

ORIGINAL SUBMISSION



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**GRN 000661**

August 1, 2016

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

**Subject: GRAS Notification for Fucoidan Concentrate from *Fucus vesiculosus***

Dear Sir/Madam:

Pursuant to proposed 21 CFR 170.36 (62 FR 18960; April 17, 1997), Marinova Pty. Ltd. (Marinova), Australia, through Soni & Associates Inc. as its agent, hereby provides notice of a claim that the food ingredient Fucoidan concentrate derived from *Fucus vesiculosus* described in the enclosed notification document is exempt from the premarket approval requirement of the Federal Food, Drug, and Cosmetic Act because it has been determined to be Generally Recognized As Safe (GRAS), based on scientific procedures.

As required, please find enclosed three copies of the notification. If you have any questions or require additional information, please feel free to contact me by phone at 772-299-0746 or by email at [sonim@bellsouth.net](mailto:sonim@bellsouth.net).

Sincerely,

Madhu G. Soni, Ph.D., FATS

Enclosure: Three copies of the GRAS notice





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**I. Claim of GRAS Status**

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

Marinova Pty. Ltd. (Marinova), Australia, has determined that its fucoidan concentrate derived from *Fucus vesiculosus* is Generally Recognized As Safe, and therefore, exempt from the requirement of premarket approval, under the conditions of its intended use. This determination is based on scientific procedures as described in the following sections, under the conditions of fucoidan concentrate's intended use in food, among experts qualified by scientific training and expertise.

Signed, (b) (6)  
(b) (6)

Date August 1, 2016

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

Agent for:

Marinova Pty. Ltd.  
Cambridge, Tasmania  
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**B. Name and Address:**

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**C. Common or usual name of the GRAS substance:**

The common name of the substance of this GRAS assessment is fucoidan derived from *Fucus vesiculosus*. Fucoidan for food uses will be marketed as standardized powder under the trade name Maritech® with product identifier *F. vesiculosus*.

**D. Conditions of use:**

Fucoidan derived from *F. vesiculosus* and marketed by Marinova under the trade name Maritech® is intended for use in food categories such as Baked goods (bread, cake, noodles); Soups; Snack Foods; Imitation Dairy Products; and Seasonings & Flavors at use levels up to 30 mg/serving (reference amounts customarily consumed; 21 CFR 101.12). The intended uses of fucoidan in the above mentioned food categories is estimated to result in the mean and 90th percentile intake for the total population of 135.45 and 249.66 mg/person/day, respectively. Foods that are intended for infants and toddlers, such as infant formulas or foods formulated for babies or toddlers, and meat and poultry products that come under USDA jurisdiction are excluded from the list of intended food uses of the subject fucoidan.

**E. Basis for GRAS Determination:**

In accordance with 21 CFR 170.30, fucoidan concentrate has been determined to be Generally Recognized As Safe (GRAS) based on scientific procedures. The determination of general recognition of safety is supported by the opinion of the Expert Panelists qualified by scientific training and experience to evaluate the safety of substances directly or indirectly added to food. A comprehensive search of the scientific literature was utilized for this determination. There exists sufficient qualitative and quantitative scientific evidence, including human, animal and *in vitro* data to determine safety-in-use for fucoidan concentrate. The source material of fucoidan concentrate, brown algae *Fucus vesiculosus* also known as Bladderwrack is commonly used as a food in Japan and as one of the first reported sources of iodine, has been consumed in western cultures for centuries as an iodine supplement. Although, *F. vesiculosus* is not mentioned in the FDA regulation, several species of brown algae (seaweed) are generally recognized as safe (GRAS) as per 21 CFR §184.1120 for use in food. Fucoidan derived from *Undaria pinnatifida* has been the subject of a GRAS notification (GRN 565) by Marinova Pty Ltd. In response to this GRAS notice, FDA did not

question the conclusions that the use of fucoidan derived from *U. pinnatifida* is GRAS under the conditions of use described in the notice.

The safety determination of Maritech® fucoidan concentrate, derived from *F. vesiculosus*, for the present GRAS assessment is based on the totality of the available scientific evidence that includes human observations and a variety of preclinical and clinical studies. On the basis of scientific procedures<sup>1</sup>, Marinova considers the consumption of fucoidan concentrate (Maritech®) derived from *F. vesiculosus*, as a food ingredient to be safe at levels up to 250 mg/person/day. Based on the available safety-related information, the estimated daily intake, if ingested daily over a lifetime, the Expert Panel concluded that the intended uses of Maritech® fucoidan concentrate derived from *F. vesiculosus* as described herein are safe.

#### **F. Availability of Information:**

The data and information that forms the basis of Marinova's fucoidan derived from *F. vesiculosus* GRAS determination will be available for the Food and Drug Administration's review and copying at the following address or will be provided to the agency upon request:

Madhu G. Soni, Ph.D., FATS  
Soni & Associates Inc.,  
749 46<sup>th</sup> Square,  
Vero Beach FL, 32968  
Phone: (772) 299-0746; E-mail: [sonim@bellsouth.net](mailto:sonim@bellsouth.net)

## **II. Detailed Information About the Identity of the GRAS Substance:**

### **A. Synonyms:**

Sulfated L-Fucose algal polysaccharide;; Fucoidin; Fucan, Fucosan, or Sulfated fucan, fucose-rich polysaccharide.

### **B. Trade Name:**

The subject of this GRAS assessment will be marketed under the name Maritech®.

### **C. Physical Characteristics**

Off white to brown powder

### **D. Chemical Abstract Registry (CAS) Number**

9072-19-9 and 84696-13-9

### **E. Chemical Formula and Molecular Weight**

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<sup>1</sup> 21 CFR §170.3 Definitions. (h) Scientific procedures include those human, animal, analytical, and other scientific studies, whether published or unpublished, appropriate to establish the safety of a substance.

(C<sub>6</sub>H<sub>10</sub>O<sub>7</sub>S)<sub>n</sub>. The molecular weight of fucoidan derived from *F. vesiculosus* ranges from 30-550 kDaltons.

## F. Structure

The general chemical structure of fucoidan derived from *F. vesiculosus* is presented in Figure II.F.1.

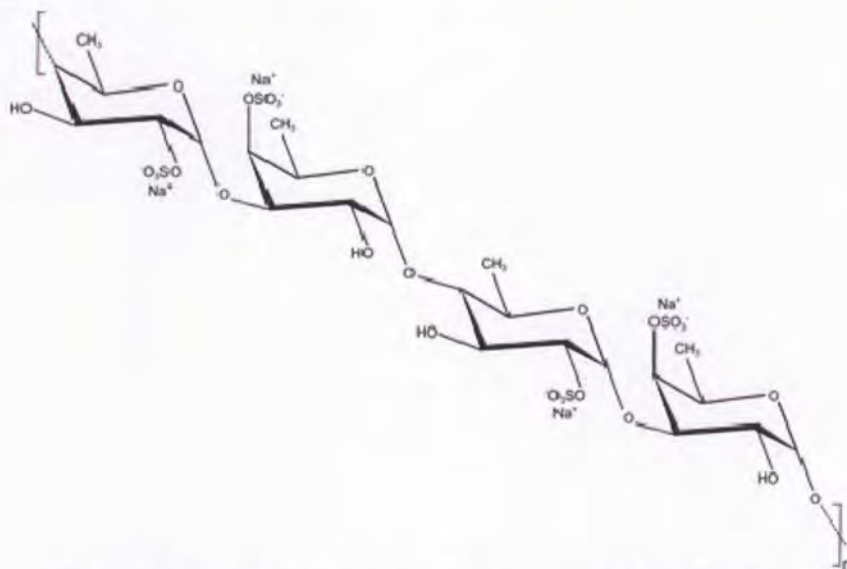


Figure II.F.1. Chemical Structure of *Fucus vesiculosus* Fucoidan

## G. Specifications

Food grade specifications of fucoidan concentrate derived from *F. vesiculosus* have been established by Marinova and are presented in Table II.G.1. To demonstrate conformance with the food-grade specifications, Marinova analyzed several batches of fucoidan derived from *F. vesiculosus*. Analytical results from five non-consecutive lots (Appendix I) for fucoidan derived from *F. vesiculosus* suggest that it is consistently manufactured to meet the standard specifications.

The product is standardized to the contents of fucoidan according to Marinova method 'C05' which determines fucoidan content based on the carbohydrate, sulfate, counterion, and acetyl content of the fucoidan polymers. The complete C05 method is provided in Appendix II. Marinova laboratory methods – including those applicable to C05 - are qualified and validated according to good laboratory practices and include the establishment of linearity, range, limits of detection and quantification (LOD/LOQ), specificity, accuracy, reproducibility and robustness.

The fucoidan content of the product derived from *F. vesiculosus* ranges from 70 to 95%. Sulfate contents of the concentrate typically comprise 20-30% by weight. The iodine content of Maritech® fucoidan derived from *F. vesiculosus* is typically less than 100 ppm (Appendix III). The average iodine content of kelp is reported to range from 1500 to 2500 ppm (Mussig

et al., 2006). Typical compositional analysis of fucoidan derived from *F. vesiculosus* is summarized in Table II.G.2.

**Table II.G.1. Typical Food Grade Specifications of Fucoidan derived from *F. vesiculosus* (Marinova, 2016)\***

Parameters	Specification	Assay method
Description	Off-white to brown powder	Visual
Moisture	<10% (105°C for 2 hours)	USP
pH value	4.0 - 7.0 (1% suspension at 25°C)	pH Meter
Solubility	Dissolves readily in water	In House
Particle size	Minimum 80% less than 300 microns	USP
Fucoidan	≥70%	Marinova Method
Pesticide residues	Complies with USP	USP
<b>Heavy metals</b>		
Arsenic (inorganic)	1 ppm Max	ICP
Lead	1 ppm Max	ICP
Cadmium	3 ppm Max	ICP
Mercury	1 ppm Max	ICP
<b>Microbiological parameters</b>		
Total Aerobic microbial count	1000 cfu/g (max)	AOAC
Yeasts and molds	100 cfu/g (max)	AOAC
Total enterobacteria count	Absent / g	AOAC
<i>Eschericia coli</i>	Absent / g	AOAC
<i>Salmonella</i> species	Absent / 10 g	USP
<i>Staphylococcus aureus</i>	Absent / g	USP
*Based on information provided by Marinova. ppm = parts per million; cfu = colony forming units		

**Table II.G.2. Typical Compositional Analysis of Fucoidan derived from *F. vesiculosus***

Component name	Amount
Fucoidan	70.0-95.0%
Alginate	2.0-5.5%
Polyphloroglucinol	0.5-2.5%
Mannitol	1-5%
Natural salts	0.5-2.5%
Other carbohydrates	0.5-1.0%

\*Based on information provided by Marinova; values are expressed as w/w

## H. Manufacturing process

Maritech® fucoidan is extracted according to current good manufacturing practices (cGMP), as outlined in Figure II.H.2, at Marinova Pty Ltd, 249 Kennedy Drive, Cambridge TAS 7170, Australia. The unique Maritech® extraction process has been specifically designed to extract fucoidan from brown seaweed without the use of solvents other than water. For the extraction of fucoidan, wild-grown seaweeds (*Fucus vesiculosus*), hand-harvested from the cleanest ocean waters are collected. The seaweed thus obtained is dried, milled and suspended in pure water. The acidification of water is carried out with food-grade acid to mildly acidic pH and the suspension is subjected to agitation. The suspension is filtered to remove seaweed residue (fucoidan rich filtrate retained) with food grade filtration aid. The filtrate thus obtained is neutralised with food-grade base to pH 5 (approximately) and subjected to secondary filtration to remove salts (mechanical only). This unique extraction process ensures the chemical constituent of the fucoidan molecule remains unaltered and equivalent as in their natural state. The secondary filtrate is concentrated, dried and milled to produce the powdered fucoidan extract.

Marinova utilizes membrane filtration technology to purify the aqueous fucoidan extracts. This process removes low molecular weight impurities without requiring any organic solvent or chemical additives which are commonly employed in precipitation processes. The purified fucoidan is flash heat-treated during the drying process, rendering disinfection via chemical or irradiation methods unnecessary. Marinova's proprietary process employs membrane filtration analogously to techniques that have been used in food processing and water purification (EPA) for many years. The membrane filters used by Marinova are compliant with applicable FDA regulations in the Code of Federal Regulations (CFR), Title 21, 3-A Sanitary Standards No. 45-02 and USDA sanitary standards and are widely used by food ingredient manufacturers, particularly for protein and carbohydrate processing.

The final product is free of allergen (milk, egg, fish, shellfish, tree nut, wheat, peanuts and soybean) contamination and arthropod and mollusk infestations and other quarantine risk material. The purification step in the Maritech® process eliminates contaminants such as salts and remaining alginate impurities from the product. This process eliminates the numerous purification steps in the commonly used extraction methods in the industry, such as disinfecting steps (hypochlorite and irradiation). In an extensive review article, Ale et al. (2011) reported that preservation of the structural integrity of the fucoidan molecules essentially depends on the extraction methodology which has a crucial, but partly overlooked, significance for obtaining the relevant structural features required for specific biological activities and for elucidating structure-function relations.

The quality assurance method for analyzing fucoidan sugars to determine purity has been developed and validated for Marinova by the Complex Carbohydrate Research Centre at the University of Georgia (US). The Maritech® extraction process is controlled using an ISO9001 and HACCP accredited quality-management system. Marinova rigorously tests its final production batches to verify adherence to quality control specifications. Maritech® complies with Organic, Kosher and Halal quality requirements. All raw materials and processing aids used in the manufacture of fucoidan are suitable food-grade materials and/or are used in accordance with applicable U.S. federal regulations for such uses. The manufacturing facility is registered with the FDA under the number: 1065 1320 208.



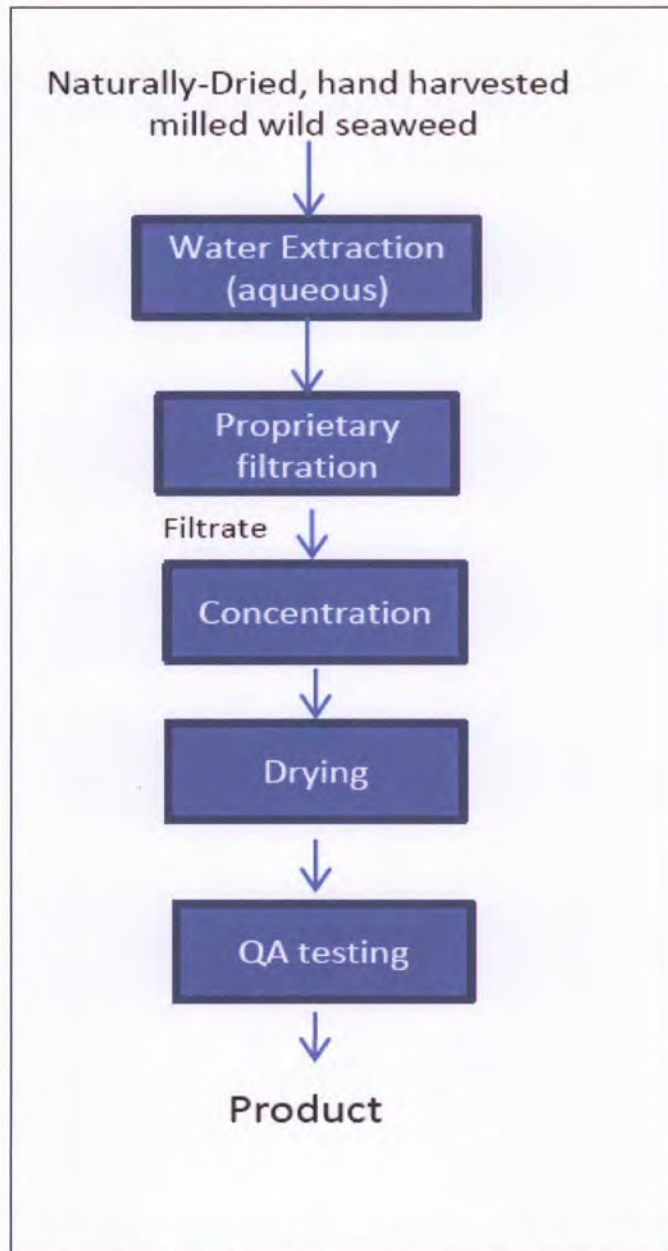


Figure II.H.2. Manufacturing Process of Fucoïdan

### III. Summary of the Basis for the Determination that Fucoïdan concentrate is GRAS

An independent panel of recognized experts, qualified by their scientific training and relevant national and international experience to evaluate the safety of food and food ingredients, was requested by Marinova to determine the Generally Recognized As Safe (GRAS) status of fucoïdan derived from *Fucus vesiculosus*. The Expert Panel consisted of the following individuals: Professor John Thomas, Ph.D., FATS (Indiana University School of Medicine);

Robert L. Martin, Ph.D. (Retired FDA Deputy Director); and Madhusudan G. Soni, PhD, FACN, FATS (Food Ingredient Safety Consultant).

A comprehensive search of the scientific databases for safety and toxicity information on fucoidan was conducted through May 2016. Additionally, safety and regulatory evaluations by national and international agencies were also searched and considered for the present assessment.

Based on a critical evaluation of the pertinent data and information summarized herein, and employing scientific procedures, the Expert Panel members have individually and collectively determined that the addition of fucoidan derived from *Fucus vesiculosus* to the foods [Baked goods (bread, cake, noodles); Soups; Snack Foods; Imitation Dairy Products; and Seasonings & Flavors at use levels up to 30 mg/serving (reference amounts customarily consumed; 21 CFR 101.12)], when not otherwise precluded by a Standard of Identity, meeting the specification cited above and manufactured in accordance with current Good Manufacturing Practice, is Generally Recognized As Safe (GRAS) under the conditions of intended use, as specified herein.

In arriving at this decision that Maritech® fucoidan derived from *Fucus vesiculosus* is GRAS, the Expert Panelists relied upon the conclusions that neither fucoidan nor any of its constituents pose any toxicological hazards or safety concerns at the intended use levels, as well as on published toxicology studies and other articles relating to the safety of the product. It is also the opinion of the Expert Panelists that other qualified and competent scientists, reviewing the same publicly available toxicological and safety information, would reach the same conclusion. The GRAS Panel did not prepare a separate report or statement, but reviewed the entire GRAS dossier.

