ORIGINAL SUBMISSION

AMIN TALATI
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ATTORNEYS AT LAW

#666

GRN 000666

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August 16, 2016

Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food And Drug Administration
5001 Campus Drive
College Park, MD 20740-3835

Re: Generally Recognized As Safe ("GRAS") Notification for Chondroitin Sodium Sulfate

Dear Sir/Madam:

Pursuant to proposed 21 CFR 170.36, our client, Gnosis S.p.A. ("Gnosis"), through Amin Talati & Upadhye, LLC as its attorneys, hereby provides notice of a claim that the food ingredient chondroitin sulfate sodium, 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.

Please do not hesitate to contact me at Ashish@AminTalati.com or 312-327-3381 if you have any questions or require additional information.

Sincerely,

(b) (6)

Ashish Talati, J.D., M.S., RAC

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AUG 17 2016
OFFICE OF
FOOD ADDITIVE SAFETY

#666

GRAS NOTIFICATION

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)

Gnosis S.p.A., Italy has determined that chondroitin sulfate sodium is Generally Recognized As Safe, consistent with Section 201(s) of the *Federal Food, Drug, and Cosmetic Act*. This determination is based on scientific procedures as described in the following sections, under the conditions of its intended use as a food ingredient. Therefore, the use of chondroitin sulfate sodium is exempt from the requirement of premarket approval.

Signed,

(b) (6)

Date Aug 16, 2016

Ashish Talati, J.D., M.S., RAC

Attorney for:

Gnosis S.p.A.
Via Lavoratori Autobianchi, 1
20832 Desio (MB), Italy

B. Name and Address of Notifier:

Marco Berna
Gnosis S.p.A.
Via Lavoratori Autobianchi
1 20832 Desio (MB)
ITLY

C. Common or Usual Name of the Notified Substance:

The common name of the substance of this notification is chondroitin sulfate sodium.

D. Conditions of Intended Use in Food

Chondroitin sulfate sodium is intended for use as a nutrient [21 CFR 170.3(o)(20)][1] in conventional foods such as beverage and beverage bases, milk and milk products, and chewing gum at use levels ranging from 50 to 200 mg/serving (reference amounts customarily consumed, 21 CFR 101.12). Chondroitin sulfate sodium is not proposed for uses in foods that are intended for infants and toddlers, such as infant formulas or foods formulated for babies or toddlers, as well as it is not intended for use in meat and poultry products that come under USDA jurisdictions. The intended use of chondroitin sulfate sodium in the above mentioned food categories, is estimated to result in a maximum daily intake of 1200 mg /person [20 mg/kg body weight (bw)/day for an individual weighing 60 kg].

E. Basis for GRAS Determination:

In accordance with 21 CFR 170.30, the intended use of chondroitin sulfate sodium has been determined to be Generally Recognized As Safe (GRAS) based on scientific procedures. The determination is supported by the opinion of the Expert Panel. A comprehensive search of the scientific literature through July 2016 was also utilized for this determination. There exists sufficient qualitative and quantitative scientific evidence, including animal and human data to determine safety-in-use for chondroitin sulfate sodium. Chondroitin sulfate as a component of connective tissue is found in human body and is commonly consumed as a dietary supplement. The safety determination of chondroitin sulfate sodium is based on the totality of available evidence.

The safety of chondroitin sulfate sodium is supported by multiple well designed human clinical trials and animal studies with chondroitin sulfate. In the published literature, several double-blind, placebo-controlled trials were found. In these studies, the use levels of chondroitin sulfate ranged from 800 to 1200 mg/day for long duration. Additionally, several animal and *in vitro* studies further corroborate the safety in use of chondroitin sulfate sodium at the intended use levels. The findings from a series of specifically designed subchronic toxicity and mutagenicity studies that followed standard regulatory guidelines, supports the safety in use of chondroitin sulfate sodium, subject of present GRAS. Based on the subchronic study, the no-observed-adverse-effect level (NOAEL) for chondroitin sulfate sodium was determined to be 1000 mg/kg bw/day, the highest dose tested. On the basis of

[1]"Nutrient supplements": Substances which are necessary for the body's nutritional and metabolic processes.

scientific procedures[2], Gnosis S.p.A. considers the consumption of chondroitin sulfate sodium, as a food ingredient to be safe at levels up to 1200 mg/person/day.

F. Availability of Information:

The data and information that forms the basis for this GRAS determination will be provided to Food and Drug Administration upon request or will be available for FDA review and copying at reasonable times at the above mentioned offices of the notifier (Section I, B) or at the offices of:

Madhu G. Soni, PhD, FATS
Soni & Associates Inc
749 46th Square
Vero Beach, FL 32068

Telephone: +1- 772-299-0746
Email: msoni@soniassociates.net

II. Detailed Information About the Identity of the Notified Substance:

Chondroitin sulfate sodium is a standardized amorphous white to off-white powder produced by fermentation of a particular strain of *E. coli* to produce the K4 polysaccharide that is further chemically transformed to chondroitin sulfate sodium. The product is chemically pure.

A. Chemical name:

Chondroitin sulfate is a glycosaminoglycan, a linear polymer constituted of random sequences of repeated disaccharide units of:
2-acetylamino-2-deoxy-4-O-sulfate-3-O-β-D-glucopyranurosyl-D-galactose
2-acetylamino-2-deoxy-6-O-sulfate-3-O-β-D-glucopyranurosyl-D-galactose
2-acetylamino-2-deoxy-4,6-O-disulfate-3-O-β-D-glucopyranurosyl-D-galactose
2-acetylamino-2-deoxy-6-O-sulfate-3-O-β-2'-O-sulfate-D-glucopyranurosyl-D-galactose

B. Chemical Abstract Registry and other Number:

Chondroitin Sulfate Sodium Salt: [CAS: 9082-07-9]
Chondroitin sulfate C: [12678-07-8], [25322-46-7]
Chondroitin sulfate mixture of regioisomers A, C, E: [9007-28-7]

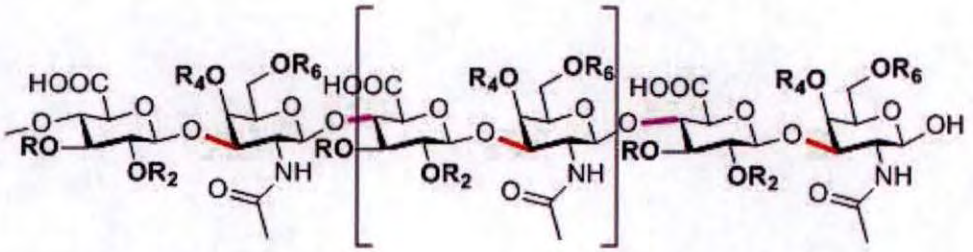
C. Chemical Formula and Molecular Weight:

Monosulfated disaccharide unit: $C_{14}H_{19}NNa_2O_{14}S$ (molecular weight = 503.3)
Bisulfated disaccharide unit: $C_{14}H_{20}NNa_3O_{17}S_2$ (molecular weight = 605.4)
Monosulfated polymer: $(C_{14}H_{19}NNa_2O_{14}S)_nH_2O$; n>1 [molecular weight = $(503.3)_n$+18]
Bisulfated polymer: $(C_{14}H_{20}NNa_3O_{17}S_2)_nH_2O$; n>1 [molecular weight = $(605.4)_n$+18]

[2] 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.

D. Structure:

The structural formula of chondroitin sulfate is presented in Figure II-D.



→[4)–β-D-GlcA-(1 →3)–β-D-GalNAc(1→]n

Chondroitin Sulfate

Chemical Formula: $(C_{14}H_{19}NNa_2O_{14}S)n\ H_2O$

Molecular Weight: (503,34)n + 18

R_4, R_6=H or SO_3Na

R_4 different from R_6

Figure II-D. Chemical Structure of Chondroitin Sulfate Sodium

E. Physical Characteristics

Chondroitin sulfate sodium is an amorphous white to off-white powder.

F. Identity and Specifications

Food grade specifications of chondroitin sulfate sodium are presented in Tables II-G.1. The product is highly pure and contains >95% chondroitin sulfate sodium. The identity of chondroitin sulfate sodium is confirmed by employing Nuclear Magnetic Resonance (NMR) spectra. Chondroitin sulfate sodium is the only main ingredient present in the product. Analytical data from three nonconsecutive manufacturing lots are presented in Appendix I.

Table II-F. Specifications of Chondroitin Sulfate Sodium (Mythocondro™)

Tests	Limits	Limits Ref.	Analytical Methods Ref.
Appearance	White to off-white powder	In-house	USP monograph
Identification: - IR - Sodium	 Positive Positive	USP monograph	USP monograph
Clarity and Color of solution (5% aqueous solution, absorbance at 420 nm)	≤ 0.35	USP monograph	USP monograph
Specific Optical Rotation (3% aqueous solution)	-7.0° to -19.0° d.b.	In-house	USP monograph
pH (1% aqueous solution)	5.5 to 7.5	USP monograph	USP monograph
Loss on drying (105 °C for 4 hours)	≤ 10.0 %	USP monograph	USP monograph
Residue on ignition	20.0 % to 30.0 % d.b.	USP monograph	USP monograph
Chloride	≤ 0.50 %	USP monograph	USP monograph
Sulfate	≤ 0.24 %	USP monograph	USP monograph
Heavy metals	≤ 0.002 %	USP monograph	USP monograph
Lead	≤ 0.5 ppm	USP <233>	USP <233>
Cadmium	≤ 0.5 ppm	USP <233>	USP <233>
Mercury	≤ 0.1 ppm	USP <233>	USP <233>
Arsenic	≤ 1.5 ppm	USP <233>	USP <233>
Electrophoretic purity: - any individual impurity	 ≤ 2 %	USP monograph	USP monograph
Microbial enumeration: - Total bacterial count - Total combined yeasts and molds - Salmonella sp. - Escherichia coli	 $\leq 10^3$ CFU/g $\leq 10^2$ CFU/g Absent / 10 g Absent / 10 g	USP monograph	USP monograph
Limit of protein	≤ 0.5 % d.b.	In-house	USP monograph
Content of chondroitin sulfate sodium	95.0 % to 105.0 % d.b.	In-house	USP monograph

G. Manufacturing process

Chondroitin sulfate sodium, the subject of this GRAS assessment, is manufactured according to current good manufacturing practices (cGMP). The manufacturing process consists of: (1) Production of K4 polysaccharide by fermentation; and, (2) Chemical transformation of K4 polysaccharide into chondroitin sulfate sodium. The fermentation stage utilizes the ability of a particular strain of *Escherichia coli* to produce the K4 polysaccharide. The production of polysaccharide is summarized in Figure II.G.1, while chemical synthesis is presented in Figure II.G.2. The production facility is registered and the FDA registration number of the food facility is 16009950712. The final product is characterized performing the same tests reported in the USP monograph of chondroitin sulfate sodium, following pharmacopeia procedures. The manufacturing process controls, along with characterization of microorganism and strain, toxigenic potentials, endotoxins, downstream processes and chemical synthesis are extensively discussed in Expert Panel Statement (see attached).

Although the microorganism [*E. coli* O5:K4:H4 strain U1-41 (ATCC23502)] is recognized as an uropathogenic strain of *E. coli* (UPEC), an extensive evaluation of the specific strain isolate used by Gnosis (GN 2284) in the fermentation for the production of K4 polysaccharide, revealed that the strain is genetically unable to produce any of the virulence factors and toxins.

