 cfu/g	10 cfu/g
Coliform bacteria	ISO 4831	absent	10 cfu/g
E. coli	ISO 7251	absent	0,3 cfu/g
Salmonella	NMKL 71	absent	Neg.
Listeria	NMKL 136	absent	Neg.
Pseudomonas	ISO 16266	absent	10 cfu/g
Toxic Heavy Metals:			
Arsenic	SW-846 6020 ICP/MS	none detected	0,10 ppm
Cadmium	SW-846 6020 ICP/MS	none detected	0,05 ppm
Lead	SW-846 6020 ICP/MS	none detected	0,10 ppm
Mercury	SW-846 7471 CVAA	none detected	0,005 ppm

* Analysis performed at Uppsala Biomedical Center, Sweden

MBR – Master Batch Record
CP – Primex Standard Test Methods
NMKL - Nordic Committee on Food Analysis
ISO – International Organization for Standardization
ICP/MS – Inductively Coupled Plasma Mass Spectrometry
CVAA – Cold Vapor Atomic Absorption

(b) (6)

Reported By: _____
Name: Vala Arnadottir
Title: Laboratory Manager
Date: 27. 7. 2011.

Methods of analysis available upon request

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primex

Certificate of analysis

Food Grade 95

Product name	ChitoClear® (Chitosan)	*Manufactured by:*
Source	*Pandalus borealis*	Primex ehf
Generic name	β(1→4) D-glucosamine / N-acetyl-D-glucosamine	Oskarsgata 7
Lot number	TM3415	580 Siglufjordur
Revision number	Rev. 03	Iceland
Date of manufacture	21.2.2009	Ph: +354 460 6900
Retest date	21.2.2012	Fax: +354 460 6909

PARAMETER	TEST METHOD	RESULTS	Comment
Dry Matter Content	CP-001	95,8 %	
Ash	CP-002	0,1 %	
Turbidity	CP-003	32 NTU	
Viscosity	CP-004 (1% chitosan)	117 cP (mPa·s)	
Solubility	CP-006	99,9 %	
Degree of Deacetylation	CP-010 (Colloidal titration)	95 %	
Protein	As total amino acids*	< 0,02 g/100g	
Sieve Analysis	CP-008	100% through 100 mesh	
Tap Density	CP-009	0,52 g/cc	
Appearance	CP-007	White powder	
Taste and odor	CP-007	No taste or smell	
Microbial:			*detection limit:*
Aerobic plate count	NMKL 86	<300 cfu/g	10 cfu/g
Yeast and mold	NMKL 98	<50 cfu/g	10 cfu/g
Coliform bacteria	ISO 4831	absent	10 cfu/g
E. coli	ISO 7251	absent	0,3 cfu/g
Salmonella	NMKL 71	absent	Neg.
Listeria	NMKL 136	absent	Neg.
Pseudomonas	ISO 16266	absent	10 cfu/g
Toxic Heavy Metals:			
Arsenic	SW-846 6020 ICP/MS	none detected	0,10 ppm
Cadmium	SW-846 6020 ICP/MS	none detected	0,05 ppm
Lead	SW-846 6020 ICP/MS	0,10 ppm	0,10 ppm
Mercury	SW-846 7471 CVAA	none detected	0,005ppm

* Analysis performed at Uppsala Biomedical Center, Sweden

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ISO – International Organization for Standardization
ICP/MS – Inductively Coupled Plasma Mass Spectrometry
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(b) (6)

Reported By:
Name: Vala Arnadottir
Title: Laboratory Manager
Date: 27. 7. 2011.

Methods of analysis available upon request

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primex

Certificate of analysis

Food Grade 95

Product name	ChitoClear® (Chitosan)	*Manufactured by:*
Source	*Pandalus borealis*	Primex ehf
Generic name	β(1→4) D-glucosamine / N-acetyl-D-glucosamine	Oskarsgata 7
Lot number	TM 3697	580 Siglufjordur
Revision number	Rev. 03	Iceland
Date of manufacture	11.11.2010	Ph: +354 460 6900
Retest date	11.11.2013	Fax: +354 460 6909

PARAMETER	TEST METHOD	RESULTS	Comment
Dry Matter Content	CP-001	95 %	
Ash	CP-002	0,2 %	
Turbidity	CP-003	29 NTU	
Viscosity	CP-004 (1% chitosan)	97 cP (mPa·s)	
Solubility	CP-006	99,8 %	
Degree of Deacetylation	CP-010 (Colloidal titration)	95 %	
Protein	As total amino acids*	<0,02 g/100g	
Sieve Analysis	CP-008	100% through 100 mesh	
Tap Density	CP-009	0,52 g/cc	
Appearance	CP-007	White powder	
Taste and odor	CP-007	No taste or smell	
Microbial:			*detection limit:*
Aerobic plate count	NMKL 86	<300 cfu/g	10 cfu/g
Yeast and mold	NMKL 98	<50 cfu/g	10 cfu/g
Coliform bacteria	ISO 4831	absent	10 cfu/g
E. coli	ISO 7251	absent	0,3 cfu/g
Salmonella	NMKL 71	absent	Neg.
Listeria	NMKL 136	absent	Neg.
Pseudomonas	ISO 16266	absent	10 cfu/g
Toxic Heavy Metals:			
Arsenic	SW-846 6020 ICP/MS	none detected	0,10 ppm
Cadmium	SW-846 6020 ICP/MS	none detected	0,05 ppm
Lead	SW-846 6020 ICP/MS	none detected	0,10 ppm
Mercury	SW-846 7471 CVAA	none detected	0,005 ppm

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MBR – Master Batch Record
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(b) (6)

Reported By: _
Name: Vala Arnadottir
Title: Laboratory Manager
Date: 25. 11. 2011.

Methods of analysis available upon request

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primex

Certificate of analysis

Food Grade 95

Product name	ChitoClear®	*Manufactured by:*
Source	*Pandalus borealis*	Primex ehf
Generic name	β(1→4) D-glucosamine / N-acetyl-D-glucosamine	Oskarsgata 7
Lot number	TM3701	580 Siglufjordur
Revision number	Rev. 03	Iceland
Date of manufacture	17.11.2010	Ph: +354 460 6900
Retest date	17.11.2013	Fax: +354 460 6909

PARAMETER	TEST METHOD	RESULTS	Comment
Dry Matter Content	CP-001	95,5 %	
Ash	CP-002	0,3 %	
Turbidity	CP-003	29 NTU	
Viscosity	CP-004 (1% chitosan)	189 cP (mPa·s)	
Solubility	CP-006	99,8 %	
Degree of Deacetylation	CP-010 (Colloidal titration)	95 %	
Protein	As total amino acids*	< 0,02 g/100g	
Sieve Analysis	CP-008	100% through 100mesh	
Tap Density	CP-009	0,53 g/cc	
Appearance	CP-007	White powder	
Taste and odor	CP-007	No taste or smell	
Microbial:			*detection limit:*
Aerobic plate count	NMKL 86	<1000 cfu/g	10 cfu/g
Yeast and mold	NMKL 98	<100 cfu/g	10 cfu/g
Coliform bacteria	ISO 4831	absent	10 cfu/g
E. coli	ISO 7251	absent	0,3 cfu/g
Salmonella	NMKL 71	absent	Neg.
Listeria	NMKL 136	absent	Neg.
Pseudomonas	ISO 16266	absent	10 cfu/g
Toxic Heavy Metals:			
Arsenic	SW-846 6020 ICP/MS	none detected	0,10 ppm
Cadmium	SW-846 6020 ICP/MS	none detected	0,05 ppm
Lead	SW-846 6020 ICP/MS	none detected	0,10 ppm
Mercury	SW-846 7471 CVAA	none detected	0,005 ppm

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(b) (6)

Reported By: _____
Name: Vala Arnadottir
Title: Laboratory Manager
Date: 27. 7. 2011.

Methods of analysis available upon request

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primex

Certificate of analysis

Food Grade 95

Product name	ChitoClear® (Chitosan)	*Manufactured by:*
Source	*Pandalus borealis*	Primex ehf
Generic name	β(1→4) D-glucosamine / N-acetyl-D-glucosamine	Oskarsgata 7
Lot number	TM 3697	580 Siglufjordur
Revision number	Rev. 03	Iceland
Date of manufacture	11.11.2010	Ph: +354 460 6900
Retest date	11.11.2013	Fax: +354 460 6909

PARAMETER	TEST METHOD	RESULTS	Comment
Dry Matter Content	CP-001	95 %	
Ash	CP-002	0,2 %	
Turbidity	CP-003	29 NTU	
Viscosity	CP-004 (1% chitosan)	97 cP (mPa·s)	
Solubility	CP-006	99,8 %	
Degree of Deacetylation	CP-010 (Colloidal titration)	95 %	
Protein	As total amino acids*	<0,02 g/100g	
Sieve Analysis	CP-008	100% through 100 mesh	
Tap Density	CP-009	0,52 g/cc	
Appearance	CP-007	White powder	
Taste and odor	CP-007	No taste or smell	
Microbial:			*detection limit:*
Aerobic plate count	NMKL 86	<300 cfu/g	10 cfu/g
Yeast and mold	NMKL 98	<50 cfu/g	10 cfu/g
Coliform bacteria	ISO 4831	absent	10 cfu/g
E. coli	ISO 7251	absent	0,3 cfu/g
Salmonella	NMKL 71	absent	Neg.
Listeria	NMKL 136	absent	Neg.
Pseudomonas	ISO 16266	absent	10 cfu/g
Toxic Heavy Metals:			
Arsenic	SW-846 6020 ICP/MS	none detected	0,10 ppm
Cadmium	SW-846 6020 ICP/MS	none detected	0,05 ppm
Lead	SW-846 6020 ICP/MS	none detected	0,10 ppm
Mercury	SW-846 7471 CVAA	none detected	0,005 ppm

* Analysis performed at Uppsala Biomedical Center, Sweden

MBR – Master Batch Record
CP – Primex Standard Test Methods
NMKL - Nordic Committee on Food Analysis
ISO – International Organization for Standardization
ICP/MS – Inductively Coupled Plasma Mass Spectrometry
CVAA – Cold Vapor Atomic Absorption

(b) (6)

Reported By: _
Name: Vala Arnadottir
Title: Laboratory Manager
Date: 25. 11. 2011.

Methods of analysis available upon request

NOTE: *Lack of colored logo indicates this document is a copy and is not controlled*

Primex ehf, Oskarsgata 7, 580 Siglufjordur, Iceland, Phone: +354 460 6900, Fax: +354 460 6909

APPENDIX D-1

Rap-RD-TP-01-003
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Rap-RD-TP-01-003: Stability Testing on reference samples of ChitoClear.

Introduction

A stability study has been described in Test protocol RD-TP-01-003. The results in the following report are from a study conducted on samples stored for proximately 34 months. For comparison are results, from earlier stability testing on three batches, conducted after 2 months of storing.

Summary

Four quality parameters of ChitoClear have been tested for changes upon storage for proximately three years. The parameters are Dry Matter Content, Viscosity, Degree of Deacetylation, Colour, Total Plate Count and Yeast and Mould. Physical parameters showed less change, after the long storage time.

Materials

Record of quality analysis of the chitosan samples.
Chitosan samples stored for 2, and 34 months:

Batch	Data after 2 months	Data after 34 months
TM888	X	X
TM890		X
TM894		X
TM896	X	X
TM897	X	X

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Storage conditions

The samples have been stored in ziplock plastic bags. The bags have been stored in a Merck plastic container with a loose lid (not airtight). Room temperature and humidity have not been determined.

Equipment

Equipment for the following test procedures:
CP-001: Dry Matter Content
CP-010: Degree of Deacetylation, Colloidal titration
CP-004: Viscosity

Procedure

The test procedures listed above have been performed according to description, and samples measured in duplicates. Samples for 'Total Plate Count' and 'Yeast and Mould' were sent to the Icelandic Fisheries Laboratories (IFL), measured according to their accredited method AB-00301.

Results

1. Dry Matter Content (PRE-10-CP-001).

Batch	Months of storage	QC-results %	Results after storage %	Difference *
TM888	2	91,8	92,0	**0,2**
-	34	91,8	90,3	-1,5
TM890	34	91,3	89,8	-1,5
TM894	34	93,6	90,4	-3,2
TM896	2	92,3	91,8	**-0,5**
-	34	92,3	89,9	-2,4
TM897	2	92,8	92,3	**-0,5**
-	34	92,8	91,0	-1,8

* Bolded differences are for samples after 2 months of storage.

Discussion:
All samples have higher moisture content than when first measured. The difference is more pronounced as the samples get older. Two batches are just out of the specification for ChitoClear (Dry Matter Content > 90 %), TM890, TM896.

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2. Degree of Deacetylation, Colloidal titration (PRE-10-CP-010).

Batch	Months of storage	QC-results %	Results after storage %	Difference *
TM888	2	99,2	97,2	**-2,0**
-	34	99,2	103,0	3,8
TM890	34	95,8	99,1	3,3
TM894	34	98,7	102,8	4,1
TM896	2	94,9	96,1	**1,2**
-	34	94,9	99,6	4,7
TM897	2	99,7	97,8	**-1,9**
-	34	99,7	101,2	1,5

* Bolded differences are for samples after 2 months of storage.

Discussion:
All samples are within specification. The results show that the deacetylation is increasing with study time. It is clear from the results that the method is not giving exactly real degree of deacetylation. This is supporting the common difference that is found between individuals and laboratories using this method.

3. Viscosity (PRE-10-CP-004).

Batch	Months of storage	QC-results cps	Results after storage cps	Difference *
TM888	2	73,0	76,0	**3,0**
-	34	73,0	73,3	0,3
TM890	34	25,0	24,7	-0,3
TM894	34	26,0	28,4	2,4
TM896	2	8,0	8,0	**0**
-	34	8,0	9,8	1,8
TM897	2	7,0	7,0	**0**
-	34	7,0	9,1	2,1

* Bolded differences are for samples after 2 months of storage.

Discussion:
Generally there is no obvious change in viscosity. But looking closer to the data. At viscosities this low (< 80 cps) we are using spindle LV-1 and speed of 60 rpm, which gives full scale viscosity range of 100 cps (according to the manual). The uncertainty of the viscometer is 1 % of that or +/- 1 cps. The torque in three of the same batches are around 10 % the values are at the edge of being measurable of the viscometer. All batches are within the general specification of ChitoClear.

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4. Total Plate Count (IFL method).

Batch	Months of storage	QC-results cfu/g	Results after storage cfu/g
TM888	2	700	<100 *
-	34	700	20
TM890	34	600	<10
TM894	34	1600	<10
TM896	2	<100	<100 *
-	34	<100	<10
TM897	2	2300	700 *
-	34	2300	<10

* Results are for samples after 2 months of storage.

Discussion:
Most batches show no growth of colonies. Compared to the QC-results of the same batch almost three years ago this is a good indication of how hostile environment chitosan is to bacteria.

5. Yeast and Mould (IFL method).

Batch	Months of storage	QC-results cfu/g	Results after storage cfu/g
TM888	2	<100	<100 *
-	34	<100	<10
TM890	34	<100	<10
TM894	34	<100	<10
TM896	2	<100	<100 *
-	34	<100	<10
TM897	2	<100	<100 *
-	34	<100	<10

* Results are for samples after 2 months of storage.

Discussion:
Unchanged results after storage.

Conclusion

The weekest links in stored chitosan is the Dry Matter Content, here the batches had relatively low numbers to begin with (most < 93 %) and with storage the Dry Matter Content decreases to values close to or just below the specification of ChitoClear.

APPENDIX D-2

Stability tests performed on chitosan solutions in various acids.

General description

- Test samples will be measured and compared to a control sample having similar degree of acetylation but medium to high viscosity (> 100 cps).
- Samples and control will be dissolved in 4 different acids:
  acetic acid,
  lactic acid,
  glycolic acid,
- Dilutions:
  4% chitosan in 2% acid
  4% chitosan in 1% acid
- Following measurements will be applied:

  Viscosity:---------- all samples will be measured at 1.00 % chitosan concentration

  Color: ---------- visual evaluation and absorption spectra

  Formation of precipitates:----- visual evaluation of the occurrence of precipitate formation and turbidity measurement.
- All samples will be measured before incubation, and after 1, 2, 3, and 4 weeks of incubation
- Incubation temperatures:
  50°C
  25°C
- Product stability will be assessed by changes in viscosity and absorption spectra as well as by visual evaluation of the solution color and formation of precipitates.

Procedures will be subject to revision depending on experience as the protocol is implemented.

Stability tests performed on chitosan solution in acetic acid.

Methods

Milled chitosan, 8 batches were used for this study. These batches had similar degree of deacetylation and medium viscosity (20-80 cps). Table 1 shows the initial parameters of different batches used. TM1565 was unclear in the beginning. All chitosan samples were dissolved in acetic acid. The following chitosan acetate solutions were made:

4% chitosan in 2% acid
4% chitosan in 1% acid

These two different solutions for all batches were kept at two different incubations temperature, at 25°C and 50°C. Samples from all solutions were collected weekly at 7 days interval or at 0, 7, 14, 21 and 28 days of incubation. Samples were diluted to 1% chitosan in 1% acetic acid in water (1% CA) before all analysis. The following analysis and evaluations for each 1% CA solution were made:

Viscosity at 25°C using Brookman viscometer. Spindle no. 1 at 60 rpm. (Primex CP-004)

Turbitity using calibrated Hach 2100AN turbitity meter (Primex CP-003).

Browning index at 400 nm using Perkin Elmer Lambda 20 UV/Vis Spectrometer.

Color of CA solutions using visual evalution.

Clearnes of CA solutions using visual evalution.

Formation of precipitates: visual evaluation of the occurrence of precipitate formation.

Results

Most changes of the parameters examined were in viscosity, browning index and color. Clearness of solutions, turbitiy and precipitate formation did not change during the incubation time at any acid concentration or temperature. Analysis of different batches showed the same pattern of changes in viscosity and browning index (Figure 1 and 2).

Therefore TM1558 and TM1559 will be shown as examples for all the batches (Fig.3 and 4). The reduction in viscosity was most at 50°C in 2% acetic acid, followed by 50°C in 1% acetic acid. The reduction was less at 25°C and the lowest reduction was in 1% acetic acid at 25°C (Fig. 3 and 4). Chitosan dissolved in 1% acetic acid showed less reduction at both temperatures. The browning index showed the same pattern (Fig. 3 and 4). More browning occured at 50°C than 25°C and in both regimes the 1% acetic acid solutions showed less browning rate than in 2% acetic acid.
The reason for the masking effect of 1% acetic acid concentration on chitosan is most likly because 4% chitosan in 1% acetic acid is not completely dissolved as in 2% acetic acid. Undissolved chitosan is much more stable and does not brake or brown so easily. Browning effect of different acids will be interesting to comapair, since acetic acid is known to cause browning and Maillard reaction faster than most other organic acids.

Table 1. Color, clearness, viscosity, browning index, turbitity and appearance of 1% chitosan acetate solution of different TM-batches.

Lots	Color	Clear-ness	Visc. (mPas)	B.I. 400	Turbit. (NTU)	Notes
TM1558	PY	UC	51,5	85	20	fine needles
TM1559	PY	UC	74,7	75	17	fine needles
TM1560	PY	UC	36,5	98	25	fine needles
TM1561	PY	UC	33,0	87	18	fine needles
TM1562	PY	C	28,3	95	19	a few fine needles
TM1563	PY	C	21,4	103	24	fine needles
TM1564	PY	UC	23,6	111	22	many fine needles
TM1565	PY	UC	26,9	173	1200	many fine needles and particles

Color: PY = pale yellow solution
Clearness: C = clear, UC = unclear solution
B.I. = browning index = absorbance at 400 nm in mOD.
Turbit. = turbitity

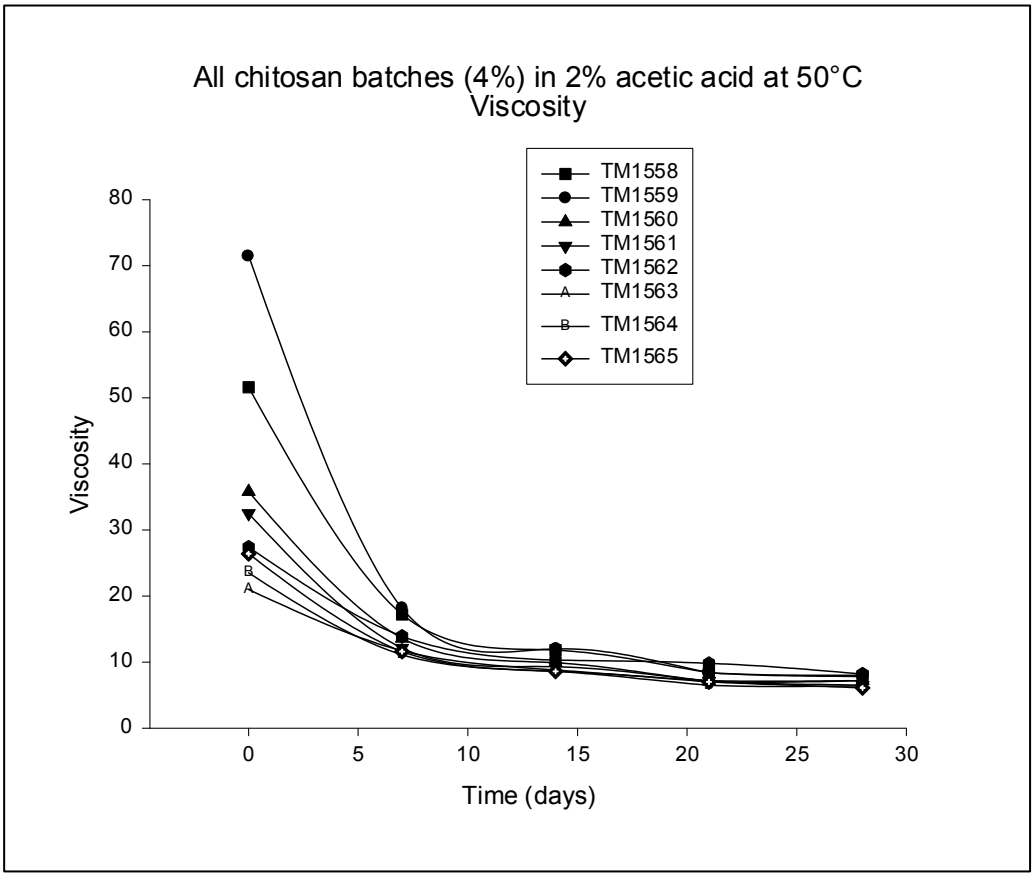


Figure 1. Changes in viscosity of 4% chitosan in 2% acetic acid at 50°C.

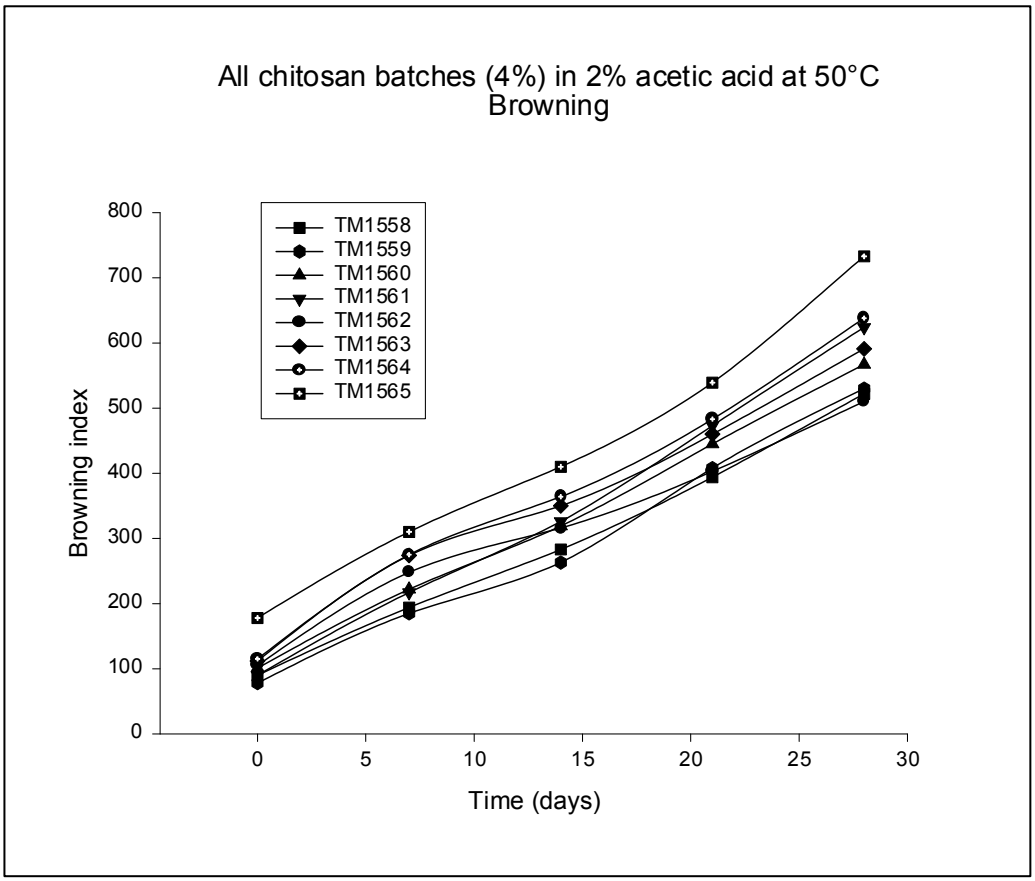
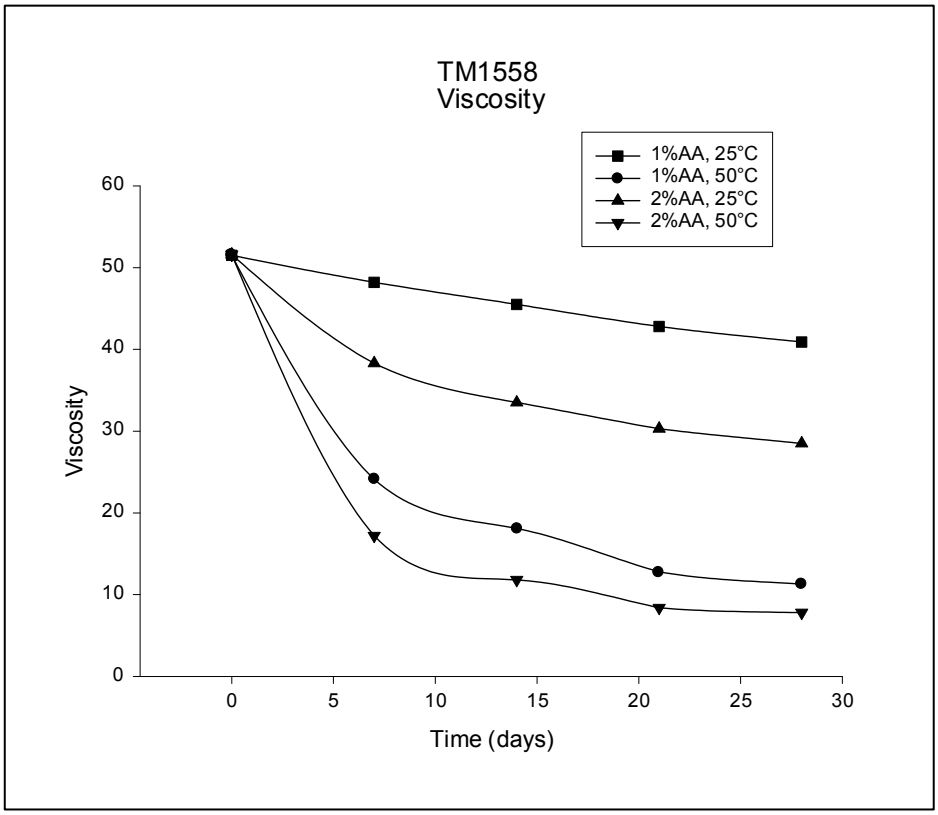


Figure 2. Browning of 4% chitosan in 2% acetic acid at 50°C.