**IV. Basis for a Conclusion that Fucoidan from *Fucus vesiculosus* is GRAS for its Intended Use.**

**EXPERT PANEL STATEMENT**

**DETERMINATION OF THE GENERALLY RECOGNIZED AS SAFE  
(GRAS) STATUS OF**

**FUCOIDAN FROM *FUCUS VESICULOSUS***

**AS A FOOD INGREDIENT**

Prepared for:  
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249 Kennedy Drive  
Cambridge, Tasmania 7170  
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Prepared by:  
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**Panel Members**

Robert L. Martin, Ph.D.  
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Madhusudan G. Soni, PhD, FACN, FATS

**DETERMINATION OF THE GENERALLY RECOGNIZED AS SAFE (GRAS)  
STATUS OF**

**FUCOIDAN FROM *FUCUS VESICULOSUS*  
AS A FOOD INGREDIENT**

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# **DETERMINATION OF THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF FUCOIDAN FROM *FUCUS VESICULOSUS* AS A FOOD INGREDIENT**

## **1. INTRODUCTION**

The undersigned, an independent panel of recognized experts (hereinafter referred to as the Expert Panel)<sup>2</sup>, qualified by their scientific training and relevant national and international experience to evaluate the safety of food and food ingredients, was convened by Soni & Associates Inc. at the request of Marinova Pty. Ltd. (Marinova), to determine the Generally Recognized As Safe (GRAS) status of Maritech® fucoidan derived from the brown seaweed, *Fucus vesiculosus* (Bladderwrack) as a food ingredient in conventional foods such as Baked goods (bread, cake, noodles), Milk (milk, yogurt) and Fruit and Vegetable Juices at use levels up to 30 mg/serving (reference amounts customarily consumed, 21 CFR 101.12). A comprehensive search of the scientific literature for safety and toxicity information on fucoidan and its source materials *F. vesiculosus* (Bladderwrack) was conducted through May 2016 and made available to the Expert Panel. The Expert Panel independently and critically evaluated materials submitted by Marinova and other information deemed appropriate or necessary. Following an independent, critical evaluation, the Expert Panel conferred on July 19, 2016 and unanimously agreed to the decision described herein.

### **1.1. Background**

Seaweeds, including various brown seaweeds are part of the food culture in Asia, notably in Japan, Philippines, and Korea, and seaweed extracts have also been used as a remedy in traditional healthcare (Ale et al., 2011). The brown color of these algae results from the dominance of the xanthophyll pigment fucoxanthin, which masks the other pigments. In recent years, much attention has been focused on functional polysaccharides isolated from natural sources. Fucoidan is a class of sulfated, fucose rich, polysaccharides found in the fibrillar cell walls and intercellular spaces of brown seaweeds of the class Phaeophyceae (Senthilkumar et al., 2013). Fucoidan-containing sulfated polysaccharides (FCSP) may also contain galactose, mannose, xylose, glucose and/or glucuronic acid, usually in minor amounts. It was first isolated in 1913 from marine brown algae and was named “fucoidin” (Kylin, 1913). As per IUPAC nomenclature it is named “fucoidan” but is also called fucan, fucosan or sulfated fucan (Bertheau and Mulloy, 2003). In several recent scientific studies, fucoidans from seaweed have been investigated for their potential biological functions such as antioxidant activity, antitumor, immunomodulatory, antiviral, antithrombotic, anticoagulant, anti-inflammatory and antilipidemic activity (Fitton, 2011; Senthilkumar et al., 2013). The production of well characterized reproducible fucoidan fractions on a commercial scale has become possible in recent years, thus making specific uses of fucoidan possible. Given its beneficial properties, Marinova intends to use standardized fucoidan derived from *Fucus vesiculosus* as a food ingredient in selected food products.

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<sup>2</sup>Modeled after that described in section 201(s) of the Federal Food, Drug, and Cosmetic Act, As Amended. See also attachments (curriculum vitae) documenting the expertise of the Panel members.

## 1.2. Description

The subject of this GRAS determination, Maritech® fucoidan derived from *Fucus vesiculosus* (Bladderwrack), is a standardized spray dried polysaccharide extract. It is an off-white to brown colored powder without any characteristic taste and odor. It will be marketed under the trade name Maritech® with product identifiers including, *F. vesiculosus* extract and 'Synergy'. General descriptive parameters and properties of fucoidan manufactured by Marinova are summarized in Table 1.

**Table 1. General Descriptive Characteristics of Fucoidan**

Parameter	Description
Botanical source	<i>Fucus vesiculosus</i>
Synonym of source	Bladderwrack, black tang, rockweed, bladder fucus, sea oak, black tany, cut weed
CAS No.	9072-19-9 and 84696-13-9
Appearance	Powder
Color	Off-white to brown
Odor	Bland
Taste	Bland
Storage	Store in well sealed containers in a cool position. Protect from light, moisture and heat
Shelf life	Five years in the original pack

\*Based on information provided by Marinova

The hierarchical classification of *F. vesiculosus* is presented in Table 2. *F. vesiculosus*, commonly known as bladderwrack, is found on the coasts of the North Sea, the western Baltic Sea, and the Atlantic and Pacific Oceans. In addition to bladderwrack, it is also known by the common names black tang, rockweed, bladder fucus, sea oak, black tany, cut weed, dyers fucus, red fucus, and rock wrack. It is a small brown seaweed measuring up to 100 cm in length and is easily recognized by the small gas-filled vesicles which occur in pairs one on either side of a central midrib running along the centre of the strap-like frond.

**Table 2. Classification of *Fucus vesiculosus***

	<i>Fucus vesiculosus</i>
Kingdom	Chromista
Phylum	Heterokontophyta or Ochrophyta
Class	Phaeophyceae
Order	Fucales
Family	Fucaceae
Genus	<i>Fucus</i>
Species	<i>Fucus vesiculosus</i>

## 1.3. Typical Specifications and Composition

Food grade specifications of fucoidan have been established by Marinova and are presented in Table 3. To demonstrate conformance with the food-grade specifications, Marinova analyzed several batches of fucoidan derived from *F. vesiculosus*. Analytical results from five non-consecutive lots (Appendix I) for fucoidan derived either from *F. vesiculosus* suggest that it is consistently manufactured to meet the standard specifications. The product is standardized to the contents of fucoidan according to Marinova method 'C05' which determines fucoidan content based on the carbohydrate, sulfate, counterion, and acetyl content of the fucoidan

polymers. The peak molecular weights of Maritech® *F. vesiculosus* fucoidan is of the order of 30-250 kDa. Sulfate contents of Maritech® fucoidan derived from *F. vesiculosus* typically comprise 20-30% by weight. The fucoidan content ranges from 70 to 95%. The iodine content of Maritech® fucoidan derived from *F. vesiculosus* is typically less than 100 ppm. The iodine content has been verified across numerous batch analyses over a period of 12 years and are summarised in Appendix III. The average iodine content of kelp is reported to range from 1500 to 2500 ppm (Mussig et al., 2006). Typical compositional analysis of Maritech® fucoidan derived from *F. vesiculosus* is summarized in Table 4.

**Table 3. Typical Food Grade Specifications of Fucoidan (Marinova, 2016)\***

Parameters	Specification	Assay method
Description	Off-white to brown powder	Visual
Moisture	<10% (105°C for 2 hours)	USP
pH value	4.0 - 7.0 (1% suspension at 25°C)	pH Meter
Solubility	Dissolves readily in water	In House
Particle size	Minimum 80% less than 300 microns	USP
Fucoidan	≥70%	Marinova Method
Pesticide residues	Complies with USP	USP
<b>Heavy metals</b>		
Arsenic (inorganic)	1 ppm Max	ICP
Lead	1 ppm Max	ICP
Cadmium	3 ppm Max	ICP
Mercury	1 ppm Max	ICP
<b>Microbiological parameters</b>		
Total Aerobic microbial count	1000 cfu/g (max)	AOAC
Yeasts and molds	100 cfu/g (max)	AOAC
Total enterobacteria count	Absent / g	AOAC
<i>Eschericia coli</i>	Absent / g	AOAC
<i>Salmonella</i> species	Absent / 10 g	USP
<i>Staphylococcus aureus</i>	Absent / g	USP

\*Based on information provided by Marinova. ppm = parts per million; cfu = colony forming units

**Table 4. Typical Compositional Analysis of Fucoidan derived from *F. vesiculosus***

Component name	Amount
Fucoidan	70.0-95.0%
Alginate	2.0-5.5%
Polyphloroglucinol	1.0-25%
Mannitol	1-5%
Natural salts	0.5-2.5%
Other carbohydrates	0.5-1.0%

\*Based on information provided by Marinova; values are expressed as w/w



#### 1.4. Manufacturing/Extraction Process

Fucoidan is extracted according to current good manufacturing practices (cGMP), as outlined in Figure 1, at Marinova Pty Ltd, 249 Kennedy Drive, Cambridge TAS 7170, Australia. The unique Maritech® extraction process has been specifically designed to extract fucoidan from brown seaweed without the use of solvents other than water. For the extraction of fucoidan, wild-grown seaweeds, hand-harvested from the cleanest ocean waters are collected. The seaweed thus obtained is dried, milled and suspended in pure water. The acidification of water is carried out with food-grade acid to mildly acidic pH and the suspension is subjected to agitation. The suspension is filtered to remove seaweed residue (fucoidan rich filtrate retained) with food grade filtration aid. The filtrate thus obtained is neutralised with food-grade base to pH~5 and subjected to secondary filtration to remove salts (mechanical only). This unique extraction process ensures the chemical constituent of the fucoidan molecule remains unaltered and equivalent as in their natural state. The secondary filtrate is concentrated, dried and milled to produce the powdered fucoidan extract.

Marinova utilizes membrane filtration technology to purify the aqueous fucoidan extracts. This process removes low molecular weight impurities without requiring any organic solvent or chemical additives which are commonly employed in precipitation processes. The purified fucoidan is flash heat-treated during the drying process, rendering disinfection via chemical or irradiation methods unnecessary. Marinova's proprietary process employs membrane filtration analogously to techniques that have been used in food processing and water purification (EPA) for many years. The membrane filters used by Marinova are compliant with applicable FDA regulations in the Code of Federal Regulations (CFR), Title 21, 3-A Sanitary Standards No. 45-02 and USDA sanitary standards and are widely used by food ingredient manufacturers, particularly for protein and carbohydrate processing.

The final product is free of allergen (milk, egg, fish, shellfish, tree nut, wheat, peanuts and soybean) contamination and arthropod and mollusk infestations and other quarantine risk material. The purification step in the Maritech® process eliminates contaminants such as salts and remaining alginate impurities from the product. This process eliminates the numerous purification steps in the commonly used extraction methods in the industry, such as disinfecting steps (hypochlorite and irradiation). In an extensive review article, Ale et al. (2011) reported that preservation of the structural integrity of the fucoidan molecules essentially depends on the extraction methodology which has a crucial, but partly overlooked, significance for obtaining the relevant structural features required for specific biological activities and for elucidating structure-function relations.

The quality assurance method for analyzing fucoidan sugars to determine purity has been developed and validated for Marinova by the Complex Carbohydrate Research Centre at the University of Georgia (US). The Maritech® extraction process is controlled using an ISO9001 and HACCP accredited quality-management system. Marinova rigorously tests its final production batches to verify adherence to quality control specifications. Maritech® complies with Organic, Kosher and Halal quality requirements. All raw materials and processing aids used in the manufacture of fucoidan are suitable food-grade materials and/or are used in accordance with applicable U.S. federal regulations for such uses. The manufacturing facility is registered with the FDA under the number: 1065 1320 208.

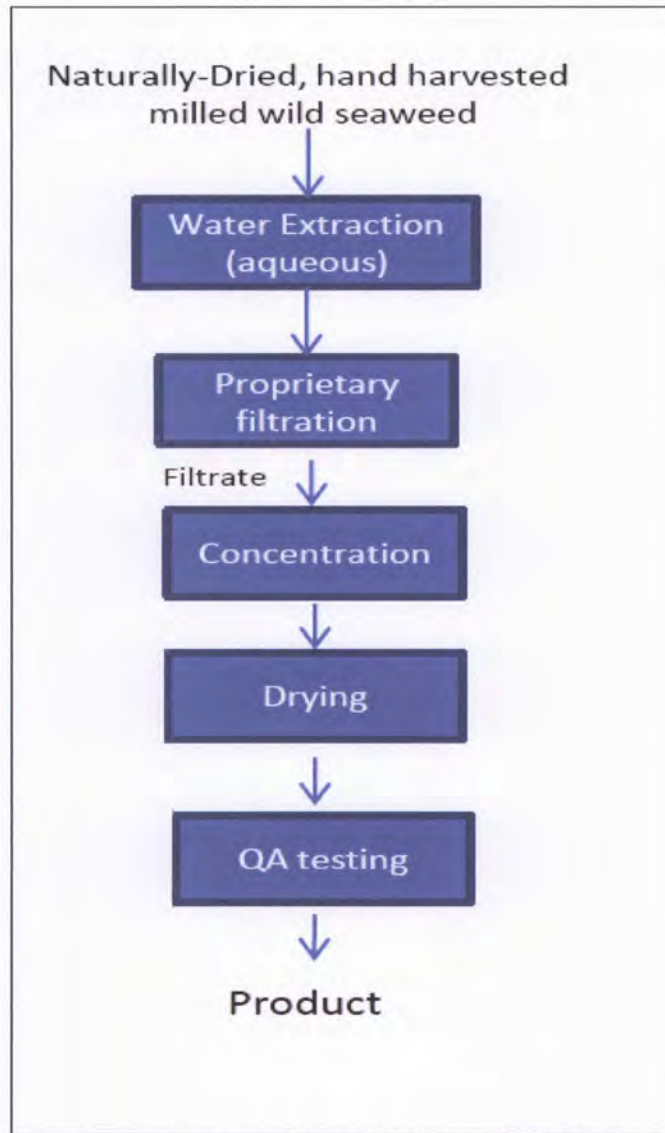


Figure 1. Manufacturing Process of Fucoidan

### 1.5. Chemical Structure

In general, fucoidan is a type of polysaccharide that contains substantial percentages of L-fucose and sulfate ester groups, mainly derived from brown seaweed (Li et al., 2008a). In recent years, fucoidan has been extensively studied for its chemical structure and biological activities. The available data thus show that the term “fucoidan” has been used for several different chemical structures and, *vice versa*, and the term covers a diverse family of fucose-containing sulfated polysaccharides. Ale et al. (2011) suggested to use the term fucose-containing sulfated polysaccharides (FCSPs) rather than fucoidan as a collective term for these polysaccharides. In the case of sulfated polysaccharides, the position of sulfate groups is important to the biological activities. The representative chemical structure of fucoidan derived from *F. vesiculosus* is presented in Figure 2. Fucoidan derived from *F. vesiculosus* as well as from several other species of brown seaweed, have simple chemical compositions, mainly being

composed of fucose and sulfate. However, the chemical compositions of most fucoidans are complex.

The most studied fucoidans have a backbone built of (1→3)-linked  $\alpha$ -L-fucopyranosyl residues or of alternating (1→3)- and (1→4)-linked  $\alpha$ -L-fucopyranosyl residues. It also includes sulfated galactofucans with backbones built of (1→6)- $\beta$ -D-galacto- and/or (1→2)- $\beta$ -D-mannopyranosyl units with fucose or fuco-oligosaccharide branching, and/or glucuronic acid, xylose or glucose substitutions (Ale et al., 2011). These fucopyranosyl residues may be substituted with short fucoside side chains or sulfate groups at C-2 or C-4 (Senthilkumar et al., 2013). In addition to fucose and sulfate, fucoidan also contains other monosaccharides (galactose, xylose, etc.) and uronic acids, even acetyl groups and protein. *F. vesiculosus* fucoidan is a high fucose sulfated fucoidan. Based on the published literature, the most common source of fucoidan used for experimental studies is derived from *F. vesiculosus*. Other common fucoidans are sourced from edible species such as *Cladosiphon okamuranus*, *Laminaria japonica* and *U. pinnatifida*.

*F. vesiculosus* fucoidans are polymeric compounds with broad polydispersities. Fucoidan derived from *F. vesiculosus* lacks the galactose content commonly found in fucoidan from *U. pinnatifida* and also does not have acetyl functional groups. Maritech® fucoidan, the subject of the present GRAS assessment derived from *F. vesiculosus* is typically over 70% pure.

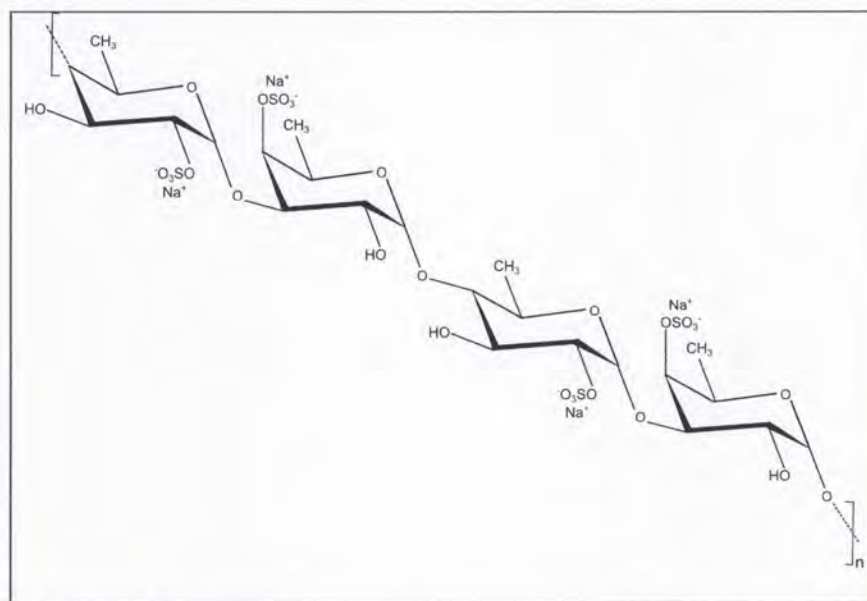


Figure 2. Chemical structure of fucoidan derived from *F. vesiculosus*

## 1.6. Regulatory History

Fucoidan, as such, is not regulated by the US FDA. However, the Code of Federal Regulation (CFR) describes several species of brown algae. As per 21 CFR §184.1120, brown algae (seaweed) is generally recognized as safe (GRAS) for use in food under the following specific limitations: added at a level not to exceed current good manufacturing practice (cGMP) to spices, seasonings, and flavorings as a flavor enhancer or adjuvant. The “specific limitations” under this regulation invokes §184.1(b)(2) according to which any use of these algae other than

that mentioned in the regulation will require a food additive petition. The regulation is for brown algae specifically and not for the constituents derived or purified from it. *F. vesiculosus*, source material for fucoidan is not mentioned in the FDA regulation. The available evidence suggests that this species of source material is commonly consumed as food in Asian countries and the product of this GRAS determination is a water extract; no chemicals are used. The European Commission has listed *Fucus vesiculosus* as a food or food ingredient that has been consumed to a significant degree before 15 May 1997. Fucoidan from *Fucus vesiculosus* is also referenced in the BELFRIT list, recently submitted to the European Commission<sup>3</sup> to amend the initial 1997 novel foods decree. Thus its access to the market is not subject to the Novel Food Regulation.