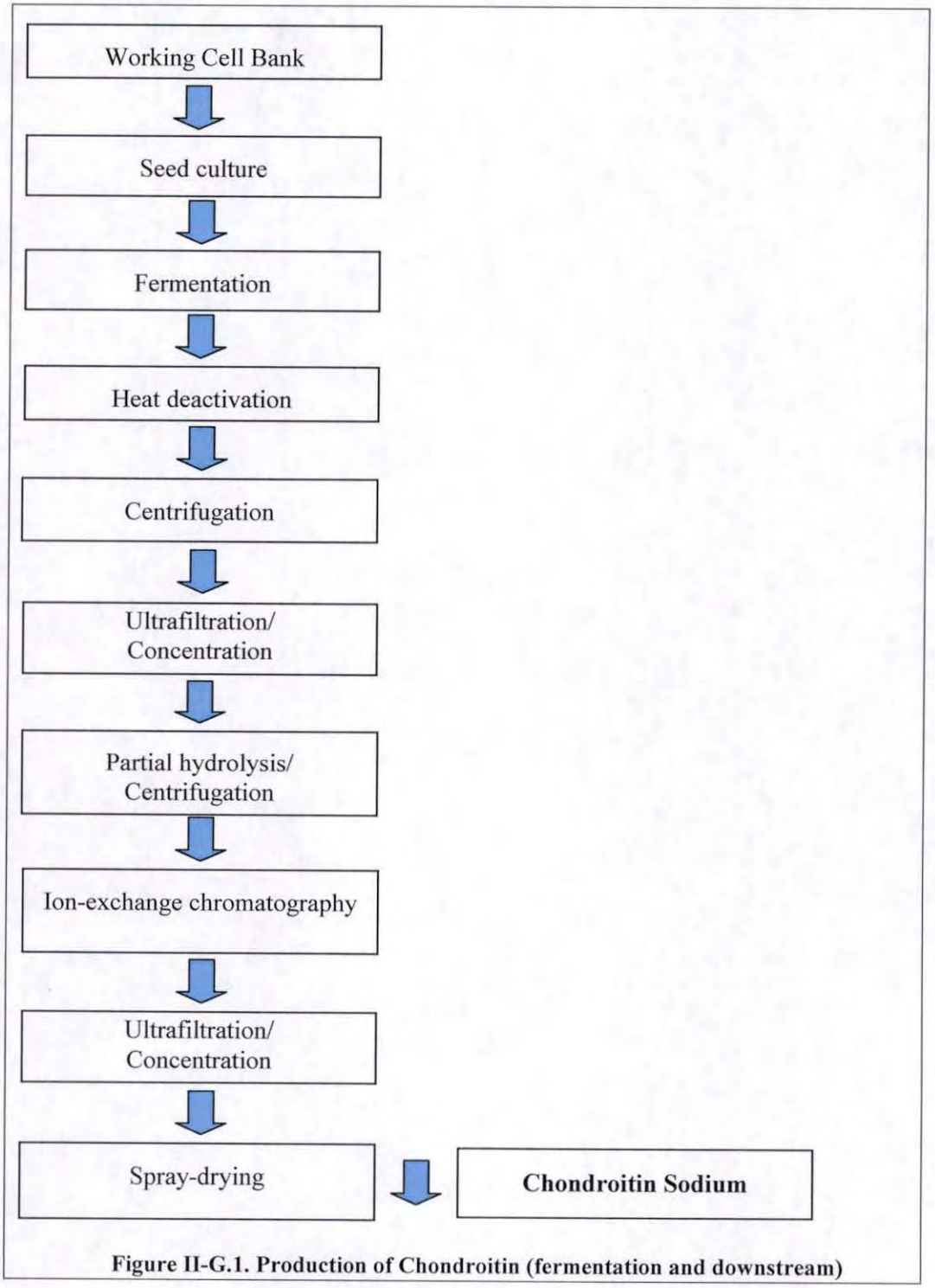


Figure II-G.1. Production of Chondroitin (fermentation and downstream)

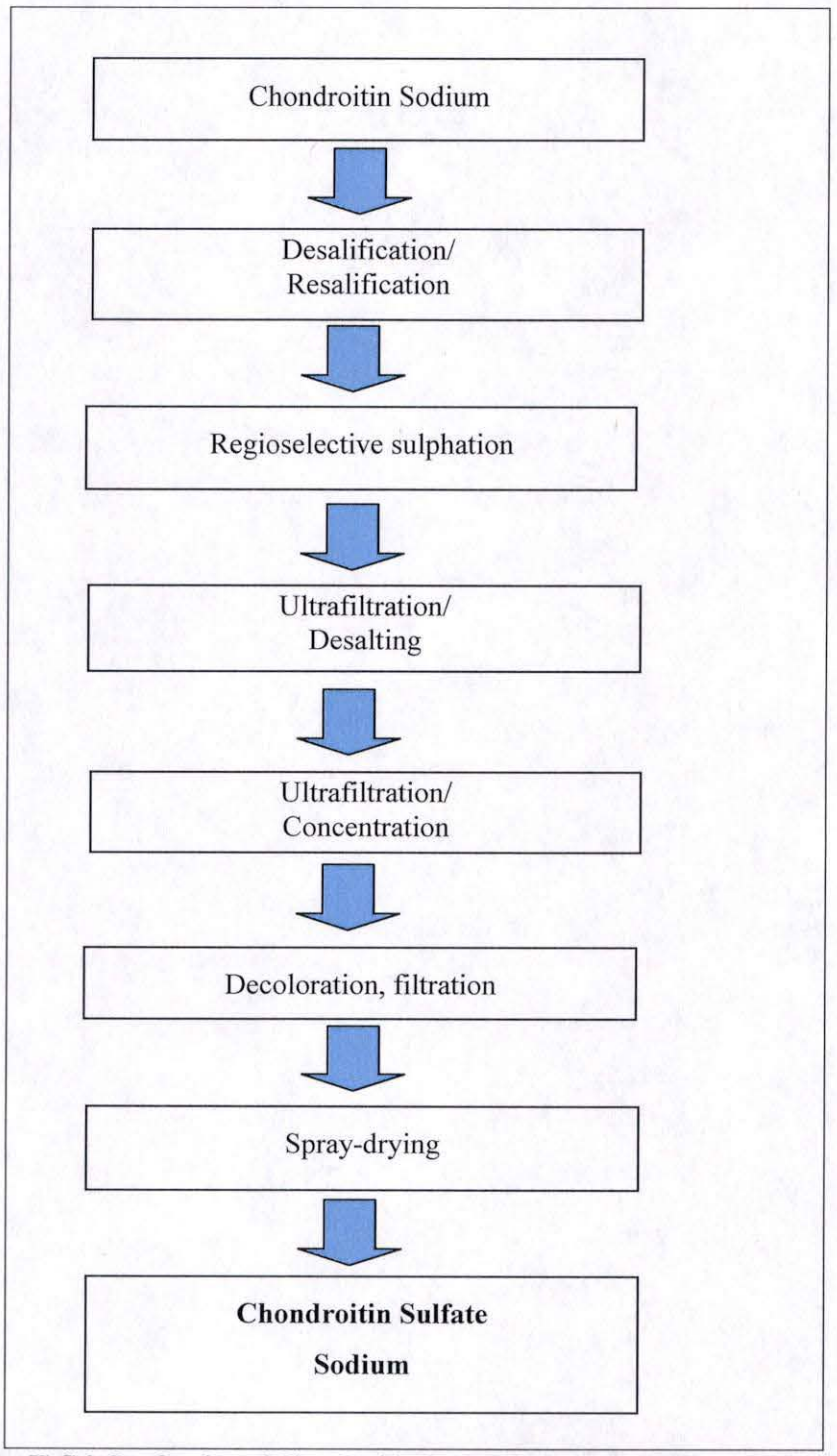


Figure II.G.2. Production of Chondroitin Sulfate Sodium (Chemical Synthesis)

The processing agents and materials used in the production of chondroitin sulfate sodium are high grade pure chemicals. Gnosis S.p.A. has established quality control measures to make sure that the individual reactions in the production process are optimized; the solvents and reactants are monitored to ensure the product complies with the appropriate food regulations. The preparation procedure assures a consistent and high-quality product.

H. Intended Technical Effects

Chondroitin sulfate sodium is intended for addition to selected foods as a as a nutrient [21 CFR 170.3(o)(20)]. The use of chondroitin sulfate sodium is intended for the general population at the levels identified in this document for addition to the following food categories: beverage and beverage bases, milk and milk products, and chewing gum. It is recognized that there are Standard of Identity requirements for some of the foods, and as such, Gnosis S.p.A. does not intend to refer them by the commonly recognized names.

III. Summary of the Basis for the Notifier's Determination that Chondroitin Sulfate Sodium is GRAS

The determination that chondroitin sulfate sodium is GRAS is based on scientific procedures. A comprehensive search of the scientific literature for safety and toxicity information on chondroitin sulfate sodium was conducted through June 2016 and was utilized for this assessment. Based on a critical evaluation of the pertinent data and information summarized here and employing scientific procedures, it is determined that the addition of chondroitin sulfate sodium to the selected foods described in this notice and at use levels ranging from 50 to 200 mg/serving (in accordance with established reference amounts customarily consumed, 21 CFR 101.12) meeting the specification cited above and manufactured according to current Good Manufacturing Practice, is GRAS under the conditions of intended use as specified herein.

In coming to this decision that chondroitin sulfate sodium is GRAS, Gnosis S.p.A. relied upon the conclusions that neither chondroitin sulfate sodium nor any of its degradation products 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. Other qualified and competent scientists, reviewing the same publicly available toxicological and safety information, would reach the same conclusion.

IV. Basis for a Conclusion that Chondroitin Sulfate Sodium is GRAS for its Intended Use.

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 convened to determine the safety of chondroitin sulfate sodium used as a food ingredient to provide consumers with a source of chondroitin sulfate sodium in their diets. Based on a critical evaluation of the pertinent data and information summarized herein, the Expert Panel members have individually and collectively determined by scientific procedures that the addition of chondroitin sulfate sodium in beverage and beverage bases, milk and milk products, and chewing gum at use levels up to 200 mg/serving (reference amounts customarily consumed, 21 CFR 101.12) when not otherwise precluded by a Standard of Identity as described here and resulting in the 90th percentile all-user estimated intake of 1200 mg/person/day is GRAS. It is also their opinion that other qualified and competent scientists, reviewing the same publicly available toxicological and safety information, would reach the same conclusion (see attached Expert Panel Statement).

EXPERT PANEL STATEMENT

DETERMINATION OF THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF CHONDROITIN SULFATE SODIUM AS A NUTRIENT

Prepared for

Gnosis S.p.A.
Via Lavoratori Autobianchi, 1
20832 Desio (MB), Italy

Expert Panel Members

Robert L. Martin, Ph.D.
John A. Thomas, Ph.D., FATS, DATS
Madhusudan G. Soni, PhD, FACN, FATS

August 2016

EXPERT PANEL STATEMENT
DETERMINATION OF THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF

CHONDROITIN SULFATE SODIUM
AS A NUTRIENT

TABLE OF CONTENTS

DETERMINATION OF THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF CHONDROITIN SULFATE SODIUM AS A NUTRIENT

1. INTRODUCTION

The undersigned, an independent panel of recognized experts (hereinafter referred to as the Expert Panel)[1], 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 Gnosis S.p.A., Italy, to determine the Generally Recognized As Safe (GRAS) status of the use of chondroitin sulfate sodium (Mythocondro™) derived from a non-animal source as a nutrient [21 CFR 170.3(o)(20)][2] in conventional foods such as Beverage and beverage bases, Milk and milk products, , and Chewing gum at use levels ranging from 50 to 200 mg/serving (reference amounts customarily consumed, 21 CFR 101.12). A comprehensive search of the scientific literature for safety and toxicity information on chondroitin sulfate sodium was conducted through June 2016 and made available to the Expert Panel. The Expert Panel independently and critically evaluated materials submitted by Gnosis S.p.A. and other information deemed appropriate or necessary. Following an independent, critical evaluation, the Expert Panel conferred on August 12, 2016 and unanimously agreed to the decision described herein.

1.1. Background

Chondroitin sulfate is an amino sugar polymer, made up of glucuronic acid and galactosamine, which belongs to the class of large polymers known as glucosaminoglycans or mucopolysaccharides (Harris et al., 2005). It is a major component of the extracellular matrix. Chondroitin sulfate is an important structural component of cartilage and is responsible for most of its resistance to compression. Additionally, because of its negative charges, chondroitin sulfate plays regulatory roles by readily interacting with proteins in the extracellular matrix. Chondroitin sulfate proteoglycans are also prominent components of the extracellular matrix in the central nervous system and are assumed to play important roles in controlling neuronal differentiation and development, as well as the nervous system response to injury (Gu et al., 2009). Chondroitin sulfate is commonly used in dietary supplements to promote joint health. The concept that orally administered chondroitin sulfate, along with glucosamine, helps support joint function has also been recognized for decades (Hathcock and Shao, 2007). As chondroitin constitutes the majority of the glycosaminoglycans in articular cartilage, it has been claimed to help support joint and cartilage function (Stuber et al., 2011). In addition to its use as a dietary supplement, chondroitin sulfate is a minor component of gelatin. Thus, chondroitin sulfate is found in almost all gelatin-derived products (food, cosmetics, pharmaceuticals and photographic material) (NIEHS, 2002). Gnosis S.p.A. intends to market a well-characterized powder form of chondroitin sulfate sodium salt (Mythocondro™) as a food ingredient, obtained by a fermentation process followed by a chemical synthesis. The cation usually associated with chondroitin sulfate is sodium. Hence, it is commonly referred to as chondroitin sulfate sodium.

[1]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.