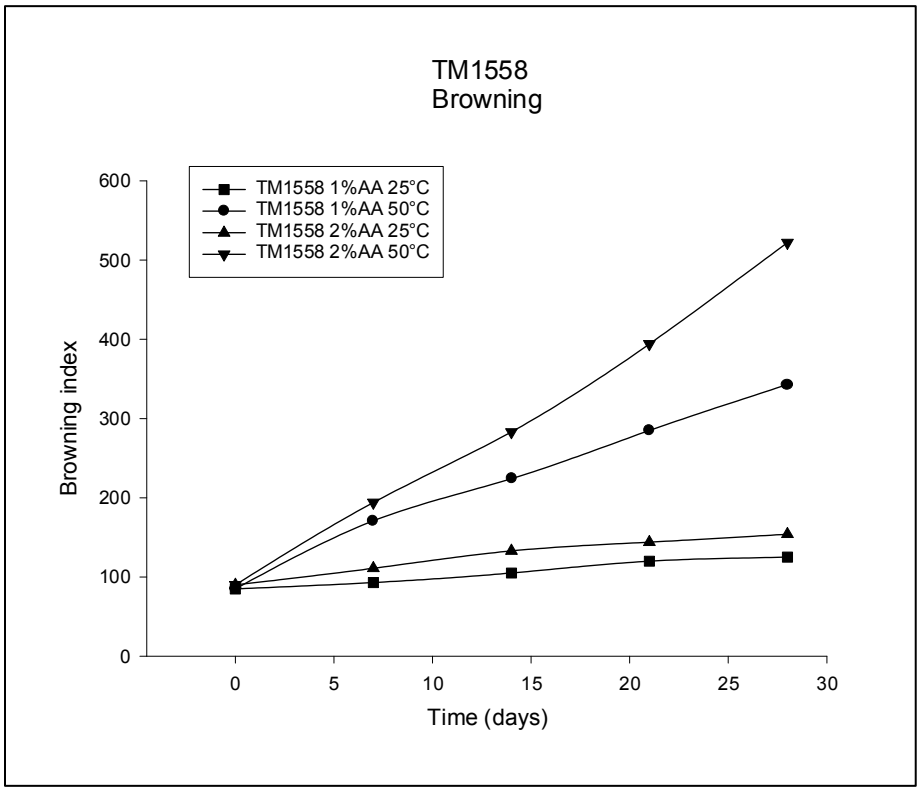
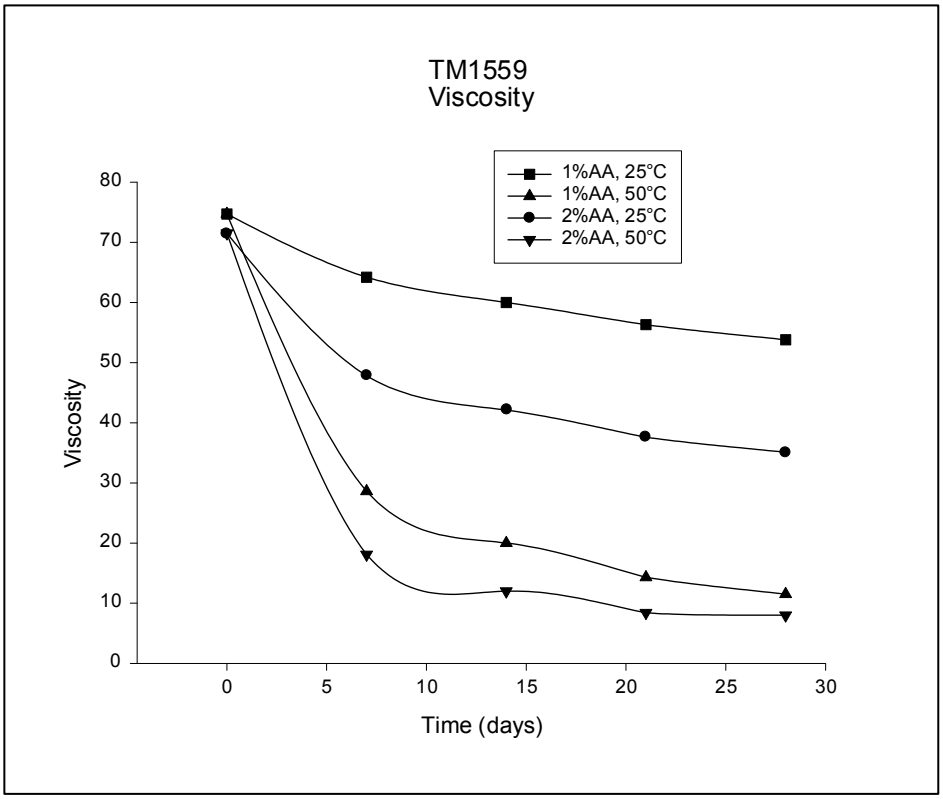


Figure 3. Changes in viscosity and browning of 4% chitosan (TM1558) in 1% and 2% acetic acid at 25 and 50°C.



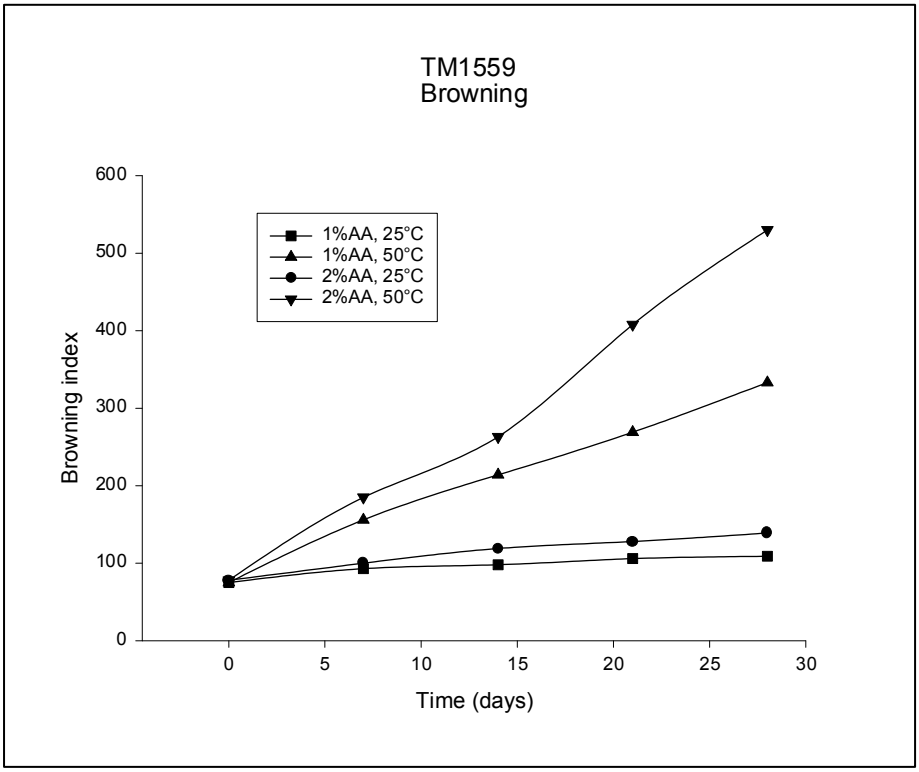


Figure 4. Changes in viscosity and browning of 4% chitosan (TM1559) in 1% and 2% acetic acid at 25 and 50°C.

Stability tests performed on chitosan solution in lactic acid.

Methods

Milled chitosan, 8 batches were used for this study. These batches had similar degree of deacetylation and medium viscosity (20-80 cps). Table 1 shows the initial parameters of different batches used. TM1565 was unclear in the beginning. All chitosan samples were dissolved in lactic acid. The following chitosan lactate solutions were made:
4% chitosan in 2% lactic acid
4% chitosan in 1% lactic acid

These two different solutions for all batches were kept at two different incubations temperature, at 25°C and 50°C. Samples from all solutions were collected weekly at 7 days interval or at 0, 7, 14, 21 and 28 days of incubation. Samples were diluted to 1% chitosan in 1% lactic acid in water (1% CL) before all analysis. The following analysis and evaluations for each 1% CL solution were made:

Viscosity at 25°C using Brookman viscometer. Spindle no. 1 at 60 rpm. (Primex CP-004)
Turbitity using calibrated Hach 2100AN turbitity meter (Primex CP-003).
Browning index at 400 nm using Perkin Elmer Lambda 20 UV/Vis Spectrometer.
Color of CL solutions using visual evalution.
Clearnes of CL solutions using visual evalution.
Formation of precipitates: visual evaluation of the occurrence of precipitate formation.

Results

Most changes of the parameters examined were in viscosity, browning index and color. After 7 days there was a striking difference in 4% chitosan in 1% lactic acid at 50°C only. Solution of 1% CL made from this regime (all 8 bathces) were very cloudy and unclear. This was neither observed in 4% chitosan in 2% lactic acid at 50°C nor 4% chitosan in 1% lactic acid at 25°C. This was not apparent in 4% chitosan in 1% acetic acid at 50°C. Therefore it looks like when chitosan is partly dissolved in lactic acid and heated to 50°C for a week, some irreversible changes in solubility occur only with lactic acid, not acetic acid.

Another deviation from acetic acid was the 4% chitosan in 1% lactic acid at 25°C. In this regime there was an increase in viscosity in many batches in the first 7 or 14 days, followed by a reduction. This was not obseved in acetic acid.Transparent beads were formed in 1% lactic acid at 25°C that were not observed in 2% lactic acid at 25°C or in 1% acetic acid at 25°C. This was not always followed by an increase in turbitity.

In summary the chitosan polymer behaved differently in lactic acid compared to that of acetic acid. This is likely to different structure of these two organic acids.

Table 1. Color, clearness, viscosity, browning index, turbitity and appearance of 1% chitosan lactate solution of different TM-batches.

Lots	Color	Clear-ness	Visc. (mPas)	B.I. 400	Turbit. (NTU)	Notes
TM1558	PY	UC	50,1	78	17	fine needles
TM1559	PY	UC	74,7	70	14	fine needles
TM1560	PY	UC	43,5	98	23	fine needles
TM1561	PY	UC	25,0	85	18	fine needles
TM1562	PY	C	29,2	97	17	a few fine needles
TM1563	PY	C	27,2	101	22	fine needles
TM1564	PY	UC	24,0	110	19	many fine needles
TM1565	PY	UC	28,9	165	549	many fine needles and particles

Color: PY = pale yellow solution; Clearness: C = clear, UC = unclear solution; B.I. = browning index = absorbance at 400 nm in mOD. Turbit. = turbitity

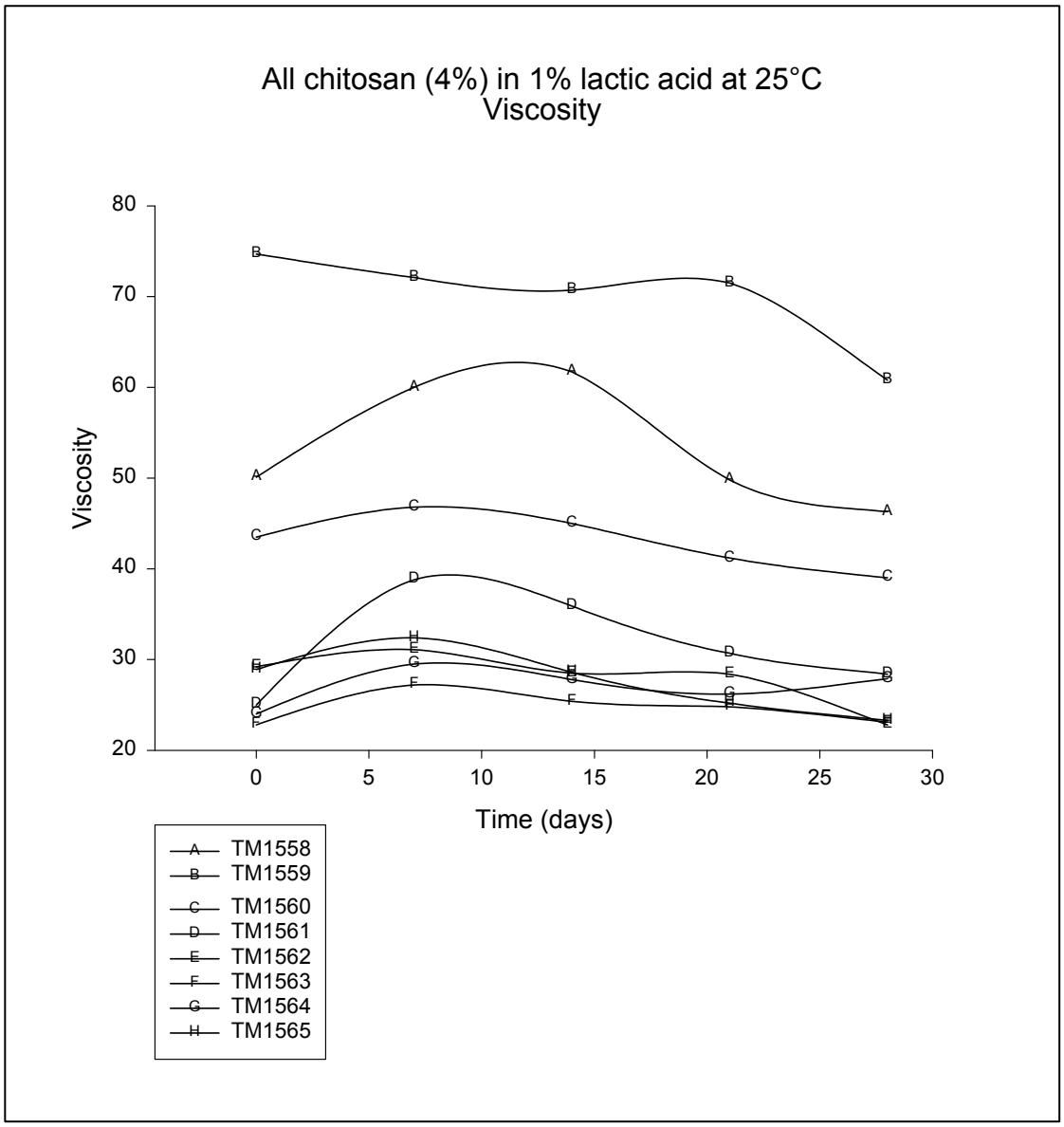
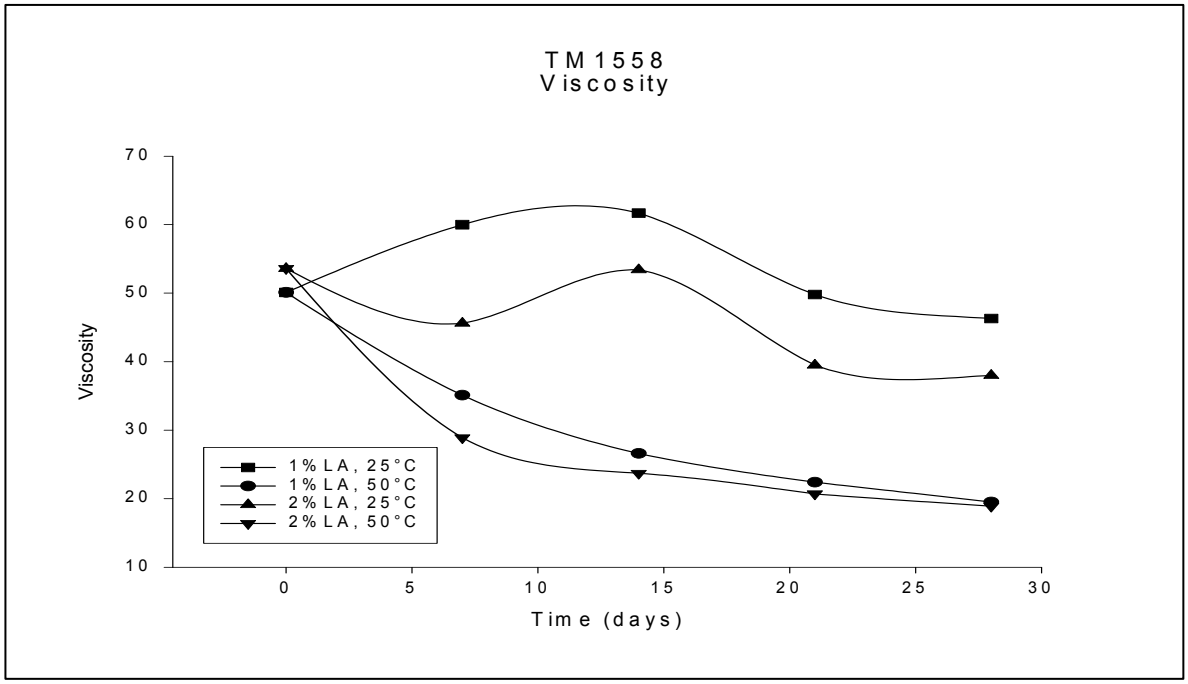


Figure 1. Changes in viscosity of 4% chitosan in 1% lactic acid at 25°C.



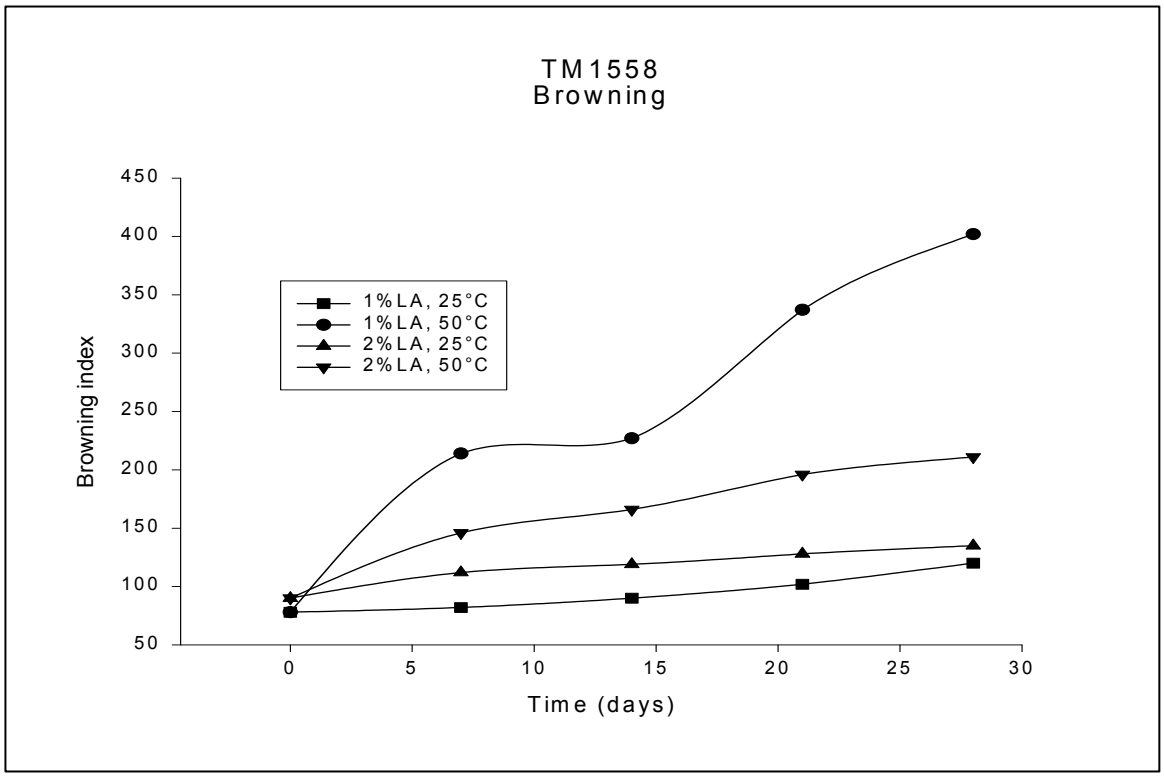
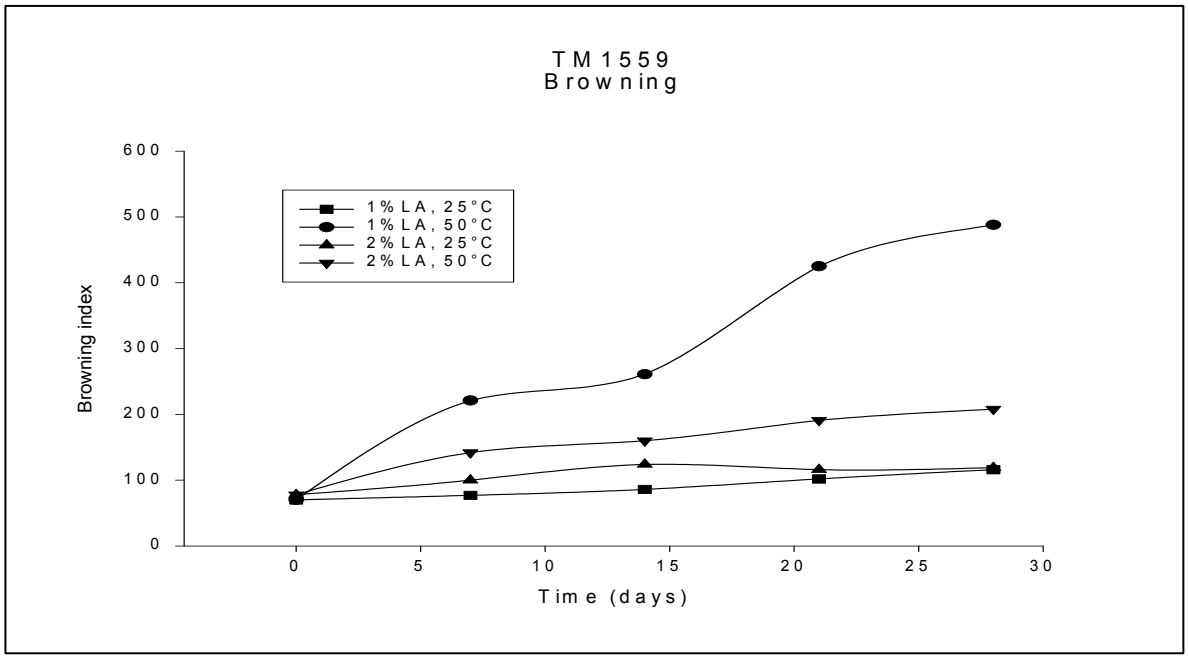


Figure 2. Changes in viscosity and browning of 4% chitosan (TM1558) in 1% and 2% lactic acid at 25 and 50°C.



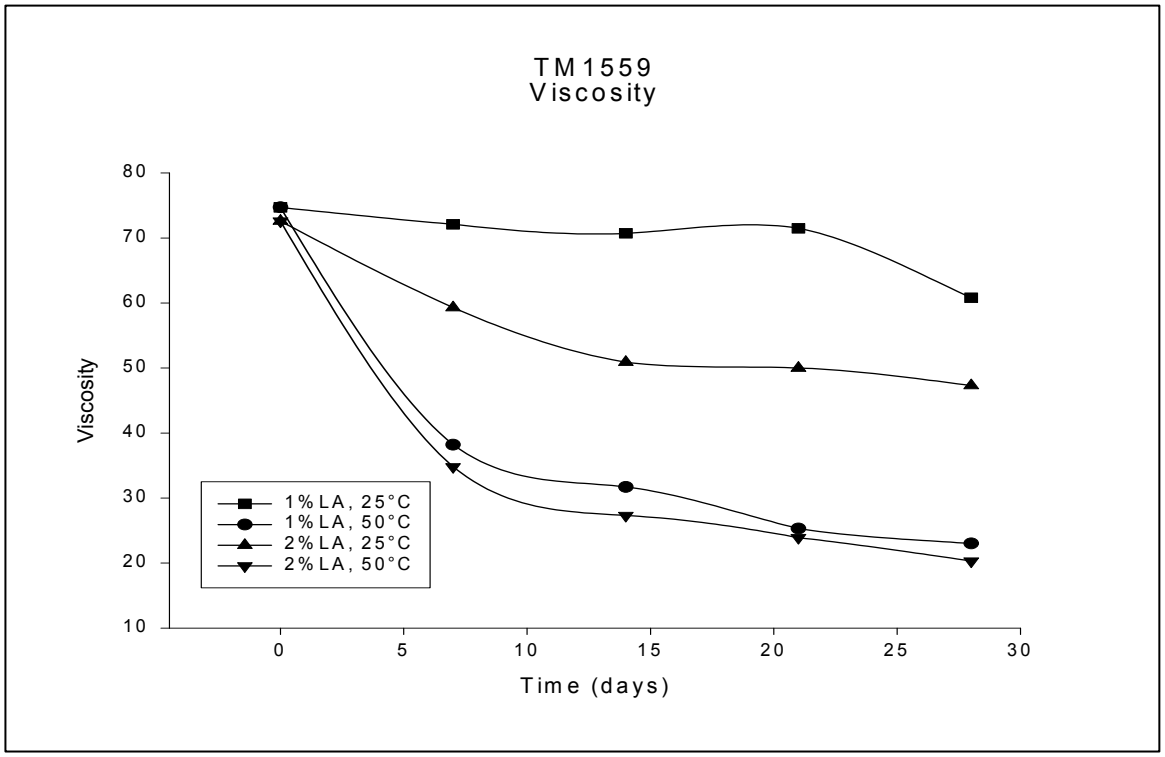


Figure 3. Changes in viscosity and browning of 4% chitosan (TM1559) in 1% and 2% lactic acid at 25 and 50°C

Stability tests performed on chitosan solution in glycolic acid.