Fucoidans derived from different brown seaweeds are commonly marketed in the United States as a dietary supplement under different names<sup>4</sup>. These products are regulated under the Dietary Supplement Health and Education Act (DSHEA, 1994) in the US. Recently, on January 23, 2013, FDA has accepted and filed a New Dietary Ingredient Notification (NDIN) on *Laminaria hyperborean* extract. The recommended daily dose in the notification states "a daily dose of 200 to 300 mg ProtaSea fuciodan [sic] per day, equivalent to 3.3 to 5 mg/kg/day for a 60 kg individual" (FDA, 2013). Marinova Pty Ltd in 2014 also submitted a GRAS notification (GRN 565) for Maritech® fucoidan derived from *Undaria pinnatifida* with a total daily dose of 250 mg per day with no questions raised by the FDA in response.

### 1.7. Uses and Consumption

Seaweed as a staple item of the diet has been used in Japan, Korea and China since prehistoric times. Seaweed is a popular food in Japan and estimates of seaweed consumption in this country range from 4.3 to 7.3 g/person/day (Toyokawa, 1978; Teas, 1981; Fujiwara-Arasaki et al., 1984; Teas et al., 2009) to 25% of the diet (SCOGS, 1973). The National Nutrition Survey in Japan reported the daily intake of seaweeds to be about 14.3 g per adult person, with women eating more than men (Fukuda et al., 2007). In Japan and other East Asian countries, some form of seaweed is used with almost every meal: as a garnish, in soup, as a vegetable, in sweet cakes and jellies, in sauces, as a tea and in salads. It is also incorporated into flour, which is used to make noodles, a common dietary item in Japan.

The consumption of *F. vesiculosus* has been documented as early as 1937 (Kiple and Ornelas, 2000). This publication lists algae known to be used as food at the time of publication and cites two references for *Fucus* species for use as a food in Alaska and in Greenland. In recent years, consumption of *F. vesiculosus* is becoming widespread throughout western cultures as a source of iodine in dietary supplements, where it is most often referred to by its common name: bladderwrack. A major source of dietary seaweed among Japanese populations is the edible brown kelp, wakame (*U. pinnatifida*) and kombu (*Laminaria japonica*) (Skibola, 2004). Additionally, *F. vesiculosus* is also commonly used as a food in Japan. It can be stored, dried, and made into a nutritious tea, and/or added to soups and stews in flakes or powder form for flavor.

The typical carbohydrates in brown algae varieties consist of fucoidan, laminaran, cellulose, alginates, and mannitol. Fucoidans are considered as an important component of brown algae and constitute up to 25-30% of the algae dry weight, depending on the specific

<sup>3</sup> Available at: <http://ec.europa.eu/growth/tools-databases/tris/en/index.cfm/search/?trisaction=search.detail&year=2015&num=162&mLang=EN>

<sup>4</sup> Several products can be found under name fucoidan at Ebay website: <http://www.ebay.com/bhp/fucoidan>

seaweed species (Myers et al., 2011). The three most popular seaweed products in Japan are nori (*Porphyra*), wakame (*Undaria*) and kombu (*Laminaria*). The available information also indicates that the seaweed consumed can be red, green, and brown. The daily intake of seaweed has been reported to range from 4.3 to 14.3 g/person/day. Assuming that half of the seaweed consumed is brown algae that contains approximately 25% fucoidan, the daily likely intake of fucoidan from intake of seaweed can range from 0.54 to 1.79 g/person/day. The available information indicates that human beings, particularly in Japan, are regularly exposed to fucoidan from dietary intake of seaweed. The available evidence suggests that *F. vesiculosus*, a species of seaweed, used as a source material for Maritech® fucoidan, the subject of the current GRAS assessment, is commonly consumed as food in Asian countries. Additionally, Maritech® fucoidan is obtained by water extraction processes, without the use of any solvents.

## **1.8. Intended Use Levels and Food Categories**

Marinova intends to use Maritech® fucoidan as a food ingredient at use levels up to 30 mg/serving in Baked goods; Soups; Snack Foods; Imitation Dairy Products; and Seasonings & Flavors (Table 5). The subcategories of the broad top-level food categories such as baked goods, snack foods and imitation dairy products categories are as follows: Baked goods include biscuits, croissants, bagels, tortillas, soft bread sticks, soft pretzels, corn bread, hush puppies; Snack foods include all varieties, chips, pretzels, popcorns, extruded snacks, fruit-based snacks (e.g., fruit chips,) grain-based snack mixes; and the imitation dairy product include imitation ice-cream, yogurt. Although some foods with standards of identity are included in the list of foods, at present, the use of Maritech® fucoidan is intended for foods without a standard of identity. Additionally, foods that are intended for infants and toddlers, such as infant formulas or foods formulated for babies or toddlers, as well as meat and poultry products that come under USDA jurisdictions are excluded from the list of intended food uses of the subject fucoidan preparation.

### **1.8.1. Estimated Daily Intake from the Intended Uses**

#### **1.8.1.1. Use of USDA Data**

Estimates of possible daily intake of fucoidan from the "maximum" intended use levels of the final product preparation have been determined using USDA's Continuing Survey of Food Intakes by Individuals (CSFII) data. The CSFII survey is designed to measure the kinds and amounts of foods eaten by Americans. The USDA CSFII survey data were used to estimate mean and high (90<sup>th</sup> percentile) per capita levels of consumption of fucoidan from the chosen food categories. In these estimates the serving size of food category or product is based on the Reference Amounts Customarily Consumed per Eating Occasion (21 CFR 101.12) and other related information. Based on USDA CSFII surveys (Smiciklas-Wright et al., 2002) for quantities of foods consumed daily, the mean and 90<sup>th</sup> percentile consumption of fucoidan from the proposed uses in Baked goods; Soups; Snack Foods; Imitation Dairy Products; and Seasonings & Flavors were determined (Table 5). The CSFII data provides intake of several different types of baked goods. In Table 5, values for biscuits are included. Similarly, for imitation dairy products, yogurt is used as a representative category. The intended use of fucoidan at levels up to 30 mg *per* serving will result in mean and 90<sup>th</sup> percentile intake of 135.45 and 249.66 mg/person/day, respectively.

**Table 5. Intended Use Levels and Possible Daily Intake of Fucoidan Based on USDA Data<sup>1</sup>**

Food category	Consumption of food product (g/day)		Use levels/ serving (mg)	Serving size; RACC (g)	Daily intake by adult (mg/person)	
	Mean	90 <sup>th</sup> %			Mean	90 <sup>th</sup> %
Baked Goods <sup>2</sup>	64	118	30	55	34.91	64.36
Soups	398	697	30	245	48.73	85.35
Snack Foods	41	84	25	30	34.17	70.00
Imitation Dairy Products <sup>3</sup>	157	266	25	225	17.44	29.55
Seasonings & Flavors	0.01*	0.02*	10	0.5	0.20	0.40
<b>Total (mg/person/day)</b>					<b>135.45</b>	<b>249.66</b>

<sup>1</sup>The daily intake calculations are based on USDA data (CSFII) and mean portion size; <sup>2</sup>Biscuits intake is used to represent baked good. <sup>3</sup>Includes yogurt as representative for this category. \*As CSFII did not report consumption, Market Research Corporation of America data (MRCA, 1965) was used. Serving size is based on Reference Amounts Customarily Consumed per Eating Occasion (21 CFR 101.12) and other related information.

### 1.8.2. Consumption Summary

The estimated intake of fucoidan from its natural presence in seaweed appears to range from 540 to 1790 mg/person/day. The higher intakes of fucoidan are likely to be in countries such as Japan where seaweed is commonly consumed. Based on the USDA CSFII database, the intended use of fucoidan at levels up to 30 mg/serving in food categories such as in Baked goods; Soups; Snack Foods; Imitation Dairy Products; and Seasonings & Flavors will result will result in mean and 90<sup>th</sup> percentile intakes of 135.45 and 249.66 mg/person/day, respectively. For safety assessment purposes, the high intake of 250 mg fucoidan/person/day (4.17 mg/kg bw/day) is considered.

For the safety assessment purposes, we used the estimated daily intake determined using the CSFII data. This method is commonly used for food ingredient intake assessment purposes. In addition to CSFII survey data there are other methods such as National Health and Nutrition Examination Survey (NHANES) data set. The NHANES is a program of studies designed to assess the health and nutritional status of adults and children in the United States. For use of NHANES dataset, one needs technical and analytical expertise and can be expensive. The use of recent NHANES data is unlikely to make a significant difference in the estimated daily intake as compared to the commonly used CSFII data. The estimates using the CSFII dataset reflect anticipated human exposure and are highly conservative. In general, experience suggests that the actual consumption will be much less.

### 1.9. Common Knowledge of Safe Use

There is common knowledge of a long history of human consumption of seaweeds. For centuries seaweeds have been traditionally collected by man for its use as food, therapeutics and fertilizer. The available information indicates that since prehistoric times, seaweed has been present as a staple item of the diet in Japan, Korea and China. About 21 species of seaweed are used in everyday cookery in Japan, six of them since the 8<sup>th</sup> century (Guiry, 2013). Until recently seaweed accounted for over 10% of the Japanese diet. Seaweeds still play a wide and varied role in modern life and are used as a food resource and a source of industrial and other chemicals.

Among different seaweeds in Japan, the most important food species are Nori (*Porphyra* species), Kombu (*Laminaria* species), and Wakame (*U. pinnatifida*). In Japan, *U. pinnatifida* is a more important crop than *Laminaria* both in value and production.

*F. vesiculosus* is perhaps the best-known species that contains a number of useful compounds. This species is common in the North Atlantic south to the Canary Islands. The plants consist of a flattened, dichotomously-branched thallus which has a small stipe and a holdfast. This species is also commonly used as a food in Japan, and is present in diets, generally at lower levels in Europe and North America. It can be stored dried, and makes a nutritious tea, and added to soups and stews in flakes or powder form for flavor. Seaweeds containing fucoidans have been used as foods such as sea vegetables for centuries (Udani and Hesslink, 2012). As discussed earlier, fucoidan is an important component of brown algae and may constitute up to 25-30% of the dried biomass. The available information demonstrates that there is common knowledge of consumption of seaweed, including *F. vesiculosus* that is the source of Maritech® fucoidan in the present application.

## 2. SAFETY RELATED DATA

The safety of fucoidan is supported by several lines of evidence including multiple human clinical trials, as well as by a variety of animal and *in vitro* experimental studies that further corroborate the human observations. Because of its known health benefits and historical use, there has been considerable effort to elucidate the biological role of fucoidan, one of the active constituents of brown seaweed, in the human body. As a result, the literature is full of information on seaweed and fucoidan. In the published literature, over 2,500 preclinical *in vitro* and *in vivo* studies with seaweed and over 1,000 studies with fucoidan have appeared. Additionally, several human clinical trials can be found in the published literature. Relevant biological and toxicological studies on fucoidan derived from *F. vesiculosus* as well as from other brown seaweed sources are included in the following section. The safety data from animal studies is extensive. Efforts have been made to present both the data supporting the safety as well as any data on the toxicity of fucoidan. The efficacy data of brown seaweed and its extract are also extensive and thus, only relevant studies are briefly mentioned in the following sections for the sake of completeness and to support the safety of fucoidan.

As the present GRAS assessment is for fucoidan derived from *F. vesiculosus*, an attempt has been made in the following sections to first present safety related information of fucoidan derived from this source separately following which the safety of fucoidan from other sources is discussed. The safety related animal and human studies of fucoidan derived from *F. vesiculosus* as well as from some other sources are summarized in Table 6. These studies are further discussed in the individual safety section of the particular source organism.

**Table 6. Summary of Toxicity studies of Fucoidan from Various Sources**

Fucoidan Characteristics	Details of Studies	Dose	Species	Results	Reference
<b>Fucoidan derived from <i>F. vesiculosus</i> (also includes <i>Macrocystis pyrifera</i>, <i>L. japonica</i>)</b>					
85% Fucoidan	115 participants with osteoarthritis (96 moderate, 17 mild and 2 severe); 96 with moderate osteoarthritis analyzed	300 mg/person/day for 12 weeks	Human	No treatment related adverse effects; No significant changes in liver, renal, hemopoietic function	Myers et al., 2016
75% total fucoidan	Full blood count, clinical biochemistry	0.1 and 1 g for 84 days	Human	No toxicity	Myers et al., 2010
75% total fucoidan	Full blood count, clinical biochemistry	0.1 and 1 g for 28 days	Human	No toxicity	Myers et al., 2011
<b>Fucoidan derived from <i>U. pinnatifida</i></b>					
53% total sugar, 7.4% sulfate, 27% Uronic acid, 54% fucose, 35% galactose	Short-term <i>in vivo</i> toxicity; Ames test; Bone marrow micronucleus	1000 mg/kg body weight per day for 28 days	Sprague Dawley rats	Not toxic at 1000 mg/kg bw/day; Increase in ALT at 2000 mg/kg; No genotoxicity	Chung et al., 2010
64.4 ± 6.0% fucose, 31.9 ± 4.7% galactose, 3.6 ± 1.3% mannose, and 31.7 ± 2.2% sulfate	Genotoxicity: Bacterial mutation; Bone marrow Micronucleus formation	Up to 2000 mg/kg/bw orally	Sprague Dawley rats	Fucoidan presents no significant genotoxic concern.	Kim et al., 2010a
64.4 ± 6.0% fucose, 31.9 ± 4.7% galactose, 3.6 ± 1.3% mannose, and 31.7 ± 2.2% sulfate	Toxicity measures- body weight, ophthalmoscopy urinalysis, hematology, and histopathology; Clotting parameters- Prothrombin time or activated partial thromboplastin time	Up to 1350 mg/kg bw/day for 4 weeks orally	Sprague Dawley rats	No change to prothrombin time or activated partial thromboplastin time	Kim et al., 2010b
75% fucoidan	Full blood count, clinical biochemistry	3 g per day for 12 days	Human	No toxicity noted	Irhimeh et al., 2005, 2007, 2009
<b>Fucoidan derived from <i>L. japonica</i></b>					
Fucoidan Fraction MW average “10–300 kD”	Oral dosing in experimental model; Toxicity by clinical	Escalation doses up to 20 mg/kg	Dogs with hemophi	No clinical toxicity	Prasad et al., 2008



	observation		lia A; Rats		
Fluorescent labeled fucoidan	Subcutaneous	5 mg/kg	Rat	Half life 83 min	Prasad et al., 2008
MW average 189 kDa Fucose 28% sulfate 29% total sugars 48%	Acute and sub-chronic toxicity	300, 900 and 2500 mg/kg bw/day for 6 months	Wistar Rats	Not toxic at 300 mg/kg bw/day Prolonged clotting at 900 and 1200 mg/kg bw	Li et al., 2005
<b>Fucoidan derived from <i>Cladisiphon</i> sp.</b>					
85% fucoidan	Patients with HTLV1 were treated clinically	6 g per day up to 13 months	Human	Four patients diarrhea; No other toxicity noted; Reduction in viral load	Araya et al., 2011

## 2.1. Safety of *F. vesiculosus* Fucoidan

### 2.1.1. Human Studies

Findings from some of the clinical studies of fucoidan derived from *F. vesiculosus* are summarized in Table 7. In a double-blind randomized placebo-controlled trial, Myers et al. (2016) investigated the effects of fucoidan (subject of this GRAS assessment) derived from *F. vesiculosus* in individuals with osteoarthritis (study completed by Myers et al., 2012 and report provided by Marinova). In this study, 144 healthy volunteers aged between 18 and 85 years with a diagnosis of osteoarthritis of the knees (n=122 with moderate arthritis- main study, n=18 with mild arthritis- mild sub-study and n=4 with severe arthritis- severe sub-study) were enrolled. The study was completed by 115 participants (96 moderate, 17 mild and 2 severe). The main analysis was undertaken in 96 participants with moderate osteoarthritis who completed the study. For this study, an aqueous extract of *F. vesiculosus* delivered as a dried powder (150 mg) was used in capsule form. Participants randomized to the active group took two capsules (300 mg) per day (morning and night) for 12 weeks. The placebo group received an identical capsule containing microcellulose. In addition to the Comprehensive Osteoarthritis Test, weight, blood pressure, pulse, and clinical laboratory measures (full blood count, liver function tests as well as urea, creatinine and electrolytes) were measured at baseline and week 12. Adverse events and any changes to treatment were recorded at each clinic visit. Safety was assessed by measuring cholesterol, liver function, renal function and hemopoietic function over the course of the study and closely monitoring adverse events (Myers et al., 2012; 2016).