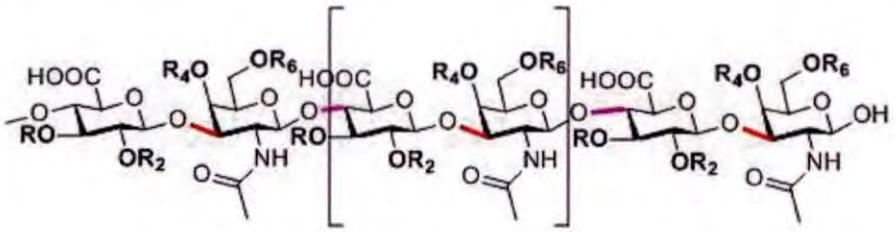
[2]"Nutrient supplements": Substances which are necessary for the body's nutritional and metabolic processes.

1.2. Description, Manufacturing Process and Specifications

The subject of this GRAS determination, chondroitin sulfate sodium, is a white to off-white powder. The structure of chondroitin sulfate is shown in Figure 1. General descriptive parameters and properties of chondroitin sulfate sodium are summarized in Table 1. Gnosis S.p.A. intends to market chondroitin sulfate sodium under the trade name Mythocondro™.

Table 1: General Descriptive Characteristics of Chondroitin Sulfate Sodium

Parameter	Description
Chemical name	Chondroitin sulfate is a glycosaminoglycan, a linear polymer constituted of random sequences of repeated disaccharide units of: 2-acetylamino-2-deoxy-4-O-sulfate-3-O-β-D-glucopyranurosyl-D-galactose 2-acetylamino-2-deoxy-6-O-sulfate-3-O-β-D-glucopyranurosyl-D-galactose 2-acetylamino-2-deoxy-4,6-O-disulfate-3-O-β-D-glucopyranurosyl-D-galactose 2-acetylamino-2-deoxy-6-O-sulfate-3-O-β-2'-O-sulfate-D-glucopyranurosyl-D-galactose
Trade name	Mythocondro™
CAS number	Chondroitin sulfate C [12678-07-8], [25322-46-7] Chondroitin sulfate mixture of regioisomers A, C, E [9007-28-7]
Molecular formula	Monosulfated disaccharide unit $C_{14}H_{19}NNa_2O_{14}S$ Bisulfated disaccharide unit $C_{14}H_{20}NNa_3O_{17}S_2$ Monosulfated polymer $(C_{14}H_{19}NNa_2O_{14}S)_nH_2O$ n > 1 Bisulfated polymer $(C_{14}H_{20}NNa_3O_{17}S_2)_nH_2O$ n > 1
Molecular weight	Monosulfated disaccharide unit 503.3 Bisulfated disaccharide unit 605.4 Monosulfated polymer $(503.3)_n+18$ n > 1 Bisulfated polymer $(605.4)_n+18$ n > 1
Physical state	Amorphous powder
Solubility	Freely soluble in water, practically insoluble in acetone and ethanol (96 per cent).
Color	White to off-white.
Storage	Preserve in tight containers, far from humidity.
Functional uses	Dietary supplement ingredient



→[4)–β-D-GlcA-(1→3)–β-D-GalNAc(1→]$_n$

Chondroitin Sulfate

Chemical Formula: $(C_{14}H_{19}NNa_2O_{14}S)n\ H_2O$

Molecular Weight: (503.34)n + 18

R_4,R_6=H or SO_3Na

R_4 different from R_6

Figure 1: Chemical Structure of Chondroitin Sulfate.

1.2.1. Identity and Specifications

Typical food-grade specifications of chondroitin sulfate sodium from Gnosis S.p.A. are summarized in Table 2. The product is a highly purified chemical substance with 95% to 105% purity on dry basis. The identity of chondroitin sulfate sodium is confirmed by employing Nuclear Magnetic Resonance (NMR) spectra. Chondroitin sulfate sodium is the only main ingredient present in the product. The product is tested according to the specifications reported in Table 2 and analyzed according to the procedures described in the USP monograph of chondroitin sulfate sodium, where relevant. Analytical results of three non-consecutive batches demonstrate that chondroitin sulfate sodium is consistently manufactured to meet the standard specifications (Appendix I). It is noted that Gnosis S.p.A's chondroitin sulfate sodium meets the specifications listed in the USP.

Table 2. Specifications of Chondroitin Sulfate Sodium (Mythocondro™)

Tests	Limits	Limits Ref.	Analytical Methods Ref.
Appearance	White to off-white powder	In-house	USP monograph
Identification: - IR - Sodium	 Positive Positive	USP monograph	USP monograph
Clarity and Color of solution (5% aqueous solution, absorbance at 420 nm)	≤ 0.35	USP monograph	USP monograph
Specific Optical Rotation (3% aqueous solution)	-7.0° to -19.0° d.b.	In-house	USP monograph
pH (1% aqueous solution)	5.5 to 7.5	USP monograph	USP monograph
Loss on drying (105 °C for 4 hours)	≤ 10.0 %	USP monograph	USP monograph
Residue on ignition	20.0 % to 30.0 % d.b.	USP monograph	USP monograph
Chloride	≤ 0.50 %	USP monograph	USP monograph
Sulfate	≤ 0.24 %	USP monograph	USP monograph
Heavy metals	≤ 0.002 %	USP monograph	USP monograph
Lead	≤ 0.5 ppm	USP <233>	USP <233>
Cadmium	≤ 0.5 ppm	USP <233>	USP <233>
Mercury	≤ 0.1 ppm	USP <233>	USP <233>
Arsenic	≤ 1.5 ppm	USP <233>	USP <233>
Electrophoretic purity: - any individual impurity	 ≤ 2 %	USP monograph	USP monograph
Microbial enumeration: - Total bacterial count - Total combined yeasts and molds - Salmonella sp. - Escherichia coli	 ≤ 10^3 CFU/g ≤ 10^2 CFU/g Absent / 10 g Absent / 10 g	USP monograph	USP monograph
Limit of protein	≤ 0.5 % d.b.	In-house	USP monograph
Content of chondroitin sulfate sodium	95.0 % to 105.0 % d.b.	In-house	USP monograph

1.3. Manufacturing Process

Generally, chondroitin sulfate used in many applications, including dietary supplements, is of animal origin and obtained by extraction from tissues of several animals such as bovine and porcine, avian and cartilaginous fishes. The animal origin poses potential consumer safety problems associated with the possible presence of transmissible infective agents (bacteria residues, viruses and prions) such as those causing spongiform encephalopathy in bovines (BSE), foot-and-mouth disease, influenza spread in birds, possible allergic reactions from fish sources, and other animal diseases (this possibility has now been reduced by a stricter selection of raw materials and by specific chemical treatments capable of inactivating prion agents) or a restriction use related to religious issues. Additionally, animal-derived chondroitin sulfate, if not controlled appropriately, can lead to products with highly variable structures and lack of reproducibility. Furthermore, there is also the possibility of contamination by other polysaccharides along with the presence of nucleic acids, proteins and other (macro)-molecules. Lack of control can also introduce structural modifications on the chondroitin sulfate backbone such as desulfation and/or depolymerization producing a polysaccharide having poor or no biological properties.

The above described potential issues for the production of chondroitin sulfate have prompted the search for alternative manufacturing processes. One such process involves the production by fermentation starting from the K4 capsular polysaccharide of *Escherichia coli* (Volpi, 2003; Volpi 2004, Volpi, 2007) followed by chemical synthesis. This new alternative approach is able to specifically produce a non-sulfated polysaccharide backbone having the same primary structure of chondroitin, i.e., disaccharides composed of GlcA $\beta(1\rightarrow3)$ GalNAc linked to each other by $\beta(1\rightarrow4)$ bonds by large-scale fermentation of *E. coli* K4. The purified non-sulfated chondroitin possessing a specific molecular mass is further sulfated by a chemical process able to introduce a specific number of sulfate groups in specific positions. This new method of preparing non-animal chondroitin sulfate shows homogeneous structure and physico-chemical properties, in particular the presence of sulfate groups in defined positions, a constant charge density and molecular mass parameters. The chemical step allows for the production of chondroitin sulfate possessing a carbohydrate structure and physico-chemical properties largely similar and comparable to animal-derived samples.

The subject of this GRAS determination, chondroitin sulfate sodium, is manufactured according to current good manufacturing practices (cGMP), as presented in Figures 2 and 3. The manufacturing process consists of two main stages:

- Production of K4 polysaccharide by fermentation; and
- Chemical transformation of K4 polysaccharide into chondroitin sulfate sodium

The fermentation stage utilizes the ability of a particular strain of *Escherichia coli* to produce the K4 polysaccharide. The production of polysaccharide is summarized in Figure 2, while the chemical synthesis is presented in Figure 3. The production facility is registered and the FDA registration number of the food facility is 16009950712. The final product is characterized performing the same tests reported in the USP monograph of chondroitin sulfate sodium, following pharmacopeia procedures.

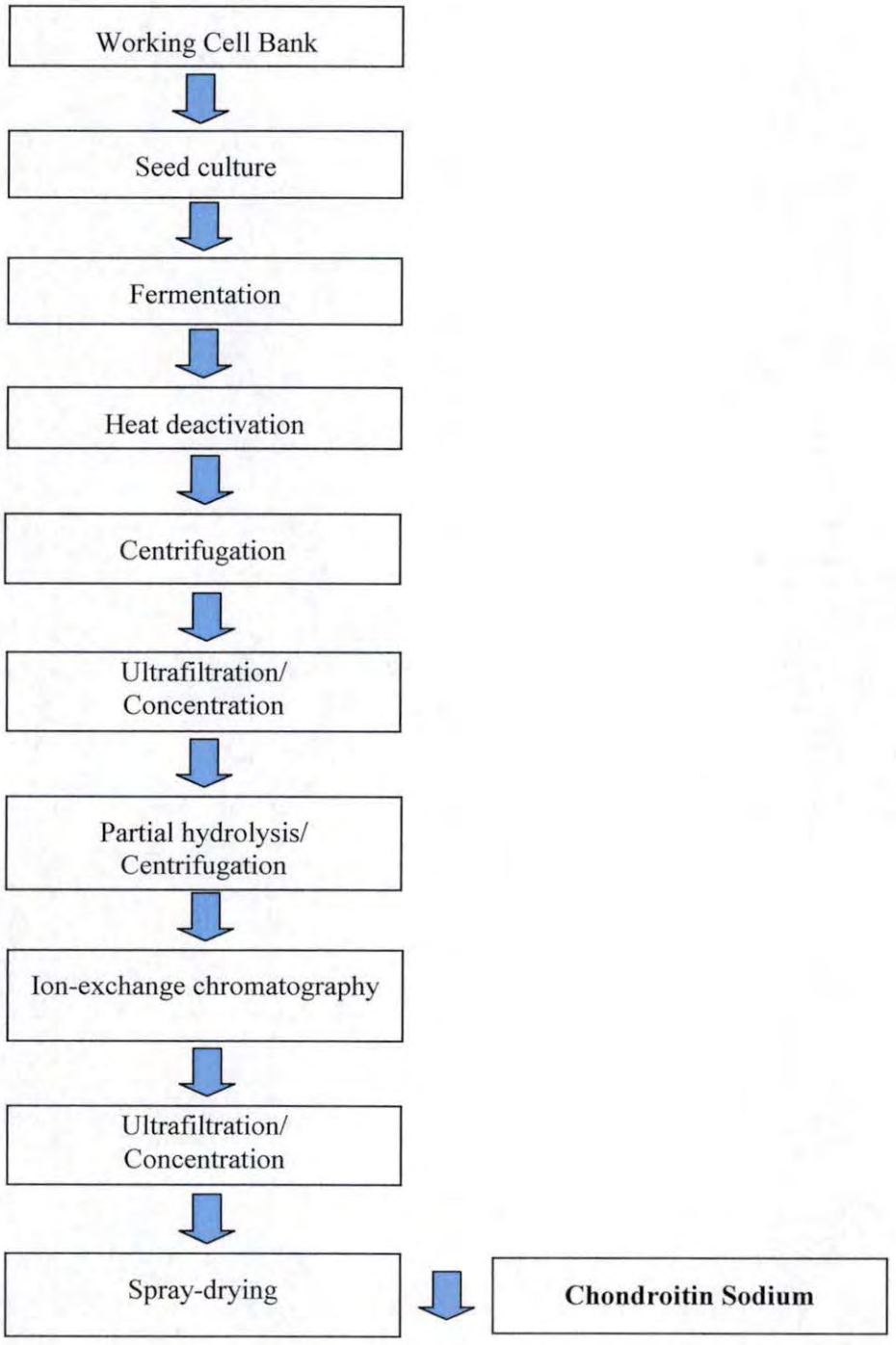


Figure 2. Production of Chondroitin (fermentation and downstream)

1.3.1. Microorganism Characteristics

The microorganism used in the fermentation step for the production of K4 polysaccharide is derived from *E. coli* O5:K4:H4 strain U1-41 (ATCC23502) and is recognized as an uropathogenic strain of *E. coli* (UPEC). The strain was purchased from the CCUG (Culture Collection, University of Goteborg), where it is deposited as CCUG 11307 (ATCC 23502) and it was used to prepare a Reference Cell Bank (RCB) stored in the internal collection of Gnosis S.p.A. under the accession number GN2284. The taxonomic position of strain GN2284 is summarized in Table 3. The strain GN2284 is a rod-shaped bacterium, Gram-negative, aerobic and facultative anaerobe, non-spore-forming and grows at the optimum temperature of 37 °C. The strain GN2284 is vancomycin resistant (5 mg/L) and sensitive to antibiotics active against Gram-negative bacteria (e.g., Ampicillin, Kanamycin). The strain GN2284 is not genetically modified.