Methods

Milled chitosan, 8 batches were used for this study. These batches had similar degree of deacetylation and medium viscosity (20-80 cps). Table 1 shows the initial parameters of different batches used. TM1565 was unclear in the beginning. All chitosan samples were dissolved in glycolic acid. The following chitosan glycolate solutions were made:
4% chitosan in 2% glycolic acid
4% chitosan in 1% glycolic acid

These two different solutions for all batches were kept at two different incubations temperature, at 25°C and 50°C. Samples from all solutions were collected weekly at 7 days interval or at 0, 7, 14, 21 and 29 days of incubation. Samples were diluted to 1% chitosan in 1% glycolic acid in water (1% CG) before all analysis. The following analysis and evaluations for each 1% CG solution were made:

Viscosity at 25°C using Brookman viscometer. Spindle no. 1 at 60 rpm. (Primex CP-004)
Turbitity using calibrated Hach 2100AN turbitity meter (Primex CP-003).
Browning index at 400 nm using Perkin Elmer Lambda 20 UV/Vis Spectrometer.
Color of CG solutions using visual evalution.
Clearnes of CG solutions using visual evalution.
Formation of precipitates: visual evaluation of the occurrence of precipitate formation.

Results

As in chitosan lactate solution most changes of the parameters examined were in viscosity, browning index and color. From 7 days onwards there was a striking difference in 4% chitosan in 1% glycolic acid at 25°C and 50°C. Solution of 1% CG made from these two regimes (all 8 bathces) were cloudy and unclear. This was not observed in 4% chitosan in 2% glycolic acid at 25°C and 50°C. Therefore it looks like when chitosan is partly dissolved in glycolic acid and stored at 25°C to 50°C for a week, some irreversible changes in solubility occur only with glycolic acid, not acetic acid.

Another deviation from acetic acid was the 4% chitosan in 1% glycolic acid at 25°C. In this regime there was an increase in viscosity in mainly two batches in the first 7 days (Fig. 1), followed by a reduction. This was not apparent in any other regime. Similar behaviour was observed in lactic acid but not in acetic acid.Cloudy beads were formed in 1% glycolic acid at 25°C that were not observed in 2% glycolic acid at 25°C or in 1% acetic acid at 25°C. This was not always followed by an increase in turbitity. Similar baviour was also observed in lactic acid but not in acetic acid.

In summary the chitosan polymer behaved differently in glycolic acid compared to that of acetic acid (AA), but similar to that of lactic acid (LA). Then comparing the three acids the structure of AA is different to that of LA and GA. LA and GA are more polar at the non-carbolic end than AA.

Table 1. Color, clearness, viscosity, browning index, turbitity and appearance of 1% chitosan glycolate solution of different TM-batches.

Lots	Color	Clear-ness	Visc. (mPas)	B.I. 400	Turbit. (NTU)	Notes
TM1558	PY	UC	47,1	78	16	fine needles
TM1559	PY	C	61,0	76	15	fine needles
TM1560	PY	UC	34,4	92	22	fine needles
TM1561	PY	UC	28,9	83	17	fine needles
TM1562	PY	C	24,5	96	19	a few fine needles
TM1563	PY	UC	19,2	97	19	fine needles

TM1564	PY	UC	20,1	102	19	many fine needles
TM1565	PY	UC	22,7	160	387	many fine needles and particles

Color: PY = pale yellow solution; Clearness: C = clear, UC = unclear solution; B.I. = browning index = absorbance at 400 nm in mOD. Turbit. = turbitity

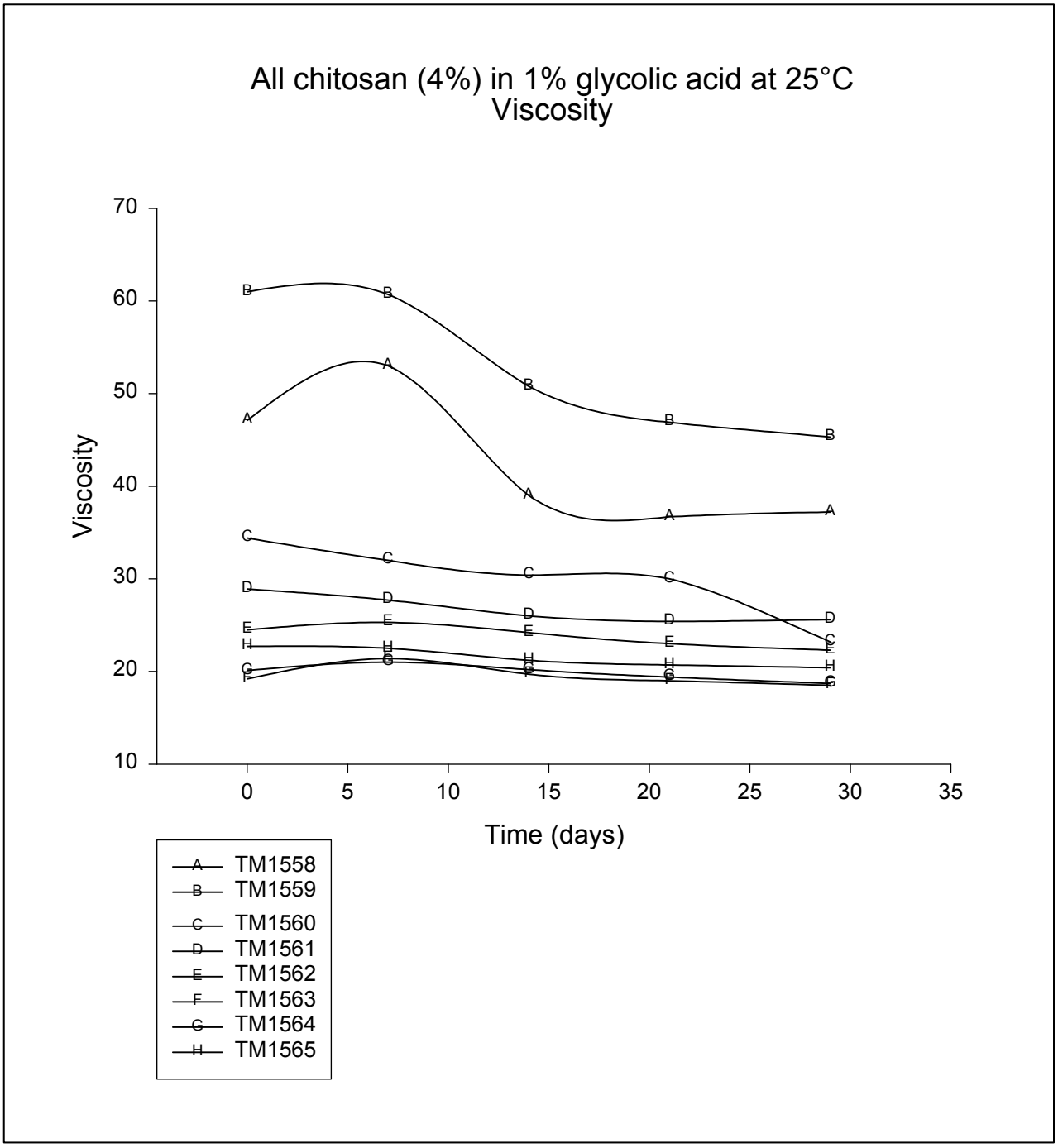


Figure 1. Changes in viscosity of 4% chitosan in 1% glycolic acid at 25°C. An increase in viscosity was observed at day 7 in A (TM1558), E (TM1562), F (TM1563) and G (TM1564).

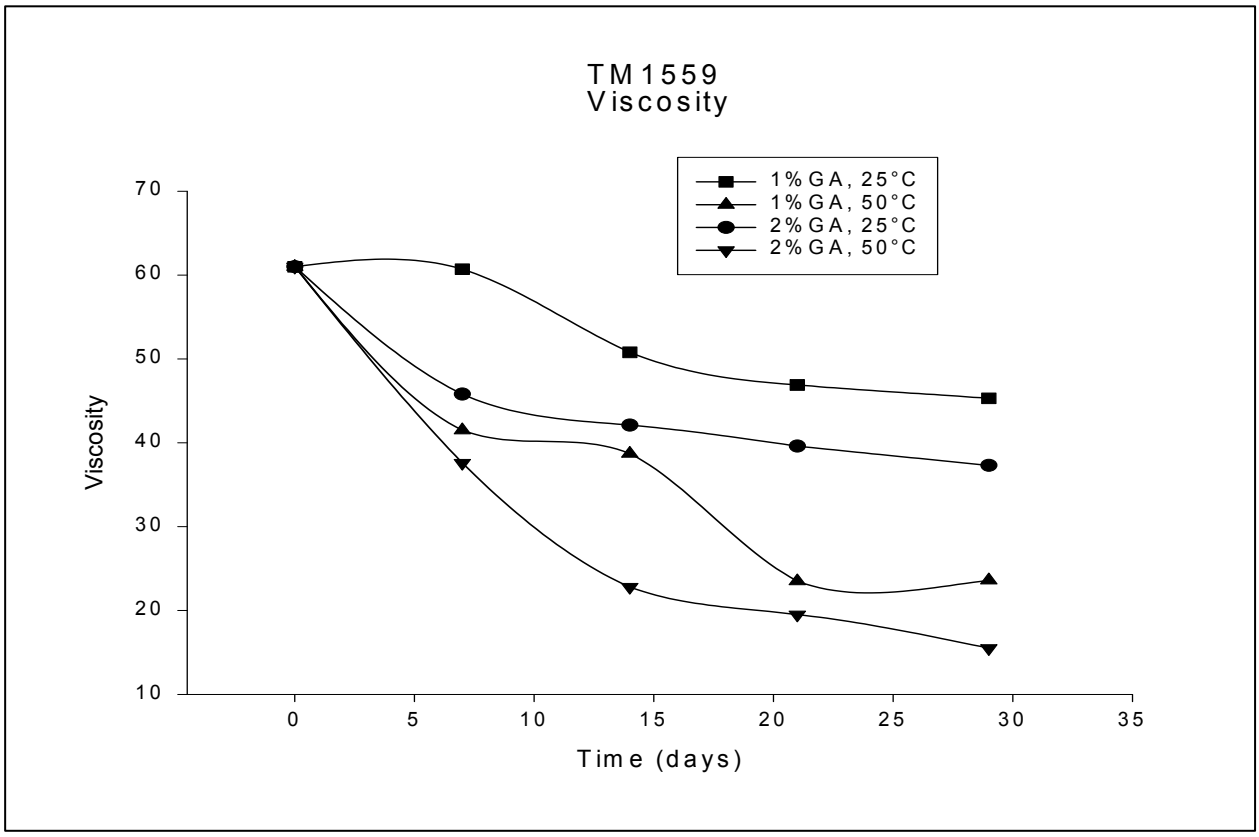
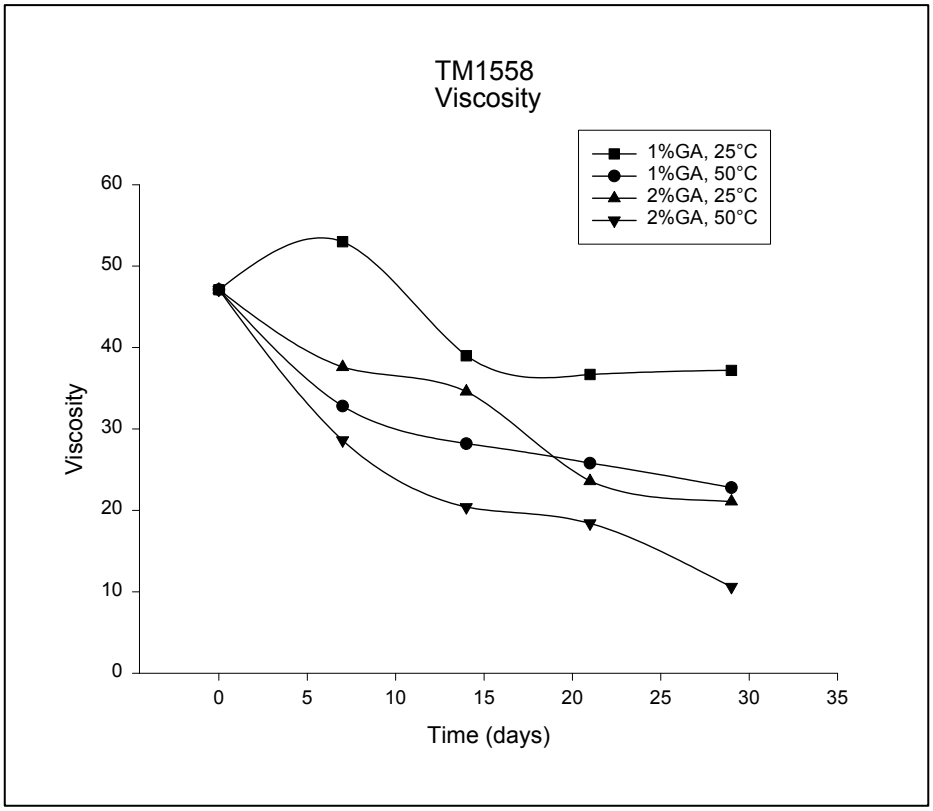
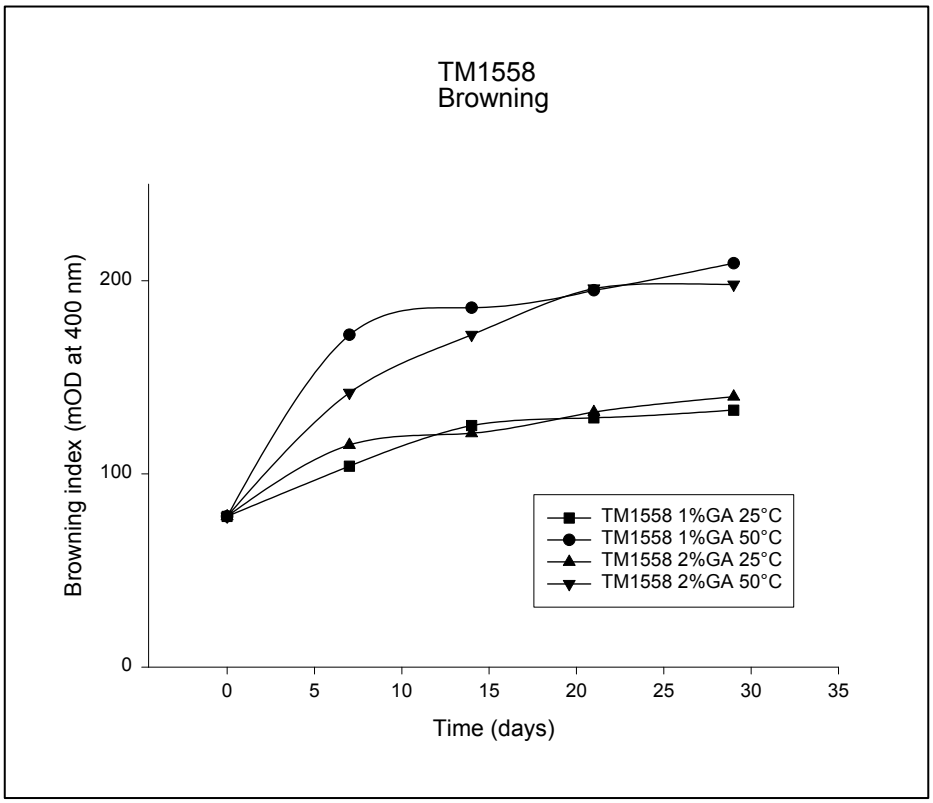


Figure 2. Changes in viscosity and browning of 4% chitosan (TM1558) in 1% and 2% glycolic acid at 25 and 50°C.





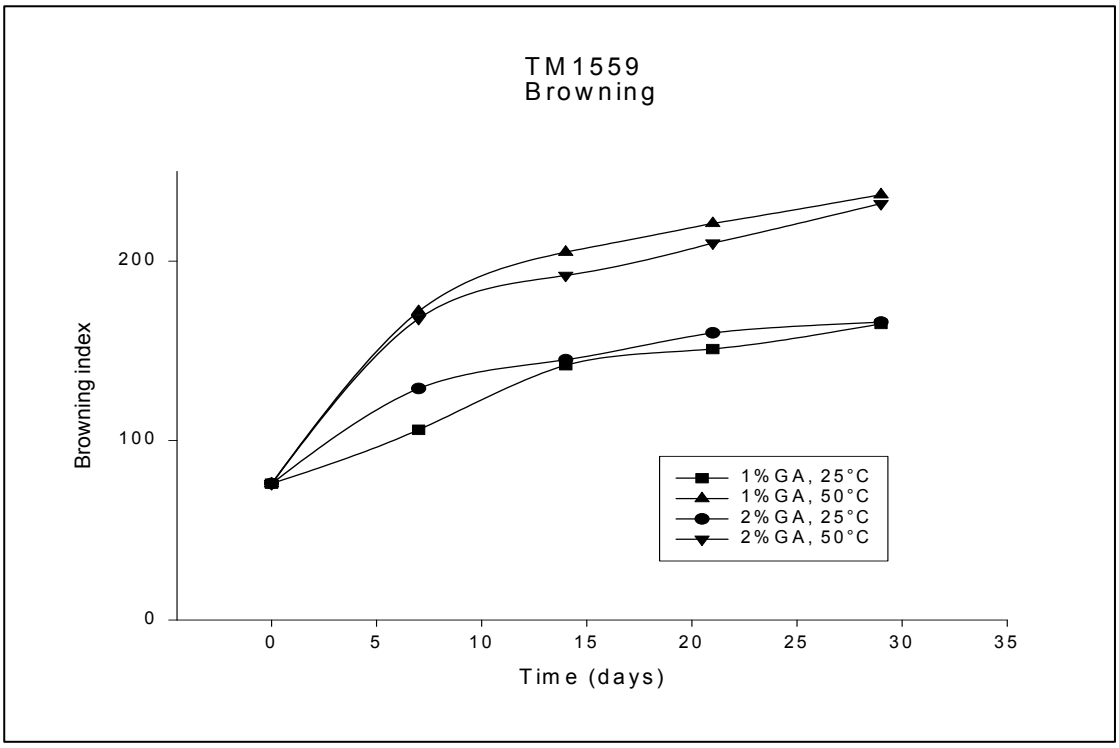


Figure 3. Changes in viscosity and browning of 4% chitosan (TM1559) in 1% and 2% glycolic acid at 25 and 50°C

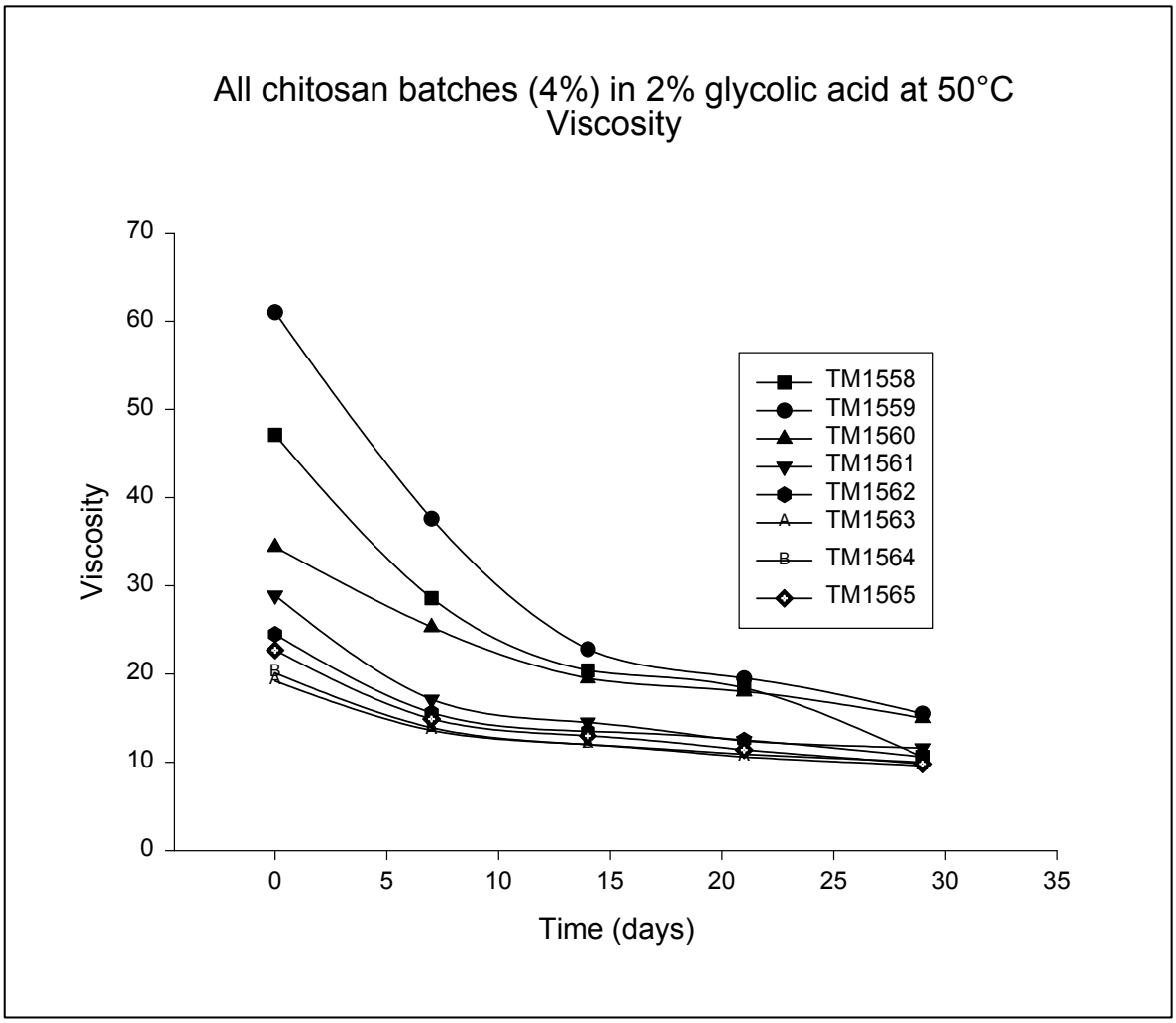


Figure 4. Changes in viscosity of all 4% chitosan batches in 2% glycolic acid at 50°C

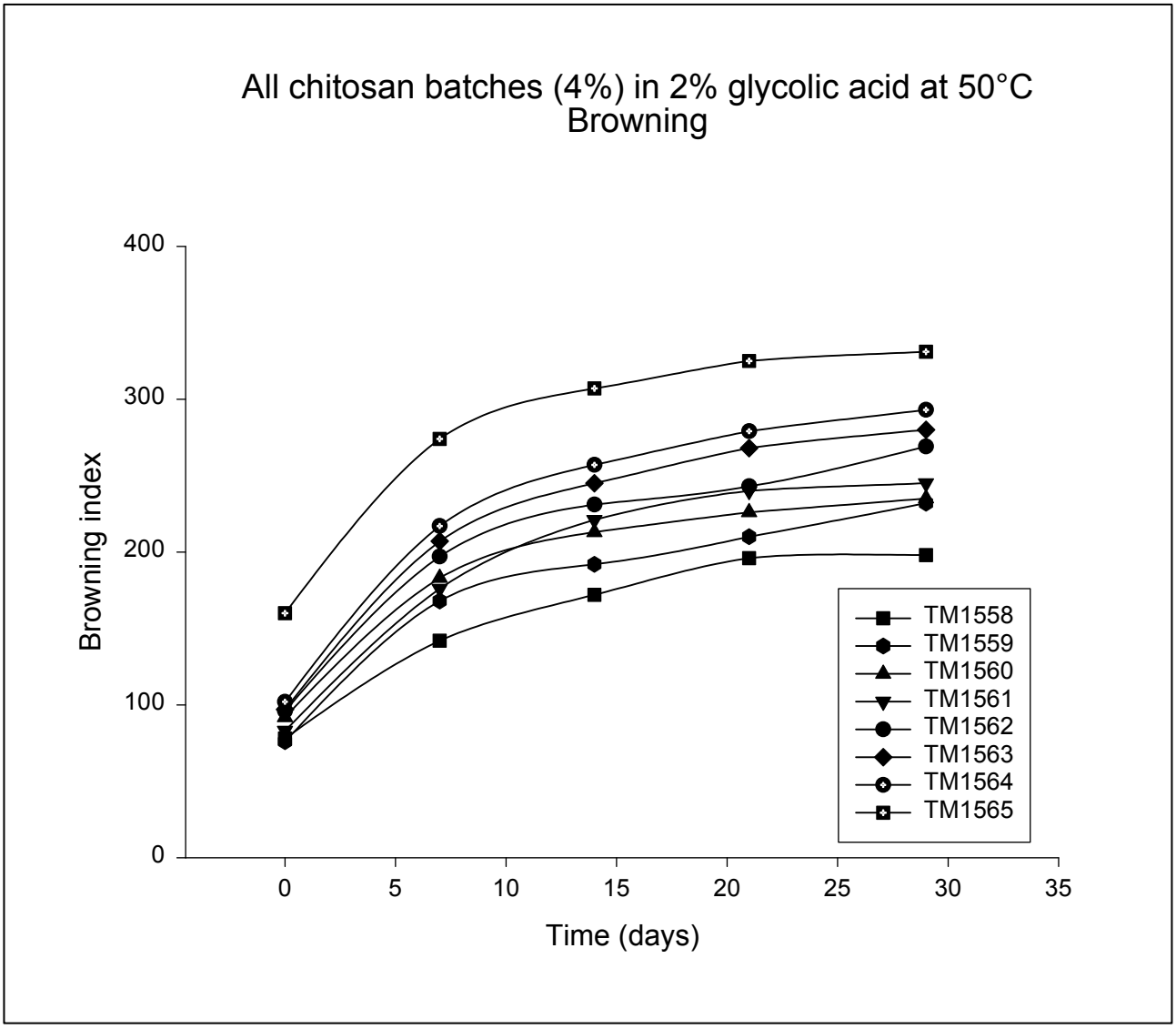


Figure 5. Changes in browning of all 4% chitosan batches in 2% glycolic acid at 50°C