In total 29 participants withdrew from the study (Myers et al., 2012; 2016). The withdrawal rate from the main study was 21% (26 out of 122 initially enrolled); 5% in the mild sub-study (1 out of 18 initially enrolled); and 50% in the severe sub-study (2 out of 4 initially enrolled). One participant was withdrawn due to a serious adverse event. The major reason for withdrawal was pain effecting 15 participants (14 from the main study and 1 in the severe sub-study). Ten of the 15 individuals withdrawing due to pain were on the placebo (this included 9/14 in the main study). Non-compliance with study protocols led to the withdrawal of 7

participants; and, inability to attend due to work commitments to the withdrawal of 3 participants. Diarrhea led to the withdrawal of 2 participants (one active and one placebo group); and nausea to the withdrawal of 1 participant (active group). Overall, there were three serious adverse events which were unlikely to be associated with the study treatment as all three participants were receiving placebo. All other adverse events recorded were mild and self limiting. There were no changes in the blood parameters that were of any clinical significance during the course of the study. The results of this study suggest that intake of fucoidan derived from *F. vesiculosus* is safe at levels of 300 mg/day for 12 weeks (Myers et al., 2012; 2016). The Primary Investigator for the trial reported that the drop-out rate for this study was typical for a study of its type, with an elderly cohort suffering chronic pain.

**Table 7. Human Clinical Studies of Fucoidan Derived from *F. vesiculosus***

Source	Dose	Duration	Subjects	Findings	Reference
<i>F. vesiculosus</i>	300 mg	Daily for 12 weeks	Osteoarthritis of the knees (115); 96 with moderate osteoarthritis analyzed	No adverse effects, including changes in liver, renal, hemopoietic function	Myers et al., 2016
<i>F. vesiculosus</i>	0.1 and 1 g	Daily for 28 days	Healthy adults (10)	No adverse effects. No changes in blood hemopoietic, hepatic, or renal function	Myers et al., 2011
<i>F. vesiculosus</i>	0.1 and 1 g	Daily for 84 days	Healthy adults (12)	No adverse effects. No changes in liver, renal, hemopoietic function	Myers et al., 2010

In an earlier published study, Myers et al. (2010) investigated the effects of seaweed extract nutrient complex on osteoarthritis. In this open label combined phase I and II pilot trial, Maritech® extract formulation, containing extracts from three different species of brown algae, plus nutrients was investigated for acute safety and efficacy. Twelve volunteers (5 females- 62±11 years and seven males- 57±9 years) with knee osteoarthritis were randomized to receive either 100 mg (n = 5) or 1000 mg (n = 7) of the formulation per day for 12 weeks. The formulation contained *F. vesiculosus* (85% w/w), *Macrocystis pyrifera* (10% w/w) and *Laminaria japonica* (5% w/w) plus vitamin B6, zinc and manganese. Fucoidan is a major component of Maritech® seaweed extracts. In addition to efficacy parameters, safety measures included full blood count, serum lipids, liver function tests, urea, creatinine and electrolytes determined at baseline and week 12. Additionally, all adverse events were recorded. Eleven participants completed 12 weeks and one completed 10 weeks of the study. The preparation was well tolerated and the six adverse events noted were unlikely to be related to the treatment. The first event, influenza occurred prior to the commencement of the trial and the participant withdrew. Two participants had hypertension, one baseline and one at week 4. Both participants had a history of hypertension. One participant had a chest infection at week 12, one had root canal work at week 12, and one participant had hyperacidity at week 12 with a history of gastric acidity at baseline. There were no changes in blood parameters measured over the course of the study with the exception of an increase in serum albumin which was not clinically significant. There were no changes in the cholesterol, liver function, renal function, and hemopoietic

function that were of any clinical significance during the course of the study. The results of this study suggest that intake of the formulation at a dose level of up to 1000 mg/day for 12 weeks did not cause any adverse effects.

In yet another published study, Myers et al. (2011) attempted to determine whether a seaweed nutrient complex containing extracts from three different species of brown algae plus nutrients is safe to administer and has biological potential as an immune modulator. The formulation used in this study was similar to that described in the above study (Myers et al., 2010). In this open label study, volunteers (n = 10) were randomized to receive the study formulation at either a 100 mg (n = 5) or 1000 mg (n = 5) dose over 4 weeks. The participants comprised nine females (mean age 33.4 years and one male age 45 years). The reports of adverse events were mild and self-limiting. Two participants who experienced an adverse event (hay fever n = 1; head cold n = 1) occurred in the last 2 days of the study and were not considered to be related to the medication. The descriptive statistics for blood safety measures (full blood count, liver function tests, and determination of urea, creatinine, electrolytes, cholesterol, and triglycerides) on days 1 and 28 did not reveal any significant changes except for potassium. However, the change was small and well within the clinical reference range so was assessed as not of clinical significance. The preparation was found to be safe over the 4 weeks at both doses tested.

### **2.1.2. Genotoxicity Studies**

Leite-Silva et al. (2007) assessed the genotoxic and antigenotoxic potential of three different concentrations (0.25, 0.5 and 1.0 mg/mL) of *F. vesiculosus* aqueous extract in cultured human lymphocytes. Genotoxicity was measured by the frequencies of chromosome aberrations and the induction of DNA damage as detected by the Comet assay. Exposure of the lymphocyte cultures to 0.25, 0.5 and 1.0 mg/mL *F. vesiculosus* aqueous extract had no effect on the chromosome aberration frequency or on the extent of DNA damage. The antigenotoxic effects of the extract were tested in the lymphocyte cultures at levels of 15 µg/mL of doxorubicin, either alone or combined with the different concentrations of the extract that was added to the cultures before, simultaneously with or after the doxorubicin. A reduction in doxorubicin-induced chromosome aberrations and DNA damage was detected by the Comet assay only when lymphocytes were pre-treated with the extract. The results of these investigations demonstrate that *F. vesiculosus* aqueous extract is not genotoxic in cultured human lymphocytes and indicate that when added to lymphocyte cultures before doxorubicin it has antigenotoxic activity against doxorubicin-induced DNA damage. Although the amount of fucoidan in the extract was not reported, it is likely to be present. In another study, Ruperez et al. (2002) reported that the antioxidant activity of the *F. vesiculosus* aqueous extract is due to the sulfated-polysaccharide fucoidan.

### **2.1.3. Animal Studies**

Lean et al. (2015) investigated the effect of fucoidan and fucoidan/polyphenol extracts on acute colitis in a mouse model. Mice that received Maritech® Synergy fucoidan extract containing 40.2% carbohydrates, 21.8% sulfates and 26.2% polyphenols or a high purity fucoidan extract containing 59.5% carbohydrates and 26.6% sulfates in an oral dose equivalent to 400 mg per kg per day demonstrated a reduction in colitis-induced weight loss and a delay in colitis-induced diarrhea and blood-in-stool amongst other symptom reductions.

Zaragoza et al. (2008) investigated the adverse effects of two extracts from *F. vesiculosus* containing 28.8% polyphenols or 18% polyphenols plus 0.0012% fucoxanthin. The LD<sub>50</sub> by the oral route in rats for extracts 1 and 2 ranged between 1000 and 2000 and >2000 mg/kg, respectively, while with female mice, they ranged between 1000 and 2000 and >750 mg/kg, respectively. In the 4 week toxicity study, five groups of seven male and seven female rats were daily administered either 1% carboxymethylcellulose (control group) or extract 1 or 2 at two doses via gavage. The doses used were 200 and 750 mg/kg bw/day as the low dose and the high dose, respectively. At the end of the treatment period, blood was collected and the most important organs were removed, weighed, and subjected to histological examination. The hematological and clinical chemistry parameters were evaluated. The overall results obtained from the 4 week toxicity study indicate that even at the daily dose of 750 mg/kg bw for 4 weeks, no relevant signs of toxicity occurred with the two *F. vesiculosus* extracts studied. The levels of fucoidan in the extracts were not mentioned.

Based on the results of a study in rats, Choi et al. (2010) suggested that anti-ulcer properties of fucoidan might contribute in protecting the inflammatory cytokine-mediated oxidative damage to gastric mucosa. In this study, the effects of fucoidan (obtained from Sigma Chemical Co., St. Louis, MO; most probably derived from *F. vesiculosus*) on aspirin-induced ulcers in adult Wistar rats were evaluated. For these investigations, in addition to three other groups, one group of rats received only fucoidan via gavage at a dose level of 20 mg/kg bw/day for 14 days. As compared to control group, fucoidan treated rats did not show any adverse effects as evaluated by serum levels of aspartate (AST) and alanine (ALT) transaminases, BUN and total cholesterol. Additionally, histopathological evaluation of gastric tissue of fucoidan treated rats did not reveal any toxicity. The results of this study show that oral administration of fucoidan at a dose level of 20 mg/kg/day for 14 days did not reveal any toxic effects.

## **2.2. Safety of Fucoidan from Other Sources**

### **2.2.1. Human Studies**

In addition to fucoidan derived from *F. vesiculosus*, fucoidan from other seaweeds, such as *Undaria*, *Laminaria* and *Cladosiphon*, has also been investigated for efficacy and safety. Some of these studies are summarized in Table 8.

In a randomized double-blinded placebo-controlled trial in a non-seaweed consuming population, Teas et al. (2009) investigated the effects of seaweed (*U. pinnatifida*) consumption in 13 men (age 47.4 ± 9.9 years) and 14 women (age 45.6 ± 12.2 years) with at least one symptom of the metabolic syndrome. Subjects were assigned to either Group 1 (1 month placebo, followed by 1 month of 4 g/day seaweed) or Group 2 (1 month of 4 g/day seaweed, followed by 1 month of 6 g/day of seaweed). Blood pressure, weight, waist circumference, inflammation biomarkers, and lipids were measured monthly. In Group 2, systolic blood pressure decreased after a month of 6 g/day seaweed, primarily in subjects with high-normal baseline blood pressure. Waist circumference changed only for women participants, with a 2.4 cm decrease in Group 1 after treatment with placebo. In Group 2, women had a mean decrease of 2.1 cm after 4 g/day and a further 1.8 cm decrease after 1 month 6 g/day seaweed. No other changes were observed. Serum markers of inflammation (nitric oxide and C-reactive protein) and all other serum parameters did not change significantly. Only minor adverse side effects were noted. As *U. pinnatifida* has been reported to contain 8-12% fucoidan, the subjects in this study received approximately 400 and

600 mg fucoidan per day. The investigators suggested that consumption of 4 to 6 g/day seaweed, typical for most people in Japan, may be associated with low metabolic syndrome prevalence.

**Table 8. Human Clinical Studies of Fucoidan from Other Sources**

Source	Dose	Duration	Subjects	Findings	Reference
<i>U. pinnatifida</i>	4-6 g seaweed (400-600 mg fucoidan)	Daily for 1 to 2 months	One symptom of metabolic syndrome (27; 13M; 14F)	No significant adverse effects noted	Teas et al., 2009
<i>U. pinnatifida</i>	3 g of 75% fucoidan	Daily for 12 days	Healthy adults (20)	No changes in platelet indices. aPTT change not considered to be clinically significant.	Irhimeh et al., 2009
<i>Laminaria japonica</i>	400 mg	Daily for 5 weeks	Healthy adults (13)	Antithrombotic effect	Ren et al., 2013.
<i>Cladosiphon</i>	100 mg	Daily for 3 weeks	Adults with Gastric ulcer	Improved rate of healing	Juffrie et al., 2006.
<i>Cladosiphon</i>	4 g	Daily	Colorectal cancer patients. 20 (10 on fucoidan)	Reduced fatigue Increased chemo cycles.	Ikeguchi et al., 2011.
<i>Cladosiphon</i>	6 g	Daily for 6-13 months	Adults with HTLV1 associated myelopathy	Lowered proviral load in patients. No disease progression	Araya et al., 2011.
<i>Cladosiphon</i>	0.83 g	Daily for 12 months	Adults with Hepatitis		Mori et al., 2012
<i>Cladosiphon</i>	4.05 g	Daily for 2 weeks	Healthy normal adults	Normal lab values except favorable change in cholesterol.	Abe et al., 2013

In a series of extensive investigations, Irhimeh and colleagues studied the effects of fucoidan derived from *U. pinnatifida* on blood coagulation related parameters. In a pilot study in human subjects, Irhimeh et al. (2009) investigated the safety and clinical effects of fucoidan (provided by Marinova) ingestion on hemostasis as well as its *in vitro* anticoagulant activity. In this single-blinded clinical trial, 20 human volunteers (age 23 to 58 years; average 40 years) were allocated to both the placebo group (n=10) or to the active treatment group (n=10) who ingested 3 g of 75% fucoidan, daily, for 12 days. Venous blood samples were collected using citrate and EDTA anticoagulant-containing tubes. Platelet indices, activated partial thromboplastin time, antithrombin-III, thrombin time, prothrombin time, and antifactor-Xa were analyzed. The plasma fucoidan concentration for the placebo group was 0.17 mg/l, whereas it was 13.06 mg/l for the fucoidan-treated group. The activated partial thromboplastin time (aPTT) increased from 28.41 to 34.01 seconds (P=0.01) at the end of 12 days. Other parameters measured after 4 days of treatment such as thrombin time decreased from 18.62 to 17.55 seconds (P=0.04), and antithrombin-III increased from 113.5 to 117% (P=0.03). In comparison with the placebo group, there were no significant changes in any of platelet indices. The global clotting time (aPTT) change was not considered to be clinically significant. The investigators also suggested that the effect of fucoidan on hemostasis was not obvious; probably due to low intestinal absorption.

Thus, fucoidan in the form used in this study does not appear to have an oral anticoagulant activity. However, as described below it has a strong *in vitro* anticoagulant activity.

In the *in vitro* study, fucoidan prolonged the aPTT time from 30.8 seconds for control to 172.5 seconds at 63 mg/l (Irhimeh et al., 2009). No detectable clot formation for concentrations higher than 100 mg/l was noted. The thrombin time was also prolonged but at a higher rate in which it was 15.2 seconds at baseline and went up to 240.1 seconds at 15.6 mg/l and then no clot was detected at higher concentrations. Low concentrations (ranging from 7.8 to 63 mg/l) of the 75% fucoidan had no effect on PT, but at 125 mg/l, the PT began to increase. The prothrombin ratio [international normalized ratio (INR)] was also affected and it increased but not constantly reflecting the changes in the PT. The AT-III decreased with the fucoidan treatment from 108% for control to 89% at 10,000 mg/l. Interestingly, at high levels (10,000– 50,000 mg/l) fucoidan had a strong effect on anti-Xa assay, whereas at low levels the effect was not obvious. Fucoidan increased activated partial thromboplastin time, thrombin time, and prothrombin time, whereas antithrombin-III decreased. These observations show a prominent anticoagulant activity of fucoidan. The investigators concluded that fucoidan has anti-coagulant activity *in vitro*, but that the *in vivo* activity is weak, probably due to poor absorption (Irhimeh et al., 2009).

In another study, Irhimeh et al. (2007) examined the effects of orally ingested *U. pinnatifida* fucoidan on the peripheral blood stem cells, the expression of cell surface receptor CXCR4, and plasma levels of stromal derived factor-1 (SDF-1), interleukin IL-12, and interferon IFN-g. For these investigations, six volunteers took 3 g of guar gum (placebo), another six volunteers took 3 g of whole *Undaria* containing 10% w/w fucoidan, and another 25 volunteers took 3 g of 75% w/w fucoidan daily for 12 days. No adverse effects were reported, and none of the volunteers exhibited toxicity when 3 g of the placebo, guar gum, or 10% fucoidan, or 75% Maritech® fucoidan extracts were taken orally per day for 12 days. Fucoidan ingestion caused mild leukopenia and lymphopenia but had no effect on neutrophils. A nonsignificant decrease in the total number of leukocytes in the peripheral blood was noted when 10% fucoidan was ingested, but when 75% fucoidan was ingested the decrease was significant after 12 days. Following ingestion of fucoidan, CD34+ cells increased significantly from 1.64 to 1.84 cells/mL after 4 days. The proportion of CD34+ cells that expressed CXCR4 increased from 45 to 90% after 12 days, the plasma level of SDF-1 increased from 1978 to 2010 pg/mL, and IFN-g level increased from 9.04 to 9.89 pg/mL. Oral administration of fucoidan significantly amplified the CXCR4+ progenitor stem cell population.

In yet another study, Irhimeh et al. (2005) investigated the bioavailability of fucoidan using a monoclonal antibody methodology. For this study, healthy volunteers (age 20-46 years; average 29) were divided into three groups. The first group (placebo) of volunteers (n = 6) took 3 g of guar gum as placebo. The second group (n = 6) took 3 g of Galactofucan sulfate (GFS®) containing 10% fucoidan. The third group (n = 40) took 3 g of GFS containing 75% fucoidan (provided by Marinova Pty. Ltd.) daily for 12 days. Citrated venous blood was collected. Plasma samples were quantified for the presence of fucoidan using the described competitive ELISA method. The plasma concentration of fucoidan in the placebo group was 0 mg/l, while in volunteers who received 10% or 75% fucoidan, it was 4.00 and 12.99 mg/l, respectively. The investigators concluded that small quantities of orally administered fucoidan may cross the intestinal wall as whole molecules probably by the process of endocytosis.

In a clinical trial in healthy human volunteers, Abe et al. (2013) investigated the effects of excessive intake of fucoidan extracted from Okinawa Mozuku (*Cladosiphon okamuranus*) on

the changes in blood and urine parameters and also the alteration in abdominal and fecal states. The test foods composed of 4.05 g fucoidan in 150 mL (5 times the typical fucoidan usage). Twenty healthy adults (4 men and 16 women with a mean age of  $22.9 \pm 1.4$  years) participated in this study. The subjects ingested the test food for 2 weeks. The subjects were instructed to record test food ingestion, stool frequency, and the presence and severity of specific abdominal or any other symptoms every week on a questionnaire sheet. Blood and urine samples were collected before (baseline) and after 2 weeks of ingestion, for measurements of all common parameters for such tests. Blood biochemical examinations revealed a statistically significant reduction in total-cholesterol and LDL-cholesterol, while chloride showed significant increase. Although significant, the blood chloride levels were within the normal range and were not considered biologically significant. Kidney and liver related parameters did not show any significant differences. There were some changes from baseline among laboratory analysis (blood cells, hepatic and urine tests) but no statistically significant changes were noted for subjects. No adverse events or laboratory abnormalities were observed. In addition, no adverse effect attributable to the ingestion of the fucoidan was reported in the subject interviews about the abdominal and fecal states. The results of this study show that daily administration of fucoidan at levels of 4.05 g for two weeks is safe for human consumption.