Table 3. Taxonomic Position of *E. coli* strain GN2284

Domain	Prokaryota
Kingdom	Bacteria
Phylum	Proteobacteria
Class	Proteobacteria Gamma
Order	Enterobacteriales
Family	Enterobacteriaceae
Genus	Escherichia
Species	*Escherichia coli*
Strain	*ATCC23502*

1.3.1.1. Strain Characterization

In order to evaluate the colony morphology, the strain GN2284 was cultivated on solid media and the appearance of the colonies was recorded. The biochemical characterization of the strain GN2284 was obtained by performing the API 20E test (Biomerieux). The genotypic characterization of the strain GN2284 was achieved by analyzing Region II of the capsule gene cluster as pathogenic *Enterobacteria* possess a genomic island required to produce an outer membrane known as capsular polysaccharide. In particular, the strain GN2284 holds a specific group of genes in the capsule cluster known as Region II, which is a 13.4-kb locus, including 7 open reading frames (ORFs), comprising the *kfoC* gene which is a characteristic gene of the strain GN2284 because it encodes the chondroitin synthase enzyme. Therefore genomic analysis of the *kfoC* gene is helpful to characterize the strain GN2284. Detailed descriptions of morphological, biochemical and genotypic analysis for the strain have been completed (Appendix III).

1.3.1.2. Toxigenic Potential

As the uropathogenic strain U1-41 is classified as a biological agent in Risk Group 2 (defined as "an agent that can cause diseases in humans and may pose a risk to workers, is unlikely to spread to the community; are usually available effective measures of prophylaxis or treatment" in the Legislative Decree No. 81 dated April 9, 2008, Title X, Annex XLVI, Italian law), the toxigenic potential of strain GN2284 has been evaluated through the presence/absence of the genetic determinants for the known toxins and virulence factors of *Escherichia coli*. A

culture of *E. coli* GN2284 has been sent to the *E. coli* Reference Center (ECRC), Animal Diagnostic Laboratory, Department of Veterinary and Biomedical Sciences, Pennsylvania State University, which is an AAVLD (American Association of Veterinary Laboratory Diagnosticians) accredited laboratory and is a recognized authority in the field of *E. coli*, being the largest American repository of *E. coli* strains holding more than 70,000 strains isolated from animals, humans, and the environment. The outcome of their investigation should, therefore, be considered an independent and authoritative assessment of the toxigenic potential of strain GN2284.

E. coli is a major component of the normal intestinal flora of animals. Many different serotypes belonging to this species can be isolated from the feces of both healthy and non-healthy animals. Most naturally-occurring *E. coli* strains are non-pathogenic, but some have acquired genes coding for virulence factors and are known to cause both enteric and extra-intestinal diseases. The pathogenicity of these strains is generally determined by the presence of genes, encoding adhesins and/or toxins that can be located on either the chromosome or plasmid(s). Current knowledge of the virulence attributes determining the pathogenicity of *E. coli* and state-of-the-art molecular methods have been used to assess the potential virulence of strain GN2284.

ECRC has carried out a search for genes that encode the virulence factors of *E. coli* by using established laboratory procedures. In particular, Polymerase Chain Reaction (PCR) has been used to detect the possible presence in strain GN2284 of the genes listed below, known to confer pathogenicity to *E. coli* strains: STa: heat stable type A; STb: heat stable type B; LT: heat labile enterotoxin; Stx-I: Shiga toxin 1 associated with EHEC and STEC strains; Stx-II: Shiga toxin 2 associated with EHEC and STEC strains; CNF-1: cytotoxic necrotizing factor 1 associated with urinary tract infection (UTI) in humans and animals; CNF-2: cytotoxic necrotizing factor 2 associated with UTI in humans and animals; Hly A, E, D: hemolysins; K88: fimbriae (adhesion factor); K99: fimbriae (adhesion factor); 987P: fimbriae (adhesion factor); F18: fimbriae (adhesion factor); F1845: fimbriae (adhesion factor); CS31A: fimbriae (adhesion factor); BFP: Bundle-Forming Pilus; EAE: intimin-gamma; ISS: Increased Serum Survivability; TSH: Temperature Sensitive Hemagglutination; and K1: capsular antigen.

Briefly, template DNA was isolated from a bacterial suspension by heating it at 99°C for 10 min. This extraction method allows the recovery of total DNA, i.e., both chromosomal and plasmidial (if present). The thermolysed suspension was micro-centrifuged and the DNA-containing supernatant was collected and used for the various PCR reactions. PCRs were carried out according to the procedure standardized and established by ECRC in their Standard Operating Procedures (SOPs). Suitable negative and positive controls consisting of appropriate DNA preparations were used to validate results. Control DNAs were extracted from the following *E. coli* strains: positive controls- B41; 80-2575; ATCC 43895; 7.2635; B26a; 0.0015 (Oct 09); 1.2925; 4.0811; and 5.2675; and negative control- K12. The primers used for the PCRs were as described in the SOPs.

The tests results obtained using the established procedures were evaluated for any toxigenic potentials of *E. coli* strain GN 2284. None of the genes searched for were found to be present in the total DNA extracted from strain GN2284, showing that the strain in question is genetically unable to produce any of the virulence factors and toxins listed above.

1.3.1.3. Endotoxins

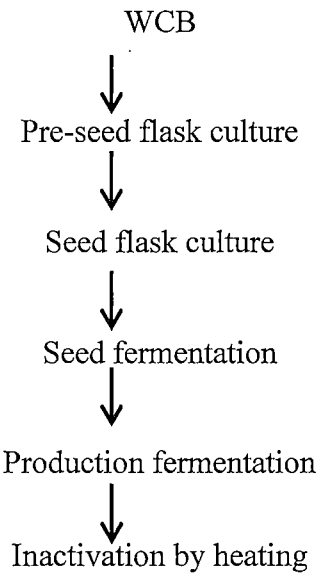
Endotoxins are toxic substances bound to the bacterial cell wall and released when the bacterium ruptures or disintegrates. Endotoxins are unavoidably present in all *E. coli* strains, being a part of the lipopolysaccharide (LPS) that is, in turn, an integral part of the outer membrane of all Gram-negative organisms. This issue is evaluated in downstream process, where the clearance of endotoxin during the purification steps is considered to ensure that the final product is safe.

1.3.2. Cell Banks

A two-tier cell banking system is employed with two aims: (a) minimize the number of generations occurring from the original material to the full-scale fermentation, and (b) ensure that all production batches are inoculated with identical cell populations. The first stage is the Master Cell Bank (MCB) that is fully characterized to ensure its identity with the biological material present in the Reference Cell Bank (RCB). The MCB contains a number of identical frozen cultures, each of which is used to prepare a Working Cell Bank (WCB). Each WCB is validated by comparison with the MCB from which it has been originated, and contains a number of identical frozen cultures, each of which is used to start a production batch.

1.3.3. Fermentation Process

Full-scale production consists of a number of successive cultivation steps, the last of which is the production phase, as shown in the following diagram. All cultivation stages are carried out under strictly aseptic conditions, with the cultivation media and the cultivation equipment being steam-sterilized according to validated procedures. Before removal from the fermenter, the culture is inactivated by heating.



1.3.4. Downstream Process

The heat-deactivated harvest broth is subjected to a biomass separation step by means of centrifugation through a continuous discharge disk-centrifuge. To facilitate biomass sedimentation, the culture is diluted with water in an approximate ratio of 1:1 prior to centrifugation. Standard yield of K4 polysaccharide after centrifugation steps: $\geq$ 95%. The supernatant obtained from the centrifugation step is subjected to an ultrafiltration (UF) step through a polysulphone membrane with a molecular weight cut-off (MWCO) of 30 kDa in order to remove all low-molecular weight impurities, while the K4 polysaccharide is retained by the membrane. At first the volume of the supernatant is reduced, while the permeate is discharged. Eventually, to facilitate the removal of the impurities from the concentrated solution, a dialysis step is carried out. Practically all the K4 polysaccharide is recovered in the concentrate; standard yield of the ultrafiltration step is: $\geq$ 98%.

The ultrafiltration dialyzed product is subjected to a partial acid hydrolysis at pH 3.8 (obtained by addition of acetic acid) at 80°C for 4 - 7 h. At the end of this phase, the pH is adjusted to 4.5 with NaOH solution. The main function of this step is to hydrolyze the glycosidic bonds linking K4 and *E. coli* lipopolysaccharide (LPS) in a complex with molecular weight (MW) greater than 150 - 200 kDa. Hydrolysis is the major crucial step of the process: it reduces the MW of the high-MW impurities where the majority of them are present in the outer cell envelope of *E. coli*. Without a suitable hydrolysis step, the UF dialyzed product looks milky, with a high dry matter content. If hydrolysis is too much stressed, however, strong depolymerization occurs, compromising the finished product quality and the global recovery. This is shown by the decrease of average MW and by an increase in the MW dispersion. Hydrolysis causes the partial degradation of contaminating polysaccharides as well, such as the *E. coli* LPS, particularly to O-antigenic portion of the LPS molecule. Hydrolysis has to be carried out by keeping chondroitin (C0S/K4-d) and LPS MW controlled. This is achieved both by SEC-HPLC analysis and by a centrifugation test with the aid of a bench top centrifuge. The clarification of the ultrafiltration product is possible only after the C0S-LPS complex (MW $\geq$ 150 – 200 kDa) has been destroyed and a 50–90 kDa C0S peak and a low MW LPS peak (10 – 15 kDa) are formed. As soon as the ultrafiltration product becomes clear, the hydrolysis is stopped. LPS degradation is likely to modify the colloidal state of the ultrafiltration product, thus allowing the suspended solid separation. This is appreciable with raw floccules formation. During hydrolysis it is thus necessary to maintain a very gentle stirring to facilitate the flocculation process.

The hydrolyzed product is subjected to a second separation step by means of centrifugation in order to eliminate the precipitated LPS and the suspended solid (as cell debris) still present after hydrolysis. The standard yield of chondroitin (C0S) after centrifugation steps is $\geq$ 98%.

The clarified solution of chondroitin (C0S) is purified by ion-exchange chromatography utilizing a strong anion exchange resin (Amberlite IRA 958-CL) in the Cl^- form. Negatively-charged chondroitin is bound by the positively-charged quaternary ammonium functional groups of the resin while, non-charged or positively-charged molecules are not bound and are lost with the flow through. Some impurities are also bound by the resin, but are lost during the subsequent elution phase. The standard yield of chondroitin (C0S) after chromatographic steps is approximately 90%. The final ultrafiltration and dialysis step is carried out on the C0S-rich

pooled fractions in order to remove the low-MW impurities that may be present. UF is carried out through a polysulphone membrane with a MWCO of 30 kDa. The step yield is about 90% and the CE purity of the chondroitin (C0S) is > 95%. The product has to be further concentrated by evaporation resulting in a recovery of close to 100%. The chondroitin sodium salt is isolated as a solid by spray-drying.

1.3.5. Chemical Synthesis

The synthetic process to manufacture chondroitin sulfate sodium is performed in two steps using chondroitin sodium salt as starting material. The first step involves desalification in which sodium is completely removed. In this process positively-charged cations (Na^+) are exchanged with H^+ and an acidic aqueous solution of chondroitin, with a pH between 2.0–2.5, is recovered after filtration. A stoichiometric amount of an alkyl-ammonium hydroxide is added to the acidic solution. The solution is checked for the completion of the salification and is immediately dried (spray dried or freeze dried) and recovered in the form tetralkylammonium salt of chondroitin. This is followed by regioselective sulphation carried out by using dimethylformamide. An ultrafiltration step is carried out on the aqueous solution of chondroitin sulfate obtained after the regioselective sulphation step. Organic and inorganic low-MW impurities that may be present are removed using the steps -- dialysis, decolorization using charcoal and filtration. Bacterial endotoxin content is checked. To the dialyzed chondroitin aqueous solution an inorganic salt (i.e., sodium chloride) is added until reaching a neutral saline solution with a 0.2M salt concentration. Chondroitin sulfate sodium is recovered as a solid by spray-drying.