Appendix
Table 1

Dupoint stability tests. 4□ chitosan in 1□ or 2□ acid

Lots	Acid	Acid conc (%)	Temp (°C)	Time (days)	Color	Clear-ness	Visc. (mPas)	B.I. 400	Turbit. (NTU)	Notes
TM1558	acetic	1	25	0	PY	UC	51,5	85	20	fine needles
TM1558	acetic	1	25	7	PY	UC	48,2	93	19	fine needles
TM1558	acetic	1	25	14	PY	UC	45,5	105	18	fine needles
TM1558	acetic	1	25	21	PY	UC	45,8	120	19	fine needles
TM1558	acetic	1	25	28	PY	UC	40,9	125	19	fine needles
TM1558	acetic	1	50	0	PY	UC	51,5	85	20	fine needles
TM1558	acetic	1	50	7	Y	C	24,1	171	21	fine needles

TM1558	acetic	1	50	14	Y	C	18,1	224	20	fine needles
TM1558	acetic	1	50	21	Y	C	12,8	285	20	fine needles
TM1558	acetic	1	50	28	Y	C	11,3	343	21	fine needles
TM1558	acetic	2	25	0	PY	UC	51,6	90	22	fine needles
TM1558	acetic	2	25	7	PY	UC	38,3	111	24	fine needles
TM1558	acetic	2	25	14	PY	UC	33,5	133	25	fine needles
TM1558	acetic	2	25	21	PY	UC	30,3	144	24	fine needles
TM1558	acetic	2	25	28	PY	UC	28,5	154	24	fine needles
TM1558	acetic	2	50	0	PY	C	51,6	90	22	fine needles
TM1558	acetic	2	50	7	Y	C	17,2	194	22	fine needles
TM1558	acetic	2	50	14	Y	C	11,8	283	21	fine needles
TM1558	acetic	2	50	21	Y	C	8,4	394	19	fine needles
TM1558	acetic	2	50	28	Y	C	7,8	522	20	fine needles
TM1559	acetic	1	25	0	PY	UC	74,7	75	17	fine needles
TM1559	acetic	1	25	7	PY	UC	64,2	93	15	fine needles
TM1559	acetic	1	25	14	PY	UC	60,0	98	16	fine needles
TM1559	acetic	1	25	21	PY	UC	56,3	106	16	fine needles
TM1559	acetic	1	25	28	PY	UC	53,8	109	16	fine needles
TM1559	acetic	1	50	0	PY	UC	74,7	75	17	fine needles
TM1559	acetic	1	50	7	Y	C	28,6	156	19	fine needles
TM1559	acetic	1	50	14	Y	C	20,0	214	18	fine needles
TM1559	acetic	1	50	21	Y	C	14,3	269	18	fine needles
TM1559	acetic	1	50	28	Y	C	11,5	333	19	fine needles
TM1559	acetic	2	25	0	PY	UC	71,5	78	19	fine needles
TM1559	acetic	2	25	7	PY	UC	47,8	100	21	fine needles
TM1559	acetic	2	25	14	PY	C	42,1	119	19	fine needles
TM1559	acetic	2	25	21	PY	C	37,6	128	20	fine needles
TM1559	acetic	2	25	28	PY	C	35,1	139	20	fine needles
TM1559	acetic	2	50	0	PY	UC	71,5	78	19	fine needles
TM1559	acetic	2	50	7	Y	C	18,1	185	19	fine needles
TM1559	acetic	2	50	14	Y	C	12,0	263	18	fine needles
TM1559	acetic	2	50	21	Y	C	8,4	408	15	fine needles
TM1559	acetic	2	50	28	Y	C	8,0	530	17	fine needles
TM1560	acetic	1	25	0	PY	UC	36,5	98	25	fine needles
TM1560	acetic	1	25	7	PY	UC	33,7	117	25	fine needles
TM1560	acetic	1	25	14	PY	UC	32,6	131	26	fine needles
TM1560	acetic	1	25	21	PY	UC	31,1	139	26	fine needles
TM1560	acetic	1	25	28	PY	UC	29,0	146	26	fine needles
TM1560	acetic	1	50	0	PY	UC	36,5	98	25	fine needles
TM1560	acetic	1	50	7	Y	UC	21,4	208	26	fine needles
TM1560	acetic	1	50	14	Y	C	16,7	253	31	fine needles
TM1560	acetic	1	50	21	Y	C	12,9	322	32	fine needles
TM1560	acetic	1	50	28	Y	C	12,1	379	32	fine needles
TM1560	acetic	2	25	0	PY	UC	35,8	101	28	fine needles
TM1560	acetic	2	25	7	PY	UC	26,7	134	32	fine needles
TM1560	acetic	2	25	14	PY	C	25,0	153	32	fine needles
TM1560	acetic	2	25	21	PY	C	22,2	161	32	fine needles

TM1560	acetic	2	25	28	PY	C	21,3	174	32	fine needles
TM1560	acetic	2	50	0	PY	UC	35,8	101	28	fine needles
TM1560	acetic	2	50	7	Y	UC	13,6	222	30	fine needles
TM1560	acetic	2	50	14	Y	C	9,9	319	28	fine needles
TM1560	acetic	2	50	21	Y	C	7,1	445	24	fine needles
TM1560	acetic	2	50	28	Y	C	7,2	567	27	fine needles
TM1561	acetic	1	25	0	PY	UC	33,0	87	18	fine needles
TM1561	acetic	1	25	7	PY	UC	31,0	109	18	fine needles
TM1561	acetic	1	25	14	PY	UC	29,6	122	19	fine needles
TM1561	acetic	1	25	21	PY	UC	27,7	130	19	fine needles
TM1561	acetic	1	25	28	PY	UC	28,0	135	19	fine needles
TM1561	acetic	1	50	0	PY	UC	33,0	87	18	fine needles
TM1561	acetic	1	50	7	Y	UC	19,7	190	22	fine needles
TM1561	acetic	1	50	14	Y	UC	15,9	251	22	fine needles
TM1561	acetic	1	50	21	Y	UC	11,9	314	21	fine needles
TM1561	acetic	1	50	28	Y	UC	10,6	385	21	fine needles
TM1561	acetic	2	25	0	PY	UC	32,5	90	20	fine needles
TM1561	acetic	2	25	7	PY	UC	25,1	121	22	fine needles
TM1561	acetic	2	25	14	PY	UC	22,5	139	22	fine needles
TM1561	acetic	2	25	21	PY	UC	20,4	148	22	fine needles
TM1561	acetic	2	25	28	PY	UC	19,6	161	22	fine needles
TM1561	acetic	2	50	0	PY	UC	32,5	90	20	fine needles
TM1561	acetic	2	50	7	Y	UC	12,1	217	22	fine needles
TM1561	acetic	2	50	14	Y	UC	9,3	326	20	fine needles
TM1561	acetic	2	50	21	Y	UC	7,1	473	18	fine needles
TM1561	acetic	2	50	28	Y	UC	6,5	624	19	fine needles
TM1562	acetic	1	25	0	PY	C	28,3	95	19	a few fine needles
TM1562	acetic	1	25	7	PY	C	29,1	129	22	a few fine needles
TM1562	acetic	1	25	14	PY	C	28,8	140	24	a few fine needles
TM1562	acetic	1	25	21	PY	C	31,2	147	23	a few fine needles
TM1562	acetic	1	25	28	PY	C	28,4	157	23	a few fine needles
TM1562	acetic	1	50	0	PY	C	28,3	95	19	a few fine needles
TM1562	acetic	1	50	7	Y	C	23,1	221	30	a few fine needles
TM1562	acetic	1	50	14	Y	C	20,1	263	29	a few fine needles
TM1562	acetic	1	50	21	Y	C	17,5	299	30	a few fine needles
TM1562	acetic	1	50	28	Y	C	16,3	349	30	a few fine needles
TM1562	acetic	2	25	0	PY	C	27,4	105	24	a few fine needles
TM1562	acetic	2	25	7	PY	C	23,4	153	26	a few fine needles
TM1562	acetic	2	25	14	PY	C	21,2	161	27	a few fine needles
TM1562	acetic	2	25	21	PY	C	18,9	174	28	a few fine needles
TM1562	acetic	2	25	28	PY	C	18,4	194	27	a few fine needles
TM1562	acetic	2	50	0	PY	C	27,4	105	24	a few fine needles
TM1562	acetic	2	50	7	Y	C	13,9	248	27	a few fine needles
TM1562	acetic	2	50	14	Y	C	10,3	316	27	a few fine needles
TM1562	acetic	2	50	21	Y	C	9,8	403	24	a few fine needles
TM1562	acetic	2	50	28	Y	C	8,2	510	26	a few fine needles
TM1563	acetic	1	25	0	PY	C	21,4	103	24	fine needles

TM1563	acetic	1	25	7	PY	C	22,9	139	25	fine needles
TM1563	acetic	1	25	14	PY	C	21,6	154	26	fine needles
TM1563	acetic	1	25	21	PY	C	21,0	164	27	fine needles
TM1563	acetic	1	25	28	PY	C	20,8	168	27	fine needles
TM1563	acetic	1	50	0	PY	C	21,4	103	24	fine needles
TM1563	acetic	1	50	7	Y	C	18,2	244	34	fine needles
TM1563	acetic	1	50	14	Y	C	16,2	301	32	fine needles
TM1563	acetic	1	50	21	Y	C	13,9	330	32	fine needles
TM1563	acetic	1	50	28	Y	C	12,9	374	32	fine needles
TM1563	acetic	2	25	0	PY	C	21,0	112	26	fine needles
TM1563	acetic	2	25	7	PY	C	18,9	168	31	fine needles
TM1563	acetic	2	25	14	PY	C	17,3	181	33	fine needles
TM1563	acetic	2	25	21	PY	C	15,6	196	35	fine needles
TM1563	acetic	2	25	28	PY	C	15,9	204	34	fine needles
TM1563	acetic	2	50	0	PY	C	21,0	112	26	fine needles
TM1563	acetic	2	50	7	Y	C	11,7	274	32	fine needles
TM1563	acetic	2	50	14	Y	C	8,8	350	30	fine needles
TM1563	acetic	2	50	21	Y	C	7,2	460	27	fine needles
TM1563	acetic	2	50	28	Y	C	7,2	591	28	fine needles
TM1564	acetic	1	25	0	PY	UC	23,6	111	22	many fine needles
TM1564	acetic	1	25	7	PY	UC	24,1	138	22	many fine needles
TM1564	acetic	1	25	14	PY	UC	23,6	148	23	many fine needles
TM1564	acetic	1	25	21	PY	UC	22,6	161	23	many fine needles
TM1564	acetic	1	25	28	PY	UC	22,5	167	23	many fine needles
TM1564	acetic	1	50	0	PY	UC	23,6	111	22	many fine needles
TM1564	acetic	1	50	7	Y	UC	18,4	238	28	many fine needles
TM1564	acetic	1	50	14	Y	UC	14,9	288	28	many fine needles
TM1564	acetic	1	50	21	Y	UC	12,4	334	28	many fine needles
TM1564	acetic	1	50	28	Y	UC	11,5	390	28	many fine needles
TM1564	acetic	2	25	0	PY	UC	23,5	115	23	many fine needles
TM1564	acetic	2	25	7	PY	UC	19,7	166	27	many fine needles
TM1564	acetic	2	25	14	PY	UC	17,9	177	29	many fine needles
TM1564	acetic	2	25	21	PY	UC	16,0	188	29	many fine needles
TM1564	acetic	2	25	28	PY	UC	16,3	202	28	many fine needles
TM1564	acetic	2	50	0	PY	UC	23,5	115	23	many fine needles
TM1564	acetic	2	50	7	Y	UC	11,1	275	28	many fine needles
TM1564	acetic	2	50	14	Y	UC	8,6	364	28	many fine needles
TM1564	acetic	2	50	21	Y	UC	6,5	483	26	many fine needles
TM1564	acetic	2	50	28	Y	UC	6,4	638	27	many fine needles
TM1565	acetic	1	25	0	PY	UC	26,9	173	1200	many fine needles and particles
TM1565	acetic	1	25	7	PY	UC	26,5	200	389	many fine needles and particles
TM1565	acetic	1	25	14	PY	UC	24,3	206	523	many fine needles and particles
TM1565	acetic	1	25	21	Y	UC	23,5	214	643	many fine needles and particles
TM1565	acetic	1	25	28	Y	UC	23,2	216	621	many fine needles and particles
TM1565	acetic	1	50	0	PY	UC	26,9	173	1200	many fine needles and particles
TM1565	acetic	1	50	7	Y	UC	16,3	306	476	many fine needles and particles
TM1565	acetic	1	50	14	Y	UC	13,0	363	533	many fine needles and particles

TM1565	acetic	1	50	21	Y	UC	10,3	418	576	many fine needles and particles
TM1565	acetic	1	50	28	Y	UC	9,4	505	561	many fine needles and particles
TM1565	acetic	2	25	0	PY	UC	26,4	178	750	many fine needles and particles
TM1565	acetic	2	25	7	PY	UC	21,4	228	576	many fine needles and particles
TM1565	acetic	2	25	14	PY	UC	18,9	229	560	many fine needles and particles
TM1565	acetic	2	25	21	PY	UC	17,1	231	891	many fine needles and particles
TM1565	acetic	2	25	28	PY	UC	17,1	259	621	many fine needles and particles
TM1565	acetic	2	50	0	PY	UC	26,4	178	750	many fine needles and particles
TM1565	acetic	2	50	7	Y	UC	11,6	310	825	many fine needles and particles
TM1565	acetic	2	50	14	Y	UC	8,6	410	1015	many fine needles and particles
TM1565	acetic	2	50	21	Y	UC	7,0	539	1638	many fine needles and particles
TM1565	acetic	2	50	28	Y	UC	6,1	733	1112	many fine needles and particles

APPENDIX E

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Amino acid analysis of proteins and peptides

Standard hydrolysis

All incoming samples are labeled with a unique id-number and are entered into our database. Based on sample type and concentration a method is assigned to the sample describing for the analyst how the sample should be processed and analyzed.

Hydrolysis takes place in 2 mL 6 M HCl / 0.1% phenol at 110°C in thoroughly evacuated and individually sealed heavy glass test tubes. The hydrochloric solution is either dispensed by itself or together with the internal standard, norleucine, to the test tubes. The internal standard compensates for all unspecific losses during the analysis process. Hydrolysis is generally continued for 24 hours.

Non standard treatments

There are variations on this standard protocol depending on the nature of the sample such as hard to hydrolyze sequences and easily chlorinated amino acids. The former case is solved by 48 or 72h hydrolysis and the latter by raising phenol to 1%. For some amino acid derivatives protective groups have to be removed before hydrolysis.

Unfortunately, free cysteine coelutes with proline and will completely compromise its value. If there is no free cysteine the value will be correct and this is the reason we report this value for all ordinary samples and the investigator self has to decide how to use this value. One way to circumvent the cysteine/proline problem is to oxidize the sample before hydrolysis and form cysteic acid from both cystine and cysteine. The oxidized product, cysteic acid, elutes well ahead of all other amino acids and can easily be quantified. Oxidation will thus improve the cysteine and proline values but will also make methionines more reliably estimated. Methionine is subjected to partial oxidation during hydrolysis and might unless oxidized give erroneously low recovery. Our laboratory performs oxidations on samples as a special service (se appendix for method). The oxidized sample is run without internal standard and is normalized back to the standard 24h hydrolysis.

Tryptophan is possible to analyze with the standard hydrolysis method but its recovery will vary considerably due to its sensitivity to oxidation during hydrolysis. If this amino acid is to be analyzed our laboratory uses 3M mercaptoethanesulfonic as hydrolyzing agent and normalize this result back to the standard 24h hydrolysis analysis. Oxidation will not result in a measurable compound as for methionine and cysteine.

If norleucine is present in the sequence of the sample, another amino acid must be used as internal standard. It is of course critical that the choice of internal standard does not interfere with the analysis of the sample.

Weighing and pipetting

Dry samples: At present we use a Mettler/Toledo MX5 scale which is calibrated and tuned once a year by Mettler/Toledo. The samples are weighed in a subtractive way by recording

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the weight of the weighing ship before and after transfer of sample to the test tube. The difference is calculated and taken as the amount sample actually transferred to the hydrolysis tubes.

Suspensions and solutions: The appropriate volume is pipetted or weighed directly into to the hydrolysis tube. The pipettes are calibrated and maintained twice a year by Mettler-Toledo (ISO 17025-accredited lab for pipettes). Highly concentrated samples will have a higher error in this procedure since a small deviation in pipetted volume corresponds to more material than for a less concentrated sample. Weighing is usually not an option since the density "always" is unknown but is certainly not 1.000.

At this stage of processing a print-out of the sample data is done and a new person checks that the withdrawn sample amount as well as sample name, lot info etc was correctly entered into the database by comparison with logs and accompanying letters.

Solubilization of hydrolysates

After hydrolysis the tubes are cooled, opened and the hydrochloric acid is evaporated. The dried samples are dissolved in an appropriate volume of sample buffer pH 2.2 followed by transfer to an autosampler vial. If high amounts of mainly tyrosine is expected an ultrasonic bath is used to ease the solubilisation. With the ordinary setup sample volumes ranging from 5 to 100 uL can be injected to the column.

Instruments

Presently the laboratory has access to three different analyzers. All analyzers uses ninhydrin for post column detection and separation of amino acids is by ion-exchange chromatography. Two analyzers are Biochrom model 20 and the third is a Biochrom model 30. The laboratory obtains ninhydrin and all physiological buffers from Biochrom but we prepare the other buffers ourselfs. The Biochrom 30 is under a once a year preventive maintenance schedule by a Biochrom service engineer.

Two instrument are run with a standard high-resolution program using sodium citrate buffers and the third instrument uses a lithium citrate system for analysis of mainly physiological samples and hard to resolve substances.

Integrations and data acquiring are made with the software EZChrom Elite (Hewlett Packard) as recommended by Biochrom.

Calculations

The software EZChrom Elite is used for integration of chromatograms in a semiautomatic manner. Every chromatogram is closely inspected with special regard to the baseline and is, if judged necessary, manually adjusted. After this inspection a working report is printed out and selected data is transferred to a database.

A second round of adjustment is done on the printed working report. This is done manually and written down on the working report. After this step a second person creates the customer reports by importing sample data into an Excel spreadsheet template. The written changes on the working report are transferred to the customer report via a separate worksheet in the

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template for documentation purposes. The person creating the customer report doubles as inspector of the analysis process.

If several analyses have been conducted on one sample, for example 24h and 72h hydrolysis, the different runs are normalized against each other by using an interactive spreadsheet template for visualization of all possible quotients together with the resulting % rsd. The whole operation is printed out and saved both in paper and electronic format. The normalized values are written down on the working copy. Factors compensating the known hydrolysis losses of serine, threonine and glucosamine are used in the report.

Amino acid standards

The software used for integration of the chromatograms is calibrated by running a standard mixture with known concentrations of amino acids. This concentration is determined partly by comparison against the amino acid Standard Reference Material 2389 from National Institute of Standards & Technology (NIST) and partly by the traditional standard for the laboratory which is based on Sigma 085K8800.

Hydrolysis of highly purified peptides will function as a sign that the standards relative levels are accurate. For this purpose NIST also supplies peptides which are more thoroughly characterized. The laboratory routinely perform analysis on starting compounds for peptide synthesis and as these are highly purified and characterized this also helps to backup the standards true-level of concentration (accuracy).

Quality system

In order to maintain the quality and detect deviations as early as possible several different parameters are supervised. With the use of the databases it is possible to make charts over such parameters as internal standards found to expected ratio, the quality of the columns by listing resolution or peak width and monitoring of integrated area/nmole for detection of malfunction of instruments.

We have a "Q"-sample which is analyzed both under standard conditions and as oxidized sample each week. This is done by in turn all personnel thus giving the laboratory's intermediate variation. The trends are visualized in Shewhart plots with warning and alarm limits. Between-lab variation is obtained by running NIST standard reference material, for example bovine serum albumin 927d.

As mentioned above one instrument is still under preventive maintenance and pipettes and scales are regularly maintained and calibrated. A schedule governs that all stock solutions of internal standards are checked regularly. Our incubators are under continuous electronic temperature monitoring with recording of values and so is the relative humidity in the area around the scales.

Reports together with all other printed material are saved for ten years in a locked archive in the laboratory domain. Electronic data, such as raw data from integrations, customer reports and all databases are copied nightly by the IBM Tivoli-system managed by Uppsala University.

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The laboratory is gradually harmonized with the ISO 17025 system and SOP:s and other quality documents are either in preliminary form or under production.

Appendix

Excerpts from Eur Pharmacopeia 6th edition (6.6)

The following paragraphs are copied from the pharmacopeia and are presented together with comments in italics on how our laboratory performs the protocol.

METHOD 1

Acid hydrolysis using hydrochloric acid containing phenol is the most common procedure used for protein/peptide hydrolysis preceding amino acid analysis. The addition of phenol to the reaction prevents the halogenation of tyrosine.

Hydrolysis solution. 6 M hydrochloric acid containing 0.1 per cent to 1.0 per cent of phenol.

Procedure

Liquid phase hydrolysis: Place the protein or peptide sample in a hydrolysis tube, and dry (the sample is dried so that water in the sample will not dilute the acid used for the hydrolysis). Add 200 µl of hydrolysis solution per 500 µg of lyophilised protein. Freeze the sample tube in a dry ice-acetone bath, and flame seal in vacuo. Samples are typically hydrolysed at 110 °C for 24 h in vacuo or in an inert atmosphere to prevent oxidation. Longer hydrolysis times (e.g., 48 h and 72 h) are investigated if there is a concern that the protein is not completely hydrolysed.

We use 2 ml 6 M HCl/0.1% phenol as standard but 1% phenol if Cys + Tyr in peptide samples. Proteins use the 0.1% phenol. Hydrolysis for 72h for Val-Ile, Val-Val, Ile-Ile and then in combination with a withdrawn aliquot after 24h. Proteins are hydrolyzed for 24h if nothing else is requested.

METHOD 2

Tryptophan oxidation during hydrolysis is decreased by using mercaptoethanesulfonic acid as the reducing acid.

Hydrolysis solution. 2.5 M mercaptoethanesulfonic acid solution.

We use the acid as supplied and that means 3 M +-0.1M for 24h. We neutralize with NaOH before adding sample buffer.

METHOD 4

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Cysteine/cystine and methionine oxidation is performed with performic acid before the protein hydrolysis.

Oxidation solution. Use performic acid freshly prepared by mixing 1 volume of hydrogen peroxide solution (30 per cent) and 9 volumes of anhydrous formic acid and incubating at room temperature for 1 h.

Procedure. Dissolve the protein/peptide sample in 20 µl of anhydrous formic acid and heat at 50 °C for 5 min; then add 100 µl of the oxidation solution. Allow the oxidation to proceed for 10-30 min. In this reaction, cysteine is converted to cysteic acid and methionine is converted to methionine-sulphone. Remove the excess reagent from the sample in a vacuum centrifuge. The oxidised protein can then be acid hydrolysed using Method 1 or Method 2. This technique may cause modifications to tyrosine residues in the presence of halides.

We make the performic acid as above but use incubation at room temperature for 2h and then we chill the acid on ice before use. The sample is dissolved directly with 200 uL ice-cold performic acid and routinely oxidized for 2 to 4 hours on ice unless it is known beforehand that o/n incubation is needed. After oxidation we dilute 10-fold with water and freeze-dry.

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APPENDIX F-1

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Title: Efficacy of freezing, frozen storage and edible antimicrobial coatings used in combination for control of Listeria monocytogenes on roasted turkey stored at chiller temperatures

Author(s): Jiang, Z (Jiang, Zheng); Neetoo, HD (Neetoo, Hudaa); Chen, HQ (Chen, Haiqiang)

Source: FOOD MICROBIOLOGY Volume: 28 Issue: 7 Pages: 1394-1401 DOI: 10.1016/j.fm.2011.06.015 Published: OCT 2011

Times Cited in Web of Science: 0

Total Times Cited: 0

Abstract: The presence and growth of Listeria monocytogenes on ready-to-eat (RTE) turkey is an important food safety issue. The antilisterial efficacy of four polysaccharide-based edible coatings (starch, chitosan, alginate and pectin) incorporating sodium lactate (SL) and sodium diacetate (SD) as well as commercial preparations Opti.Form PD4, NowvaGARD (TM) CB1, Protect-M and Guardian (TM), NR100 were compared against L monocytogenes on roasted turkey. Pectin coating treatments incorporating SL/SD, Opti.Form PD4 with or without Protect-M, and NovaGARD (TM) CB1 displayed higher antimicrobial efficacy against.
L. monocytogenes than the other antimicrobials and coating materials. In the second phase of the study, it was investigated whether frozen storage could enhance the antilisterial effectiveness of pectin coating treatments on chilled roasted turkey. Inoculated roasted turkey samples coated with pectin-based treatments were frozen for up to 4 weeks and subsequently stored at 4 degrees C for 8 weeks. Frozen storage significantly enhanced the antilisterial activity of various coating treatments; with selected treatments reducing the L monocytogenes populations by as much as 1.1 log CFU/cm(2) during the subsequent 8-week chilled storage. This study demonstrates that pectin-based antimicrobial edible coatings hold promise in enhancing the safety of RTE poultry products and frozen storage has the potential to enhance their effectiveness. (C) 2011 Elsevier Ltd. All rights reserved.

Accession Number: WOS:000295252300020

ISSN: 0740-0020

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Title: Effect of the molecular weight and concentration of chitosan in pork model burgers

Author(s): Sayas-Barbera, E (Sayas-Barbera, E.); Quesada, J (Quesada, J.); Sanchez-Zapata, E (Sanchez-Zapata, E.); Viuda-Martos, M (Viuda-Martos, M.); Fernandez-Lopez, F (Fernandez-Lopez, F.); Perez-Alvarez, JA (Perez-Alvarez, J. A.); Sendra, E (Sendra, E.)

Source: MEAT SCIENCE Volume: 88 Issue: 4 Pages: 740-749 DOI: 10.1016/j.meatsci.2011.03.007 Published: AUG 2011

Times Cited in Web of Science: 0

Total Times Cited: 0

Abstract: Chitosan of high and low molecular weights was added at 0%, 0.25%, 0.5% and 1% concentrations to a burger model system. Burgers were evaluated by physicochemical analysis, cooking characteristic and storage stability. The antioxidant activity of chitosan was studied in vitro. The addition of chitosan influenced pH and color properties, in molecular weight and concentration dependent ways. Cooking properties were significantly affected by the chitosan. High molecular weight chitosan improved all cooking characteristics compared with control samples. Low molecular weight chitosan increased the shelf life of burgers, enhanced the red color and reduced total viable counts, compared with control and high molecular weight chitosan samples. The antioxidant activity of chitosan was dependent on molecular weight and

concentration. The results indicate that high molecular weight chitosan (HMWC) improves all cooking characteristics and antioxidant activity while low molecular weight chitosan extends the red color and reduces total viable counts. (C) 2011 Elsevier Ltd. All rights reserved.