Mori et al. (2012) examined the effects of fucoidan extracted from the marine alga, *Cladosiphon okamuranus* Tokida on hepatitis C virus (HCV) activity in humans. In this open-label uncontrolled study, 15 patients (7 men and 8 women; age,  $66.1 \pm 11.1$  years; range, 42-86) with chronic hepatitis C, and HCV-related cirrhosis and hepatocellular carcinoma were treated with fucoidan (0.83 g/day) for 12 months. The clinical symptoms, biochemical tests, and HCV RNA levels were assessed before, during, and after treatment. Fucoidan was found to be effective in lowering HCV RNA level in this study, although its effect was temporary. No adverse events were observed in any patients, suggesting that daily oral administration of fucoidan for 12 months is safe and tolerable. In another similar open label study, 13 patients with HAM/TSP<sup>5</sup> were treated with 6 g fucoidan (source not clear but base on supplier appears to be derived from *C. okamuranus*) daily for 6-13 months (Araya et al., 2011). Fucoidan therapy resulted in a 42.4% decrease in the HTLV-1 proviral load without affecting the host immune cells. During the treatment, no exacerbation was observed. Four patients with HAM/TSP developed diarrhea, which improved immediately after stopping fucoidan administration. No other adverse effects of fucoidan were noted.

### 2.2.2. Genotoxicity Studies

Chung et al. (2010) investigated potential adverse effects of fucoidan derived from *U. pinnatifida*. These investigators studied genotoxicity by an *in vitro* Salmonella Ames test and an *in vivo* micronucleus test with ICR mice, while short-term toxicity was evaluated following 28-day oral repeated administration tests in rats. Fucoidan used in these studies was isolated by hydrolyzing *U. pinnatifida* in 0.05 M or 0.5 M HCl at 80°C for 30 min and then neutralizing it with 1 m NaOH. After desalting by gel filtration, the hydrolysate was lyophilized. The chemical composition of the prepared fucoidan was reported as 27% uronic acid, 53% monosaccharides and 7.4% sulfate.

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<sup>5</sup>HAM/TSP = Human T-lymphotropic virus type-1 (HTLV-1), a human retrovirus that causes HTLV-1-associated myelopathy/tropical spastic paraparesis.

The Ames test was performed with *Salmonella typhimurium* strains TA100 (base pair mutation) and TA98 (frame shift mutation). Fucoidan was tested at levels of 0, 125, 250, 375, 500 µg/plate (Chung et al., 2010). Additionally, *Salmonella* antimutagenicity test (Ames test) was also conducted with 4-Nitroquinoline- 1-oxide (4NQO, 0.15 µg/plate). As compared with the spontaneous revertant colonies formed in the control group, fucoidan treatment did not induce significant dose dependent revertant colonies at any of the doses tested, in either *S. typhimurium* TA 98 or TA 100 strains. Additionally, fucoidan treatments exhibited dose-dependent antimutagenic effects in both TA98 and TA100 against 4NQO as a mutagen. The maximum inhibition of mutagenicity ratio with fucoidan treatment was 76%.

For the *in vivo* bone marrow micronucleus assay, fucoidan was administered orally (0, 250, 500, 1000, 2000 mg/kg) to 5 week-old ICR male mice (Chung et al., 2010). Mitomycin C was administered (2 mg/kg) as a positive control. After 36 hours of treatment, the animals were euthanized and bone marrow cells were processed for measurement of micronucleated polychromatic erythrocyte percentage ratio (MNPCEs) per polychromatic erythrocytes (PCEs). In total, 500 polychromatic erythrocytes and/or normochromatic erythrocytes (NCEs) were scored for %PCEs/(PCEs + NCEs). During the course of experiment, no lethality or significant clinical symptoms were noted. As compared with the negative control groups, fucoidan administration at the doses tested caused no significant change in %MNPCE or %PCE (PCE + NCE), while as expected mitomycin C administration, the positive control, caused significant changes in %MNPCE and %PCE (PCE + NCE). The findings from the Ames test and micronucleus assay suggest that fucoidan is not genotoxic.

Kim et al. (2010a) investigated the potential genotoxic effects of fucoidan extracted from Sporophyll of *U. pinnatifida* using a test battery of three different methods. In a reverse mutation assay using four *S. typhimurium* strains (TA98, TA100, TA1535, and TA1537) and *Escherichia coli* (WP2uvrA), fucoidan (312.5, 625, 1250, 2500 and 5000 µg fucoidan/plate) did not increase the number of revertant colonies in any tester strain regardless of metabolic activation by S9 mix. Fucoidan did not cause more than a twofold increase in the mean number of revertants per plate compared to the negative control. The positive controls for each strain resulted in the expected increase in the number of revertant colonies. These data indicate no evidence of gene mutagenic potential under the conditions used in this test for fucoidan.

The chromosomal aberration assay was conducted using a Chinese hamster lung cell line (CHL/IU), both in the presence and absence of S9 metabolic activation (Kim et al., 2010a). The chromosome aberration was assessed via two different procedures; a 6-hour exposure followed by an 18-hour recovery (with or without metabolic activation), and a 24-hour continuous exposure. The publication reports that fucoidan (313-5000 µg/mL) produced dose-related changes in cytotoxicity but did not change the number of cells with numerical aberrations. Fucoidan did not cause chromosomal aberration in short tests with S9 mix or in the continuous (24 hour) test. It appears that the authors have wrongly described the fucoidan dose (313-5000 µg/mL) in the results section, while the Table 2 of the article provides correct values. Given this, the correct concentrations of fucoidan used in the chromosomal aberration assay should be 1.25, 2.5 and 5.0 mg/ml. There are some additional discrepancies between the data provided in the Table 2 of the article and the description provided under result section 3.2 of the article. The results provided in Table suggest that fucoidan did not cause chromosomal aberration in short tests with S9 mix or in the continuous (24 hour) test.



For the *in vivo* micronucleus assay, mice received a gavage dose of fucoidan at dose levels of 0, 500, 1000, and 2000 mg/kg bw/day, or positive control cyclophosphamide at a dose level of 70 mg/kg bw/day (Kim et al., 2010a). No signs of toxicity or gross behavioral changes, significant weight changes or death were noted. At 24 hours after the final dose, mice were euthanized and their femurs removed. Bone marrow cells were collected by washing the femur cavity with FBS to prepare a slide for MN test. There were no statistically significant treatment-related differences in incidences of MNPCEs or the PCE/NCE ratio, whereas the positive control induced significant increased incidence of MNPCEs. The results of this assay demonstrate that fucoidan did not induce chromosome aberrations in mouse bone marrow cells.

Song et al. (2012) investigated the potential genotoxicity of low molecular weight fucoidan (LMF) using a standard battery of tests (bacterial reverse mutation assay, chromosomal aberrations assay, and mouse micronucleus assay). LMF was prepared by acid hydrolysis of high molecular weight fucoidan (HMF) extracted from sporophylls of *U. pinnatifida*. In a reverse mutation assay using four *S. typhimurium* strains and *E. coli*, LMF did not increase the number of revertant colonies in any tester strain regardless of metabolic activation by S9 mix, and did not cause chromosomal aberration in short tests with the S9 mix or in the continuous (24 hour) test. In the bone marrow micronucleus test in ICR mice that were administered via oral gavage at doses up to 2000 mg/kg bw/day, no significant or dose-dependent increases in the frequency of micronucleated polychromatic erythrocytes was noted. Based on the results of these studies, the investigators concluded that the LMF was not genotoxic under the conditions of the reverse mutation assay, chromosomal aberrations assay, or mouse micronucleus assay.

### 2.2.3. Animal Toxicity Studies

In addition to the above described genotoxicity studies, Chung et al. (2010) also performed a 28-day repeat dose oral toxicity study with fucoidan derived from *U. pinnatifida*. In this study, Sprague-Dawley rats (5/sex/group) were administered (via gavage) fucoidan at a dose level of 0 (vehicle), 250, 500, 1000, 2000 mg/kg bw/day for 28 days. The health condition, clinical symptoms and mortality of the animals were checked twice daily, and body weights, feed consumption and water consumption were recorded once a week. At termination, blood samples were collected for hematological and biochemical parameter measurements. At necropsy, the animals were euthanized and tissues were collected, weighed and detailed pathological and morphological observation was performed. No specific clinical symptom was observed in any animal. There was no significant difference or trend in body weight, or water or feed consumption among the groups. At doses up to 1000 mg/kg bw/day, no significant differences in hematological or biochemical parameters were noted. However, at 2000 mg/kg bw/day, ALT and triglyceride levels in male groups were significantly higher, and the total and HDL cholesterol levels in the 2000 mg/kg bw/day female groups were significantly higher (data not presented in the publication). Additionally, MCHC in the 2000 mg/kg bw/day female groups was significantly lower. Fucoidan administration at levels up to 1000 mg/kg bw/day did not cause any significant difference in organ weight or pathology. However, thyroid weights at 2000 mg/kg bw/day were significantly increased in both sexes. No liver histopathological finding was observed in any of the fucoidan-treated groups, compared with those of the control. Although the liver morphology was normal, based on the increase in liver enzyme (ALT) in male rats at the highest dose levels, the investigators indicated that fucoidan is safe at doses up to 1000 mg/kg bw/day. The no-effect-level for clotting time was 1000 mg/kg for 28 days (Chung et al., 2010).

Kim et al. (2010b) also tested the toxicity of fucoidan extracted from the Sporophyll of *U. pinnatifida* in a 4-week oral trial in Sprague–Dawley (SD) rats. The study was conducted in compliance with the test guidelines from the Korea Food and Drug Administration (KFDA) under Good Laboratory Practice regulations for Nonclinical Laboratory Studies. The chemical composition of fucoidan was reported as 64.4±6.0% fucose; 31.9±4.7% galactose, 3.6±1.3% mannose, and 31.7±2.2% sulfate. Fucoidan (0, 150, 450, and 1350 mg/kg bw/day) was administered daily by oral gavage to age-matched male and female rats (10/sex/group) for 28 days. No animals died during the experimental period, and fucoidan did not produce toxic signs in any animals. Fucoidan treatment did not alter weight gain or feed and water intake. Ophthalmologic examinations were normal. Fucoidan did not consistently change hematology or serum biochemistry values. No increase in the activity of serum toxicity marker enzymes (ALT, AST) was noted. However, BUN levels in the female groups (11.9±1.2) treated with fucoidan decreased compared with the control group (13.9±1.3). BUN levels in the male groups treated with fucoidan did not change. Fucoidan administration did not significantly affect any other hematology or biochemical parameters, including prothrombin or Activated Partial Thromboplastin Time values in any treatment groups. Gross examination revealed no abnormalities. Significantly lower values in the relative weights of the liver were observed in male (2.85±0.11) and female (2.83±0.1) rats treated with a high dose of fucoidan (1350 mg/kg) compared to control males (3.02±0.08) and female (2.87±0.1), but absolute liver weights were not different. High-dose fucoidan decreased the absolute and relative right adrenal weights. However, these changes showed no dose-dependent responses and no histopathological findings, and were therefore judged to be unrelated to fucoidan administration. Histological examinations did not reveal any pathological signs. Fucoidan administration did not affect the liver tissue or produce morphological variation in its lining. Fucoidan did not change other vital organs (lung, heart, spleen, and liver). The results of this study show that fucoidan is not toxic when orally administered at 150, 450, and 1350 mg/kg bw/day for 4 weeks, and does not have anticoagulant activity, reducing concern about adverse effects related to excess bleeding.

Li et al. (2005) investigated the acute and subchronic (6 months) toxicity of varying levels of fucoidan extracted from *L. japonica* in Wistar rats after oral administration. For the preliminary acute toxicity study, several doses of fucoidan (0, 100, 500, 1000, 2000 and 4000 mg/kg bw) were tested and in the main study dose of 4000 mg/kg bw was employed. No signs of toxicity or gross behavioral changes were noted at 4000 mg/kg dose. Gross and microscopic examinations also did not reveal any evidence of toxicity. The LD<sub>50</sub> appears to be greater than 4000 mg/kg bw.

For the repeat-dose long term toxicity study, rats (10/sex/group) were administered (via gavage) fucoidan at a dose level of 0 (vehicle), 300, 900, and 2000 mg/kg bw/day for 6 months (Li et al., 2005). Two additional groups of rats (10/sex/group), one control and another high dose recovery groups were also included. The results showed that no significant toxicological changes were noted at the dose level of 300 mg/kg bw/day fucoidan administered to rats. However, when the dose was increased to 900 and 2500 mg/kg bw/day, the clotting time was significantly prolonged. Except this observation, no other signs of toxicity were noted. Based on these results, the investigators concluded that the no adverse effect level of fucoidan from *L. japonica* is 300 mg/kg bw/day.

In another study, Gideon and Rengasamy (2008) investigated the toxicity of fucoidan from *Cladosiphon okamuranus* in rats after oral administration for 3 months. In this study,

fucoïdan was administered to Wistar rats via oral gavage at dose levels of 0, 300, 600, 1200, 2400 and 4000 mg/kg bw, 6 days per week for 90 days. No significant toxicological effects were noted at dose levels of 300 or 600 mg/kg/day. At fucoïdan doses of 1,200 mg/kg/day and above, prolonged clotting time was noted. Given these effects, 600 mg/kg/day was considered to be the NOAEL in this study.

### 2.3. Similarity/Differences in Fucoïdians

The fucoïdan used in the above Section 2.2 safety studies is either derived from other sources such as *Undaria*, *Laminaria* and *Cladosiphon*, or is extracted by employing different processes. Hence, it is important to understand whether the safety studies conducted with other fucoïdians or by employing different extraction process is applicable to those with Maritech® *F. vesiculosus* fucoïdan.

Cumashi et al. (2007) has compared similarities between fucoïdians from different species, and this includes fucoïdan from *Fucus vesiculosus*, although not *Laminaria japonica* or *Undaria pinnatifida*. The fucoïdan extracts were isolated on a lab scale, and are therefore slightly different to those prepared by Marinova. Some natural variation would be expected due to environmental factors such as location, water source, etc. However, the comparison between fucoïdan from different species remains valid, showing broad trends and some subtle differences in *in vitro* studies. There are several papers that compare fucoïdan from one or two species, but not a specific paper that compares the three quoted, as related to a particular bioactivity. In a recent study by Zhang (2015), immune-modulation effects, such as promoting activation of dendritic cells (DCs), natural killer (NK) cells and T cells, and enhancing anti-viral and anti-tumor responses of *Undaria*, *Fucus* and *Macrocystis* fucoïdan were investigated following *ex vivo* exposure and delivered via intravenous (iv) and intraperitoneal (ip) routes. There are differences between the fucoïdians in the degree of biological response they elicit, however it is important to note that the fucoïdan extracts in this paper were delivered by iv and ip administration, and as such, the serum concentrations are much higher than achievable from oral delivery, which accentuates any subtle differences in the bioactivity. Orally delivered fucoïdians of different species do not achieve such high serum concentrations, thus any potential differences in biological activities would be minor.

In an attempt to understand similarity and differences between fucoïdians derived from different sources, the composition of *F. vesiculosus* fucoïdan has been comprehensively investigated by Marinova. The typical composition of *F. vesiculosus* fucoïdan is compared with fucoïdan derived from other sources such as *Undaria* and *Laminaria* in Table 9. In broad terms, the *Undaria pinnatifida* and *Fucus vesiculosus* fucoïdan extracts produced by Marinova contain similar compositions with approximately 50% carbohydrates, 25-30% sulfates and 7% cations. The *U. pinnatifida* extract described by Chung et al. (2010) was reported to contain 53% monosaccharides (presumably following hydrolysis), of which, 54% were fucose and 35% galactose. Unfortunately, little detail is provided as to the analytical methodology used; however, the relatively high uronic acid content of 27% and low sulfate content of 7.4% suggest that the extract was of an intermediate purity, containing co-extracted alginic acid. Based on the reported parameters, the fucoïdan extract utilized in the Chung et al. study would be expected to contain approximately 40-50% fucoïdan.

**Table 9. Typical Compositional Analysis of Fucoïdan from *Fucus*, *Undaria* and *Laminaria*\***

Parameters	<i>Undaria pinnatifida</i>	<i>Fucus vesiculosus</i>	<i>Laminaria japonica</i>
<b>Carbohydrates</b>			
Fucose (%w/w)	29.3	49.7	26.5
Galactose (%w/w)	21.2	2.9	3.3
Xylose (%w/w)	0.0	3.0	0.8
Mannose (%w/w)	0.9	0.3	3.5
Glucose (%w/w)	0.9	0.0	0.6
Arabinose (%w/w)	0.0	0.0	0.2
Rhamnose (%w/w)	0.0	0.0	0.7
Uronic acid (%w/w)	4.3	6.8	17.0
<b>Others</b>			
SO <sub>4</sub> (%w/w)	25.2	27.0	24.0
Acetyl (%w/w)	2.5	0.0	0.5
Cations (%w/w)	7.0	6.8	10.0

\*Based on information provided by Marinova

The toxicity studies of polyphenol rich extracts from *Fucus vesiculosus* by Zaragoza et al. (2008) also serve as a useful comparator. The extracts studied by Zaragoza et al. (2008) contained 25-30% polyphenol and 10-20% carbohydrates –consistent with a lower purity *Fucus vesiculosus* fucoïdan extract. The safety of the extracts at high doses in a rat model was verified.

#### **2.4. Absorption, Distribution, Metabolism and Excretion (ADME)**

The bioavailability of fucoïdan extracts from a number of species has been investigated in both animal and human studies with reports indicating little to no systemic uptake of fucoïdan following oral administration. In an *in vitro* investigation of the digestibility of fucoïdan from *U. pinnatifida* by human fecal bacteria, Michel et al. (1996) demonstrated a lack of degradation, suggesting that the ingested fucoïdan remains unaltered within the gut. In a rat model, (Tokita et al., 2010) serum analysis indicated the presence of trace amounts of fucoïdan, originating from *Cladosiphon okamuranus*. In this study, analysis of the serum and plasma fucoïdan demonstrated that the fucoïdan itself had remained unchanged. The highest reported uptake of fucoïdan in human studies was reported by Irhimeh et al. (2005), who suggested that up to 1% of the oral dose had been absorbed, through analysis of serum with a non-specific antibody-based assay. Thus, literature reports of fucoïdan pharmacokinetics suggest that only small amounts (up to 1% of the oral dose) of fucoïdan are absorbed.