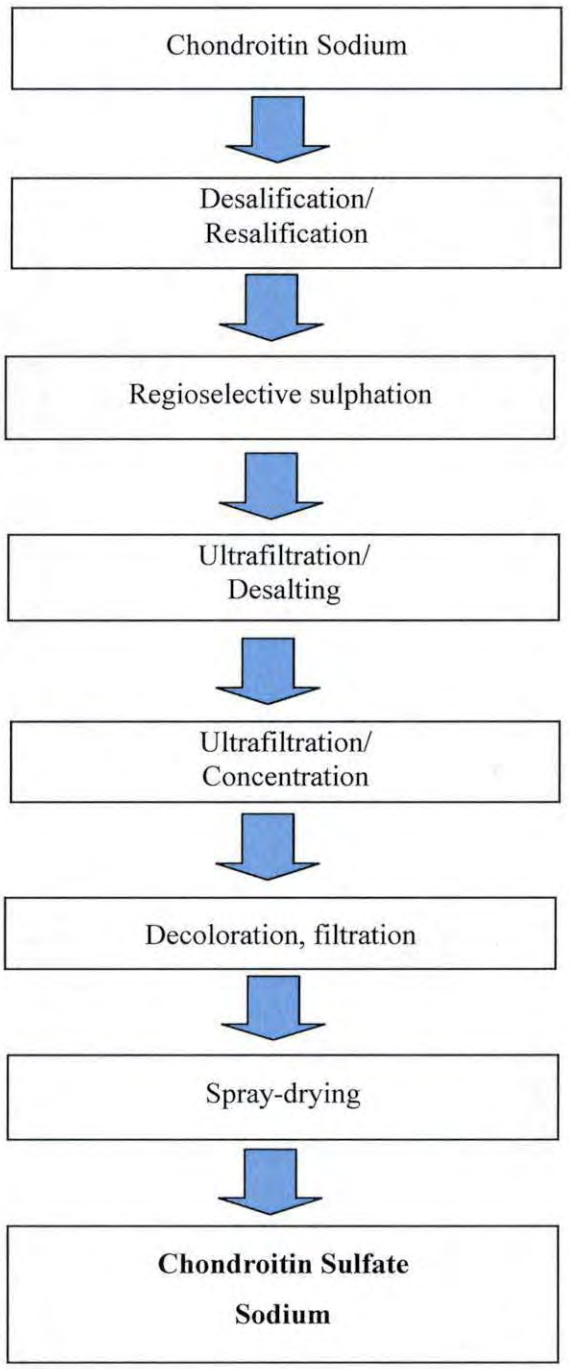


Figure 3. Production of Chondroitin Sulfate Sodium (Chemical Synthesis)

1.4. Chemistry and Biological Activity

As mentioned earlier, chondroitin sulfate belongs to a family of heteropolysaccharides called glycoaminoglycans or mucopolysaccharides. In the form of proteoglycans, glycosaminoglycans comprise the ground substance in the extracellular matrix of connective tissue. Chondroitins consisting of an alternating sequence of D-glucuronate and N-acetyl-D-galactosamine-4/6-sulfate residues linked through alternating bonds. The disaccharide units are

joined to one another by a β1-4 linkage. The residues are joined by a β1-3 linkage. Chondroitin is a mixture of different forms. The most common ones are chondroitin-4-sulfate, also known as chondroitin sulfate A, chondroitin-6-sulfate also known as chondroitin sulfate C, and dermatan sulfate, also known as chondroitin sulfate B (Foot and Mulholland, 2005; Simanek et al., 2005; Lamari and Karamanos, 2006).

The molecular weight of chondroitin sulfate ranges from 5,000 to 50,000 D and contains approximately 15 to 150 basic units of D-glactosamine and D-glucuronic acid (Hendler and Rorvik, 2002). In humans, chondroitin sulfate is found in cartilage, bone, cornea skin and arterial wall. This type of chondroitin is referred to as chondroitin sulfate A. The amino group of galactosamines in the basic unit of this type is acetylated yielding N-acetyl-galactosamine. There is a sulfate group esterified to the 4-position in N-acetyl-glactosamine. Another type, chondroitin sulfate C is primarily found in fish and shark cartilage, but also in humans. This type is also made up of repeating units of D-glacatosamine and D-glucuronic acid. The amino group of D-galactosamine is acetylated to give N-acetyl-galactosamine, and in the case of chondroitin sulfate C, the sulfate group is esterified to the 6-position of N-acetyl-galactosamine. Yet another type, chondroitin sulfate B is also known as dematan sulfate. It is commonly found in skin and also in heart valves, tendons and arterial walls. It is made up of repeating units of D-glactosamine and either L-iduronic acid and D-glucuronic acids. Its molecular weight ranges from 15,000 to 40,000D.

Glycoproteins are macromolecules that are made of a protein and a carbohydrate. In the extra cellular matrix of connective tissue, glycoproteins known as proteoglycans form the ground substance (Hendler and Rorvik, 2002). Proteoglycans are polyanionic substances of high-molecular weight. These substances contain many different types of heteropolysaccharide side-chains covalently linked to a polypeptide-chain backbone. The polysacchrides, which include chondroitin sulfate and hyaluronic acid, make up as much as 95% of the proteoglycan structure. These proteoglycans are claimed to play an important role in nervous systems. The polysaccharide groups in proteoglycans are called glycosaminoglycans and include hyaluronic acid, chondroitin sulfate, dermatan sulfate, keratan sulfate, heparin and heparan sulfate. Both chondroitin sulfate and hyaluronic acid are vital for the structure and function of articular cartilage and are fundamental components of aggrecan found in articualr cartilage. In the progression of degenerative joint disease or osteoarthritis aggrecan synthesis is decreased leading to the loss of cartilage resiliency and the pain along with other symptoms common of osteoarthritis.

1.5. Current Uses

As a major component of cartilage, chondroitin sulfate helps cushion the joints. Chondroitin sulfate is believed to provide structure, hold water and nutrients, and promote elasticity in cartilage. These properties have made chondroitin sulfate a popular dietary supplement for people seeking to nourish their joint tissues. Chondroitin is marketed alone or in combination with other joint supplements, usually with glucosamine (another compound believed key to cartilage formation and repair). Used with glucosamine, chondroitin sulfate supports cartilage and joint function. Over 40 million Americans have been reported to suffer from osteoarthritis (Adebowale et al., 2000). The market potential for glucosamine/chondroitin sulfate as a dietary supplement is large. In a period between July 1998 and May 1999, retail sales

in the US were estimated at more than half billion dollars (Adebowale et al., 2000). It is likely that the current sales are much higher than those reported for the year 1998-1999.

The principal source of human exposure to chondroitin sulfate occurs from its use as a dietary supplement. In clinical trials, a dose of 1200 mg/day of chondroitin sulfate has been used (Adebowale et al., 2000). However, for a short-term use the recommended dose appears to be 1200-1800 mg/day, with a maintenance dose of 600-750 mg/day, to promote joint health. As dietary supplements, chondroitin sulfate-containing products are regulated under the Dietary Supplement Health and Education Act (DSHEA, 1994). Chondroitin sulfate is a popular dietary supplement. It is commonly sold as chondroitin sulfate, in capsules or tablets. It is often used in combination with glucosamine. The usual dose is 1200 mg daily in divided doses (Hendler and Rorvik, 2002). There is no dietary reference intake (DRI) for chondroitin sulfate. There are no major dietary sources of chondroitin. A search of the Dietary Supplement Label Database[3] showed 22 products with either chrondroitin sulfate as the name of the product or listed as an ingredient in the dietary product[4].

1.6. Intended Use Levels and Food Categories

Gnosis S.p.A. intends to use chondroitin sulfate sodium salt as a food ingredient in the following food categories mentioned in 21 CFR 170.3(n): (3) Beverages and beverages bases; (6) Chewing gum, including all forms; (10) Dairy product analogs; (20) Frozen dairy desserts and mixes; (30) Milk, whole and skim; (31) Milk products. The available information from USDA intake estimates of different food products database indicate that the above food categories mentioned in 21 CFR 170.3(n) will fit under three broad categories such as Beverage and beverage bases, Milk and milk products, and Chewing gum (Table 4). The intended use of chondroitin sulfate sodium will be 200 mg/serving for beverages and milk products, while for chewing gum it will be 50 mg/serving. The intake analysis is performed using average intake of the proposed foods reported in Smiciklas-Wright et al. (2002) and USDA (1997). The intended use of chondroitin sulfate sodium at 200 mg (beverages and milk products) and 50 mg (chewing) *per* serving will result in average and high (90^{th} percentile) intake of 595.33 and 1186.66 mg chondroitin sulfate sodium/person/day, respectively. For safety assessment purposes the high intake of 1186.66 or approximately 1200 mg/person/day is considered as appropriate.

It is recognized that there are Standard of Identity requirements for some of these foods (i.e., milk and milk products), located in Title 21 of the Code of Federal Regulations, and as such, Gnosis S.p.A. does not intend to refer to them by the commonly recognized names. Chondroitin sulfate sodium is not proposed for uses in foods that are intended for infants and toddlers, such as infant formulas or foods formulated for babies or toddlers, as well as it is not intended for use in meat and poultry products that come under USDA jurisdictions.

[3] Available at: http://www.dsld.nlm.nih.gov/dsld/
[4] Available at: http://www.dsld.nlm.nih.gov/dsld/rptQSearch.jsp?item=chrondroitin+sulfate&db=adsld

Table 4. Intended Use Levels and Possible Daily Intake of Chondroitin Sulfate Sodium

Food category	Mean consumption of food product (g/day)	Use levels (mg)		Serving size (g)	Daily intake by adult (mg/person)	
		Per serving	(mg/kg)		Average	High (90th %)
Beverage and beverage bases[1]	333	200	2083	240	277.50	555.00
Milk and milk products[2]	274	200	2083	240	228.33	456.66
Chewing gum[3]	7	50	62500	3-5 (~4)	87.50	175.00
Total (mg/person/day)					**593.33**	**1186.66**

*The daily intake calculations are based on USDA data. The high (90th percentile) consumption of chondroitin sulfate was determined by multiplying the corresponding mean total intake value by a factor of two on the grounds that the 90th percentile consumption rarely exceeds the mean by more than a factor of two (FDA, 2006). Serving size is based on Reference Amounts Customarily Consumed per Eating Occasion (21 CFR 101.12). [1]For Beverages- soft drinks is considered (Source 1-page 242); [2]Milk and milk products includes- milk, milk drinks, yogurt, desserts and dairy analogs (Source 2- page 29); [3] chewing gum (Source 2- page 31).
Source 1 = Smiciklas-Wright et al. (2002); Source 2 = USDA (1997)

2. DATA PERTAINING TO SAFETY

Chondroitin sulfate has been extensively investigated for its effects in multiple human clinical trials, animal studies and in *in vitro* experimental studies. Given its potential health benefits, uses, and as an endogenous constituent of human body there has been considerable effort to elucidate the biological role of chondroitin sulfate. A simple search of databases such as PubMed has revealed over 14,000 articles. However, animal safety studies were very limited, while in several clinical human studies, along with efficacy, safety or tolerability of chondroitin sulfate has been investigated. Additionally, in several clinical studies, chondroitin sulfate has been investigated together with glucosamine. In the following section, relevant safety related information from animal and human clinical studies of chondroitin sulfate are summarized. Efforts have been made to present both the data supporting the safety as well as any data on the adverse effects of chondroitin. Gnosis has undertaken gentotoxicity and subchronic toxicity studies to support the safety of chondroitin sulfate, the subject of this GRAS assessment. In some publications, chondroitin sulfate sodium is referred to as chondroitin sulfate or chondroitin. In this GRAS document, the term that was stated in the article is used in discussing the article. The Expert committee is aware of this usage and has taken it into account in its evaluation.

2.1. Specific Safety Studies

In a series of specifically designed studies with chondroitin sulfate sodium (the subject of this present GRAS assessment), subchronic toxicity, allergenicity, potential *in vitro* genotoxic effects, and *in vivo* bioavailability in humans was investigated. The chondroitin sulfate sodium used in these studies was produced by Gnosis S.p.A. Italy. The complete reports of all these studies were provided by Gnosis S.p.A. Recently, findings from these studies were published by Miraglia et al. (2016). These studies are described in the following sections. These studies and publication supports the safety and GRAS status of chondroitin sulfate sodium.