Accession Number: WOS:000291180900022

ISSN: 0309-1740

Record 5 of 34

Title: Chitosan inhibits premature browning in ground beef

Author(s): Suman, SP (Suman, S. P.); Mancini, RA (Mancini, R. A.); Joseph, P (Joseph, P.); Ramanathan, R (Ramanathan, R.); Konda, MKR (Konda, M. K. R.); Dady, G (Dady, G.); Yin, S (Yin, S.)

Source: MEAT SCIENCE Volume: 88 Issue: 3 Pages: 512-516 DOI: 10.1016/j.meatsci.2011.02.002 Published: JUL 2011

Times Cited in Web of Science: 0

Total Times Cited: 0

Abstract: Our objective was to evaluate the effect of chitosan on premature browning in refrigerated ground beef patties stored in different packaging systems. Ground beef patties (15% fat) with chitosan (1% w/w) or without chitosan (control) were individually packaged either in vacuum (VP), aerobic packaging (AP), carbon monoxide modified atmosphere packaging (LO-0X; 0.4% CO + 19.6% CO(2) + 80% N(2)), or high-oxygen modified atmosphere packaging (HI-Ox; 80% O(2)+ 20% CO(2)), and stored for 0.1. or 3 days at 1 degrees C. At the conclusion of storage, raw surface redness was evaluated, patties were cooked to internal end-point temperatures of either 66 degrees C or 71 degrees C, and internal cooked color was measured. The incorporation of chitosan increased ($P<0.05$) the interior redness of patties stored in AP, VP, and LO-OX, but not in HI-OX. The results of the present study suggest that the incorporation of 1% chitosan minimizes premature browning in ground beef patties stored under AP, VP, and LO-OX. (C) 2011 Elsevier Ltd. All rights reserved.

Accession Number: WOS:000289822800029

ISSN: 0309-1740

Record 6 of 34

Title: Partial substitution of nitrite by chitosan and the effect on the quality properties of pork sausages

Author(s): Garcia, M (Garcia, Mario); Beldarrain, T (Beldarrain, Tatiana); Fornaris, L (Fornaris, Leonid); Diaz, R (Diaz, Raul)

Source: CIENCIA E TECNOLOGIA DE ALIMENTOS Volume: 31 Issue: 2 Pages: 481-487 Published: APR-JUN 2011

Times Cited in Web of Science: 0

Total Times Cited: 0

Abstract: The aim of the study was to evaluate the influence of partial nitrite replacement by chitosan on the quality of Ham Visking (a type of pork sausages). Five Ham Visking formulations were elaborated modifying the sodium nitrite (0.011; 0.016 or 0.0212%) and chitosan concentrations (0.25 or 0.5%) in the products. Sausages were stored at 4 degrees C and physicochemical, microbiological, and sensorial evaluations were performed in order to estimate their shelf life. Chitosan can be used in pork sausages without affecting ensory attributes such as color although the panelists detected textural differences among the samples with chitosan, which suggests that there is some influence of deacetylation degree of chitosan on the textural behavior of sausages which still needed to be explained for a successful application of chitosan

in meat products. The reduction of residual sodium nitrite did not affect the color and flavor of such products, but the use of chitosan increasedsignificantly the shelf life of sausages.

Accession Number: WOS:000293654300031

ISSN: 0101-2061

Record 7 of 34

Title: Use of Chitosan in Turkish Sausage (Sucuk) Production and Effects on Quality

Author(s): Gokmen, M (Gokmen, Mukadderat); Gurbuz, U (Gurbuz, Umit)

Source: KAFKAS UNIVERSITESI VETERINER FAKULTESI DERGISI Volume: 17 Pages: S67-S71 Supplement: A Published: MAR-APR 2011

Times Cited in Web of Science: 0

Total Times Cited: 0

Abstract: This study aims to investigate the effect of chitosan, natural polysaccharide, use in different proportions (0.05%, 0.1%, 0.5% and 1%) on the quality of Turkish sausage production. In the study, as a control group, the first group was added only 0.05% nitrate. Microbiological analysis (total aerobic mesophilic bacteria, Enterobacteriaceae, coliform and Escherichia coli, sulphite-reducing clostridia, mold-yeast count) was done in the four different stages of experimental sausage production, (meat [DN(1)], after mixing [DN(2)], after filling [DN(3)], after ripening [DN(4)]) and on the 1, 7, 15, 30 and 60 days of the storage. Sensory qualities of experimental sausage samples (flavor, color, appearance and texture) were evaluated in the DN4. It was then determined that a little amount of chitosan addition (0.05%, 0.1% and 0.5%) into the production of Turkish sausage affected the microbiological and sensory quality positively. However, addition of much larger amounts (such as 1%) affected the sensory quality in a negative way. Moreover, it was determined that higher amounts of chitosan applications (0.5% and 1%) created technological problems.

Accession Number: WOS:000290955200012

ISSN: 1300-6045

Record 8 of 34

Title: Sensory evaluation and inhibition of Listeria monocytogenes in bovine pate added of chitosan from Mucor rouxii

Author(s): Bento, RA (Bento, Roberta Albuquerque); Stamford, TLM (Montenegro Stamford, Tania Lucia); Stamford, TCM (Montenegro Stamford, Thayza Christina); de Andrade, SAC (Cardoso de Andrade, Samara Alvachian); de Souza, EL (de Souza, Evandro Leite)

Source: LWT-FOOD SCIENCE AND TECHNOLOGY Volume: 44 Issue: 2 Pages: 588-591 DOI: 10.1016/j.lwt.2010.08.016 Published: MAR 2011

Times Cited in Web of Science: 1

Total Times Cited: 1

Abstract: This study aimed to assess the efficacy of chitosan from Mucor rouxii UCP 064 in inhibiting Listeria monocytogenes in bovine meat pate at 4 degrees C The influence of the chitosan addition on sensory aspects of the product was also evaluated The addition of chitosan from M rouxii at 5 mg/g to bovine meat pate decreased the counts of L monocytogenes from approximately 7 to 3 log cfu/g after 6 days of storage at 4 C In pate without chitosan the counts were over 7 log cfu/g already after two days of storage Sensory evaluation suggested that addition of chitosan in pate would be acceptable to consumers although some negative influence on flavor and taste was found From these results chitosan from fungi could be considered

a possible alternative compound to control L monocytogenes in ready-to-eat meat products (C) 2010 Elsevier Ltd All rights reserved

Accession Number: WOS:000284723800033

ISSN: 0023-6438

Record 9 of 34

Title: Packaging-specific influence of chitosan on color stability and lipid oxidation in refrigerated ground beef

Author(s): Suman, SP (Suman, S. P.); Mancini, RA (Mancini, R. A.); Joseph, P (Joseph, P.); Ramanathan, R (Ramanathan, R.); Konda, MKR (Konda, M. K. R.); Dady, G (Dady, G.); Yin, S (Yin, S.)

Source: MEAT SCIENCE Volume: 86 Issue: 4 Pages: 994-998 DOI: 10.1016/j.meatsci.2010.08.006 Published: DEC 2010

Times Cited in Web of Science: 2

Total Times Cited: 2

Abstract: We examined the influence of chitosan on lipid oxidation and color stability of ground beef stored in different modified atmosphere packaging (MAP) systems. Ground beef patties with chitosan (1%) or without chitosan (control) were packaged either in high-oxygen MAP (HIOX; 80% O(2)+20% CO(2)), carbon monoxide MAP (CO; 0.4% CO+19.6% CO(2)+80% N(2)), vacuum (VP), or aerobic packaging (PVC) and stored at 1 C. Chitosan increased (P<0.05) redness of patties stored in PVC and CO, whereas it had no effect (P>0.05) in HIOX. Chitosan patties demonstrated lower (P<0.05) lipid oxidation than controls in all packaging. Control patties in PVC and HIOX exhibited greater (P<0.05) lipid oxidation than those in VP and CO, whereas chitosan patties in different packaging systems were not different (P>0.05) from each other. Our findings suggested that antioxidant effects of chitosan on ground beef are packaging-specific. (C) 2010 The American Meat Science Association. Published by Elsevier Ltd. All rights reserved.

Accession Number: WOS:000283456300017

ISSN: 0309-1740

Record 10 of 34

Title: Review of Antimicrobial and Antioxidative Activities of Chitosans in Food

Author(s): Friedman, M (Friedman, Mendel); Juneja, VK (Juneja, Vijay K.)

Source: JOURNAL OF FOOD PROTECTION Volume: 73 Issue: 9 Pages: 1737-1761 Published: SEP 2010

Times Cited in Web of Science: 10

Total Times Cited: 10

Abstract: Interest in chitosan, a biodegradable, nontoxic. non-antigenic. and biocompatible biopolymer isolated from shellfish, arises from the fact that chitosans are reported to exhibit numerous health-related beneficial effects, including strong antimicrobial and antioxidative activities in foods The extraordinary interest in the chemistry and application in agriculture, horticulture. environmental science, industry. microbiology, and medicine is attested by about 17.000 citations on this subject in the Scopus database A special need exists to develop a better understanding of the role of chitosans in ameliorating foodborne illness To contribute to this effort, this overview surveys and interprets our present knowledge of the chemistry and antimicrobial activities of chitosan in solution, as powders, and in edible films and coating against foodborne pathogens, spoilage bacteria, and pathogenic viruses and fungi in several food categories These include produce, fruit juices, eggs and dairy, cereal, meat, and seafood products. Also covered are antimicrobial activities of chemically modified and nanochitosans, therapeutic properties. and possible

mechanisms of the antimicrobial. antioxidative. and metal chelating effects Further research is suggested in each of these categories. The widely scattered data on the multifaceted aspects of chitosan microbiology, summarized in the text and in 10 tables and 8 representative figures. suggest that low-molecular-weight chitosans at a pH below 6.0 presents optimal conditions for achieving desirable antimicrobial and antioxidative-preservative effects in liquid and solid foods We are very hopeful that the described findings will be a valuable record and resource for further progress to improve microbial food safety and food quality

Accession Number: WOS:000281617700021

ISSN: 0362-028X

Record 11 of 34

Title: Application of chitosan-incorporated LDPE film to sliced fresh red meats for shelf life extension

Author(s): Park, SI (Park, Su-il); Marsh, KS (Marsh, Kenneth S.); Dawson, P (Dawson, Paul)

Source: MEAT SCIENCE Volume: 85 Issue: 3 Pages: 493-499 DOI: 10.1016/j.meatsci.2010.02.022 Published: JUL 2010

Times Cited in Web of Science: 1

Total Times Cited: 1

Abstract: Chitosan lactate was impregnated as an antimicrobial additive into low density polyethylene (LDPE) with different concentrations. The antimicrobial effectiveness was tested with three pathogenic bacteria, specifically Listeria monocytogenes, Escherichia colt and Salmonella enteritidis. Also, these chitosan incorporated films were applied on red meat surfaces to determine the effectiveness of chitosan on color shelf life extension and microbial growth inhibition. Chitosan was exposed to 0.1% peptone water containing the three pathogens in separate tests and inhibited microbial growth a higher levels with increasing concentration of chitosan in the film matrix. Oxygen permeability was not affected by the incorporation of chitosan, while the water vapor permeability increased with the addition of chitosan. Film elongation decreased with the addition of chitosan. When chitosan incorporated films were applied on fresh red meat, microorganisms on the meat surface were not inhibited but significant extension of red color shelf life were observed in refrigerated, sliced red meats. (c) 2010 Elsevier Ltd. All rights reserved.

Accession Number: WOS:000278344300017

ISSN: 0309-1740

Record 12 of 34

Title: Combined Chitosan-Thyme Treatments with Modified Atmosphere Packaging on a Ready-to-Cook Poultry Product

Author(s): Giatrakou, V (Giatrakou, V.); Ntzimani, A (Ntzimani, A.); Savvaidis, IN (Savvaidis, I. N.)

Source: JOURNAL OF FOOD PROTECTION Volume: 73 Issue: 4 Pages: 663-669 Published: APR 2010

Times Cited in Web of Science: 0

Total Times Cited: 0

Abstract: In the present study, natural antimicrobials chitosan and thyme, and their combination, were evaluated for their effect on the shelf life of a ready-to-cook (RTC) chicken-pepper kebab (skewer) stored under modified atmosphere packaging (MAP) conditions at 4 +/- 0.5 degrees C for 14 days. The following treatments were examined: control samples stored under aerobic packaging (A), samples stored under MAP (M), samples treated with 1.5% chitosan (vol/wt) and stored under MAP (M-CH), samples treated with 0.2% thyme essential oil (vol/wt) (M-T), and samples treated with 1.5% chitosan (vol/wt) and 0.2% thyme essential oil (vol/wt) and stored under MAP (M-CH-T). Treatment M-CH-T significantly affected aerobic

plate counts and counts of lactic acid bacteria. Pseudomonas spp., Brochothrix thermosphacta, Enterobacteriaceae, and yeasts and molds during the entire storage period. Similarly, lipid oxidation of the RTC product was retarded (M-CH-T treatment) during storage, whereas redness was maintained in M-T. M-CH, and M-CH-T samples. Based primarily on sensory data (taste attribute), M-CH and M-T treatments extended RTC product shelf life by 6 clays, whereas M-CH-T treatment resulted in a product with a shelf life of 14 days that maintained acceptable sensory characteristics (shelf life of the control was 6 days).

Accession Number: WOS:000276417100007

ISSN: 0362-028X

Record 13 of 34

Title: Effects of Chitosan with Different Molecular Weight and Nitrite Addition on the Residual Nitrite Contents and Self-life of Emulsified Sausage during Cold Storage

Author(s): Park, WY (Park, Woong-Yeoul); Kim, YJ (Kim, Young-Jik)

Source: KOREAN JOURNAL FOR FOOD SCIENCE OF ANIMAL RESOURCES Volume: 30 Issue: 2 Pages: 269-276 Published: APR 2010

Times Cited in Web of Science: 0

Total Times Cited: 0

Abstract: The objective of this study was to determine the effects of residual nitrite contents, chitosan with different molecular weight and nitrite addition on emulsified sausage during cold storage. Six types of sausages were evaluated: control, 0.5% 50 kDa chitosan (T1), 0.5% 200 kDa chitosan (T2), 150 ppm nitrite (T3), 0.5% 50 kDa chitosan+150 ppm nitrite (T4), and 0.5% 200 kDa chitosan+150 ppm nitrite (T5). Each type of sausage was tested in triplicate and assigned to one of four storage periods: 0, 10, 20 and 30 days. As the storage time increased, the presence of chitosan and nitrite resulted in decreased residual nitrite value and increased pH (in control and T2), TBARS (thiobarbituric acid reactive substance) values, and total plate counts (TPC). Values for pH, TBARS, residual nitrite and total plate counts decreased significantly in response to the addition of chitosan and nitrite relative to the control ($p<0.05$). T5 was redder than the control (higher CIE a*) at 30 d; however, no difference in the CIE L* and b* values was observed. T5 was significantly ($p<0.05$) more effective at delaying lipid oxidation when compared to the other treatment groups. T5 presented TPC that was significantly lower ($p<0.05$) than the other groups after three days of storage. In addition, the use of chitosan and nitrite in combination had much better antioxidant and antimicrobial effectiveness than other treatment groups. In conclusion, this study demonstrates that the addition of 0.5% 200 kDa chitosan and 150 ppm nitrite in combination with emulsified sausages tended to improve antioxidative and antimicrobial effects during storage when compared to other treatment groups.

Accession Number: WOS:000277347400014

ISSN: 1225-8563

Record 14 of 34

Title: Effect of chitosan and thyme oil on a ready to cook chicken product

Author(s): Giatrakou, V (Giatrakou, V.); Ntzimani, A (Ntzimani, A.); Savvaidis, IN (Savvaidis, I. N.)

Source: FOOD MICROBIOLOGY Volume: 27 Issue: 1 Pages: 132-136 DOI: 10.1016/j.fm.2009.09.005 Published: FEB 2010

Times Cited in Web of Science: 4

Total Times Cited: 4

Abstract: The present study examined the effect of natural antimicrobials: chitosan, thyme and their

combination, on the shelf-life of a Ready to Cook (RTC) chicken-pepper kebab (skewer) stored under aerobic conditions at 4 +/- 0.5 degrees C for a period of 12 days. Treatments examined in the present study were the following: A (control samples, untreated), A-CH (chitosan: 1.5% v/w), A-T (thyme essential oil; 0.2% v/w) and A-CH-T (chitosan; 1.5% v/w and thyme essential oil; 0.2% v/w). The shelf-life of the samples was determined using both microbiological and sensory analyses. Among the microorganisms examined, pseudomonads were the most resistant group towards the combined application of chitosan and thyme oil (ca. 1.5 log cycle reduction) while Lactic acid bacteria (LAB), Brochothrix thermosphacta and Enterobacteriaceae were the most sensitive to the combined action of these two agents (2-3 log cycle reduction). Yeasts-moulds were also part of the natural microbial association of the RTC product, with A-CH-T treatment suppressing effectively their growth during the entire period of storage. Treatments A-CH and A-CH-T resulted in lower pH values as compared to the control (A) samples. Of the treatments examined in the present study, A-CH-T, gave a "spicy", desirable and pleasant (organoleptically acceptable) RTC product. Based primarily on sensory data (taste attribute) A-CH, A-T and A-CH-T treatments extended the product's shelf-life by ca. 4 and 6 days, respectively, as compared to the control sample. (C) 2009 Elsevier Ltd. All rights reserved.

Accession Number: WOS:000272436000020

ISSN: 0740-0020

Record 15 of 34

Title: Effects of pH and chitosan on beef emulsion properties

Author(s): Kurt, S (Kurt, Sukru)

Source: INTERNATIONAL JOURNAL OF FOOD SCIENCE AND TECHNOLOGY Volume: 45 Issue: 1 Pages: 140-146 DOI: 10.1111/j.1365-2621.2009.02113.x Published: JAN 2010

Times Cited in Web of Science: 1

Total Times Cited: 1

Abstract: P>Effects of pH (3.75-8.25) and chitosan (0-0.36%) on emulsion capacity (EC), emulsion stability (ES) and apparent yield stress values of emulsion (raw emulsion; AYSe) and emulsion gel (cooked emulsion; AYSg) of beef were studied by using a model system. Chitosan increased ES, AYSe and AYSg values of beef in the acidic medium. However, EC increased with increasing pH and chitosan levels until a critical point, which was determined to be at pH 5.87 and a chitosan level of 0.218%. The interaction effects of chitosan and pH on ES, AYSe and AYSg were also found to be significant ($P < 0.01$).

Accession Number: WOS:000272655500018

ISSN: 0950-5423

Record 16 of 34

Title: Effects of Various Fiber Additions on Lipid Digestion during In Vitro Digestion of Beef Patties

Author(s): Hur, SJ (Hur, S. J.); Lim, BO (Lim, B. O.); Park, GB (Park, G. B.); Joo, ST (Joo, S. T.)

Source: JOURNAL OF FOOD SCIENCE Volume: 74 Issue: 9 Pages: C653-C657 DOI: 10.1111/j.1750-3841.2009.01344.x Published: NOV-DEC 2009

Times Cited in Web of Science: 3

Total Times Cited: 3

Abstract: The purpose of this study was to examine the effect of various fiber additions on lipid digestion during the in vitro digestion of beef patties. The control patties were prepared with 90.5% lean meat and 9.5% tallow. Treatments consisted of 90% lean meat with 9.5% tallow and either 0.5% cellulose, 0.5%

chitosan, or 0.5% pectin. The beef patties were then passed through an in vitro digestion model that simulated the composition of the mouth, stomach, and small intestine juices. The change in structure and properties of the lipid droplets was monitored by laser scanning confocal fluorescence microscopy. In general, there was a decrease in lipid droplet diameter as the droplets moved from mouth to stomach to small intestine. The amount of free fatty acid dramatically increased after in vitro digestion in all beef patties. The amount of free fatty acid was, however, lower in beef patties containing chitosan and pectin than other beef patties after in vitro digestion. Beef patties containing various fibers had lower thiobarbituric acid-reactive substances (TBARS) values than samples with no fibers. Among the samples to which fibers were added, chitosan and pectin had lower TBARS than beef patties with cellulose. The cholesterol content decreased after in vitro digestion in all beef patties but was not different among the beef patties before and after in vitro digestion. These results enhance our understanding of the physicochemical and structural changes that occur to ground beef within the gastrointestinal tract.

Accession Number: WOS:000271959100006

ISSN: 0022-1147

Record 17 of 34

Title: The Combined Effect of Moderate Pressure and Chitosan on Escherichia coli and Staphylococcus aureus Cells Suspended in a Buffer and on Natural Microflora of Apple Juice and Minced Pork

Author(s): Malinowska-Panczyk, E (Malinowska-Panczyk, Edyta); Kolodziejska, I (Kolodziejska, Ilona); Murawska, D (Murawska, Dorota); Wolosewicz, G (Wolosewicz, Gabriel)

Source: FOOD TECHNOLOGY AND BIOTECHNOLOGY Volume: 47 Issue: 2 Pages: 202-209 Published: APR-JUN 2009

Times Cited in Web of Science: 4

Total Times Cited: 4

Abstract: The effect of chitosan and pressure of 193 MPa at -20 degrees C on Escherichia coli and Staphylococcus aureus cells suspended in a buffer at pH=5.8 as well as on the natural microflora of minced pork and apple juice has been evaluated. Immediately after pressure treatment of the tested bacteria in the presence of chitosan, the synergistic antimicrobial effect was higher against E. coli than against S. aureus, which amounted to additional 3.6 and 0.7 log cycles, respectively, compared to either treatment acting alone. However, incubation of S. aureus cells for 20 h at 37 degrees C after pressure treatment in the presence of chitosan led to complete inactivation of these bacteria. The combined effect of moderate pressure and chitosan did not decrease the total bacterial or psychrophilic and psychrotrophic count in minced pork in comparison with meat treated only with one factor, but the growth of psychrophilic and psychrotrophic bacteria was inhibited during storage up to 8 days at 5 degrees C. In apple juice, the combined effect of moderate pressure and chitosan only slightly increased the inactivation of bacterial population. However, during storage of samples at 5 degrees C for 15 days, the total bacterial count was about 1 log cycle lower than after the pressure treatment, while psychrophiles and psychrotrophs were not detected in 1 mL of the samples. The yeasts in apple juice were inactivated after pressure treatment alone. Synergistic reduction of moulds amounted to 2 log cycles and increased during storage at 5 degrees C. After 5 days, moulds were not detected in 1 mL of the samples.

Accession Number: WOS:000267212100012

ISSN: 1330-9862

Record 18 of 34

Title: Edible chitosan films on ready-to-eat roast beef for the control of Listeria monocytogenes

Author(s): Beverlya, RL (Beverlya, Richelle L.); Janes, ME (Janes, Marlene E.); Prinyawlwatkula, W (Prinyawlwatkula, Witoon); No, HK (No, Hong K.)

Source: FOOD MICROBIOLOGY Volume: 25 Issue: 3 Pages: 534-537 DOI: 10.1016/j.fm.2007.11.002 Published: MAY 2008

Times Cited in Web of Science: 20

Total Times Cited: 20

Abstract: The use of chitosan as an edible film was evaluated for its antimicrobial activity against Listeria monocytogenes (LM) on the surface of ready-to-eat (RTE) roast beef. L. monocytogenes, decimally diluted to give an initial inoculation of > 6.50 log CFU/g, was inoculated onto the surface of RTE roast beef cubes, and air-dried. The samples were dipped into chitosan (high or low molecular weights) solutions dissolved with acetic or lactic acid at 0.5% (w/v) or 1% (w/v) then bagged and refrigerated at 4 degrees C. The bacterial counts were determined on days 0, 7, 14, 2 1, and 28. The samples were spread plated onto modified Oxford agar plates and incubated at 37 degrees C for 48 h. An initial 6.50 log CFU/g of L. monocytogenes inoculated onto the surface of the non-coated RTE roast beef increased too > 10 log CFU/g by day 28. On day 14, L. monocytogenes counts were significantly different for all the chitosan-coated samples from the control counts by 2-3 log CFU/g and remained significantly different on day 28. Our results have shown that the acetic acid chitosan coating were more effective in reducing L. monocytogenes counts than the lactic acid chitosan coating. Our study indicated that chitosan coatings could be used to control L. monocytogenes on the surface of RTE roast beef. (C) 2007 Elsevier Ltd. All rights reserved.

Accession Number: WOS:000254994400012

ISSN: 0740-0020

Record 19 of 34

Title: Chitosan and mint mixture: A new preservative for meat and meat products

Author(s): Kanatt, SR (Kanatt, Sweetie R.); Chander, R (Chander, Ramesh); Sharma, A (Sharma, Arun)

Source: FOOD CHEMISTRY Volume: 107 Issue: 2 Pages: 845-852 DOI: 10.1016/j.foodchem.2007.08.088 Published: MAR 15 2008

Times Cited in Web of Science: 22

Total Times Cited: 25

Abstract: Meat is prone to both microbial and oxidative spoilage and therefore it is desirable to use a preservative with both antioxidant and antimicrobial properties. Mint extract alone had good antioxidant activity but poor antimicrobial activity, while chitosan alone showed poor antioxidant activity with excellent antimicrobial properties. Therefore, the potential of chitosan and mint mixture (CM), as a preservative for meat and meat products, was investigated. Addition of chitosan to mint extract did not interfere with the antioxidant activity of mint. In the case of 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, the IC50 value for CM (17.8 mu g/ml) was significantly (p <= 0.05) lower than that for mint extract (23.6 mu g/ml). CM efficiently scavenged superoxide and hydroxyl radicals. The antimicrobial activities of CM and chitosan were comparable against the common food spoilage and pathogenic bacteria, the minimum inhibitory concentration being 0.05%. CM was more effective against Gram-positive bacteria. The shelf life of pork cocktail salami, as determined by total bacterial count and oxidative rancidity, was enhanced in CM-treated samples stored at 0-3 degrees C. (C) 2007 Elsevier Ltd. All rights reserved.