#### **2.5. Allergenicity**

A search of published information did not reveal any evidence of allergies following consumption of fucoïdan. No allergic reactions have been reported in the clinical studies performed by Marinova with fucoïdan. In addition, no allergy complaints have been received by Marinova in the 10+ years that Marinova's Maritech® extracts have been produced and consumed in the marketplace. Marinova has also performed specific topical allergen testing (HRIPT) on fucoïdan derived from *F. vesiculosus*. The extract was determined to be a non-primary sensitizer, and non-photo sensitizer when applied topically (Fitton et al., 2015).

Ingesting fucoïdan is unlikely to induce any changes in allergic response. There are no known reports of allergic responses to ingested fucoïdan. However, there are reports of reduced allergic responses in animal models for topically applied fucoïdan (Yang, 2012). In addition,

fucoïdan suppressed IgE from *ex vivo* peripheral blood cells from patients with the allergic condition of atopic dermatitis. Whilst there is one additional report of reduced allergic response (Maruyama et al., 2005), this refers to intra-peritoneal (IP) delivered fucoïdan rather than edible fucoïdan. Thus the cumulative evidence from Marinova's clinical studies, combined with the lack of allergic responses to oral doses in the literature reinforces the low allergenicity of fucoïdan extracts.

## 2.6. Anticoagulant Activity

The anticoagulant activity of fucoïdan is by far the most extensively investigated. Although some of the coagulation-related (*in vitro*, animal and human) studies of fucoïdan are described in earlier sections, an attempt has been made to understand implications of these effects as it relates to the safety-in-use at the proposed uses of fucoïdan by Marinova. The *in vivo* animal safety studies in which effects of fucoïdan on coagulation were investigated are summarized in Table 10. Additional details of these studies are described in Table 6.

**Table 10. Summary of Hematological Findings from Animal Toxicity Studies**

Reference	Species	Fucoïdan source	Dose levels	Findings	Comments
Kim et al. (2010b)	SD rat	Fucoïdan from <i>U. pinnatifida</i> with 64.4% fucose, 31.9% galactose, 3.6% mannose, 31.7% sulfate	0, 150, 450, 1350 mg/kg for 28 days via oral gavage (10/sex/group), 7 days/week	There was no change in prothrombin time or activated partial thromboplastin time	NOEL for clotting: 1350 mg/kg/day for 28 days
Chung et al. (2010)	SD rat	Fucoïdan from <i>U. pinnatifida</i> , 27% uronic acid, 28.6% fucose, 18.9% galactose and 8% other monosaccharides, 7.4% sulfate	0, 250, 500, 1000, 2000 mg/kg/day once/day for 28 days (5/sex/group). Hematology, clinical chemistry conducted	No evaluation of clotting time, limited number of hematological and clinical chemistry parameters	NOEL for hematology: 1000 mg/kg/day for 28 days
Li e et al. (2005)	Wistar rat	<i>L. japonica</i> , 48% total sugar, 28% fucose, 29% sulfate, MW 189 k	0, 300, 900, 2500 mg/kg/day for 180 days; oral gavage (10 or 20/sex/group)	Increased clotting time at two high doses in both sexes; recovery in high dose males, but not high dose females after one month	NOEL for clotting: 300 mg/kg/day for 180 days
Gideon and Rengasamy (2008)	Wistar rat	Extract of <i>Cladosiphon okamuranus</i> with 40.2% fucose, 3.7% uronic acid, 24.7% monosaccharides, 18.6% sulfate, MW 380 k	0, 30, 600, 1200, 2400, 4000 mg/kg for 90 days via oral gavage in water (6/sex/group), 6 days/week	Increased clotting time at $\geq 1200$ mg/kg in both sexes with greater severity in males. No effects at 600 mg/kg	NOEL for clotting: 600 mg/kg/day for 90 days

The available information from multiple *in vitro* and *in vivo* studies suggest that the sulfate content, molecular weight, and sugar composition may be related to the anticoagulant activity of fucoïdan (Colliec et al., 1991; Kim et al., 2007; Li et al., 2008a; Li et al., 2008b). In general, the higher content of sulfate has been reported to be associated with the higher anticoagulant activity. Conversely, the anticoagulant activity gradually decreased up to a point where the sulfate content was too high. This was confirmed with the use of oversulfated

fucoidans prepared by chemical sulfation of natural fucoidan where highly sulfated fucoidan showed an increase in anticoagulant activity up to a certain degree of sulfation that then gradually decreased anticoagulant activity (Li et al., 2008a).

In addition to sulfate content, molecular weight of fucoidan was also found to be closely related to the anticoagulant action of fucoidan. The available information indicates that fucoidan requires a sugar-chain long enough to bind the thrombin, so a certain minimum molecular weight is required to attain anticoagulant activity. Fucoidan extracted from *Lessonia vadosa*, which had high anticoagulant activity possessed a molecular weight of 320,000 Da (Chandia and Matsuhiro, 2008). A smaller fucoidan fraction with a molecular weight of 32,000 Da showed weak anticoagulant activity. Some studies have also shown that the anticoagulant activity may be related to the sugar composition of fucoidan (Nishino et al., 1989). Li et al. (2008a) suggested that the sugars did not affect the anticoagulant activity but the sulphate groups attached to those sugars was responsible for the altered anticoagulant activity. In another study, Li et al. (2008b) reported that uronic acid composition, although not necessary for anticoagulant activity, could improve the anticoagulant action by enhancing the sugar chain's flexibility.

In an attempt to understand any association between anticoagulant activity and the differences between fucoidan compositions, several parameters such as carbohydrates, sulfate, acetyl groups and cations were compared for fucoidan derived from *Fucus*, *Undaria* and *Laminaria* (Table 9). Over all the available information indicate that the molecular weights and sulfate contents of fucoidan derived from these three species are similar. *Fucus* and *Laminaria* are most similar in their neutral carbohydrate profile, which is primarily fucose, however *Fucus* and *Undaria* have the most similar total neutral carbohydrate content – *Laminaria* differs in its high uronic acid content. These observations indicate that coagulation-related effects of fucoidan derived from *Fucus*, *Undaria* and *Laminaria* can be similar between these species.

As discussed earlier (see section 2.2.1.), Irhimeh et al. (2009) reported that fucoidan derived from *U. pinnatifida*, given orally, had a modest but significant effect on some of the coagulation assays, in particular, the intrinsic pathway. However, the coagulation tests were still within reference ranges and unlikely in themselves to be 'clinically valuable'. It should be noted that in this study, fucoidan was administered at a dose level of 3 g/person/day. As compared to the intended use levels this dose level is much (12 times) higher.

In order to identify the structural features important for increasing fucoidan's procoagulant activity and minimizing anticoagulant activity, Zhang et al. (2014) investigated thrombin generation (CAT), anticoagulant effect (aPTT) and TFPI- inhibition as a function of fucoidan's molecular size and charge density. In this study, *F. vesiculosus* fucoidan was fractionated by charge and size as well as over- and desulfated to different degrees to yield preparations with various structural properties. The fraction's pro- and anticoagulant activities were assessed by calibrated automated thrombography (CAT) and activated partial thromboplastin time (aPTT assay). Binding to the inhibition of the anticoagulant protein tissue factor pathway inhibitor (TFPI) and the ability to activate coagulation via the contact pathway were also investigated. The results of this study show that a chain length (DP) of at least 70 sugar units (MW - 15 kD) and a charge density (OS) of at least 0.5 sulfates/unit maintain procoagulant activity, but lose most of the anticoagulant activity and cannot activate the contact pathway.

Prasad et al. (2008) investigated potential efficacy and safety of fucoidan derived from *F. vesiculosus* and *L. japonicain* in dogs with hemophilia A (hemophilia A dogs) with minimally

increased hemostasis and in treatment-naive severe hemophilia A dogs. Subcutaneous administration of fucoidan to low-FVIII dogs improved hemostasis as exhibited in thromboelastography (TEG) and cuticle bleeding time (CBT) tests. Moreover, oral administration of fucoidan to AAVFVIII dogs and treatment-naive severe hemophilia A dogs for a multi week dose escalating period yielded correction to normal ranges in both TEG and CBT end points at 5 to 15 mg/kg and 15 to 20 mg/kg dose levels, respectively. In all 3 separate studies, throughout their duration, fucoidan was well tolerated by the dogs without any adverse events. Additional pharmacologic characterization of fucoidan included intravenous pharmacokinetic analysis in rats. The findings from this study did not reveal any increase in aPTT clotting times for dogs at 15 mg/kg/day. These investigators reported that their observations along with the published findings suggest that fucoidans are well tolerated and that safe hemostasis can be achieved.

In summary, the available information related to anticoagulant activity, also described earlier in safety sections, indicates a low level of toxicity. Among the different *in vivo* safety-related studies, in five repeat dose oral studies in rats that lasted for 14 to 180 days, coagulation-related effects were investigated (Table 11). In the longer exposure period studies, the only significant and consistent effect noted was a prolongation of clotting time. Additionally, there were isolated, scattered other effects that were not observed consistently across studies. The effect on clotting time is consistent and has been widely reported in the literature. These data are summarized in Table 11. These studies also indicate that the dose to induce prolonged clotting time is related to the length of administration. This also suggests that in order to induce clotting effect, fucoidan needs to accumulate in the blood to certain critical levels. In these studies, administration of fucoidan at dose levels of 900, 1200, or >1350 mg/kg/day was required to prolong coagulation after 180, 90 or 28 days of administration, respectively. These studies also suggest that the pattern of effect and no-effect levels depend on the choice of the high dose and next lower dose where effects are not observed and the length of time of dose administration. Compared to the intake of fucoidan (4.17 mg/kg bw/day) from the proposed uses of fucoidan, the lowest dose at which fucoidan (900 mg/kg bw/day) induced prolongation of clotting time is over 200-fold higher.

**Table 11. Summary of Coagulation-Related *in vivo* Animal and Human Studies of Fucoidan**

Reference	Species	Treatment Duration	Minimum anti-coagulation dose (mg/kg bw/day)	Anti-coagulation NOAEL (mg/kg bw/day)
Irhimeh et al. (2009)	Human	4 days	Not determine	96
Chung et al. (2010)	Rat	28 days	2000 <sup>a</sup>	1000 <sup>a</sup>
Kim et al. (2010b)	Rat	28 days	>1350	1350
Gideon and Ranngasamy (2008)	Rat	90 days	1200	600
Li et al. (2005)	Rat	180 days	900	300

<sup>a</sup>Clotting time was not evaluated but mean corpuscular hemoglobin concentration was decreased at 2000 mg/kg bw/day

In regard to any possible interference with anticoagulant therapy, the available evidence indicates that fucoidan at dietary levels up to 250 mg/day would not interfere with anticoagulant therapy. However, to further mitigate any concern for individuals on anticoagulant therapy, Marinova will include a statement of caution on the label for consumers receiving anticoagulation therapy.

## 2.7. Immune Effects

The available information indicates that fucoidan from different species can cause immunomodulatory effects. Jin et al. (2014) reported that fucoidan extracted from *F. vesiculosus* can act as an adjuvant and promote antigen-specific T cell immune response *in vivo*. In another study, Zhang et al. (2015) reported that fucoidan from *U. pinnatifida* strongly delayed human neutrophil apoptosis at low concentration, while fucoidan from *F. vesiculosus* delayed human neutrophil apoptosis at higher concentration. These investigators also reported that *U. pinnatifida* showed a considerable, dose-dependent inhibiting effect on neutrophil apoptosis at concentrations between 5-100 µg/mL, and at 50 µg/mL fucoidan from *U. pinnatifida* significantly increased the production of IL-6, IL-8 and TNF-α from neutrophils. These and other *in vitro*, and *in vivo* studies suggest immune effects of fucoidan. However, it is important to consider the delivery route of fucoidan. In the above cited papers, fucoidan was delivered via systemic (ip) routes rather than oral, and reaches levels much higher than those expected after oral delivery. In the Jin et al. (2014) study fucoidan was administered via ip at a dose level of 10 mg/kg. Similarly, in the Zhang et al. (2015) study, mice were administered ip and iv fucoidan from *Undaria*, *Fucus* and *Macrocystis* at doses of 20 or 50 mg/kg. The low concentrations at which apoptosis delay was observed are still higher than those which could be achieved by oral delivery.

In an oral dose human study, Negishi et al (2013) demonstrated that 300 mg of *Undaria fucoidan* can help to restore an antibody response to the seasonal flu vaccine in an elderly population. This response was similar to the effects of optimal fruit and vegetable consumption in older people, where normal dietary intake can help achieve an effective vaccine response (Gibson et al., 2012). Thus, whilst high dosages of ip or iv delivered fucoidan appear to have significant effects on NK activity, etc., these effects result from concentrations that would not be achievable from oral delivery of the dosages considered herein. The effects observed in oral studies of fucoidan administration are comparable to that achieved from a normal healthy diet, thus reinforcing the safety of the subject fucoidan.

## 2.8. Structure and Biological Activity

Fucoidan is a complex polysaccharide composed primarily of fucose subunits, but also including galactose, xylose, mannose and other sugar monomers. Varying degrees of sulfation are present on the fucoidan backbone, which leads these molecules to possess a significant negative charge. Fucoidan can also vary in the sulfation position, linkages (between adjacent sugar monomers) and molecular weight (a large range of molecular weight fractions are typically present). A comparison of fucoidan extracts from *Fucus*, *Laminaria*, *Cladosiphon* and *Ascophyllum* genera demonstrated comparable anti-inflammatory activity irrespective of fucose and sulfate content in a rat model with IP delivery of the extracts. In the same study, *in vitro* P-selectin inhibitory activity, anticoagulant activity, tubulogenesis and carcinoma adhesion activities were found to vary between species (Cumashi et al., 2007).



The degree of sulfation (and thus the charge density) of fucoidan, was found to have an effect on the pro- and anticoagulant activities of fucoidan extracted from *Fucus vesiculosus*, with a higher degree of sulfation leading to higher activities in both properties. Molecular size was also found to affect the pro- and anticoagulant activities, with larger molecular weight fractions demonstrating greater activities akin to those of unfractionated fucoidan (Zhang and Till, 2014). Such variation in biological activity due to molecular weight has also been reported in liver fibrosis, anti-tumor and immune modulation models (Nakazo et al., 2010; Maruyama et al., 2005, 2003; Shimizu et al., 2005).

The biological activity of fucoidan extracts has been reported by numerous *in vitro* studies; however, as discussed in section 2.4 (above), the resulting low plasma concentration of fucoidan from the ingestion of the dose described in this assessment is highly unlikely to result in any significant effects that preclude the safety of the extracts.

Fucoidan is a soluble fiber that is thought to be unchanged by fermentation with human gut microbiota (Michel et al., 1996). Fucoidan may have effects similar to other soluble fibers, such as oat glucans. These would not be expected to greatly alter gut microorganisms.

## 2.9. Safety of Other Components

The compositional analysis of fucoidan derived from *Fucus vesiculosus* (Table 4) suggest that in addition to fucoidan, the final product contains- Alginate 2.0-5.5%; Polyphloroglucinol 1.0-25%; Mannitol 1-5%; Natural salts 0.5-2.5%; and Other carbohydrates 0.5-1.0%. As described earlier, the unique extraction process (use of water as extraction solvent) employed by Marinova ensures that the chemical constituent of the fucoidan molecule remains unaltered and equivalent as in their natural state. This process also ensures that no other products are formed during the processing. The remaining product constituents including alginate, polyphloroglucinol, mannitol, and other carbohydrates are naturally present in kelp that has a safe history of human consumption.

Alginates or salts of alginic acid are natural polyuronide constituents of certain brown algae. The FDA has affirmed alginates such as sodium alginate, potassium alginate, calcium alginate and ammonium alginate as GRAS. Sodium alginate is regulated by the FDA under 21 CFR 184.1724 as a texturizer, formulation aid, stabilizer, thickener, firming agent, flavor adjuvant, emulsifier, flavor enhancer, surface active agent. As compared to the permitted use levels of sodium alginate and other alginates, the resulting use (1.95 mg/serving) or intake of alginate (16.25 mg/person/day) from the proposed uses of fucoidan is very small and is considered as safe.

Plant polyphenols have been extensively studied and are abundant in the food-supply, and are most commonly consumed in large quantities from fruit and vegetables, green tea, black tea, red wine, coffee, chocolate, olives, and extra virgin olive oil (D'Archivio, M et al. (2010). "Bioavailability of the Polyphenols: Status and Controversies". *Int. J. Mol. Sci.* 11 (4): 1321–1342.). More specifically, studies such as those of Zaragoza (2008) have reported on the specific safety of polyphenolic-rich extracts from *Fucus vesiculosus* in animal models.

Another constituent of fucoidan, mannitol, is allowed as a food additive for use as food, or in contact with food on an interim basis pending additional study. The FDA has reviewed the safety of the uses of mannitol in food and concluded that certain uses of mannitol are safe as codified under 21 CFR 180.25. In recent actions, FDA has amended the regulation at 21 CFR

180.25 to provide for additional methods of manufacture of mannitol. While it is not clear if the FDA considered any additional uses of mannitol at that time, it appears that the FDA continued to accept the safety assessment that less than 20 g/person/day is safe. The resulting intake of mannitol (0.007 g/person/day) from the proposed uses of fucoidan is very small and is considered as safe. Similarly, the intake of other constituents of fucoidan, such as natural salts and other carbohydrates, from the proposed use is very small and is considered as safe.

### 3. Common Knowledge Elements for a 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 information that is present in public domain and studies published in scientific journals. Several of the studies, particularly animal and human clinical studies reviewed for this safety assessment have been published in the peer reviewed scientific journals as reported in Table 6 and Section 2.1 and 2.2. 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 elements:<sup>6</sup> (A) 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 (B) 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 the National Academy of Sciences. To be sure, the Expert Panel of the present GRAS assessment recognizes that the safety of fucoidan derived from *Fucus vesiculosus* is supported by its presence in the human diet, particularly in Japan and other East Asian countries. Additionally, many scientific studies have been conducted and published.