2.1.1. Specific Subchronic Toxicity Study

In a dose-response study, (Miraglia et al., 2016; Rossiello and Oberto, 2013) adverse effects, if any, of chondroitin sulfate sodium were investigated in rats. The study was conducted in accordance with the Principles of Good Laboratory Practice for the testing of Chemicals OECD [ENV/MC/CHEM(98)17] and as per the guidelines from European Parliament Directive 2004/10/EC. This study was designed in agreement with the procedures described in: - OECD Guideline no. 408 'Repeated Dose 90-day Oral Toxicity Study in Rodents' adopted 21 September 1998. For this study, Sprague Dawley rats (10/sex/group; 5-6 weeks old) were gavaged daily with chondroitin sulfate sodium at dose levels of 0 (control), 250 (low dose), 500 (mid dose), or 1000 (high dose) mg/kg body weight (bw)/day for 90 consecutive days. Two additional groups of animals (5/sex/group) for the recovery study received 0 (control-R) and 1000 (high dose R) mg/kg bw/day of the extract for 90 days, followed by no additional treatment for 28 days.

Throughout the study period the animals were observed for clinical signs of toxicity and mortality/morbidity (twice daily); detailed clinical examinations (including neurotoxicity), and body weight and feed consumption (weekly) were recorded. Once during weeks 12 or 13 of treatment and once during week 4 of recovery, an evaluation of sensory reactivity to stimuli of different modalities (e.g., auditory, visual and proprioceptive stimuli), an assessment of grip strength, and motor activity was performed. During week 13, blood samples and overnight urine samples were collected. During week 4 of the recovery period blood samples were also taken. Hematological and clinical chemistry parameters were investigated. Urine analysis was performed. Serum immunoglobulin levels were determined at pre-dosing and during week 13. Organ weights were recorded and over 40 tissues and organs were harvested at the necropsy and fixed in 10% buffered neutral formalin. Histopathological examination was carried out on full set of tissues collected from the high dose and control groups. All gross lesions from all rats irrespective of group were also evaluated for histological changes (Miraglia et al., 2016; Rossiello and Oberto, 2013).

There were no signs of adverse effects or mortality noted in any animals during the course of the study. Neurotoxicity assessment, such as weekly detailed clinical observations of animals at removal from the cage and in an open arena, and the sensory reactivity to different stimuli, as well as the motor activity performed at the end of treatment and/or recovery period, did not show relevant differences between the groups. No toxicological changes in body weight were observed during the study in treated animals, compared to controls. The mean feed consumption was comparable in all the dose groups of both the sexes. Ophthalmoscopy did not reveal any treatment-related lesions. No changes of toxicological significance were noted in hematological parameters and no changes were recorded at coagulation test at the examination performed at the end of the treatment period (Miraglia et al., 2016; Rossiello and Oberto, 2013).

There were no treatment-related biologically or toxicologically significant changes in clinical chemistry parameters. No toxicological significance was attributed to the changes recorded in two animals treated at 1000 mg/kg/day (increase of aspartate aminotransferase- AST and triglycerides) or to the statistically significant increases of chloride and sodium in females dosed with 1000 mg/kg/day, decreases of phosphorus in those treated with 250 mg/kg/day and potassium in those receiving 500 and 1000 mg/kg/day, since the changes were of minimal

severity or incidence and/or not dose-related, therefore considered unrelated to treatment. Increases in alanine aminotransferase (ALT) was seen in one male and one female dosed with 1000 mg/kg/day. In addition, AST and bilirubin were increased in some treated females. Changes recorded for ALT and bilirubin were not observed during the dosing phase (at week 13). Therefore, they were considered unrelated to treatment. The increment of AST was insufficient in severity to be considered of toxicological significance. The above noted significant changes in clinical chemistry parameters following the administration of chondroitin sulfate sodium were not observed in both sexes, lacked correlating changes in other clinical parameters, were of small magnitude, were not noted in a dose-related manner, or were not associated with microscopic changes in the related organs and hence they were considered as incidental changes/biological variations and not treatment-related adverse effects No changes were recorded in urinalysis at the examination performed at the end of the treatment period (Miraglia et al., 2016; Rossiello and Oberto, 2013).

At termination, no differences in body weight and organ weights were seen in either sex. There were no treatment-related macroscopic or histopathological findings in any of the groups. All the gross and histopathological changes observed were considered as spontaneous and incidental to rats of this particular strain and age. Based on the results of this study, the no-observed effect level (NOAEL) of the chondroitin sulfate sodium is determined as 1000 mg/kg bw/day, the highest dose tested (Miraglia et al., 2016).

The estimated maximum daily intake of 1200 mg chondroitin sulfate sodium/person from its intended uses is approximately 50-fold lower than the demonstrated safe levels of intake in subchronic toxicity study.

2.1.2. Specific Allerginicity Study

As part of the above described subchronic toxicity study (Section2.1.1.), additional investigations were performed to determine potential allergenicity, if any, of chondroitin sulfate sodium (Mosiello and Oberto, 2013). For this, immunoglobulin IgG, IgA and IgE in serum from rats treated repeatedly with chondroitin sulfate sodium for 13 weeks by the oral route (dose levels: control, 250, 500 and 1000 mg/kg bw/day) were measured. No significant differences between pre-test and Week 13 data, nor between control and treated groups data were observed, with the exception of IgG in females dosed with 1000 mg/kg bw/day. In these animals, IgG from Week 13 samples were significantly lower at statistical analysis than controls at 13 weeks. In general the direction of this finding is not considered a concern. In addition, most of the IgG from pre-test phase samples were similar to those recorded during Week 13, therefore the decrease of IgG was considered irrelevant. IgG values in females dosed with 1000 mg/kg bw/day after 13 weeks of treatment in fact, were no significantly different from pre-dose values in the same group of animals and their values are considered to be normal values in the population of rats.

The investigators concluded that no inflammation or immunological processes occurred following chondroitin sulfate sodium administration. The findings from this study corroborate the safety of chondroitin sulfate sodium.

2.1.3. Specific Genotoxicity Studies

In three separate studies, the mutagenic effects of non-animal chondroitin sulfate sodium, the subject of this GRAS assessment, were investigated (Miraglia et al., 2016). These methods included a bacterial reverse mutation test (Ames test) using *Salmonella typhimurium* strains and *Escherichia coli* WP2 strains, an *in vitro* mammalian chromosomal aberration study in Chinese Hamster Ovary Cells (CHO), and a mutation in mouse lymphoma cell assay (Fluctuation Method). The results of these experiments indicate that chondroitin sulfate sodium is unlikely to be genotoxic. These studies support the safety of chondroitin sulfate sodium.

2.1.3.1. Ames Assay

The potential of non-animal chondroitin sulfate sodium, the subject of this GRAS assessment, to induce gene mutations in comparison to vehicle control was examined according to the plate incorporation test and the pre-incubation test using *S. typhimurium* (TA1535, TA1537, TA98, TA100) and *E. coli* (WP2 uvrA) strains, as measured by reversion of auxotrophic strains to prototrophy (Miraglia et al., 2016; Bisini and Oberto, 2011). The study was conducted as per the OECD Guideline for the testing of chemicals No. 471 (Adopted July 1997) and ICH S2A Genotoxicity: Specific Aspects of Regulatory Tests, Step 5. The test was performed in the presence and absence of a rat liver metabolizing system. The following substances were used as positive controls: sodium azide, 9-aminoacridine, 2-nitrofluorene, 2-aminoanthracene, methylmethanesulphonate. A preliminary toxicity test was undertaken in order to select the concentrations of chondroitin sulfate sodium to be used in the main assays. Chondroitin sulfate sodium was assayed in the toxicity test at a maximum concentration of 5000 µg/plate and at four lower concentrations spaced at approximately half-log intervals: 1580, 500, 158 and 50.0 µg/plate. No toxicity was observed with any tester strain at any dose level, in the absence or presence of S9 metabolism.

Two experiments were performed including negative and positive controls in the absence and presence of an S9 metabolizing system. Three replicate plates were used at each test point. The first experiment was performed using a plate-incorporation method. Chondroitin sulfate sodium was assayed at the maximum dose level of 5000 µg/plate and at four lower dose levels spaced by two-fold dilutions: 2500, 1250, 625 and 313 µg/plate. As no increases in revertant numbers were observed at any concentration tested, a pre-incubation step was included for all treatments. Chondroitin sulfate sodium did not induce two-fold increases in the number of revertant colonies in the plate incorporation or pre-incubation assay, at any dose level, in any tester strain, in the absence or presence of S9 metabolism. It is concluded that chondroitin sulfate sodium does not induce reverse mutation in *S. typhimurium* or *E. coli* in the absence or presence of S9 metabolism (Miraglia et al., 2016; Bisini and Oberto, 2011).

2.1.3.2. Chromosomal Aberration Assay

Chondroitn sulfate sodium was tested for its ability to cause chromosomal damage in Chinese hamster ovary cells (CHO), following *in vitro* treatment in the absence and presence of S9 metabolic activation (Miraglia et al., 2016; Ciliutti and Oberto, 2012). The study was designed to comply with the experimental methods indicated in: Test method B.10 '*in vitro* mammalian chromosome aberration test' described in Council Regulation (EC) No. 440/2008, and OECD Guideline No. 473 (Adopted: 21st July 1997). For this assay, two main experiments were performed. For the first experiment, both in the absence and presence of S9 metabolism,

the chondroitin sulfate sodium was assayed at the dose levels of 5000, 2500, 1250, 625, 313, 156, 78.1 and 39.1 μg/ml. Cyclophosphamide and Mitomycin-C were used as positive controls. Both in the absence and presence of S9, the cells were treated for 3 hours and harvested at 20 hours, corresponding to approximately a 1.5 cell cycle. As negative results were obtained, a second assay in the absence of S9 metabolism was performed with the same dose levels using a continuous treatment until harvest at 20 hours. Following treatment with chondroitin sulfate sodium, no statistically significant increases in the number of cells bearing aberrations, including or excluding gaps, were observed at any dose level or sampling time in the absence or presence of S9 metabolic activation. The results of these experiments suggest that chondroitin sulfate sodium does not induce chromosomal aberrations in Chinese hamster ovary cells after *in vitro* treatment (Miraglia et al., 2016; Ciliutti and Oberto, 2012).

2.1.3.3. Mutation on Mouse Lymphoma Cells

Using a fluctuation method, chondroitin sulfate sodium was examined for mutagenic activity by assaying for the induction of 5-trifluorothymidine resistant mutants in mouse lymphoma L5178Y cells after *in vitro* treatment, in the absence and presence of S9 metabolic activation (Miraglia et al., 2016; Salvador and Oberto, 2012). The mutation assay method used in this study is based on the identification of L5178Y colonies which have become resistant to a toxic thymidine analogue trifluorothymidine (TFT). This analogue can be metabolized by the enzyme thymidine kinase (TK) into nucleosides, which are used in nucleic acid synthesis resulting in the death of TK-competent cells. TK-deficient cells, which are presumed to arise through mutations in the TK gene, are unable to metabolize trifluorothymidine and thus survive and grow in its presence.

For the present experiments, a preliminary cytotoxicity assay was performed. Chondroitin sulfate sodium was assayed at a maximum dose level of 5000 μg/ml both in the absence and presence of S9 metabolism (Miraglia et al., 2016; Salvador and Oberto, 2012). A wide range of lower dose levels were included in the treatment series: 2500, 1250, 625, 313, 156, 78.1, 39.1 and 19.5 μg/ml. No relevant toxicity was noted at any dose level using the short treatment time in the absence or presence of S9 metabolic activation. Mild toxicity was noted at the highest dose level tested (5000 μg/ml), reducing the relative survival to 49% of the concurrent negative control value, while slight toxicity (relative survival: 63%) in the absence of S9 metabolic activation, using the long treatment time. No relevant toxicity was observed over the remaining dose levels tested. Two independent assays for mutation to 5-trifluorothymidine resistance were performed using the following dose levels: 1. Treatment time 3 hours - Dose levels 5000, 2500, 1250, 625 and 313 μg/ml- in the absence and presence of S9; 2a. Treatment time 24 hours- Dose levels 5000, 2500, 1250, 625 and 313 μg/ml- in the absence of S9; and 2b. Treatment time 3 hours- Dose levels 5000, 4000, 3200, 2560 and 2048 μg/ml- in the presence of S9. Methylmethanesulfonate was used as a positive control. No relevant increases in mutant frequencies were observed following treatment with the test item, in the absence or presence of S9 metabolism. The results of these experiments suggest that chondroitin sulfate does not induce mutation at the TK locus of L5178Y mouse lymphoma cells *in vitro* in the absence or presence of S9 metabolic activation (Salvador and Oberto, 2012).