Accession Number: WOS:000251760400033

ISSN: 0308-8146

Record 20 of 34

Title: Control of Listeria monocytogenes on ham steaks by antimicrobials incorporated into chitosan-coated plastic films

Author(s): Ye, M (Ye, Mu); Neetoo, H (Neetoo, Hudaa); Chen, H (Chen, Haiqiang)

Source: FOOD MICROBIOLOGY Volume: 25 Issue: 2 Pages: 260-268 DOI: 10.1016/j.fm.2007.10.014 Published: FEB 2008

Times Cited in Web of Science: 26

Total Times Cited: 26

Abstract: Contamination of ready-to-eat (RTE) meat products such as ham steaks with Listeria monocytogenes has been a concern for the meat processing industry. The objective of this study was to evaluate the antilisterial efficacy of chitosan-coated plastic films alone or incorporating five generally recognized as safe (GRAS) antimicrobials. Effect of chitosan-coated plastic film on the growth of L. monocytogenes was first investigated in an aqueous system of culture medium broth and chitosan-coated films were able to inhibit the growth of L. monocytogenes in a concentration-dependent manner. However, chitosan-coated plastic films were not able to control the growth of L. monocytogenes on ham steaks. Therefore, five GRAS antimicrobials were subsequently incorporated into chitosan-coated plastic films to enhance their antilisterial effectiveness. Ham steaks were surface-inoculated with a five-strain cocktail of L. monocytogenes and then packaged in chitosan-coated plastic films containing 500 IU/cm(2) of nisin, 0.01 g/cm(2) of sodium lactate (SL), 0.0025 g/cm(2) of sodium diacetate, 0.003 g/cm(2) of potassium sorbate (PB), or 0.001 g/cm(2) of sodium benzoate (SB). The samples were stored at room temperature (ca. 20 degrees C) for 10 days. Incorporating antimicrobials into chitosan-coated plastic films slowed down or inhibited the growth of L. monocytogenes. The chitosan-coated plastic film containing SL was the most effective antimicrobial film and its efficacy against L. monocytogenes on ham steaks was evaluated during 12-week storage at 4 degrees C. The film showed excellent long-term antilisterial effect with the counts of L. monocytogenes being slightly lower than the initial inoculum. Chitosan-coated plastic films containing 0.001 g/cm(2) of SL have a potential to be used on ham steaks to control L. monocytogenes. (C) 2007 Elsevier Ltd. All rights reserved.

Accession Number: WOS:000252988700006

ISSN: 0740-0020

Record 21 of 34

Title: Applications of chitosan for improvement of quality and shelf life of foods: A review

Author(s): No, HK (No, H. K.); Meyers, SP (Meyers, S. P.); Prinyawiwatkul, W (Prinyawiwatkul, W.); Xu, Z (Xu, Z.)

Source: JOURNAL OF FOOD SCIENCE Volume: 72 Issue: 5 Pages: R87-R100 DOI: 10.1111/j.1750-3841.2007.00383.x Published: JUN-JUL 2007

Times Cited in Web of Science: 50

Total Times Cited: 52

Abstract: Chitosan is a modified, natural biopolymer derived by deacetylation of chitin, a major component of the shells of crustacean. Recently, chitosan has received increased attention for its commercial applications in the biomedical, food, and chemical industries. Use of chitosan in food industry is readily seen due to its several distinctive biological activities and functional properties. The antimicrobial activity and film-forming property of chitosan make it a potential source of food preservative or coating material of natural origin. This review focuses on the applications of chitosan for improvement of quality of various foods from agriculture, poultry, and seafood origin.

Accession Number: WOS:000247780200006
ISSN: 0022-1147

Record 22 of 34

Title: Effect of rosemary extract, chitosan and alpha-tocopherol on microbiological parameters and lipid oxidation of fresh pork sausages stored at 4 degrees C

Author(s): Georgantelis, D (Georgantelis, Dimitrios); Ambrosiadis, I (Ambrosiadis, Ioannis); Katikou, P (Katikou, Panagiota); Blekas, G (Blekas, Georgios); Georgakis, PA (Georgakis, Spyridon A.)

Source: MEAT SCIENCE Volume: 76 Issue: 1 Pages: 172-181 DOI: 10.1016/j.meatsci.2006.10.026 Published: MAY 2007

Times Cited in Web of Science: 32

Total Times Cited: 35

Abstract: The effect of rosemary extract, chitosan and alpha-tocopherol, added individually or in combination, on microbiological parameters and lipid oxidation of fresh pork sausages stored for 20 days at 4 degrees C was investigated. Microbiological determinations included the enumeration of Enterobacteriaceae, Pseudomonas spp., total viable count, yeasts and moulds and lactic acid bacteria, while pH values were also determined. Lipid oxidation was evaluated through measurement of primary (peroxide value) and secondary (malondialdehyde concentration) oxidation products. Chitosan addition resulted in significant ($P < 0.05$) inhibition of microbial growth, while the lowest microbial counts were obtained in the samples containing both chitosan and rosemary, indicating a possible synergistic effect. Chitosan and its combinations with either alpha-tocopherol or rosemary, and especially the latter combination, showed also the most intense antioxidative effect ($P <= 0.05$), at least when compared to alpha-tocopherol alone or the controls, while use of rosemary extract alone had a comparable ($P > 0.05$) antioxidative effect with chitosan and its combinations. Shelf life of samples containing chitosan was almost doubled compared to the remaining samples. In conclusion, the best antimicrobial and antioxidative effects were obtained from the combination of chitosan with the rosemary extract. (c) 2006 Elsevier Ltd. All rights reserved.

Accession Number: WOS:000245260300020
ISSN: 0309-1740

Record 23 of 34

Title: Effect of rosemary extract, chitosan and alpha-tocopherol on lipid oxidation and colour stability during frozen storage of beef burgers

Author(s): Georgantelis, D (Georgantelis, Dimitrios); Blekas, G (Blekas, Georglos); Katikou, P (Katikou, Panagiota); Ambrosiadis, I (Ambrosiadis, Ioannis); Fletouris, DJ (Fletouris, Dimitrios J.)

Source: MEAT SCIENCE Volume: 75 Issue: 2 Pages: 256-264 DOI: 10.1016/j.meatsci.2006.07.018 Published: FEB 2007

Times Cited in Web of Science: 27

Total Times Cited: 32

Abstract: The effect of rosemary extract, chitosan and a-tocopherol, added individually or in combination, on lipid oxidation and colour stability of frozen (-18 degrees C) beef burgers stored for 180 days was investigated. The burgers' lipid oxidation and appearance were evaluated through measurement of primary (conjugated dienes and peroxide value) and secondary (malondialdehyde) oxidation products, together with visual assessment and instrumental measurement of colour. Chitosan alone and in combination with either rosemary or alpha-tocopherol had the best antioxidative effect ($P < 0.05$) compared to individual use of rosemary or alpha-tocopherol, while the best results were obtained with the combination of chitosan and

rosemary. The differences of antioxidative effects, however, between individual use of rosemary or alpha-tocopherol as compared to the controls were also significant (P < 0.05). Chitosan added individually or in combination with either rosemary or alpha-tocopherol had also a noteworthy effect on the burgers' appearance as it contributed to red colour retention for a much longer period (P < 0.05) compared all other treatments and the controls. In conclusion, the best antioxidative effects were obtained with the combination of chitosan and rosemary extract. (c) 2006 Elsevier Ltd. All rights reserved.

Accession Number: WOS:000242776500009

ISSN: 0309-1740

Record 24 of 34

Title: Chitosan protects cooked ground beef and turkey against Clostridium perfringens spores during chilling

Author(s): Juneja, VK (Juneja, Viiay K.); Thippareddi, H (Thippareddi, Harsnavardhan); Bari, L (Bari, Latiful); Inatsu, Y (Inatsu, Yasuhiro); Kawamoto, S (Kawamoto, Shinichi); Friedman, M (Friedman, Mendel)

Source: JOURNAL OF FOOD SCIENCE Volume: 71 Issue: 6 Pages: M236-M240 DOI: 10.1111/j.1750-3841.2006.00109.x Published: AUG 2006

Times Cited in Web of Science: 11

Total Times Cited: 11

Abstract: We investigated the inhibition of Clostridium perfringens spore germination and outgrowth by the biopolymer chitosan during abusive chilling of cooked ground beef (25% fat) and turkey (7% fat) obtained from a retail store. Chitosan was mixed into the thawed beef or turkey at concentrations of 0.5%, 1.0%, 2.0%, or 3.0% (w/w) along with a heat-activated 3-strain spore cocktail to obtain a final spore concentration of 2 to 3 log(10) CFU/g. Samples (5 g) of the ground beef or turkey mixtures were then vacuum-packaged and cooked to 60 degrees C in 1 h in an temperature-controlled water bath. Thereafter, the products were cooled from 54.4 to 7.2 degrees C in 12, 15, 18, or 21 h, resulting in 4.21, 4.51, 5.03, and 4.70 log(10) CFU/g increases, respectively, in C.perfringens populations in the ground beef control samples without chitosan. The corresponding increases for ground turkey were 5.27, 4.52, 5.11, and 5.38 log(10) CFU/g. Addition of chitosan to beef or turkey resulted in concentration- and time-dependent inhibition in the C.perfringens spore germination and outgrowth. At 3%, chitosan reduced by 4 to 5 log(10) CFU/g C.perfringens spore germination and outgrowth (P <= 0.05) during exponential cooling of the cooked beef or turkey in 12, 15, or 18 h. The reduction was significantly lower (P < 0.05) at a chilling time of 21 h, about 2 log(10) CFU/g, that is, 7.65 log(10) CFU/g (unsupplemented) compared with 5.59 log(10) CFU/g (3% chitosan). The results suggest that incorporation of 3% chitosan into ground beef or turkey may reduce the potential risk of C.perfringens spore germination and outgrowth during abusive cooling from 54.4 to 7.2 degrees C in 12, 15, or 18 h.

Accession Number: WOS:000240286800033

ISSN: 0022-1147

Record 25 of 34

Title: Effect of chitosan coating and vacuum packaging on the quality of refrigerated grilled pork

Author(s): Yingyuad, S (Yingyuad, Songchai); Ruamsin, S (Ruamsin, Sompoch); Reekprkhon, D (Reekprkhon, Dhaweerat); Douglas, S (Douglas, Supaporn); Pongamphai, S (Pongamphai, Suwassa); Siripatrawan, U (Siripatrawan, Ubonrat)

Source: PACKAGING TECHNOLOGY AND SCIENCE Volume: 19 Issue: 3 Pages: 149-157 DOI:

10.1002/pts.717 Published: MAY-JUN 2006

Times Cited in Web of Science: 10

Total Times Cited: 10

Abstract: The effects of chitosan coating and vacuum packaging on the quality and shelf-life of retail packaged grilled pork in a PVDC/nylon pouch during refrigerated storage at 2 +/- 1 degrees C were investigated. Grilled pork without chitosan coating and packaged in 100% air was used as the control sample. Firmness, pH, colour, peroxide values, microbial population and shelf-life (as determined by sensory characteristics) of the samples were determined during the storage. The results showed that colour, peroxide values and microbial population coincided with sensory characteristics. The delayed growth of microbes in vacuum packaging can be attributed to inhibition by low levels of O-2. Chitosan coating was shown to minimize oxidation, as reflected by the peroxide values, colour changes and microbial counts of the samples. Vacuum-packaged chitosan-coated grilled pork showed negligible microbial growth and was found to be organoleptically acceptable throughout the storage period. Chitosan coating along with vacuum packaging provided a type of active packaging to maintain quality and extend the shelf-life of grilled pork. Copyright (C) 2006 John Wiley & Sons, Ltd.

Accession Number: WOS:000238917700003

ISSN: 0894-3214

Record 26 of 34

Title: Application of chitosan films enriched with oregano essential oil on Bologna - Active compounds and sensory attributes

Author(s): Chi, S (Chi, S); Zivanovic, S (Zivanovic, S); Penfield, MP (Penfield, MP)

Source: FOOD SCIENCE AND TECHNOLOGY INTERNATIONAL Volume: 12 Issue: 2 Pages: 111-117 DOI: 10.1177/1082013206063845 Published: APR 2006

Times Cited in Web of Science: 14

Total Times Cited: 15

Abstract: Chitosan films prepared with oregano essential oil were applied on bologna slices. Release of the essential oil compounds during film preparation and application on the meat product and consumer acceptability of bologna enriched with oregano essential oil were tested. Oregano essential oil compounds were quantified by gas chromatography mass spectroscopy (GCMS) after extraction from the film-forming solution, films before and after application on bologna and from bologna slices before and after application of the films. The results indicated that the concentration of components of the essential oil sharply decreased during film preparation, e.g. from 757.7 ppm carvacrol in film-forming solution to 2.1 ppm in dried films. No carvacrol was detected in the films after application on bologna for 5 days at 4 degrees C, mainly due to its diffusion into bologna. It seemed that the moisture and high lipid content of bologna helped the diffusion of the oregano essential oil from the chitosan film matrix into the product. Sensory evaluation suggested that addition of 45 ppm or less of oregano oil to bologna would be acceptable to consumers. Results support the potential use of chitosan-oregano essential oil films as an antimicrobial packaging material for processed meat.

Accession Number: WOS:000236542200003

ISSN: 1082-0132

Record 27 of 34

Title: Effectiveness of antimicrobial food packaging materials

Author(s): Cooksey, K (Cooksey, K)

Source: FOOD ADDITIVES AND CONTAMINANTS Volume: 22 Issue: 10 Pages: 980-987 DOI: 10.1080/02652030500246164 Published: OCT 2005

Times Cited in Web of Science: 31

Total Times Cited: 32

Abstract: Antimicrobial additives have been used successfully for many years as direct food additives. The literature provides evidence that some of these additives may be effective as indirect food additives incorporated into food packaging materials. Antimicrobial food packaging is directed toward the reduction of surface contamination of processed, prepared foods such as sliced meats and Frankfurter sausages (hot dogs). The use of such packaging materials is not meant to be a substitute for good sanitation practices, but it should enhance the safety of food as an additional hurdle for the growth of pathogenic and/or spoilage microorganisms. Studies have focused on establishing methods for coating low-density polyethylene film or barrier films with methyl cellulose as a carrier for nisin. These films have significantly reduced the presence of Listeria monocytogenes in solutions and in vacuum packaged hot dogs. Other research has focused on the use of chitosan to inhibit L. monocytogenes and chlorine dioxide sachets for the reduction of Salmonella on modified atmosphere-packaged fresh chicken breasts. Overall, antimicrobial packaging shows promise as an effective method for the inhibition of certain bacteria in foods, but barriers to their commercial implementation continue to exist.

Accession Number: WOS:000233295600010

Conference Title: 3rd International Symposium on Food Packaging - Ensuring the Safety, Quality and Traceability of Foods

Conference Date: NOV 17-19, 2005

Conference Location: Barcelona, SPAIN

ISSN: 0265-203X

Record 28 of 34

Title: Development of shelf-stable intermediate-moisture meat products using active edible chitosancoating and irradiation

Author(s): Rao, MS (Rao, MS); Chander, R (Chander, R); Sharma, A (Sharma, A)

Source: JOURNAL OF FOOD SCIENCE Volume: 70 Issue: 7 Pages: M325-M331 DOI: 10.1111/j.1365-2621.2005.tb11475.x Published: SEP 2005

Times Cited in Web of Science: 6

Total Times Cited: 6

Abstract: Shelf-stable intermediate-moisture (IM) meat products were developed using a combination of hurdles such as reduced a(w), active edible coating of chitosan, and irradiation. Chitosan prepared from chitin had a viscosity of 16 cP, molecular Weight of 17.54 kDa, and a degree of deacetylation (DD) of 74%. The nitrogen content of the chitosan was estimated to be 7.56%. The antioxidant activity of chitosan increased upon irradiation without significantly affecting its antimicrobial property. The effect of irradiated chitosan coating in terms of its antimicrobial and antioxidant properties in IM meat products immediately after irradiation and during storage was assessed. The a(w) of meat products such as mutton sheek kababs and streaky bacon was first reduced to 0.85 +/- 0.02. The products were then coated with chitosan and irradiated (4 kGy). No viable bacteria or fungi were detected in chitosan-coated, irradiated products. In contrast, IM meat products that were not subjected to gamma radiation showed visible fungal growth within 2 wk. The chitosan-coated products showed lower thiobarbituric acid-reactive substances (TBARS) than the noncoated samples for up to 4 wk of storage at ambient temperature. The studies thus clearly indicated the potential use of chitosan coating for the preparation of safe and stable meat products.

Accession Number: WOS:000232216900013
ISSN: 0022-1147

Record 29 of 34

Title: Effect of irradiated chitosan on the rancidity of radiation-processed lamb meat

Author(s): Kanatt, SR (Kanatt, SR); Chander, R (Chander, R); Sharma, A (Sharma, A)

Source: INTERNATIONAL JOURNAL OF FOOD SCIENCE AND TECHNOLOGY Volume: 39 Issue: 9 Pages: 997-1003 DOI: 10.1111/j.1365-2621.2004.00868.x Published: NOV 2004

Times Cited in Web of Science: 13

Total Times Cited: 14

Abstract: We report here the production of irradiated chitosan and its novel use as a natural antioxidant for minimising lipid peroxidation of radiation-processed lamb meat. Antioxidant activity of chitosan isolated from shrimp waste was determined by the beta-carotene bleaching assay and 1,1-Diphenyl-2-picrylhydrazyl (DPPH) scavenging activity. Irradiation of chitosan at 25 kGy dose of gamma radiation resulted in a six-fold increase in its antioxidant activity as compared to the non-irradiated chitosan as measured by DPPH assay. Similarly the reducing power of irradiated chitosan was 6-fold greater than autoclaved chitosan. The suitability of irradiated chitosan for controlling lipid oxidation of radiation-processed meat was also investigated. Irradiated chitosan when added to meat before radiation processing was found to be more effective in minimising lipid perioxidation than non-irradiated chitosan as measured by TBA number and carbonyl content. TBA values of irradiated meat containing irradiated chitosan decreased by 88% (leg) and 54% (rib) as compared to the corresponding sample without chitosan. Further development of post-processing rancidity was reduced by 39% (leg) and 59% (rib) in the samples treated with chitosan as compared to non-treated controls even after a week of storage at 0-3degreesC.

Accession Number: WOS:000225025600011
ISSN: 0950-5423

Record 30 of 34

Title: Antimicrobial edible films and coatings

Author(s): Cagri, A (Cagri, A); Ustunol, Z (Ustunol, Z); Ryser, ET (Ryser, ET)

Source: JOURNAL OF FOOD PROTECTION Volume: 67 Issue: 4 Pages: 833-848 Published: APR 2004

Times Cited in Web of Science: 118

Total Times Cited: 127

Abstract: Increasing consumer demand for microbiologically safer foods, greater convenience, smaller packages, and longer product shelf life is forcing the industry to develop new food-processing, cooking, handling, and packaging strategies. Nonfluid ready-to-eat foods are frequently exposed to postprocess surface contamination, leading to a reduction in shelf life. The food industry has at its disposal a wide range of nonedible polypropylene- and polyethylene-based packaging materials and various biodegradable protein- and polysaccharide-based edible films that can potentially serve as packaging materials. Research on the use of edible films as packaging materials continues because of the potential for these films to enhance food quality, food safety, and product shelf life. Besides acting as a barrier against mass diffusion (moisture, gases, and volatiles), edible films can serve as carriers for a wide range of food additives, including flavoring agents, antioxidants, vitamins, and colorants. When antimicrobial agents such as benzoic acid, sorbic acid, propionic acid, lactic acid, nisin, and lysozyme have been incorporated into edible films, such films retarded surface growth of bacteria, yeasts, and molds on a wide range of products, including meats and cheeses. Various antimicrobial edible films have been developed to minimize growth of spoilage and pathogenic

microorganisms, including Listeria monocytogenes, which may contaminate the surface of cooked ready-to-eat foods after processing. Here, we review the various types of protein-based (wheat gluten, collagen, corn zein, soy, casein, and whey protein), polysaccharide-based (cellulose, chitosan, alginate, starch, pectin, and dextrin), and lipid-based (waxes, acylglycerols, and fatty acids) edible films and a wide range of antimicrobial agents that have been or could potentially be incorporated into such films during manufacture to enhance the safety and shelf life of ready-to-eat foods.

Accession Number: WOS:000220705800030

ISSN: 0362-028X

Record 31 of 34

Title: Novel combinations of chitosan, carnocin and sulphite for the preservation of chilled pork sausages

Author(s): Roller, S (Roller, S); Sagoo, S (Sagoo, S); Board, R (Board, R); O'Mahony, T (O'Mahony, T); Caplice, E (Caplice, E); Fitzgerald, G (Fitzgerald, G); Fogden, M (Fogden, M); Owen, M (Owen, M); Fletcher, H (Fletcher, H)

Source: MEAT SCIENCE Volume: 62 Issue: 2 Pages: 165-177 Article Number: DOI: 10.1016/S0309-1740(01)00243-1 Published: OCT 2002

Times Cited in Web of Science: 28

Total Times Cited: 33

Abstract: The aim of this study was to develop novel preservation systems for fresh pork sausages based on combinations of chitosan (polymeric beta-1,4-N-acetylglucosamine) carnocin (a bacteriocin produced by Carnobacterium piscicola) and low concentrations of sulphite. Two pilot-scale trials showed that 0.6% chitosan combined with low sulphite (170 ppm) retarded the growth of spoilage organisms more effectively (3-4 log cfu/g) than high levels (340 ppm) of sulphite alone at 4 C for up to 24 days. Microbial counts for frozen sausages showed that the preservative efficacy of the chitosan/sulphite combination was maintained following frozen storage. Carnocin did not protect sausages from spoilage but in a challenge trial, it reduced the numbers of Listeria innocua by up to 2.0 log cfu/g in the first 5 days of chill-storage. Sulphite was degraded rapidly within the first 3 days of storage in all the sausages that contained only this preservative but levels decreased less rapidly and persisted for longer in the presence of chitosan. Results of Quantitative Descriptive Analysis using 31 trained panellists reflected the gradual deterioration of all the sausages during storage. The batch containing chitosan and sulphite deteriorated less rapidly and was judged to be more acceptable for a longer period than all the other batches. (C) 2002 Elsevier Science Ltd. All rights reserved.

Accession Number: WOS:000177493500004

ISSN: 0309-1740

Record 32 of 34

Title: Quality characteristics of reduced-fat Chinese-style sausage as related to chitosan's molecular weight

Author(s): Lin, KW (Lin, KW); Chao, JY (Chao, JY)

Source: MEAT SCIENCE Volume: 59 Issue: 4 Pages: 343-351 DOI: 10.1016/S0309-1740(01)00084-5 Published: DEC 2001

Times Cited in Web of Science: 24

Total Times Cited: 31

Abstract: Microbiological and physicochemical characteristics of reduced-fat (approximately 22%) Chinese-style sausages containing 0.1% chitosan (in 1% lactic acid solution) with three different molecular weights [low molecular weight chitosan (CHL), MW = 150 kDa; medium molecular weight chitosan (CHM), MW =

600 kDa; high molecular weight chitosan (CHH), MW = 1250 kDa] were demonstrated. Sausages were also subject to oven heating or deep-fat frying for sensory evaluation. Treatments containing chitosan were lower in pH at any storage periods than the control (CONT). Generally speaking, total plate counts and lactic acid bacteria counts of all treatments slightly increased, with CHL being the lowest. However, all bacterial counts were lower than 7 [log colony forming unit (CFU)/g] at the end of storage. Results from sensory evaluation showed that deep-fat fried sausages from treatments CHL and CHM were higher in overall acceptability. This study demonstrated that the addition of chitosan to reduced-fat Chinese-style sausage resulted in no detrimental effect on textural properties. (C) 2001 Elsevier Science Ltd. All rights reserved.

Accession Number: WOS:000171504700001

ISSN: 0309-1740

Record 33 of 34

Title: Inhibition of surface spoilage bacteria in processed meats by application of antimicrobial films prepared with chitosan

Author(s): Ouattara, B (Ouattara, B); Simard, RE (Simard, RE); Piette, G (Piette, G); Begin, A (Begin, A); Holley, RA (Holley, RA)

Source: INTERNATIONAL JOURNAL OF FOOD MICROBIOLOGY Volume: 62 Issue: 1-2 Pages: 139-148 DOI: 10.1016/S0168-1605(00)00407-4 Published: DEC 5 2000

Times Cited in Web of Science: 127

Total Times Cited: 134

Abstract: A 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, and were applied onto bologna, regular cooked ham, or pastrami. At various times during storage, packages were opened and the amounts of antimicrobial agents remaining in the chitosan matrix were measured. Regardless of film composition or meat product type, 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 h of storage. Addition of lauric acid, but not cinnamaldehyde, to the chitosan matrix generally reduced the release of acetic acid significantly (P less than or equal to 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 bacterial growth were tested against indigenous lactic acid bacteria and Enterobacteriaceae, and against Lactobacillus sakei or Serratia liquefaciens, surface-inoculated onto the meat products. 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. Strongest inhibition was observed on drier surfaces (bologna), onto which acid release was slower, and with films containing cinnamaldehyde, as a result of its greater antimicrobial activity under these conditions. (C) 2000 Elsevier Science B.V. All rights reserved.

Accession Number: WOS:000165915300016

ISSN: 0168-1605

Record 34 of 34

Title: EFFECT OF CHITOSAN IN MEAT PRESERVATION

Author(s): DARMADJI, P (DARMADJI, P); IZUMIMOTO, M (IZUMIMOTO, M)

Source: MEAT SCIENCE Volume: 38 Issue: 2 Pages: 243-254 DOI: 10.1016/0309-1740(94)90114-7

Published: 1994

Times Cited in Web of Science: 87

Total Times Cited: 99

Abstract: The effect of chitosan in meat preservation, including microbiological, chemical, sensory and color qualities, was examined. In liquid medium, chitosan (0.01%) inhibited the growth of some spoilage bacteria such as Bacillus subtilis IFO 3025, Escherichia coli RB, Pseudomonas fragi IFO 3458 and Staphylococcus aureus IAM 1011. At higher concentrations (0.1 and 1.0%) it inhibited the growth of the meat starter cultures, Lactobacillus plantarium IAM 1216, Pediococcus pentosaceus IAM 12296 and Micrococcus varians IFO 3765. In meat, during incubation at 30-degrees-C for 48 h or storage at 4-degrees-C for 10 days, 0.5-1.0% chitosan inhibited the growth of spoilage bacteria, reduced lipid oxidation and putrefaction, and resulted in better sensory attributes. Chitoosan also had a good effect on the development of the red color of meat during storage.