### 4. RISK ASSESSMENT

There is sufficient qualitative and quantitative scientific evidence, including human and animal data, to determine safety-in-use of Maritech® fucoidan derived from *Fucus vesiculosus* (Bladderwrack) as a food ingredient in conventional foods. The intended use of fucoidan by Marinova will result in an estimated daily 90<sup>th</sup> percentile intake of 250 mg fucoidan/person/day (4.17 mg/kg bw/day). The available scientific evidence suggests that fucoidan will be as safe as the seaweeds from which it is derived. There is common knowledge of a significant history of human consumption of seaweeds, including *F. vesiculosus*, as a staple item of diet. The available information suggest that daily intake of seaweed can range from 4.3 to 14.3 g/person/day, while that of fucoidan can range from 0.54 to 1.79 g/person/day. This background exposure information suggests that intake of fucoidan from its proposed uses is likely to be safe.

In addition to the background exposure, several scientific studies, particularly on fucoidan derived from *F. vesiculosus* further supports the safety. Following oral consumption, small quantities of orally administered fucoidan may cross the intestinal wall as whole molecules probably by the process of endocytosis. The safety of fucoidan derived from *F. vesiculosus* is

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<sup>6</sup>62 FR 18938 (17 April 1997) which is accessible at <http://www.gpo.gov/fdsys/pkg/FR-1997-04-17/html/97-97-9706.htm>.

supported by a double-blind randomized placebo-controlled clinical trial in which subjects were given 300 mg fucoidan/day for 12 weeks. In this study no adverse events or treatment related changes in liver function, renal function and hemopoietic function were noted. In addition to this human study, other human studies as well as animal and genotoxicity studies further support the safety of fucoidan. The available information indicate that orally administered fucoidan derived from different sources unlikely to achieve high serum concentrations, thus any potential differences in biological activities would be minor. Additional animal, human and genotoxicity studies, including those with fucoidan derived from other seaweeds, particularly from *U. pinnatifida* further supports the safety. In a dose-response 4 week toxicity study in rats, administration of fucoidan derived from *U. pinnatifida* at dose levels up to 1350 mg/kg bw/day did not reveal any adverse effects, including any anticoagulant activity, reducing concern about adverse effects related to excess bleeding.

In *in vitro* studies, fucoidan is known for its anticoagulant activity. In the special case of haemophilic blood, fucoidan may act as a procoagulant, by promoting the extrinsic clotting pathway *in vitro* (Zhang et al., 2014). In a normal situation, oral intake of fucoidan does not lead to appreciable effects on global clotting in humans. In clinical studies using fucoidan, there have been no reports of adverse effects on clotting, even at high levels of intake. Animal toxicity studies also demonstrate very little effect of high dosage on clotting parameters. Clotting parameters were changed at 900 mg/kg oral *L. japonica* fucoidan in a rat model (Li et al., 2005) (but not at 300 mg/kg). In a short-term rat study, fucoidan derived from *U. pinnatifida*, at a dose level of 1350 mg/kg/day for 28 days did not cause any changes in clotting parameters (Kim et al., 2010b). In the NDIN (FMC, 2012) that has been accepted and filed by FDA (2013), anti-coagulation effects of fucoidans have been extensively described and assessed. The notifier concluded that at a dose of 200-300 mg/day, fucoidan dose would be anticipated to be safe.

The safety of fucoidan derived from *F. vesiculosus* is supported by:

- The long history of safe dietary exposure to Bladderwrack (*F. vesiculosus*), going back thousands of years.
- The absence of toxic effects in *in vitro* and *in vivo* toxicity studies at significant multiples of anticipated human exposure.
- Lack of adverse effects in animal studies and human studies.

The safety assessment of fucoidan derived from *F. vesiculosus* is based on the totality of available evidence, including background exposure and experimental studies. A variety of experimental studies with animals and human clinical trials and comparison with data from other studies in which fucoidan derived from other sources is used further corroborate the safety. In a clinical trial, administration of fucoidan derived from *F. vesiculosus* at doses of up to 300 mg/day for 12 weeks did not cause any significant adverse effects. Compared to the safe dose in humans, the estimated daily intake of fucoidan from the intended uses of the extract at the 90<sup>th</sup> percentile of 250 mg/person (4.17 mg/kg bw/day) is lower. The estimated daily intake, if ingested daily over a lifetime, is considered safe.

## 5. SUMMARY

Marinova has developed a process to manufacture standardized fucoidan derived from *F. vesiculosus*. It is produced without the use of solvents other than water and its production occurs in accordance with standard operating procedures, using starting materials and processing

materials that meet appropriate food grade specifications. The final product derived from *F. vesiculosus* contains  $\geq 70\%$  fucoidan. Other components in these two products include: alginate 2.0-5.5%; polyphloroglucinol 1.0-25%; mannitol 1-5%; natural salts 0.5-2.5%; and other carbohydrates 0.5-1.0%. The iodine content of fucoidan is typically less than 100 ppm and is over 15-fold lower than kelp.

Marinova intends to use fucoidan as a food ingredient in baked goods, soups, snack foods, imitation dairy products, and seasonings & flavors at use levels up to 30 mg fucoidan *per* serving. The intended use of fucoidan in these food categories will result in an estimated daily intake for “users only” at the 90<sup>th</sup> percentile of approximately 250 mg/person (4.17 mg/kg body weight/day). Such exposure is considered safe based on the following elements:

- Seaweeds such as Bladderwrack, the source material of fucoidan, has a very long history of safe use in humans and animals, and is recommended as part of a healthy human diet in Japan.
- In *in vitro* and *in vivo* studies fucoidan is not genotoxic.
- No adverse effects of fucoidan in a human clinical study at dose levels 300 mg/day for 12 week were noted.
- Safety studies of fucoidan (derived from other species) support the safe uses at proposed levels.

On the basis of scientific procedures<sup>7</sup> and history of exposure from natural sources, the consumption of fucoidan, derived from *F. vesiculosus*, as an added food ingredient is considered safe at levels up to 250 mg/day. The intended uses are compatible with current regulations, *i.e.*, fucoidan will be used in baked goods, soups, snack foods; imitation dairy products; and seasonings & flavors at use levels up to 30 mg fucoidan *per* serving and it is produced according to current good manufacturing practices (cGMP).

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<sup>7</sup> 21 CFR §170.3 Definitions. (h) Scientific procedures include those human, animal, analytical, and other scientific studies, whether published or unpublished, appropriate to establish the safety of a substance.

## 6. CONCLUSION

Based on a critical evaluation of the publicly available data summarized herein, the Expert Panel members whose signatures appear below, have individually and collectively concluded that consumption of fucoidan, extracted from *F. vesiculosus*, using Marinaova's Maritech® process, as a food ingredient in selected food products (baked goods, soups, snack foods; imitation dairy products; and seasonings & flavors) at levels of up to 30 mg/serving (reference amounts customarily consumed, 21 CFR 101.12) when not otherwise precluded by a Standard of Identity as described in this monograph and resulting in the 90<sup>th</sup> percentile estimated intake of up to 250 mg/person/day (4.17 mg/kg body weight/day for an individual weighing 60 kg) is safe and GRAS.

It is also our opinion that other qualified and competent scientists reviewing the same publicly available toxicological and safety information would reach the same conclusion. Therefore, we have also concluded that fucoidan extracted from *F. vesiculosus* using Marinaova's Maritech® process, when used as described, is GRAS based on scientific procedures.

### Signatures

(b) (6)

Robert L. Martin, Ph.D.

July 19, 2016

Date

(b) (6)

John A. Thomas, Ph.D., F.A.T.S., D.A.T.S.

July 22, 2016

Date

(b) (6)

Madhusudan G. Soni, Ph.D., F.A.C.N., F.A.T.S.

July 26, 2016

Date

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**8. APPENDIX I**

**Analytical data from five non-consecutive lots of  
Fucoïdan derived from *Fucus vesiculosus***

## Certificate of Analysis

### Organic Maritech® *Fucus vesiculosus* Extract

Batch number: FVF2011544  
 Botanical name: *Fucus vesiculosus*  
 Fresh/dry: Dry  
 Origin of seaweed: Canada

Common name: Bladderwrack  
 Manufacture date: August 2011  
 Expiry date: August 2016  
 Extract solvent: 100% water

**Storage conditions:** Store in well sealed containers under cool conditions. Protect from light, moisture and heat.

Test	Specification	Result
Appearance:	Off white to brown powder	Light brown powder
Odour and taste:	Bland odour and taste	Pass
Moisture:	<10% (105°C for 2 hours)	9.35%
pH:	4.0 - 7.0 (1% suspension at 25°C)	4.64
Particle size:	Minimum 80% less than 300 microns	95%
<b>Total Heavy metals<sup>1</sup>:</b>		
Inorganic arsenic	< 2ppm	0.06 ppm
Total arsenic	No specification	2.8 ppm
Lead	< 1 ppm	0.64 ppm
Mercury	< 1 ppm	< 0.01 ppm
Cadmium	< 3 ppm	0.62 ppm
Copper	No specification	5.4 ppm
<b>Microbiology:</b>		
Total aerobic microbial count	<10,000 CFU/g	Complies
Yeast and mould count	<100 CFU/g	Complies
Total enterobacteria count	Absent/g	Complies
<i>Eschericia coli</i>	Absent/g	Complies
<i>Salmonella species</i> <sup>1</sup>	Absent/10g	Complies
<i>Staphylococcus aureus</i>	Absent/g	Complies
<b>Pesticide residues<sup>1</sup>:</b>	Complies with USP	Complies
<b>Phytonutrients<sup>2</sup>:</b>		
Fucoidan	≥ 90%	95.1%

**Notes:**

1. Tested annually
2. Methods available for review

**Quality Approval:**

**Date:**

Dr Damien Stringer MRACI CChem  
 Product Development Manager

**Release Approval:**

**Date:**

Dr Vicki Gardiner FRACI CChem  
 Operations Manager

Issued: 1/11/2011



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## Certificate of Analysis

### Organic Maritech® *Fucus vesiculosus* Extract

Batch number: FVF2012503  
 Botanical name: *Fucus vesiculosus*  
 Fresh/dry: Dry  
 Manufactured: Australia

Common name: Bladderwrack  
 Manufacture date: February 2012  
 Expiry date: February 2017  
 Extract solvent: 100% water

**Storage conditions:** Store in well sealed containers under cool conditions. Protect from light, moisture and heat.

Test	Specification	Result
Appearance:	Off white to brown powder	Light brown powder
Odour and taste:	Bland odour and taste	Complies
Moisture:	<10%	8.12%
pH:	4.0 - 7.0 (1% suspension at 25°C)	4.70
Particle size:	80% less than 300 microns	98%
<b>Heavy metals<sup>1</sup>:</b>		
Inorganic Arsenic:	< 2 ppm	<0.05 ppm
Total Arsenic:	No Specification	2.2 ppm
Lead:	< 1 ppm	0.9 ppm
Mercury:	< 1 ppm	< 0.01 ppm
Cadmium:	< 3 ppm	1.0 ppm
Copper:	No Specification	5.4 ppm
<b>Microbiology:</b>		
Total aerobic microbial count	<10,000 CFU/g	Complies
Yeast and mould count	<100 CFU/g	Complies
Total enterobacteria count	Absent /g	Complies
<i>Eschericia coli</i>	Absent /g	Complies
<i>Salmonella species</i> <sup>1</sup>	Absent /10g	Complies
<i>Staphylococcus aureus</i>	Absent /g	Complies
Pesticide residues <sup>1</sup> :	Complies with USP	Complies
<b>Phytonutrients<sup>2</sup>:</b>		
Fucoidan	≥ 90%	94.7%

Notes:  
 1. Tested annually  
 2. Methods available for review

Quality Approval:

Date:

Dr Sam Karpiniec MRACI CChem  
 Senior Chemist

Release Approval:

Date:

Dr Damien Stringer MRACI CChem  
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Issued: 09/08/2013



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## Certificate of Analysis

### Maritech® *Fucus vesiculosus* Extract

Batch number: FVF2014505  
 Botanical name: *Fucus vesiculosus*  
 Fresh/dry: Dry  
 Manufactured: Australia

Common name: Bladderwrack  
 Manufacture date: February 2014  
 Expiry date: February 2019  
 Extract solvent: 100% water

**Storage conditions:** Store in well sealed containers under cool conditions. Protect from light, moisture and heat.

Test	Specification	Result
Appearance:	Off white to brown powder	Medium brown powder
Odour and taste:	Bland odour and taste	Complies
Moisture:	<10%	9.11%
pH:	4.0 - 7.0 (1% suspension at 25°C)	4.89
Particle size:	80% less than 300 microns	94%
<b>Heavy metals<sup>1</sup>:</b>		
Inorganic Arsenic:	< 2 ppm	< 0.05 ppm
Total Arsenic:	No Specification	3.5 ppm
Lead:	< 1 ppm	0.66 ppm
Mercury:	< 1 ppm	0.018 ppm
Cadmium:	< 3 ppm	0.89 ppm
Copper:	No Specification	11 ppm
<b>Microbiology:</b>		
Total aerobic microbial count	<10,000 CFU/g	Complies
Yeast and mould count	<100 CFU/g	Complies
Total enterobacteria count	Absent /g	Complies
<i>Eschericia coli</i>	Absent /g	Complies
<i>Salmonella species</i> <sup>1</sup>	Absent /10g	Complies
<i>Staphylococcus aureus</i>	Absent /g	Complies
Pesticide residues <sup>2</sup> :	Complies with USP	Complies
<b>Phytonutrients<sup>2</sup>:</b>		
Fucoidan	≥ 90%	92.8%

**Notes:**

1. Tested annually
2. Methods available for review

**Quality Approval:**

**Date:**

Dr Sam Karpiniac MRACI CChem  
 Senior Chemist

**Release Approval:**

**Date:**

Dr Damien Stringer MRACI CChem  
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## Certificate of Analysis

### Organic Maritech<sup>®</sup> *Fucus vesiculosus* Extract

Batch number: FVF2014521  
 Botanical name: *Fucus vesiculosus*  
 Fresh/dry: Dry  
 Manufactured: Australia

Common name: Bladderwrack  
 Manufacture date: July 2014  
 Expiry date: July 2019  
 Extract solvent: 100% water

**Storage conditions:** Store in well sealed containers under cool conditions. Protect from light, moisture and heat.

Test	Specification	Result
Appearance:	Off-white to brown powder	Medium-brown powder
Odour and taste:	Bland odour and taste	Complies
Moisture:	<10%	8.55%
pH:	4.0 - 7.0 (1% suspension at 25°C)	4.88
Particle size:	≥80% less than 300 microns	96%
<b>Heavy metals<sup>1</sup>:</b>		
Arsenic (Inorganic):	<2 ppm	0.06 ppm
Total arsenic	No specification	2.6 ppm
Lead	<1 ppm	0.76 ppm
Mercury	<1 ppm	<0.01 ppm
Cadmium	<3 ppm	0.86 ppm
Iodine	No specification	45 ppm
<b>Microbiology:</b>		
Total aerobic microbial count	<10,000 CFU/g	Complies
Yeast and mould count	<100 CFU/g	Complies
Total enterobacteria count	Absent /g	Complies
<i>Eschericia coli</i>	Absent /g	Complies
<i>Salmonella species</i> <sup>2</sup>	Absent /10g	Complies
<i>Staphylococcus aureus</i>	Absent /g	Complies
Pesticide residues <sup>1</sup> :	Complies with USP	Complies
<b>Phytonutrients<sup>2</sup>:</b>		
Fucoidan	≥90%	91.2%

Notes:  
 1. Tested annually  
 2. Methods available for review

**Quality Approval:**

**Date:**

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Issued: 20 Apr 2015

**Release Approval:**

**Date:**

Dr Damien Stringer MRACI CChem  
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## Certificate of Analysis

### Maritech® *Fucus vesiculosus* Extract

Batch number: FVF2014533  
 Botanical name: *Fucus vesiculosus*  
 Fresh/dry: Dry  
 Manufactured: Australia

Common name: Bladderwrack  
 Manufacture date: November 2014  
 Expiry date: November 2019  
 Extract solvent: 100% water

**Storage conditions:** Store in well sealed containers under cool conditions. Protect from light, moisture and heat.

Test	Specification	Result
Appearance:	Off white to brown powder	Medium brown powder
Odour and taste:	Bland odour and taste	Complies
Moisture:	<10%	8.27%
pH:	4.0 - 7.0 (1% suspension at 25°C)	4.46
Particle size:	80% less than 300 microns	100%
<b>Heavy metals<sup>1</sup>:</b>		
Inorganic Arsenic:	< 2 ppm	0.061 ppm
Total Arsenic:	No Specification	3.0 ppm
Lead:	< 1 ppm	0.75 ppm
Mercury:	< 1 ppm	< 0.01 ppm
Cadmium:	< 3 ppm	0.65 ppm
Copper:	No Specification	7.4 ppm
<b>Microbiology:</b>		
Total aerobic microbial count	<10,000 CFU/g	Complies
Yeast and mould count	<100 CFU/g	Complies
Total enterobacteria count	Absent /g	Complies
<i>Eschericia coli</i>	Absent /g	Complies
<i>Salmonella species</i> <sup>2</sup>	Absent /10g	Complies
<i>Staphylococcus aureus</i>	Absent /g	Complies
Pesticide residues <sup>1</sup> :	Complies with USP	Complies
<b>Phytonutrients<sup>2</sup>:</b>		
Fucoidan	≥ 90%	93.6%

**Notes:**

1. Tested annually
2. Methods available for review

**Quality Approval:**

**Date:**

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**Release Approval:**

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## **9. APPENDIX II**

### **Determination of Fucoïdan content: Marinova Laboratory C05 method**

(attached separately “**Analysis- Determination of Fucoïdan Content**” pp. 1-8)

## 10. APPENDIX III

Iodine Content of 5 non-consecutive lots

Batch Code	Iodine Content (ppm)
FVC2011007	32
FVF2014019	79
FVF2014505	28
FVF2014516	48
FVF2014533	59

**1.0 Purpose**

1.1 To determine the fucoidan content of algal extracts as the sum of fucoidan carbohydrates, sulfate, acetylation and counterions using gas chromatography, UV-Vis spectroscopy, inductively coupled plasma mass spectrometry/atomic absorption spectroscopy, and nuclear magnetic resonance spectroscopy/titrimetric methods.