2.1.4. Specific Human Bioavailability Study

In a single centre, single dose, open-label, randomized, two-way, cross-over study, bioavailability of chondroitin sulfate sodium was investigated in 24 healthy human volunteers (male and female; ages 18-65 years (Miraglia et al., 2016; Radicioni and Rusca, 2013). For bioavailability, chondroitin sulfate sodium, the subject of this GRAS determination, was compared with chondroitin sulfate sodium (reference formulation) from Now Foods, USA. The two formulations were orally administered as a single dose of 2400 mg/day under fasting conditions in two consecutive study periods, with a wash-out interval of at least 7 days. The pharmacokinetic profile of disaccharides ΔDi-6S, ΔDi-4S and ΔDi-0S and of total chondroitin sulfate in plasma was analyzed. Additionally, safety and tolerability data of test and reference formulation were collected. Blood samples were collected at pre-dose (0), 1, 2, 3, 4, 5, 6, 7, 8, 12, 16 and 24 hours post-dose for analysis. The following parameters were analyzed: Cmax: Maximum plasma concentration; Tmax: Time to achieve Cmax; AUC0-t: Area under the concentration curve from administration to the last observed concentration time t, calculated with the linear trapezoidal method; and Frel: Relative bioavailability, calculated as ratio AUC0-t (test)/ AUC0-t (reference).

The main pharmacokinetic parameters of plasma chondroitin sulfate measured and calculated before and after single dose of test and reference formulations and after subtraction of baseline are summarized in Table 5. On average, the concentration of all 4 analytes, chondroitin sulfate, and its disaccharides ΔDi-6S, ΔDi-4S and ΔDi-0S, did not vary markedly from the administration to 24 hours post-dose. Secondary peaks were found in the mean curve after single dose of both products. Indeed, the range of tmax was wide after both the test and the reference formulations. The 3 disaccharides ΔDi-6S, ΔDi-4S and ΔDi-0S showed similar concentration vs. time profiles. Nevertheless, chondroitin sulfate, ΔDi-4S and ΔDi-0S showed on average a higher extent of absorption (AUC) after the test product than after the reference. The higher bioavailability was indicated by the mean AUC0-t treatment ratios (Frel). Cmax did not differ to the same extent as AUC between treatments. tmax did not differ significantly between treatments (Miraglia et al., 2016).

Table 5. Comparative Pharmacokinetic Parameters Between Test and Reference Chondroitin Formulations

Treatment	Cmax (µg/mL)	tmax (h)	AUC0-t (µg/mLxh)	Frel (%)
Test Formulation	1.83±1.66	8.00 (3-24)	19.34±17.39	236.67±302.53
Reference Formulation	1.84±2.34	7.00 (1-24)	13.52±10.47	

In summary, the results of this comparative pharmacokinetic study showed that the bioavailability of chondroitin sulfate, ΔDi-4S and ΔDi-0S in extent of absorption (AUC) was significantly higher after the test formulation as compared to the reference formulation. The difference in rate of absorption (Cmax) was less marked, while no significant difference in tmax was noted between the formulations. The test formulation, administered in single dose, is able to yield an increase in plasma chondroitin sulfate and deriving disaccharides whose bioavailability is higher than that of the reference formulation (higher extent of absorption). The safety and tolerability of a single dose of both products was excellent.

The safety assessment during this study showed 5 adverse events that occurred to 4 subjects (16.7%). These adverse events included cases of headache, abdominal discomfort,

diarrhea, Presyncope, and neck pain. All the adverse events occurred after administration of the reference formulation, while no adverse event was reported after administration of the test formulation (chondroitin sulfate sodium). The reported events were not judged to be related to the intake of the chondroitin sulfate on the basis of the physician evaluation. No serious adverse events occurred during the study and no subject discontinued the study due to adverse events or other safety concerns. Similarly, no clinically meaningful effect on vital signs, body weight or laboratory parameters were observed. The results of this study suggest that microbial derived chondroitin sulfate sodium behaves similar to that of animal derived chrondroitin sulfate. These findings also indicate the safety studies of animal derived chrondroitin sulfate are applicable to microbial derived chondroitin sulfate sodium.

2.2. Endotoxin Levels

The presence of bacterial endotoxins in the intermediate and finished product was monitored using the Limulus amebocyte lysate (LAL) test on different fractions throughout the production process. Although some values were variable through the production process, bacterial endotoxins in the finished spray-dried MYTHOCONDRO were well below the expected values established by Gnosis. The results for the chondroitin sodium intermediate and the finished product are summarised in Table 6.

Table 6. Analysis of Endotoxins for 3 Batches of MYTHOCONDRO Through the Manufacturing Process

Fraction	Internal Expected Value (EU/mg)	Analytical Data (EU/mg)		
		Batch no.		
		11/13-24	RB142052-1	RB142052-2
Chondroitin sodium intermediate	$\leq 10^3$	6.7×10^2	5.8×10^2	6.0×10^2
		GN47 13D13DS	GN47 22+23L13DS	GN47 24M13DS
Finished spray-dried MYTHOCONDRO	$\leq 10^2$	2.7	1.0	2.0

EU = endotoxin units.

Gnosis wishes to maintain the internal specification for residual bacterial endotoxins at ≤ 100 Endotoxin units/mg. While regulatory threshold levels for endotoxin contamination in foods currently do not exist, a number of studies regarding the endotoxin levels in some foods consumed by humans were identified. Specifically, typical ranges of endotoxin load have been reported for drinking water (O'Toole et al., 2008), cow's milk (Gehring et al., 2008), and infant formula powder (Townsend et al., 2007) which support the safety of the present endotoxin specification.

In a study to determine the endotoxin levels in water from Australia, drinking water from 12 distribution and 4 reservoir sites were reported to contain endotoxin levels ranging from < 4 to 119 EU/mL and 43 to 83 EU/mL, respectively, using a chromogenic Limulus Amebocyte Lysate (LAL) test (O'Toole et al., 2008). In a comparative study to determine whether differences in endotoxin load between milk samples consumed by non-farming vs. farming families could contribute to protective effects reported for farm milk, milk endotoxin was

measured in a total of 376 milk samples with the kinetic chromogenic LAL test, wherein the mean endotoxin content ranged from 100 to 1,000 EU/mL milk; however, some milk samples consumed by farming families derived from farm milk contained endotoxin levels reaching 10,000,000 EU/mL milk (Gehring et al., 2008). In infant formula, analysis of 75 samples from 7 countries reported endotoxin level ranging between 40 to 5.5 x104 EU/g (Townsend et al., 2007).

Considering that the endotoxin specification and content in MYTHOCONDRO ($\leq$ 100 EU/mg; ranging from 1.0 to 2.7 EU/mg, respectively) are well within the range of endotoxin levels reported in infant formula, endotoxins are not considered to be a safety concern in Gnosis' final product.

2.3. Studies from Literature

2.3.1. Absorption, Distribution, Metabolism and Elimination (ADME)

In a study in rats and dogs, Conte et al. (1995) investigated biochemical and pharmacokinetics aspects of chondroitin sulfate following oral treatment. Chondroitin sulfate was found to be partially absorbed from the gut, both as intact chondroitin sulfate and as lower molecular weight fractions of depolymerised material. In another study with radio-labelled chondroitin sulfate, Palmeri et al. (1991) reported that following ingestion, chondroitin is found in the plasma and in tissues such as the liver, kidneys and cartilage. Partially depolymerised chondroitin sulfate was found to be excreted in the urine (Conte et al., 1991).

In a study in six subjects, Baici et al. (1992) reported that oral consumption of 2 g of chondroitin sulfate (64% chondroitin sulfate A and 32% chondroitin sulfate C) by 18 subjects did not produce measurable changes in the total serum concentration of glycosaminoglycans, suggesting that chondroitin sulfate is not absorbed. The possibility that low molecular weight, desulfated oligomers and monomers may be produced and absorbed could not be ruled out. Baici et al. (1992) described the results of studies conducted by other investigators including Palmieri et al. (1990), Conte et al. (1991), and others. Palmeieri et al (1990) reported that over 70% of the radioactivity administered orally to rats and dogs is absorbed. Conte et al. (1991) reported that the absolute bioavailability of the glycosaminoglycan was 13.2% of the administered dose of chondroitin sulfate. In another study described by Biaci et al. (1992), it was reported that following administration of $^{35}SO_4$-chondroitin sulfate orally to rats only a small portion of the radioactivity was absorbed. The remaining radioactivity was excreted in the feces. When $^{35}SO_4$-chondroitin sulfate was administered orally to rats pretreated with antibiotics to depress the bacterial flora, almost all the radioactivity was found in the feces. It was concluded that sulfatases present in the intestinal bacterial flora were responsible for sulfate splitting from the chondroitin sulfate chain, and that no intact chondroitin sulfate can be absorbed through the intestinal wall.

Palmieri et al. (1990) dosed Wistar rats and dogs orally with 16 mg/kg bw of a mixture of tritiated chondroitin sulfate A and C (MW 14,000 D). More than 70% of the radioactivity was absorbed. Plasma levels showed a rapid increase after oral administration, followed by a large plateau with a maximum at the 14th and 28th h in the rat and in the dog, respectively. Radioactivity was found in tissues, and urine was the main route of excretion. However, the known lability of tritium coupled with the use of a chromatographic gel size that could not

distinguish compounds in the molecular weight range of concern raise doubts that the measured radioactivity can be equated to chondroitin sulfate. However, as summarized in a review article by Bali et al. (2001), other studies support that chondroitin sulfate is absorbed in the intestinal tract. In general, oral administration of 2 or 3 g of chondroitin sulfate to humans produced an increase in the concentration of chondroitin sulfates in the blood after 3-6 hours.

2.3.2. Human Observations

In a number of clinical studies, the effects of chondroitin sulfate alone or in combination with glucosamine on osteoarthritis and certain other health endpoints has been extensively investigated. These studies did not reveal adverse effects. While these studies were not specifically designed to assess toxicity, the absence of adverse effects provides support for the safety. In these studies, use levels of chondroitin sulfate primarily ranged from 800 to 1200 mg/day. Some of these clinical trials were double-blind, placebo-controlled and as these trials are considered the least likely to result in bias, they provide an opportunity to assess the safety and tolerability in a fairly diverse population. Collectively, these studies appear to be of sufficient quality and consistency and demonstrate that chondroitin sulfate did not cause adverse effects and was well tolerated. Some of these studies are described below. Additional details of these studies along with findings from review articles are summarized in Appendix II[5].

In an extensive and critical review of clinical studies of glucosamine and chondroitin sulfate, Hathcock and Shao (2007) assessed safety of chondroitin sulfate through quantitative risk assessment, and identification of a potential safe upper level of intake. For the risk assessment, "Observed Safe Level"[6] (OSL) methodology was utilized. These authors noted that the absence of a systematic pattern of adverse effects in humans in response to orally administered chondroitin sulfate precludes the selection of a NOAEL or lowest observed adverse effect level (LOAEL). Therefore, by definition, the usual approach to risk assessment for identification of a tolerable upper level of intake (UL) could not be used. Instead, the newer method described as the OSL or Highest Observed Intake (HOI) was utilized. The OSL risk assessments indicate that based on the available published human clinical trial data, the evidence for the absence of adverse effects is strong for chondroitin sulfate at supplemental intakes up to 1200 mg/day, and this level was identified as the OSLs for normal healthy adults. These investigators concluded that the complete absence of adverse effects at these levels supports a confident conclusion of the long-term safety.

In the review article by Hathcock and Shao (2007), several clinical trials involving the oral administration of chondroitin sulfate (Rovetta and Monteforte, 1996; Bourgeois et al., 1998; Mazieres et al., 2001; Verbruggen et al., 2002; Rovetta et al., 2004; Uebelhart et al., 2004; Clegg et al., 2006) are summarized. In these studies, the age, health conditions, dosage, duration, and monitoring and evaluation methods have differed greatly. The highest oral chondroitin sulfate dosage administered was noted as 1200 mg/day (Bourgeois et al., 1998; Verbruggen et al., 2002; Clegg et al., 2006). Additional trails have utilized dosages of 1000 mg (Mazieres et al., 2001) and 800 mg (Rovetta and Monteforte, 1996; Rovetta et al., 2004; Uebelhart et al., 2004). The

[5] Available at: http://www.ncbi.nlm.nih.gov/pubmedhealth/PMH0016482/

[6] This methodology is defined as "the highest intake with convincing evidence of safety, even if there are no established adverse effects at any level." "Observed safe level (OSL)" is a model based on observations of exposed humans, in which the highest intake with convincing evidence of safety, even if there are no established adverse effects at any level is taken without applying additional safety factors. A FAO/WHO report gave a similar definition for the highest observed intake.

number of subjects in the trials varied from 12 to 635, and the clinical monitoring capable of detecting adverse effects ranged from sparse to extensive (e.g., self-reports of possible adverse effect to clinical evaluation combined with extensive hematological and clinical chemistry indices). None of these clinical trials found any significant adverse effects (Hathcock and Shao, 2007).