Accession Number: WOS:A1994NZ44300010

ISSN: 0309-1740

APPENDIX F-2

Record 1 of 10

Title: Partial substitution of nitrite by chitosan and the effect on the quality properties of pork sausages

Author(s): Garcia, M (Garcia, Mario); Beldarrain, T (Beldarrain, Tatiana); Fornaris, L (Fornaris, Leonid); Diaz, R (Diaz, Raul)

Source: CIENCIA E TECNOLOGIA DE ALIMENTOS **Volume:** 31 **Issue:** 2 **Pages:** 481-487 **Published:** APR-JUN 2011

Times Cited in Web of Science: 0

Total Times Cited: 0

Abstract: The aim of the study was to evaluate the influence of partial nitrite replacement by chitosan on the quality of Ham Visking (a type of pork sausages). Five Ham Visking formulations were elaborated modifying the sodium nitrite (0.011; 0.016 or 0.0212%) and chitosan concentrations (0.25 or 0.5%) in the products. Sausages were stored at 4 degrees C and physicochemical, microbiological, and sensorial evaluations were performed in order to estimate their shelf life. Chitosan can be used in pork sausages without affecting ensory attributes such as color although the panelists detected textural differences among the samples with chitosan, which suggests that there is some influence of deacetylation degree of chitosan on the textural behavior of sausages which still needed to be explained for a successful application of chitosan in meat products. The reduction of residual sodium nitrite did not affect the color and flavor of such products, but the use of chitosan increasedsignificantly the shelf life of sausages.

Accession Number: WOS:000293654300031

ISSN: 0101-2061

Record 2 of 10

Title: Use of Chitosan in Turkish Sausage (Sucuk) Production and Effects on Quality

Author(s): Gokmen, M (Gokmen, Mukadderat); Gurbuz, U (Gurbuz, Umit)

Source: KAFKAS UNIVERSITESI VETERINER FAKULTESI DERGISI **Volume:** 17 **Pages:** S67-S71 **Supplement:** A **Published:** MAR-APR 2011

Times Cited in Web of Science: 0

Total Times Cited: 0

Abstract: This study aims to investigate the effect of chitosan, natural polysaccharide, use in different proportions (0.05%, 0.1%, 0.5% and 1%) on the quality of Turkish sausage production. In the study, as a control group, the first group was added only 0.05% nitrate. Microbiological analysis (total aerobic mesophilic bacteria, Enterobacteriaceae, coliform and Escherichia coli, sulphite-reducing clostridia, mold-yeast count) was done in the four different stages of experimental sausage production, (meat [DN(1)], after mixing [DN(2)], after filling [DN(3)], after ripening [DN(4)]) and on the 1, 7, 15, 30 and 60 days of the storage. Sensory qualities of experimental sausage samples (flavor, color, appearance and texture) were evaluated in the DN4. It was then determined that a little amount of chitosan addition (0.05%, 0.1% and 0.5%) into the production of Turkish sausage affected the microbiological and sensory quality positively. However, addition of much larger amounts (such as 1%) affected the sensory quality in a negative way. Moreover, it was determined that higher amounts of chitosan applications (0.5% and 1%) created technological problems.

Accession Number: WOS:000290955200012

ISSN: 1300-6045

Record 3 of 10

Title: Sensory evaluation and inhibition of Listeria monocytogenes in bovine pate added of chitosan from Mucor rouxii

Author(s): Bento, RA (Bento, Roberta Albuquerque); Stamford, TLM (Montenegro Stamford, Tania Lucia); Stamford, TCM (Montenegro Stamford, Thayza Christina); de Andrade, SAC (Cardoso de Andrade, Samara Alvachian); de Souza, EL (de Souza, Evandro Leite)

Source: LWT-FOOD SCIENCE AND TECHNOLOGY **Volume:** 44 **Issue:** 2 **Pages:** 588-591 **DOI:** 10.1016/j.lwt.2010.08.016 **Published:** MAR 2011

Times Cited in Web of Science: 1

Total Times Cited: 1

Abstract: This study aimed to assess the efficacy of chitosan from Mucor rouxii UCP 064 in inhibiting Listeria monocytogenes in bovine meat pate at 4 degrees C The influence of the chitosan addition on sensory aspects of the product was also evaluated The addition of chitosan from M rouxii at 5 mg/g to bovine meat pate decreased the counts of L monocytogenes from approximately 7 to 3 log cfu/g after 6 days of storage at 4 C In pate without chitosan the counts were over 7 log cfu/g already after two days of storage Sensory evaluation suggested that addition of chitosan in pate would be acceptable to consumers although some negative influence on flavor and taste was found From these results chitosan from fungi could be considered a possible alternative compound to control L monocytogenes in ready-to-eat meat products (C) 2010 Elsevier Ltd All rights reserved

Accession Number: WOS:000284723800033

ISSN: 0023-6438

Record 4 of 10

Title: Combined Chitosan-Thyme Treatments with Modified Atmosphere Packaging on a Ready-to-Cook Poultry Product

Author(s): Giatrakou, V (Giatrakou, V.); Ntzimani, A (Ntzimani, A.); Savvaidis, IN (Savvaidis, I. N.)

Source: JOURNAL OF FOOD PROTECTION **Volume:** 73 **Issue:** 4 **Pages:** 663-669 **Published:** APR 2010

Times Cited in Web of Science: 0

Total Times Cited: 0

Abstract: In the present study, natural antimicrobials chitosan and thyme, and their combination, were evaluated for their effect on the shelf life of a ready-to-cook (RTC) chicken-pepper kebab (skewer) stored under modified atmosphere packaging (MAP) conditions at 4 +/- 0.5 degrees C for 14 days. The following treatments were examined: control samples stored under aerobic packaging (A), samples stored under MAP (M), samples treated with 1.5% chitosan (vol/wt) and stored under MAP (M-CH), samples treated with 0.2% thyme essential oil (vol/wt) (M-T), and samples treated with 1.5% chitosan (vol/wt) and 0.2% thyme essential oil (vol/wt) and stored under MAP (M-CH-T). Treatment M-CH-T significantly affected aerobic plate counts and counts of lactic acid bacteria. Pseudomonas spp., Brochothrix thermosphacta, Enterobacteriaceae, and yeasts and molds during the entire storage period. Similarly, lipid oxidation of the RTC product was retarded (M-CH-T treatment) during storage, whereas redness was maintained in M-T. M-CH, and M-CH-T samples. Based primarily on sensory data (taste attribute), M-CH and M-T treatments extended RTC product shelf life by 6 clays, whereas M-CH-T treatment resulted in a product with a shelf life of 14 days that maintained acceptable sensory characteristics (shelf life of the control was 6 days).

Accession Number: WOS:000276417100007

ISSN: 0362-028X

Record 5 of 10

Title: Influence of chitosan addition on quality properties of vacuum-packaged pork sausages

Author(s): Garcia, M (Garcia, Mario); Diaz, R (Diaz, Raul); Puerta, F (Puerta, Felicidad); Beldarrain, T (Beldarrain, Tatiana); Gonzalez, J (Gonzalez, Juan); Gonzalez, I (Gonzalez, Iris)

Source: CIENCIA E TECNOLOGIA DE ALIMENTOS **Volume:** 30 **Issue:** 2 **Pages:** 560-564 **DOI:** 10.1590/S0101-20612010000200041 **Published:** APR-JUN 2010

Times Cited in Web of Science: 0

Total Times Cited: 0

Abstract: The aim of the study was to evaluate the influence of the chitosan addition on the quality of vacuum packaged pork sausages. A variant of the product was elaborated with 1% (w/w) of chitosan in lactic acid solution at 1% (v/v) and it was compared to a control. Sausages were mechanically stuffed and manually conformed and vacuum packaged. Sausages were stored at 4 degrees C and microbiological evaluations, pH measurements, texture profile analysis and sensorial evaluation were performed. The chitosan addition in the formulation of the sausages did not reduce the microbiological counts. The pH values obtained in all samples were similar, which suggests that the chitosan addition did not influence the pH values of sausages. The added chitosan did not affect significantly ($p <= 0.05$) the results of the texture profile analysis and sensorial attributes and therefore, the overall acceptance of the sausages.

Accession Number: WOS:000280706600041

ISSN: 0101-2061

Record 6 of 10

Title: Effect of chitosan and thyme oil on a ready to cook chicken product

Author(s): Giatrakou, V (Giatrakou, V.); Ntzimani, A (Ntzimani, A.); Savvaidis, IN (Savvaidis, I. N.)

Source: FOOD MICROBIOLOGY **Volume:** 27 **Issue:** 1 **Pages:** 132-136 **DOI:** 10.1016/j.fm.2009.09.005 **Published:** FEB 2010

Times Cited in Web of Science: 4

Total Times Cited: 4

Abstract: The present study examined the effect of natural antimicrobials: chitosan, thyme and their combination, on the shelf-life of a Ready to Cook (RTC) chicken-pepper kebab (skewer) stored under aerobic conditions at 4 +/- 0.5 degrees C for a period of 12 days. Treatments examined in the present study were the following: A (control samples, untreated), A-CH (chitosan: 1.5% v/w), A-T (thyme essential oil; 0.2% v/w) and A-CH-T (chitosan; 1.5% v/w and thyme essential oil; 0.2% v/w). The shelf-life of the samples was determined using both microbiological and sensory analyses. Among the microorganisms examined, pseudomonads were the most resistant group towards the combined application of chitosan and thyme oil (ca. 1.5 log cycle reduction) while Lactic acid bacteria (LAB), Brochothrix thermosphacta and Enterobacteriaceae were the most sensitive to the combined action of these two agents (2-3 log cycle reduction). Yeasts-moulds were also part of the natural microbial association of the RTC product, with A-CH-T treatment suppressing effectively their growth during the entire period of storage. Treatments A-CH and A-CH-T resulted in lower pH values as compared to the control (A) samples. Of the treatments examined in the present study, A-CH-T, gave a "spicy", desirable and pleasant (organoleptically acceptable) RTC product. Based primarily on sensory data (taste attribute) A-CH, A-T and A-CH-T treatments extended the product's shelf-life by ca. 4 and 6 days, respectively, as compared to the control sample. (C) 2009 Elsevier Ltd. All rights reserved.

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ISSN: 0740-0020

Record 7 of 10

Title: Effect of chitosan coating and vacuum packaging on the quality of refrigerated grilled pork

Author(s): Yingyuad, S (Yingyuad, Songchai); Ruamsin, S (Ruamsin, Sompoch); Reekprkhon, D (Reekprkhon, Dhaweerat); Douglas, S (Douglas, Supaporn); Pongamphai, S (Pongamphai, Suwassa); Siripatrawan, U (Siripatrawan, Ubonrat)

Source: PACKAGING TECHNOLOGY AND SCIENCE **Volume:** 19 **Issue:** 3 **Pages:** 149-157 **DOI:** 10.1002/pts.717 **Published:** MAY-JUN 2006

Times Cited in Web of Science: 10

Total Times Cited: 10

Abstract: The effects of chitosan coating and vacuum packaging on the quality and shelf-life of retail packaged grilled pork in a PVDC/nylon pouch during refrigerated storage at 2 +/- 1 degrees C were investigated. Grilled pork without chitosan coating and packaged in 100% air was used as the control sample. Firmness, pH, colour, peroxide values, microbial population and shelf-life (as determined by sensory characteristics) of the samples were determined during the storage. The results showed that colour, peroxide values and microbial population coincided with sensory characteristics. The delayed growth of microbes in vacuum packaging can be attributed to inhibition by low levels of O-2. Chitosan coating was shown to minimize oxidation, as reflected by the peroxide values, colour changes and microbial counts of the samples. Vacuum-packaged chitosan-coated grilled pork showed negligible microbial growth and was found to be organoleptically acceptable throughout the storage period. Chitosan coating along with vacuum packaging provided a type of active packaging to maintain quality and extend the shelf-life of grilled pork. Copyright (C) 2006 John Wiley & Sons, Ltd.

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Record 8 of 10

Title: Application of chitosan films enriched with oregano essential oil on Bologna - Active compounds and sensory attributes

Author(s): Chi, S (Chi, S); Zivanovic, S (Zivanovic, S); Penfield, MP (Penfield, MP)

Source: FOOD SCIENCE AND TECHNOLOGY INTERNATIONAL **Volume:** 12 **Issue:** 2 **Pages:** 111-117 **DOI:** 10.1177/1082013206063845 **Published:** APR 2006

Times Cited in Web of Science: 14

Total Times Cited: 15

Abstract: Chitosan films prepared with oregano essential oil were applied on bologna slices. Release of the essential oil compounds during film preparation and application on the meat product and consumer acceptability of bologna enriched with oregano essential oil were tested. Oregano essential oil compounds were quantified by gas chromatography mass spectroscopy (GCMS) after extraction from the film-forming solution, films before and after application on bologna and from bologna slices before and after application of the films. The results indicated that the concentration of components of the essential oil sharply decreased during film preparation, e.g. from 757.7 ppm carvacrol in film-forming solution to 2.1 ppm in dried films. No carvacrol was detected in the films after application on bologna for 5 days at 4 degrees C, mainly due to its diffusion into bologna. It seemed that the moisture and high lipid content of bologna helped the diffusion of the oregano essential oil from the chitosan film matrix into the product. Sensory evaluation suggested that addition of 45 ppm or less of oregano oil to bologna would be acceptable to consumers. Results support the potential use of chitosan-oregano essential oil films as an antimicrobial packaging material for processed meat.

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ISSN: 1082-0132

Record 9 of 10

Title: Quality characteristics of reduced-fat Chinese-style sausage as related to chitosan's molecular weight

Author(s): Lin, KW (Lin, KW); Chao, JY (Chao, JY)

Source: MEAT SCIENCE **Volume:** 59 **Issue:** 4 **Pages:** 343-351 **DOI:** 10.1016/S0309-1740(01)00084-5 **Published:** DEC 2001

Times Cited in Web of Science: 24

Total Times Cited: 31

Abstract: Microbiological and physicochemical characteristics of reduced-fat (approximately 22%) Chinese-style sausages containing 0.1% chitosan (in 1% lactic acid solution) with three different molecular weights [low molecular weight chitosan (CHL), MW = 150 kDa; medium molecular weight chitosan (CHM), MW = 600 kDa; high molecular weight chitosan (CHH), MW = 1250 kDa] were demonstrated. Sausages were also subject to oven heating or deep-fat frying for sensory evaluation. Treatments containing chitosan were lower in pH at any storage periods than the control (CONT). Generally speaking, total plate counts and lactic acid bacteria counts of all treatments slightly increased, with CHL being the lowest. However, all bacterial counts were lower than 7 [log colony forming unit (CFU)/g] at the end of storage. Results from sensory evaluation showed that deep-fat fried sausages from treatments CHL and CHM were higher in overall acceptability. This study demonstrated that the addition of chitosan to reduced-fat Chinese-style sausage resulted in no detrimental effect on textural properties. (C) 2001 Elsevier Science Ltd. All rights reserved.

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Record 10 of 10

Title: EFFECT OF CHITOSAN IN MEAT PRESERVATION

Author(s): DARMADJI, P (DARMADJI, P); IZUMIMOTO, M (IZUMIMOTO, M)

Source: MEAT SCIENCE **Volume:** 38 **Issue:** 2 **Pages:** 243-254 **DOI:** 10.1016/0309-1740(94)90114-7 **Published:** 1994

Times Cited in Web of Science: 87

Total Times Cited: 99

Abstract: The effect of chitosan in meat preservation, including microbiological, chemical, sensory and color qualities, was examined. In liquid medium, chitosan (0.01%) inhibited the growth of some spoilage bacteria such as Bacillus subtilis IFO 3025, Escherichia coli RB, Pseudomonas fragi IFO 3458 and Staphylococcus aureus IAM 1011. At higher concentrations (0.1 and 1.0%) it inhibited the growth of the meat starter cultures, Lactobacillus plantarium IAM 1216, Pediococcus pentosaceus IAM 12296 and Micrococcus varians IFO 3765. In meat, during incubation at 30-degrees-C for 48 h or storage at 4-degrees-C for 10 days, 0.5-1.0% chitosan inhibited the growth of spoilage bacteria, reduced

lipid oxidation and putrefaction, and resulted in better sensory attributes. Chitoosan also had a good effect on the development of the red color of meat during storage.

Accession Number: WOS:A1994NZ44300010

ISSN: 0309-1740

APPENDIX G

Summary of NTP 26-week Study with Chitosan in Rats

March, 2011

The National Toxicology Program (NTP) conducted a 26-week feeding study with Chitosan in rats. They conducted this study on the basis of a nomination to the Chemical Selection Working Group as expressed in a summary document. Chitosan has many applications including industrial, pharmaceutical, agricultural and cosmetic uses as well as a diet aid. The concern noted in the Limited Summary of Data for Chemical Selection document is that a combination of uses can result in a very large daily intake by an individual.

The intended design of this study was as a standard subchronic study, extended to 26-weeks with additional analysis included to evaluate potential metabolic alterations following chitosan consumption. The source of chitosan is not specified on NTP's website. The study was conducted by adding chitosan to the diet at dose levels of 0, 1, 3 and 9% and was completed in 2007 with the animals on study for a total of 169 days[9].

PROTOCOL SUMMARY

1. GROUP DESIGNATION AND ANIMALS
    a. Five dose groups and a control group were included in the study with 30 rats/sex/group. The groups were equally divided into Core, Special A and Special B groups. The controls were untreated in this dosed feed study. During the last 1 – 3 days of the quarantine/acclimation period, animals were formally randomized to study groups.
    b. The weight range of the animals used in the study was no more than ±20% from the mean body weight, by sex, of the animals available for randomization.
    c. Male and female Sprague Dawley Rats were used in this study and were five to six weeks of age at the start of dosing.
    d. Animals are quarantined/acclimated for a minimum of 10 but less than 14 days prior to dosing.
    e. Animals were identified using a tattoo at the base of the tail.
2. HOUSING
    a. The rats were group housed by sex and dose group. There were a maximum of 5 animals per cage. Polycarbonate cages in a suspended cage rack system were used and measured 22" long x 12.5" wide x 8" high.
    b. Cages were changed twice weekly. Irradiated, heat treated hardwood bedding was used.
    c. Sentinel animals were used as part of the animal disease screen program.
3. TEST ARTICLE STORAGE AND PREPARATION
    a. The stability of the bulk test article used in the study was determined at defined intervals. The NTP has set these intervals as every 24 ± 2 weeks that the test article is in the Contract Laboratory's Facilities. The prepared dosed feed was also analyzed for test article concentration at set intervals during the study. The NTP has set these intervals as testing of the initial formulation and then every

[9] When preparing this study summary, only a brief summary of study conduct was available. It was assumed that the additional dose days were requested in the individual Scope of Work for this test article. These specifications can be found on the NTP website at the following link: http://ntp.niehs.nih.gov/files/Specifications_2006Oct1.pdf in section IX and as a special request from the NTP. At the time of preparation of this document, the study was undergoing Pathology Review.

10 ± 2 weeks. Samples were also being collected from the animal room dose at the beginning of the study. The homogeneity of the test article in the prepared feed was also determined.

b. An archival sample of each batch used was retained.

4. ANIMAL ROOM ENVIRONMENT AND HUSBANDRY
   a. A minimum of ten complete air changes were provided per hour. The temperature and relative humidity were maintained at 72˚F ± 3˚F and 35 – 65%, respectively. The light cycle was set at 12 hours light and twelve hours dark in each 24 hour period with an intensity of 30 ± 3 foot candles at 3.3 feet from the floor.
   b. The dosed feed was supplied *ad libitum* to the animals in a feeder as a powdered feed. The feeders were uniquely marked to identify the test article, dose group and control group feeders.
   c. Municipal drinking water was supplied *ad libitum*.
5. DOSING
   a. The Chitosan was administered in AIN-93M feed at concentrations of 0, 1, 3 and 9% (w/w) for up to 6 months. Animals were dosed via the feed for 7 days/week up to the time of sacrifice. Access to the feed was not restricted.
6. STUDY ENDPOINTS
   a. For the rats in the Core group, body weights and clinical observations were recorded weekly and prior to euthanasia.
   b. Feces was collected from the Special B group rats during study weeks 6, 12, 18 and 24. Calcium, fat and moisture were determined from the collected samples.
   c. Urine was collected for approximately 24 hours prior to the end of each fecal collection period. The urine was analyzed for total deoxypyridinoline and urinary creatinine.
   d. Food consumption was recorded every 3 – 4 days for the Core group rats. For the Special B group rats, food consumption was recorded every 1 – 2 days while in the metabolic cages for feces and urine collection.
   e. Blood samples were collected from the Special A and B group rats after weeks 6, 12, 18 and 24. The samples from Special A group rats were analyzed for Vitamins A, E and 1, 25 $(OH)_2$ Vitamin D. The samples from Special B group rats were analyzed for defined hematology and clinical chemistry parameters, as well as for total osteocalcin, parathyroid hormone and Vitamin K1.
   f. Survival data was recorded for each animal.
   g. At termination, livers were collected from the Special A and B group rats. The livers from the Special A group were analyzed for Vitamin A and E. The livers from the Special B group were analyzed for Vitamin K1.
   h. Femurs were collected from the Special A group at termination and analyzed for calcium, ash and moisture.
   i. Vaginal cytology was performed on all Core group females for 16 consecutive days prior to scheduled termination. Sperm morphology was evaluated on all Core group males at scheduled termination.
   j. All animals underwent a complete necropsy on the final day of the study. The necropsies of both sexes were completed within two consecutive days with one entire sex being necropsied on the same day. The order of necropsy was randomized within sex and the necropsy was initiated within five minutes after an animal was euthanized.
   k. The following organs/tissues were examined from all animals in the study:

Adrenal glands
Brain
Clitoral glands
Esophagus
Eyes
Femur
Gross lesions
Harderian glands
Heart and aorta
Intestine, large (cecum, colon, rectum)
Intestine, small (duodenum, jejunum, ileum)
Kidneys
Liver
Lungs and mainstem bronchi
Lymph nodes (mandibular, mesenteric)
Mammary gland with adjacent skin
Muscle (thigh)
Nerve (sciatic)
Nose (nasal cavity and nasal turbinates)
Oral cavity, larynx and pharynx
Ovaries
Pancreas
Parathyroid glands
Pituitary gland
Preputial glands
Prostate
Salivary glands
Seminal vesicles
Spinal cord
Spleen
Stomach (forestomach and glandular)
Testes, epididymides and vaginal tunics of testes
Thymus
Thyroid gland
Tissue masses
Tongue
Trachea
Urinary bladder
Uterus
Vagina
Zymbal glands

l. The bone lengths of the right and left tibia and right femur were measured in all Core group animals. The above tissues were collected and fixed for histopathologic examination. All tissues were fixed in 10% neutral buffered formalin with the following exceptions, which were fixed in the appropriate solutions noted for 24 to 72 hours and then transferred to 10% neutral buffered formalin; eyes (Davidson's solution), testes, vaginal tunics of testes and epididymides (modified Davidson's solution). When appropriate, the tissues/organs were fixed in their entirety but each tissue thickness did not exceed 0.5 cm. Specific fixing, trimming and histopathological evaluation instructions are outlined in the Subchronic Study Protocol available from the National Toxicology Program website.

RESULTS

The following data tables are available on the results of the study are listed on the NTP website at http://ntp-apps.niehs.nih.gov/ntp_tox/index.cfm?fuseaction=ntpsearch.searchresults&searchterm=9012-76-4

1. Survival Data – Male and Female
2. Clinical Observation Data – Male
3. Group Mean Body Weight Data – Male and Female
4. Group Non-neoplastic Diagnoses – Male and Female

Statistical analysis of the data is not available on NTP's website. Presumably results of hematology, clinical chemistry and organ weights will be provided by NTP at some future data.

Based on the available summary data tables on the NTP website it is apparent that all animals survived to the end of the study and body weight were not affected in any of the dose groups in either sex. There were many incidental findings noted but no gross or histological lesions which can be attributed to test article exposure. As listed above, individual animal data on these study parameters are provided on NTP's website.