**2.0 Scope**

This method is used to determine the total fucoidan content in seaweed extracts. It is generic and can be used with all seaweed species. The method outlines the procedures for the following:

- The determination of the total carbohydrate content and profile
- The determination of the sulfate and counter ion content
- The determination of the degree of acetylation

**3.0 Introduction**

3.1 Fucoidan is an ionic, substituted heteropolymer present in brown seaweeds. The polymer consists mostly of fucose, although some seaweed species have considerable galactose content. The carbohydrate units are substituted with acetyl ( $-OCH_2CH_3$ ) and sulfate ( $-OSO_3$ ) groups. The degree of sulfate and acetyl substitution varies from species to species and may also vary depending on how the fucoidan is treated during production. To balance the charge of the sulfate groups, counterions (mostly sodium, potassium, calcium and magnesium) are also present in the fucoidan molecule.

3.2 In order to determine the fucoidan content of a seaweed or seaweed extract, the carbohydrate profile and content, acetylation, sulfation and counterions must be known. These are determined by a variety of techniques: Gas Chromatography (GC), UV-Vis, Nuclear Magnetic Resonance (NMR), Atomic Absorption Spectroscopy (AAS), Inductively Coupled Plasma – Mass Spectrometry (ICP-MS) and titrimetric assays.

3.3 The GC method for determining the carbohydrate profile of fucoidan was developed by the Complex Carbohydrate Research Centre at the University of Georgia for Marinova Pty Ltd. It relies on the hydrolysis of polysaccharide moieties and their subsequent reduction and acetylation to produce acetylated alditol derivatives that are stable for gas chromatographic analysis. Following from the carbohydrate profile determination, the total carbohydrate content is determined by the phenol-sulfuric carbohydrate analysis reported by Dubois, validated by Marinova and outlined in Marinova Standard Operating Procedure (SOP) CP824-2, in combination with the spectroscopic determination of uronic acid content as per SOP CP824-10.

3.4 The acetyl content may be determined either titrimetrically, as per FAO methods for modified starch, (adapted and validated for fucoidan by Marinova as per SOP CP824-12) or by NMR. The NMR based acetylation content method was developed by Marinova with assistance from the NMR facility at the University of Tasmania. This technique measures the relative proportion of acetyl groups and fucose methyl groups. The resonances for these two moieties are well separated in the  $^1H$  NMR spectrum. The fucose methyl resonance is at  $\sim 1.6$ ppm and the acetyl resonance at  $\sim 2.5$ ppm. Using the fucose methyl as a reference enables monitoring of the relative degree of acetylation in a series of derivatives. The method is particularly robust since no chemical alteration of the sample is required.

3.5 The sulfate content may be determined spectrophotometrically by the method of Dodgson as adapted and validated for fucoidan by Marinova and outlined in SOP CP824-11, or by ICP-MS, as developed in conjunction with the University of Tasmania.

3.6 The counterion content of fucoidan extracts may be determined using AAS or ICP-MS techniques, which have both been validated for fucoidan extracts as per SOP CP824-19 (for AAS), or as outlined in this procedure (for ICP-MS).

#### 4.0 Responsibilities

4.1 The Operations Manager is responsible for providing technical assistance and process support. This support will include technical reports concerning laboratory trials as well as technical advice based on professional experience.

4.2 The Senior Chemist is responsible for revising the laboratory procedures and supporting documentation. The Senior Chemist is responsible for overseeing analyses and ensuring that procedures are carried out in order to meet standards and deadlines.

4.3 The Analyst is responsible for assisting the Senior Chemist in maintaining equipment, reviewing documents and meeting analytical schedules.

4.4 The Analyst is responsible for performing operations outlined in this and related procedures as well as recording tasks as they are completed in the laboratory records.

#### 5.0 Analysis

##### 5.1 Safety Precautions

5.1.1 A laboratory coat, protective eyewear and latex gloves must be worn when handling acidic materials.

5.1.2 All reagents should be used in the fume hood.

5.1.3 Due care and attention is to be given to the weighing and pipetting process to prevent accidents. All spills must be attended to immediately.

##### 5.2 Determination of carbohydrate profile by gas chromatography

###### 5.2.1 Reagents and Equipment

###### a) Standards

Prepare 1 mg/ml solutions of the following analytes

- |              |                                     |
|--------------|-------------------------------------|
| a. Arabinose | e. Mannose                          |
| b. Rhamnose  | f. Glucose                          |
| c. Fucose    | g. Galactose                        |
| d. Xylose    | h. Myo-inositol (internal standard) |

###### b) 2.0 M Trifluoroacetic acid (TFA)

2.0 M Trifluoroacetic acid is prepared by adding, for example, 1.54 ml of redistilled TFA to 8.46 ml of distilled water. Each sample requires 1000  $\mu$ L of this reagent.

###### c) Concentrated TFA

###### d) 1M ammonium hydroxide solution ( $\text{NH}_4\text{OH}$ )

Place approximately 50 ml of distilled water in a 100 ml volumetric flask. Add 5.15 ml of  $\text{NH}_4\text{OH}$ . Swirl flask to mix. Add distilled water to the line on the volumetric flask and mix well. Store in an appropriately labelled glass container.

###### e) Sodium borohydride in 1 M $\text{NH}_4\text{OH}$

Prepare 10 mg/ml solution of  $\text{NaBH}_4$  in 1.0 M  $\text{NH}_4\text{OH}$ . For example, combine 20 mg  $\text{NaBH}_4$  with 2 ml of 1.0 M  $\text{NH}_4\text{OH}$ . Mix well. Prepare this solution immediately prior to use.

**f) 0.20 M sodium carbonate**

Weigh out 2.12 g of anhydrous sodium carbonate and place in a 100 ml volumetric flask. Add distilled water to the line on the volumetric flask. Mix well and store in an appropriately labelled glass container.

**g) Methanol/acetic acid solution (MeOH:AcOH, 9:1)**

Add, for example, 1.5 ml glacial acetic acid to 13.5 ml methanol (MeOH) in a glass vial. Cap and mix well

**h) Iso-propanol (iPrOH)**
**i) Methanol (MeOH)**
**j) Dichloromethane (DCM)**
**k) Distilled Water**
**l) Pipettes**

The Eppendorf pipettes (1000  $\mu$ L and 5 mL) are used with the appropriate tips. A new tip is used for each sample.

**m) Vials**

16mL vials with Teflon lined phenolic caps.

**n) Balance and Heating Blocks**

Samples are weighed on the Mettler four-place balance. Heating blocks should be set to temperature and allowed to equilibrate prior to the commencement of the procedure.

**5.2.2 Procedure**
**a) Standard preparation**

- i. Prepare standards by adding 200  $\mu$ l of analytes a – g and 80  $\mu$ l of myo-inositol to a vial. Three standards are to be prepared. The standards should be evaporated to dryness at 50 °C using a stream of air. The dry-down will take approximately 1 hour.
- ii. Residual moisture can be co-distilled with 200  $\mu$ l *i*PrOH. This should be performed three times, taking care not to blow the dried solid out of the vial. These dry-downs take only a few minutes.
- iii. Standards should be treated as per the samples from step (viii): *Reduction*. The previous standard step (ii) can also be performed in tandem with sample steps (vi) and (vii).

**b) Sample preparation**

- i. **Hydrolysis:** Prepare 1.5 mg/ml sample stock solutions by accurately weighing ~15 mg of each sample and dissolving in 10 mL of distilled water.
- ii. Aliquot 1000  $\mu$ l of each sample stock into a screwcap vial. Add 125  $\mu$ l of 1 mg/mL myo-inositol solution as an internal standard.
- iii. Take the solutions to dryness using a stream of air while heating the solution at 50 °C.
- iv. Add 1000  $\mu$ l of 2 M TFA to each sample. Cap tightly and incubate on a heating block for 2 hours at 121°C.
- v. Remove samples and allow to cool to room temperature. Dry down with a stream of air while heating at 50 °C.
- vi. Remove residual moisture by adding 200  $\mu$ l *i*PrOH, and drying down with a stream of air, as above.

- vii. Repeat (vi) twice.
- viii. **Reduction:** Add 800  $\mu\text{l}$  of freshly prepared  $\text{NaBH}_4/\text{NH}_4\text{OH}$  solution (see 5.2.1(e)) to each sample. This must incubate at room temperature for at least 1 hour, but it is best if it can be left to incubate overnight.
- ix. Neutralise any excess borohydride by adding 200  $\mu\text{l}$  of glacial acetic acid per sample.
- x. Add 200  $\mu\text{l}$  of MeOH and dry down.
- xi. Add 400  $\mu\text{l}$  of 9:1 MeOH:AcOH solution and dry down to remove boric acid residues.
- xii. Repeat step (xi) once.
- xiii. Add 200  $\mu\text{l}$  of MeOH and dry down.
- xiv. Repeat step (xiii) twice. A crusty white residue on the sides of the tubes should result.
- xv. **O-acetylation:** add 1000  $\mu\text{l}$  of acetic anhydride to each sample and swirl.
- xvi. Add 920  $\mu\text{l}$  concentrated TFA to each sample. Cap and incubate at 50 °C for 10 minutes.
- xvii. Remove samples from the heat and allow to cool to room temperature. Add approximately 2 ml of *i*PrOH to each sample and dry down.
- xviii. Add approximately 4 ml of 0.20 M sodium carbonate to each sample. Gently shake.
- xix. Add approximately 4 ml of DCM to each sample. Cap the tube and mix gently. Release the pressure regularly by opening the cap.
- xx. Allow the phases to separate and discard the aqueous phase (top layer).
- xxi. Add 2 ml distilled water and mix gently as above.
- xxii. Again, allow the phases to separate and discard the aqueous phase.
- xxiii. Repeat steps (xxi) and (xxii) twice. On the last wash, remove the organic phases (bottom layer), transferring these into new tubes. Dry down with a stream of air.
- xxiv. Dissolve each dried sample in 800  $\mu\text{l}$  DCM. Transfer the solutions into GC vials, cap and place in the autosampler for analysis.

### 5.2.3 Analysis

#### a) **Gas Chromatography**

Gas chromatography is performed using a Hewlett Packard 6890 chromatograph fitted with a HP7673 auto-sampler, split/splitless injector and flame ionisation detector. A 30 metre, 0.25mm (i.d.) SGE BPX70 fused capillary column is used (0.25 $\mu\text{m}$  film of 70% cyanopropyl polysilphenylene-siloxane, moderate polarity). The column is operated in constant pressure mode with a nominal linear velocity of 42 cm/sec or 1.6 mL/min of nitrogen. The injector is held at 250 °C with a head pressure of 20 psi and run in splitless mode. The sample is injected in fast mode (0.5  $\mu\text{l}$ ) with dichloromethane washes pre and post injection. The flame ionisation detector is held at 280 °C and  $\text{N}_2$  or He is used as the makeup gas. The combined column/makeup gas flow is held constant at 45 mL/min. The column oven is temperature programmed as follows:

Initial temperature: 40 °C, initial time 2 minutes

Temperature program: 30 °C/min to 170 °C, then 4 °C/min to 235 °C; hold for 20 minutes

The HPChem program contains the method 'CCRC', which is used to run and analyse the samples. Each day's samples are to be stored in a new folder identified by the current date, and created under the 'Sequence Parameter' menu.

b) **Calculation of Results**

The response factors must be determined for each batch of analyses since it varies with GC instrument and operating conditions. If there has been any change to the configuration of the GC instrument, the alditol acetate derivative of each single carbohydrate standard should be run to establish its retention time which is used to assign peaks for each monosaccharide in the standards and samples.

From the weight and peak areas in the standard, the detector response factor (RF) value of each sugar can be calculated.

The relative response factor (RF) for each carbohydrate is determined from the standard chromatogram and calculated using the following formula:

$$RF = \frac{\text{weight inositol} * \text{peak area sugar}}{\text{peak area inositol} * \text{weight sugar}}$$

where all these values refer to those found in the standards.

The relative weight of each sugar in the samples is then calculated using the following formula, for example:

$$\text{weight of fucose} = \frac{\text{weight inositol} * \text{total peak area of fucose}}{RF \text{ of fucose} * \text{peak area inositol}}$$

where all these values refer to those found in the samples.

A template (see related documents) has been developed to facilitate the calculation of results. The relative monosaccharide ratios determined from this method may then be used to formulate appropriate standards for quantification using SOP CP824-2. Non-fucoidan carbohydrates identified by the GC method such as mannose (from mannitol) and glucose must be subtracted from the total carbohydrate content when determining fucoidan content.

### 5.3 Determination of degree of acetylation content

5.3.1 **The procedure for determining acetylation content via NMR is outlined below. Where NMR facilities are not available, acetylation may be determined via Marinova SOP CP824-12.**

5.3.2 A 400MHz wide bore Varian-Inova NMR spectrometer is used with a 10mm probe.

5.3.3 The sample (10 mg +/- 1mg) is dissolved in 99.9% D<sub>2</sub>O, CAS [7789-20-0] (1.0 ml, Cambridge Isotope Laboratories) in a high precision 5mm NMR tube (Wilmad 535-PP). No internal standard is added and the HDO peak is set at 4.75 ppm.

5.3.4 The spectrum is acquired without spinning with the following parameters:

Temperature      60°C  
 Pulse sequence   standard pi pulse  
 Frequency        399.683 MHz



Relaxation delay 10 seconds  
 Datapoints 128K  
 Acquisition time 10.7 seconds  
 Transients 128 to 256

5.3.5 The time domain data is Fourier transformed with a 1Hz exponential window function (line broadening), manually phased, baseline corrected by the polynomial method and drift corrected.

5.3.6 The peaks at 1.6 and 2.5 ppm are integrated (from 2.9 to 2.1 ppm and 2.1 to 1.0 ppm) and the ratio reported as the degree of acetylation.

5.3.7 The percent (weight) of acetylation can be calculated using the following formula:

$$\% \text{acetylation} = \frac{\text{molecular weight acetyl (43)}}{\text{molecular weight fucose (146)}} * \% \text{fucose content} * \frac{\text{integration of acetyl methyl}}{\text{integration of fucose methyl}} * 100$$

Where %fucose is determined from the GC and UV-Vis methods, and the molecular weight of fucose is that of the fucose unit in the polymer (ie -H<sub>2</sub>O)

#### 5.4 Determination of degree of sulfation

5.4.1 The procedure for determining sulfate content via ICP-MS is outlined below. Where ICP-MS facilities are not available, sulfate may be determined via Marinova SOP CP824-11.

##### a) Sample preparation

- i. Small amounts (1-10 mg) of sample are weighed into tin cups on a highly accurate micro-balance.
- ii. Samples are dissolved and diluted in ultra-pure water to 100 g (nominally ~100 ml) in 120 ml polycarbonate sealed containers.
- iii. A 1 g subsample is further diluted 10 fold to 10 g, with indium added as an internal standard (at a concentration level of 100 ppb) and acid added (0.1 ml high purity SEASTAR nitric acid) in 12 ml polycarbonate sample tubes.
- iv. Multiple blank samples are also taken through this sample preparation procedure (typically three).
- v. Dried sodium sulphate is used as an "in-house" standard reference material (S = 22.6 wt%) in addition to the certified sulfur standard (below).

##### b) Instrumental analytical protocol

- i. Analyses are performed using an ELEMENT magnetic sector ICP-MS (Finnigan, Bremen, Germany). This instrument allows high resolution measurements to be performed, which in this instance means that the major isotope of S (<sup>32</sup>S) can be spectrally resolved from the interfering <sup>16</sup>O<sub>2</sub> (also of nominal mass 32 amu).
- ii. The instrument is allowed to warm for ~1 hr prior to any analysis.
- iii. Sulfur standards are prepared from an externally sourced mixed multi-elemental calibration solution (100 ppm stock solution in 10% nitric, QCD Analysts, USA).
- iv. All calibration blanks and standards are prepared in 1% nitric acid, with indium present at 100 ppb [i.e. calibrants and samples are matched in terms of internal standard and acidity]. A typical calibration consists of a blank and 1 standard (100 ppb).

- v.  $^{32}\text{S}$  and  $^{115}\text{In}$  are the isotopes analysed, over 40 scans.
- vi. A 120 second sample uptake time is employed, along with a 150 second rinse with 5% nitric acid between each sample.

**c. Calculation of percentage sulfur values**

- i. The wt%S values given in the table are calculated as follows:

$$\frac{((\text{reading ppb} - \text{blank ppb}) * \text{dilution factor})}{10^7}$$

- ii. The wt% sulfate composition is give by wt%S multiplied by three.

**5.5 Determination of counterion content**

5.5.1 **The procedure for determining counterion content via ICP-MS is outlined below. Where ICP-MS facilities are not available, counterions may be determined via Marinova SOP CP824-19.**

**5.5.2 Procedure**

- i. Small amounts (1-10 mg) of sample are weighed into tin cups on a highly accurate micro-balance.
- ii. Samples are initially dissolved and diluted in ultra-pure water only.
- iii. Samples are diluted ~200,000 times in a stage (to 100 g).
- iv. Samples are prepared for final analysis with indium added as an internal standard (100 ppb) and nitric acid (1%) added.
- v. Analyses are undertaken using magnetic sector ICP-MS using three resolution modes
- vi. Sample data is given as wt%.

**5.6 Determination of fucoidan content**

The formula to determine total fucoidan content as a wt% of dry weight is:

$$\text{wt\%fucoidan (dry matter)} = \frac{\% \text{carbohydrate} + \% \text{acetylation} + \% \text{sulfate} + \% \text{cations}}{\% \text{solid}} * 100$$

Where %solid can be determined using SOP CP824-3.

**6.0 Related Documents**

GC Carbohydrate Template  
 Laboratory Results spreadsheet  
 CP824-2 Total Sugars by Spectroscopic Analysis  
 CP824-3 Determination of moisture and solid content  
 CP824-10 Uronic Acids by spectroscopic analysis  
 CP824-11 Sulfate by UV-Vis Analysis  
 CP824-12 Acetylation by Titration  
 CP824-19 Fucoidan Cations by AAS

**7.0 References**

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