STUDY RESULT TABLES

SURVIVAL DATA – MALES (n=10/dose group)

Week	0%	1%	3%	9%
1	10	10	10	10
25	10	10	10	10

SURVIVAL DATA – FEMALES (n=10/dose group)

Week	0%	1%	3%	9%
1	10	10	10	10
25	10	10	10	10

CLINICAL OBSERVATION DATA – MALES

TREATMENT GROUP (%)	NUMBER OF ANIMALS INVOLVED	ABNORMALITY OBSERVED
9	1	Ulcer/Abscess

GROUP MEAN BODY WEIGHT DATA – MALES (grams)

Week	Vehicle Control (n=10)	Vehicle Control Std. Dev.	1% Dose Group (n=10)	1% Dose Group Std. Dev.	3% Dose Group (n=10)	3% Dose Group Std. Dev.	9% Dose Group (n=10)	9% Dose Group Std. Dev.
1	267.1	34.69	275.4	39.49	272.3	36.75	253.9	20.85
2	345.5	23.29	359.2	27.36	353.5	28.92	307.1	18.76
3	387.6	27.64	403.7	32.03	397.9	32.12	349.7	23.27
4	421.2	27.61	437.7	32.25	436.4	38.88	388.0	22.00
5	445.7	33.65	464.6	34.46	463.9	42.05	412.6	26.07
6	475.2	35.46	492.8	34.73	491.1	45.93	441.9	26.59
7	496.0	38.32	513.3	34.48	514.0	45.98	463.8	31.28
8	514.5	40.88	535.5	35.75	533.8	45.72	482.5	33.55
9	528.9	40.06	553.8	39.44	548.3	45.80	498.0	37.63
10	543.4	42.00	570.3	42.40	565.5	49.81	510.8	36.77
11	553.5	44.90	584.7	41.94	578.7	55.75	521.4	37.88
12	563.4	48.88	597.9	43.55	583.8	58.10	526.9	42.15
13	578.0	47.81	611.7	44.04	602.4	62.99	543.6	42.28
14	586.6	46.70	622.2	46.48	612.7	65.73	556.9	41.41
15	596.5	47.98	630.9	47.74	620.1	61.59	564.8	40.79
16	606.8	51.55	645.3	51.17	634.2	62.99	575.1	38.38
17	613.6	54.37	656.9	52.94	645.5	67.51	584.1	38.44
18	624.4	55.89	667.3	56.37	657.4	70.77	595.4	41.02
19	632.7	59.75	676.6	59.47	663.9	71.49	599.8	44.73
20	643.4	60.67	688.6	58.12	670.3	69.07	606.3	44.36
21	653.2	62.40	700.3	60.73	676.7	70.19	611.5	46.58
22	664.5	63.61	706.7	61.17	685.6	71.68	615.3	49.96
23	666.4	65.12	704.1	63.69	689.0	73.59	611.5	50.67

GROUP MEAN BODY WEIGHT DATA –FEMALES (grams)

Week	Vehicle Control (n=10)	Vehicle Control Std. Dev.	1% Dose Group (n=10)	1% Dose Group Std. Dev.	3% Dose Group (n=10)	3% Dose Group Std. Dev.	9% Dose Group (n=10)	9% Dose Group Std. Dev.
1	187.3	15.79	185.5	14.93	186.5	16.63	183.8	13.57
2	219.8	13.63	216.9	10.77	214.1	18.39	206.4	17.33
3	233.3	12.58	229.3	12.01	231.3	20.16	221.1	19.66
4	248.00	18.07	241.1	14.70	241.6	22.44	234.2	24.50
5	257.8	18.95	252.2	15.50	250.7	26.11	243.3	23.44
6	265.6	19.74	261.9	16.41	259.2	28.96	247.7	25.56

7	273.5	21.04	268.4	18.27	267.3	30.07	258.7	28.02
8	281.4	21.45	276.2	20.47	274.1	28.87	265.7	29.02
9	287.1	22.63	284.0	21.54	280.8	29.92	267.9	28.61
10	293.9	22.71	289.4	21.57	286.4	31.75	273.6	31.79
11	299.9	23.53	294.5	20.71	292.3	31.93	278.6	32.46
12	304.5	24.41	299.9	22.46	298.5	32.21	280.8	32.73
13	311.7	28.37	303.0	24.78	303.9	31.06	284.6	32.59
14	315.8	32.30	306.6	28.16	308.7	30.97	287.6	32.77
15	320.4	34.04	310.7	27.64	313.5	28.88	290.9	32.59
16	324.5	35.89	313.5	28.67	314.7	28.22	292.6	34.26
17	326.5	36.88	317.2	26.92	318.1	28.81	296.2	33.99
18	330.0	36.42	321.3	27.39	320.7	29.53	298.6	34.33
19	327.8	34.40	324.1	29.96	321.0	31.42	296.9	36.18
20	335.0	36.84	331.9	31.12	330.4	32.41	302.2	35.67
21	339.1	37.58	337.3	33.17	336.4	35.54	306.3	38.81
22	343.2	39.47	340.0	32.82	338.9	33.90	306.3	37.86
23	345.2	41.12	340.0	31.47	339.0	33.83	309.0	42.21

GROUP NON-NEOPLASTIC DIAGNOSES – MALE

LIVER

TREATMENT GROUP (%)	NUMBER EXAMINED	NUMBER OF LESIONS OBSERVED	LESION NAME	LESION QUALIFIER	SITE DESCRIPTION
0	10	0	N/A	N/A	N/A
1	10	0	N/A	N/A	N/A
3	10	0	N/A	N/A	N/A
9	10	1	Degeneration	Cystic, Minimal	Not specified
0	10	2	Hematopoietic Cell Proliferation	Minimal	Not Specified
1	10	3	Hematopoietic Cell Proliferation	Minimal	Not Specified
3	10	3	Hematopoietic Cell Proliferation	Minimal	Not Specified
9	10	6	Hematopoietic Cell Proliferation	Minimal	Not Specified
0	10	10	Inflammation	Chronic Active, Minimal	Not Specified
1	10	10	Inflammation	Chronic	Not Specified

				Active, Minimal	
3	10	10	Inflammation	Chronic Active, Minimal	Not Specified
9	10	9	Inflammation	Chronic Active, Minimal	Not Specified
0	10	6	Fatty Change	Minimal (3)/Mild(2)/ Moderate(1)	Periportal
1	10	3	Fatty Change	Minimal (1)/Mild(2)	Periportal
3	10	6	Fatty Change	Minimal (4)/Mild(2)	Periportal
9	10	3	Fatty Change	Minimal	Periportal

PANCREAS

TREATMENT GROUP (%)	NUMBER EXAMINED	NUMBER OF LESIONS OBSERVED	LESION NAME	LESION QUALIFIER	SITE DESCRIPTION
0	10	2	Inflammation	Minimal	Not Specified
9	10	1	Inflammation	Minimal	Not Specified

FORESTOMACH

TREATMENT GROUP (%)	NUMBER EXAMINED	NUMBER OF LESIONS OBSERVED	LESION NAME	LESION QUALIFIER	SITE DESCRIPTION
0	10	3	Hyperplasia	Minimal	Epithelium
9	10	1	Hyperplasia	Minimal	Epithelium

BLOOD VESSELS

TREATMENT GROUP (%)	NUMBER EXAMINED	NUMBER OF LESIONS OBSERVED	LESION NAME	LESION QUALIFIER	SITE DESCRIPTION
0	10	0	N/A	N/A	N/A
9	10	1	Inflammation	Minimal	Not specified

HEART

TREATMENT GROUP (%)	NUMBER EXAMINED	NUMBER OF LESIONS	LESION NAME	LESION QUALIFIER	SITE DESCRIPTION

		OBSERVED			
0	10	5	Cardiomyopathy	Minimal	Not specified
9	10	2	Cardiomyopathy	Minimal/ Mild	Not specified
0	10	0	N/A	N/A	N/A
9	10	1	Mineralization	Minimal	Not specified

ADRENAL CORTEX

TREATMENT GROUP (%)	NUMBER EXAMINED	NUMBER OF LESIONS OBSERVED	LESION NAME	LESION QUALIFIER	SITE DESCRIPTION
0	10	0	N/A	N/A	N/A
9	10	1	Cytoplasmic Vacuolization	Minimal	Not specified

PARATHYROID GLAND

TREATMENT GROUP (%)	NUMBER EXAMINED	NUMBER OF LESIONS OBSERVED	LESION NAME	LESION QUALIFIER	SITE DESCRIPTION
0	10	1	Hyperplasia	Minimal	Not specified
1	10	0	N/A	N/A	N/A
3	10	0	N/A	N/A	N/A
9	10	0	N/A	N/A	N/A

PITUITARY

TREATMENT GROUP (%)	NUMBER EXAMINED	NUMBER OF LESIONS OBSERVED	LESION NAME	LESION QUALIFIER	SITE DESCRIPTION
0	10	1	Cyst	Not specified	Not specified
9	10	0	N/A	N/A	N/A

THYROID

TREATMENT GROUP (%)	NUMBER EXAMINED	NUMBER OF LESIONS OBSERVED	LESION NAME	LESION QUALIFIER	SITE DESCRIPTION
0	10	0	N/A	N/A	N/A
9	10	1	Hyperplasia	Minimal	C cell

PREPUCIAL GLAND

TREATMENT GROUP (%)	NUMBER EXAMINED	NUMBER OF LESIONS OBSERVED	LESION NAME	LESION QUALIFIER	SITE DESCRIPTION
0	10	0	N/A	N/A	N/A
9	10	3	Inflammation	Minimal (1)/Chronic active (2)	Not specified

PROSTATE

TREATMENT GROUP (%)	NUMBER EXAMINED	NUMBER OF LESIONS OBSERVED	LESION NAME	LESION QUALIFIER	SITE DESCRIPTION
0	10	7	Inflammation	Minimal (2)/Mild (5)	Not specified
1	10	9	Inflammation	Minimal (8)/Mild (1)	Not specified
3	10	10	Inflammation	Minimal (6)/Mild (4)	Not specified
9	10	10	Inflammation	Minimal (5)/Mild (5)	Not specified

TESTES

TREATMENT GROUP (%)	NUMBER EXAMINED	NUMBER OF LESIONS OBSERVED	LESION NAME	LESION QUALIFIER	SITE DESCRIPTION
0	10	0	N/A	N/A	N/A
9	10	1	Mineralization	Minimal	Not specified

MANDIBULAR LYMPH NODE

TREATMENT GROUP (%)	NUMBER EXAMINED	NUMBER OF LESIONS OBSERVED	LESION NAME	LESION QUALIFIER	SITE DESCRIPTION
0	10	1	Infiltration	Mild, plasma cell	Not specified
9	10	2	Infiltration	Minimal, plasma cell	Not specified

SPLEEN

TREATMENT GROUP (%)	NUMBER EXAMINED	NUMBER OF LESIONS OBSERVED	LESION NAME	LESION QUALIFIER	SITE DESCRIPTION

0	10	5	Hematopoietic cell proliferation	Minimal	Not specified
9	10	2	Hematopoietic cell proliferation	Minimal	Not specified

THYMUS

TREATMENT GROUP (%)	NUMBER EXAMINED	NUMBER OF LESIONS OBSERVED	LESION NAME	LESION QUALIFIER	SITE DESCRIPTION
0	10	1	Atrophy	Minimal	Not specified
9	10	0	Atrophy	N/A	N/A

SKIN

TREATMENT GROUP (%)	NUMBER EXAMINED	NUMBER OF LESIONS OBSERVED	LESION NAME	LESION QUALIFIER	SITE DESCRIPTION
0	10	0	N/A	N/A	N/A
9	10	1	Hemorrhage	Moderate	Not specified
0	10	0	N/A	N/A	N/A
9	10	1	Mineralization	Mild	Not specified
0	10	0	N/A	N/A	N/A
9	10	1	Ulcer	Marked	Not Specified

SKELETAL MUSCLE

TREATMENT GROUP (%)	NUMBER EXAMINED	NUMBER OF LESIONS OBSERVED	LESION NAME	LESION QUALIFIER	SITE DESCRIPTION
9	1	1	Inflammation	Granulomatous, moderate	Not specified

LUNG

TREATMENT GROUP (%)	NUMBER EXAMINED	NUMBER OF LESIONS OBSERVED	LESION NAME	LESION QUALIFIER	SITE DESCRIPTION
0	10	0	Hemorrhage	Minimal	Not Specified
9	10	0	N/A	N/A	N/A
0	10	2	Inflammation	Chronic Active/ Minimal	Not Specified
9	10	4	Inflammation	Chronic Active/	Not Specified

				Minimal	

NOSE

TREATMENT GROUP (%)	NUMBER EXAMINED	NUMBER OF LESIONS OBSERVED	LESION NAME	LESION QUALIFIER	SITE DESCRIPTION
0	10	0	N/A	N/A	N/A
9	10	1	Hyperplasia	Mild	Goblet Cell
0	10	1	Inflammation	Minimal	Not Specified
9	10	0	N/A	N/A	N/A

EYE

TREATMENT GROUP (%)	NUMBER EXAMINED	NUMBER OF LESIONS OBSERVED	LESION NAME	LESION QUALIFIER	SITE DESCRIPTION
0	10	0	N/A	N/A	N/A
1	1	1	Fibrosis	Mild	Choroid
9	10	0	N/A	N/A	N/A
0	10	0	N/A	N/A	N/A
1	1	1	Cataract	Moderate	Lens
9	10	0	N/A	N/A	N/A

HARDERIAN GLAND

TREATMENT GROUP (%)	NUMBER EXAMINED	NUMBER OF LESIONS OBSERVED	LESION NAME	LESION QUALIFIER	SITE DESCRIPTION
0	10	0	N/A	N/A	N/A
9	10	1	Hyperplasia	Minimal	Not Specified
0	10	2	Cellular Infiltration	Minimal, Lymphocyte	Not Specified
9	10	1	Cellular Infiltration	Minimal, Lymphocyte	Not Specified

KIDNEY

TREATMENT GROUP (%)	NUMBER EXAMINED	NUMBER OF LESIONS OBSERVED	LESION NAME	LESION QUALIFIER	SITE DESCRIPTION
0	10	1	Cyst	Mild	Cortex
1	10	0	N/A	N/A	N/A
3	10	0	N/A	N/A	N/A
9	10	0	N/A	N/A	N/A

0	10	0	N/A	N/A	N/A
1	10	0	N/A	N/A	N/A
3	10	1	Infarct	Mild	Not Specified
9	10	0	N/A	N/A	N/A
0	10	2	Mineralization	Minimal	Not Specified
1	10	4	Mineralization	Minimal	Not Specified
3	10	3	Mineralization	Minimal	Not Specified
9	10	5	Mineralization	Minimal	Not Specified
0	10	9	Nephropathy	Minimal	Not Specified
1	10	9	Nephropathy	Minimal	Not Specified
3	10	9	Nephropathy	Minimal (7)/Mild(2)	Not Specified
9	10	9	Nephropathy	Minimal (6)/Mild(3)	Not Specified
0	10	2	Dilation	Minimal/ Mild	Pelvis
1	10	0	N/A	N/A	N/A
3	10	1	Dilation	Mild	Pelvis
9	10	0	N/A	N/A	N/A
0	10	1	Inflammation	Mild	Pelvis
1	10	0	N/A	N/A	N/A
3	10	0	N/A	N/A	N/A
9	10	0	N/A	N/A	N/A

URINARY BLADDER

TREATMENT GROUP (%)	NUMBER EXAMINED	NUMBER OF LESIONS OBSERVED	LESION NAME	LESION QUALIFIER	SITE DESCRIPTION
0	10	0	N/A	N/A	N/A
9	10	1	Hyperplasia	Minimal	Transitional Epithelium

GROUP NON-NEOPLASTIC DIAGNOSES – FEMALE

LIVER

TREATMENT GROUP (%)	NUMBER EXAMINED	NUMBER OF LESIONS OBSERVED	LESION NAME	LESION QUALIFIER	SITE DESCRIPTION
0	10	1	Hematopoietic Cell Proliferation	Minimal	Not Specified

1	10	1	Hematopoietic Cell Proliferation	Minimal	Not Specified
3	10	2	Hematopoietic Cell Proliferation	Minimal	Not Specified
9	10	1	Hematopoietic Cell Proliferation	Minimal	Not Specified
0	10	9	Inflammation	Chronic Active, Minimal	Not Specified
1	10	9	Inflammation	Chronic Active, Minimal	Not Specified
3	10	9	Inflammation	Chronic Active, Minimal	Not Specified
9	10	10	Inflammation	Chronic Active, Minimal	Not Specified
0	10	7	Fatty Change	Minimal (6)/Mild(1)	Periportal
1	10	4	Fatty Change	Minimal	Periportal
3	10	4	Fatty Change	Minimal	Periportal
9	10	0	N/A	N/A	N/A

PANCREAS

TREATMENT GROUP (%)	NUMBER EXAMINED	NUMBER OF LESIONS OBSERVED	LESION NAME	LESION QUALIFIER	SITE DESCRIPTION
0	10	0	N/A	N/A	N/A
9	10	1	Atrophy	Minimal	Not Specified
0	10	1	Inflammation	Minimal	Not Specified
9	10	1	Inflammation	Chronic Active, Minimal	Not Specified

HEART

TREATMENT GROUP (%)	NUMBER EXAMINED	NUMBER OF LESIONS OBSERVED	LESION NAME	LESION QUALIFIER	SITE DESCRIPTION
0	10	1	Cardiomyopathy	Minimal	Not specified
9	10	0	N/A	N/A	N/A

PITUITARY

TREATMENT GROUP (%)	NUMBER EXAMINED	NUMBER OF LESIONS OBSERVED	LESION NAME	LESION QUALIFIER	SITE DESCRIPTION
0	10	1	Hyperplasia	Mild	Rathkes Cleft
9	10	0	N/A	N/A	N/A

CLITORAL GLAND

TREATMENT GROUP (%)	NUMBER EXAMINED	NUMBER OF LESIONS OBSERVED	LESION NAME	LESION QUALIFIER	SITE DESCRIPTION
0	10	2	Inflammation	Chronic Active, Mild	Not Specified
9	10	0	N/A	N/A	N/A

SPLEEN

TREATMENT GROUP (%)	NUMBER EXAMINED	NUMBER OF LESIONS OBSERVED	LESION NAME	LESION QUALIFIER	SITE DESCRIPTION
0	10	1	Hematopoietic cell proliferation	Minimal	Not specified
9	10	0	N/A	N/A	N/A

THYMUS

TREATMENT GROUP (%)	NUMBER EXAMINED	NUMBER OF LESIONS OBSERVED	LESION NAME	LESION QUALIFIER	SITE DESCRIPTION
0	10	1	Atrophy	Minimal	Not specified
9	10	0	N/A	N/A	N/A

BRAIN

TREATMENT GROUP (%)	NUMBER EXAMINED	NUMBER OF LESIONS OBSERVED	LESION NAME	LESION QUALIFIER	SITE DESCRIPTION
0	10	1	Developmental Malformation	Not Specified	Not specified
9	10	0	N/A	N/A	N/A

LUNG

TREATMENT GROUP (%)	NUMBER EXAMINED	NUMBER OF LESIONS OBSERVED	LESION NAME	LESION QUALIFIER	SITE DESCRIPTION
0	10	0	N/A	N/A	N/A
9	10	1	Hyperplasia	Minimal	Alveolar Epithelium
0	10	2	Cellular Infiltration	Minimal, Histiocyte	Alveolus
9	10	0	N/A	N/A	N/A
0	10	1	Mineralization	Minimal	Artery
9	10	1	Mineralization	Minimal	Artery
0	10	0	N/A	N/A	N/A
9	10	1	Mineralization	Minimal	Not Specified

NOSE

TREATMENT GROUP (%)	NUMBER EXAMINED	NUMBER OF LESIONS OBSERVED	LESION NAME	LESION QUALIFIER	SITE DESCRIPTION
0	10	1	Hyperplasia	Mild	Goblet Cell
9	10	0	N/A	N/A	N/A

HARDERIAN GLAND

TREATMENT GROUP (%)	NUMBER EXAMINED	NUMBER OF LESIONS OBSERVED	LESION NAME	LESION QUALIFIER	SITE DESCRIPTION
0	10	1	Cellular Infiltration	Minimal, Lymphocyte	Not Specified
9	10	1	Cellular Infiltration	Minimal, Lymphocyte	Not Specified

KIDNEY

TREATMENT GROUP (%)	NUMBER EXAMINED	NUMBER OF LESIONS OBSERVED	LESION NAME	LESION QUALIFIER	SITE DESCRIPTION
0	10	8	Mineralization	Minimal	Not Specified
1	10	8	Mineralization	Minimal	Not Specified
3	10	5	Mineralization	Minimal	Not Specified
9	10	6	Mineralization	Minimal	Not Specified
0	10	5	Nephropathy	Minimal	Not Specified
1	10	6	Nephropathy	Minimal	Not Specified
3	10	5	Nephropathy	Minimal	Not Specified
9	10	0	N/A	N/A	N/A

APPENDIX H

Additional Biological Effects Studies

Jun, et al. (2010) conducted a 5-week study to determine the anti-obesity properties of chitosan alone and in combination with vitamin C. Hartley guinea pigs were divided into 4 groups (6/group); control with the normal diet, F-control with the high fat diet, a chitosan group which was a high fat diet with 5% chitosan and a chito-vit C group with the high fat diet with 5% chitosan and 0.5% vitamin C. Chitosan feeding did not influence body weight gain, food intake or food efficiency ratio. The addition of vitamin C to the chitosan significantly decreased the body weight gain and food efficiency ratio, as compared to the F-controls. There were no significant differences in the relative organ weight between chitosan and control groups. The liver weight was significantly higher in the chito-vit C group as compared to the F-control and chitosan groups. There were no significant differences between the chitosan, chito-vit C and F-control groups with respect to the perirenal fat pad weights but the percentage of the epididymal fat pad was significantly lowered in the chitosan group as compared to the F-control group. There was no effect noted following the addition of vitamin C. The chitosan group had significantly increased total fecal weight, fecal fat excretion, fecal water excretion and fecal water content, as compared to the F-controls. The same parameters were significantly increased in the chito-vit C group, with the exception of the fecal water content. When compared to the chitosan group, the total fecal weight, fecal fat excretion and fecal fat content was significantly increased in the chito-vit C group. The total cholesterol, low density lipoprotein (LDL) cholesterol and triacylglycerol levels were significantly lower in the chitosan and chito-vit C groups as compared to the F-controls. There was no difference in these parameters between the chitosan and chito-vit C groups. The plasma TBARS levels in the chitosan and chito-vit C groups were significantly lower than the F-controls and the liver glutathione (GSH) level was significantly higher in the chitosan group than in the F-controls. There was no difference in these parameters between the chitosan and chito-vit C groups. No significant difference was noted in plasma and liver AEAC (radical scavenging activity) levels in the chitosan and chito-vit C groups as compared to F-controls and when compared to each other. The authors concluded that the fat-binding and water holding capacity of the chitosan may reduce the absorption of cholesterol and fat, thereby increasing total fecal weight, fecal fat excretion and fecal water excretion, which may in turn decrease body weight. Furthermore, the addition of vitamin C increased fecal fat excretion and reduced body weight gain. It should be noted that in this study the animals were fed high fat diets.

A study by Van Bennekum (2005) investigated the mechanism of serum cholesterol-lowering effects of insoluble dietary fibers and its association with intestinal cholesterol absorption and hepatic cholesterol homeostasis. Female C57BL/6 mice were fed a high fat/high cholesterol diet with or without the addition of 7.5% cholestyramine, chitosan or cellulose for three weeks. A control group was included which received the high fat/high cholesterol diet with no added fiber. Moderate body weight gains were noted in the chitosan treated group (+3.1%) and all mice were healthy and active throughout the study. The serum and liver cholesterol levels in the fiber supplemented groups were significantly lower than the control groups. Hepatic total cholesterol stores were also decreased in the fiber supplemented groups as compared to controls. There was no change in the cholesterol absorption efficiency or fecal sterol excretion in the chitosan group, when compared to the controls. The food intake was lower in all fiber supplemented groups when compared to intake in the control group. No adverse effects were reported following the three week feeding study.

Zhang, et al. (2008) investigated the hypolipidemic mechanism of chitosan in male Sprague-Dawley rats. The rats were divided into 5 groups; a normal fat control group, a high-fat control group, a positive control group and 2 chitosan groups. In one chitosan group, the chitosan was fed from the beginning and in the other chitosan group, the chitosan feeding was initiated after 2 weeks. A commercial diet with 5% cellulose was used as the base diet and the study continued for 6 weeks. The high fat group had the base diet supplemented with 12% lard and 1% cholesterol. Cholestyramine was used as the positive control agent and replaced the cellulose in the base diet for the positive control group. For the chitosan groups, the base diet was used but the cellulose was replaced with chitosan at the same level (5%). The rats fed the chitosan containing diets had reduced plasma and liver lipid levels while fecal excretion of fat and cholesterol were increased. There was an increased liver hepatic and lipoprotein lipase activity, as compared to the high fat control group. The chitosan fed animals showed similar food consumption but a reduced body weight gain, as compared to the high fat control group. No other adverse reactions were reported following exposure to chitosan at this dosage level. No significant differences were noted between the chitosan feeding at the beginning or after 2 weeks groups, although the feeding of chitosan at the beginning exhibited lower lipid levels compared to those in the group fed after the initial 2-week delay in chitosan administration.

Sumiyoshi (2006) conducted a study where male C57BL/6J mice were dosed with water soluble chitosans having an average molecular weight of 21, 46 and 130 kDa. The mice were fasted overnight, gavaged with 500 mg/kg of the chitosans (dissolved in distilled water) and then gavaged 20 seconds later with 0.2 ml of a butter emulsion. Blood was collected from the tail vein at 0, 0.5, 1, 2, 3 and 4 hours after dosing with the butter emulsion. Plasma triacylglycerol (TG) concentrations were determined and the 45 kDa chitosan was found to be most effective at inhibiting the plasma TG elevation after the oral lipid tolerance test. No adverse toxicity was noted following the chitosan dose. In another study in the same reference, mice were gavaged twice daily with 46-kDa water soluble chitosan at 100 and 300 mg/kg. Animals were dosed for 20 weeks and were maintained on a high fat diet which contained 45.0% butter, 17.1% corn starch, 10.0% sucrose, 20.0% casein, 3.0% cellulose, 3.5% mineral mixture, 1.0% vitamin mixture, 0.4% choline chloride and had a total kcal of 535.2. Control mice were given either a low fat (3.0% butter, 41.5% corn starch, 5.0% sucrose, 20% casein, 3.0% cellulose, 3.5% mineral mixture, 1.0% vitamin mixture, 0.4% choline chloride and 22.6% water, kcal 292.8) or the high fat diet for 20 weeks. After 10 weeks of dosing were completed, feces were collected at 24 hour intervals for 4 weeks and triacylglycerol, total cholesterol (TC) and bile acids were measured. Body weight and food consumption were measured once weekly. Blood was collected on week 20 and the plasma TG and TC were determined. The 46 kDa chitosan at 300 mg/kg prevented increases in body weight, white adipose tissue weights and liver TC and TG as well as increased the fecal bile acid and fat levels. Other than these changes, no adverse effects were noted which were attributable to chitosan exposure.

Hirano, et al. (1990) evaluated chitosan for use as a feed ingredient for domestic animals. Male rabbits, white leghorn hens and white broilers were used in the study and were fed chitosan supplemented diets for periods of up to 239 days. Fecal samples were collected daily to evaluate the digestibility of the chitosan. The levels of total cholesterol, triacylgycerol and free fatty acids in the serum were evaluated periodically during the studies and the levels of total cholesterol and triacylglycerol in the livers was also evaluated. The growth, appetite and general appearance were recorded and no abnormalities in these parameters were noted in hens fed 1.2 – 1.4 g chitosan/kg body weight/day and in rabbits fed 0.7 – 0.8 g chitosan/kg body weight/day. There was a decrease in the egg-laying rate and appetite when 3.6 – 4.2 g chitosan/kg body weight /day was fed for 140

days or 14 – 18 g chitosan/kg body weight/day for 56 days. The authors stated that this was due to the incomplete digestion of the chitosan at these levels. These physiological abnormalities were reversed within 1 week after chitosan consumption was stopped. The digestibility of chitosan was estimated at 41 – 83% in rabbits and 67 – 98% in hens and broilers. It was suggested by the authors that the digestibility of chitosan may increase with time due to adaptation to feeding. An increase in serum cholesterol values were noted following the feeding of a cholesterol additive diet, this was suppressed by feeding a 2% chitosan supplemented diet but not with a 1% chitosan supplemented diet.

In an *in vitro* study, Carreno-Gomez and Duncan (1997) evaluated seven soluble chitosan polymers and chitosan microspheres loaded with horseradish peroxidise (HRP) for their cytotoxicity towards B16F10 cells and the ability to lyse rat erythrocytes. Chitosan hydrochloride, chitosan hydroglutamate, chitosan hydrolactate and glycol chitosan were evaluated, each with two forms of different viscosity (with the exception of the glycol chitosan). The B16F10 cells were seeded into 96-well microtiter plates and the soluble chiotsans and controls were added to the plates at various concentrations. The cytotoxicity was evaluated by direct cell counting and trypan blue exclusion. The erythrocytes were collected from male Wistar rats and the erythrocytes were added to the soluble chitosans or chitosan microspheres using a microtitre plate assay. Hemoglobin release was evaluated spectrophotometrically with a microtitre plate reader. All tested chitosan forms showed some degree of toxicity, with the exception of the low viscosity chitosan hydrolactate in the cytotoxicity experiment.

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SUBMISSION END