In a study by Verbruggen et al. (2002), 165 subjects received orally chondroitin sulfate at a dose level of 1200 mg/day for 3 years. The monitoring included examination by three physicians. The only adverse effect noted was a single case of gastritis in one chondroitin sulfate-treated subject. As compared with placebo controlled treatment, the subject withdrawals were fewer among those treated with chondroitin sulfate. These results are consistent with the most recent NIH-sponsored glucosamine/ chondroitin arthritis intervention trial (GAIT) with chondroitin sulfate with over 1500 osteoarthritis patients who ingested 1500 mg/day glucosamine hydrochloride, 1200 mg/day chondroitin sulfate, the combination of the two, 200 mg/day of Celebrex™ (pain killer), or placebo for 24 weeks (Clegg et al., 2006). Throughout the study period adverse effects were closely monitored. In this study, a total of 635 patients were exposed to chondroitin sulfate. Results showed no significant difference in the incidence of adverse effects between any of the treatment arms. None of the clinical trials found any adverse effects on clinical chemistry (blood and urine) or hematological measurements resulting from oral chondroitin sulfate.

As effects of chondroitin sulfate has been investigated in several clinical trials, the clinical trial evidence to support joint health has been the subject of several reviews and meta-analyses (Leeb et al., 2000; McAlindon et al., 2000; McAlindon, 2001; Edelist and Evans, 2001; Richy et al., 2003). These publications focused primarily on the effects of oral chondroitin sulfate in limiting the progression of osteoarthritis. However, these are also relevant for the safety of chondroitin sulfate. The meta-analyses support the safety of oral chondroitin sulfate at 1200 mg/day, the highest intake systematically investigated.

Based on the available clinical data, Hathcock and Shao (2007) attempted to identify the OSL of chondroitin sulfate. These investigators noted a nearly complete absence of any adverse effects of chondroitin sulfate within the range of the clinical trials reviewed (800 – 1200 mg/day) suggesting that the highest level, 1200 mg/day, is not a true NOAEL and that any LOAEL is likely to be much higher. The single case of gastritis among hundreds of subjects treated suggests that this one case is not causally related to chondroitin sulfate, or that the individual had a very unusual sensitivity, and should not influence the outcome of the risk assessment. The lack of known adverse effects supports the 1200 mg/day as the OSL for oral consumption of chondroitin sulfate. Evidence from well-designed randomized, controlled human clinical trials indicates that chondroitin sulfate is safe at levels 1200 mg/day.

In another review article, Vangsness et al. (2009) critically evaluated the available double- blind, placebo-controlled, randomized controlled trials using glucosamine and chondroitin sulfate for knee osteoarthritis. These investigators noted that almost every included trial has found the safety of these compounds to be equal to placebo. Some of the relevant human clinical trials with chondroitin alone or in combination with glucosamine are summarized below and additional details, along with findings from review articles provided in Appendix II A and B. These studies did not reveal any consistent adverse effect.

In a recent comparative study of two chondroitin sulfate preparations from different origin (avian and bovine) in symptomatic osteoarthritis of the knee, Faldellon et al. (2013) compared the effect, including safety, of two products Structum® (avian, 1000 mg/day) and Chondrosulf® (bovine, 1200 mg/day) in a randomized, double-blind, double placebo, active-controlled, parallel-group study using a noninferiority design. Symptomatic osteoarthritis of the knee patients, aged 50-80 years, received either Structum® (500 mg BID) or Chondrosulf® (400 mg TID) for 24 weeks. For this study, 837 patients were randomized of which 817 were available for the full analysis dataset and 692 for the per protocol analysis. Safety was assessed by recording adverse events. The results of this study show that both products are equally effective on pain relief and functional improvement in patients with symptomatic knee osteoarthritis over a 6 month period of time. Treatments were well tolerated with a withdrawal (primarily due to gastrointestinal disturbances) of 2.4% (ten subjects) in Structum® group and 4.5% (nineteen subjects) in Chondrosulf® group from the study for safety reasons. Twenty serious adverse events were reported during the trial but none were considered being related to any of the treatments. Regarding the lack of a placebo group, the investigators noted that the safety of chondroitin sulfate is well documented in several randomized, double-blind, controlled trials.

In a long-term study, Kahan et al. (2016) investigated the effects of chondroitin sulfate on the progression of knee osteoarthritis. In this randomized, double-blind, placebo-controlled trial 622 patients with osteoarthritis were randomly assigned to receive either 800 mg chondroitin sulfate (n = 309; Age: 62.9 ± 0.5; Female: 70%) or placebo (n = 313; Age: 61.8 ± 0.5; Female: 67%) once daily for 2 years. The intent-to-treat analysis demonstrated a significant reduction in minimum joint space width loss in the chondroitin sulfate group as compared with the placebo group. The percentage of patients with radiographic progression was significantly reduced. Pain improved significantly faster in the treatment group than in the placebo group. There were no differences in safety between groups. Tolerability was assessed as very good or good by the majority of the patients in the chondroitin sulfate and placebo groups (94% and 93% of subjects, respectively, at 24 months). There were no significant differences between the groups in the frequency of adverse events during the clinical trial. Most of the adverse events were transient and mild. Gastrointestinal side effects were the most frequently reported but were similar in both groups. Adverse events were the cause of withdrawal in 16 patients (5%) in the treatment group and 17 patients (5%) in the placebo group. "Routine laboratory tests" did not show any significant abnormalities in the 2 groups.

In another randomized placebo-controlled trial, Mazierer et al. (2007) evaluated the efficacy and tolerability of chondroitin sulfate in patients with knee osteoarthritis. In this randomized placebo-controlled trial, chondroitin sulfate (1 g/day) was administered for 24 weeks. Safety was assessed by recording adverse events spontaneously reported by the patients on request at each visit. Adverse events were analyzed with regard to their number, seriousness, intensity and causal relationship with treatment and outcome. In this study, 307 patients were included; 28 patients discontinued the study because of lack of efficacy or adverse events. No significant difference was observed between treatment groups for changes in biomarkers over 24 weeks. Tolerance was considered to be satisfactory. The safety assessment included 309 patients (received the treatment of at least one dose, were not assessable for efficacy but were included in the safety analysis). Seventy five patients (49%) in the chondroitin sulfate group and 76 (49%) in the placebo group reported at least one treatment - emergent adverse event. The treatment was

prematurely discontinued because of adverse events for 13 patients in the chondroitin sulfate group and 8 patients in the placebo group. A total of 18 adverse events in 14 patients in the chondroitin sulfate group and 20 in 16 patients in the placebo group were reported. In both groups, the majority (50%) of these adverse events were related to gastrointestinal troubles including dyspepsia, nausea, vomiting, abdominal pain and diarrhea.

In a double-blind, placebo-controlled, randomized clinical trial lasted 12 months, Meisser et al. (2007) determined whether using 1500/1200 mg of glucosamine hydrochloride and chondroitin sulfate (GH/CS) is effective, both separately and combined with exercise, compared to a placebo plus exercise program in improving physical function, pain, strength, balance, and mobility in older adults with knee osteoarthritis. In this study, 89 older adults (age>/=50 years) with knee osteoarthritis randomized to either GH/CS or placebo group. Of the 89 randomized participants, 72 (81%) completed the study. The GH/CS group was not superior to the placebo group in function, pain, or mobility after both phases of the intervention (pill only and pill plus exercise). The only reported adverse event was hair loss in one participant in the GH/CS group. The results of this study suggest that chondroitin sulfate is well tolerated.

Michel et al. (2005) investigated effects of chondroitin sulfate in inhibiting cartilage loss in knee osteoarthritis. In this randomized, double-blind, placebo-controlled trial, 300 patients with knee osteoarthritis were randomly assigned to receive either 800 mg chondroitin sulfate or placebo once daily for 2 years. Of 341 patients screened, 300 entered the study and were included in the intent-to-treat analysis. The 150 patients receiving placebo had progressive joint space narrowing. In contrast, there was no change in mean joint space width for the 150 patients receiving chondroitin sulfate. Chondroitin sulfate was well tolerated, with no significant differences in rates of adverse events between the two groups. There was no statistically significant difference in the frequency of any event between the two groups. Adverse events led to the withdrawal of 9 patients from each study group. Only 2 events were judged to be possibly related to CS (abdominal pain and nausea in 1 patient each). All other adverse events were judged to be unrelated to the study drug and were most probably caused by concomitant disorders.

Moller et al. (2010) assesses the efficacy and safety of chondroitin sulfate on symptomatic knee osteoarthritis. In this randomized, double-blind, placebo-controlled clinical trial 129 patients with symptomatic knee osteoarthritis and concomitant psoriasis were randomized into two groups receiving 800 mg daily of chondroitin sulfate or placebo for 3 months. Tolerability and safety parameters investigated were the incidence and severity of adverse events reported throughout the study, changes in laboratory tests including complete blood cell count, biochemical profile and urinalysis and patient's and investigator's assessment of tolerability. After 3 months of treatment, chondroitin sulfate was more effective than placebo, relieving pain, decreasing the Lequesne index and reducing the number of patients using acetaminophen as rescue medication. Adverse events were infrequent and evenly distributed among groups. This study confirms the efficacy and safety of chondroitin sulfate as a symptomatic slow-acting drug in patients with knee osteoarthritis and shows that chondroitin sulfate improves plantar psoriasis. At the final visit, tolerability of treatment with both chondroitin sulfate and placebo was considered 'very good' by near 90% of physicians and 80% of patients. No clinically significant laboratory abnormalities or pattern changes in vital signs were observed during chondroitin sulfate treatment. Treatment compliance was above 90% in both groups at each time point, with a mean compliance rate of 97%.

In a double-blind, placebo-controlled study, Sawizke et al. (2010) evaluated the efficacy and safety of glucosamine and chondroitin sulfate, alone or in combination, as well as celecoxib and placebo on painful knee osteoarthritis over 2 years. In this study, 662 patients with knee osteoarthritis were enrolled. This subset continued to receive their randomized treatment: glucosamine 500 mg three times daily, chondroitin sulfate 400 mg three times daily, the combination of glucosamine and chondroitin sulfate, celecoxib 200 mg daily, or placebo over 24 months. Information on adverse events was collected per patient report at each visit in response to an open-ended query. Monitoring of vital signs, blood counts, serum glucose, aminotransferases, creatinine, partial thromboplastin time and urinalysis was also performed. Compared with placebo, the odds of achieving a 20% reduction in WOMAC pain were celecoxib: 1.21, glucosamine: 1.16, combination glucosamine/CS: 0.83 and chondroitin sulfate alone: 0.69, and were not statistically significant. Adverse reactions were similar among treatment groups and serious adverse events were rare for all treatments.

2.3.3. Animal Toxicity Studies

In two acute and subchronic oral toxicity studies in rats, Schauss et al. (2007) investigated safety of hydrolyzed chicken sternal cartilage containing collagen type II, chondroitin sulfate, and hyaluronic acid. The product used in the study contains a patented composition of naturally occurring hydrolyzed collagen type II (60%), chondroitin sulfate (20%), and hyaluronic acid (10%). In the acute oral toxicity study, five males and five females of Sprague-Dawley rats were administered a single dose of 5000 mg of the hydrolyzed sternal preparation/kg body weight and the animals were observed for 14 days. The dose of chondroitin sulfate in this study is 1000 mg/kg bw. All animals survived and exhibited normal body weight gain throughout the study. Macroscopic necropsy examination conducted on day 15 revealed no gross pathological lesions in any of the animals.

In the subchronic study, Sprague-Dawley rats were divided into four groups (10/sex/group) and were administered daily, either 0, 30, 300 or 1000 mg of the hydrolyzed sternal preparation/kg bw/day for over 90 days (Schauss et al., 2007). The equivalent dose of chondroitin sulfate is 0, 6, 60 and 200 mg/kg bw/day, respectively. All animals survived and showed no significant changes in their body weights and histopathology. Although some differences were observed between the treated and control animals in several parameters, they were generally not dose-related or considered to be of toxicological significance. The results of this study indicated that the hydrolyzed chicken sternal cartilage collagen, containing chondroitin sulfate, was well tolerated.

2.3.4. Other Genotoxicity Studies

Sodium chondroitin sulfate was not mutagenic in *S. typhimurium* strains TA92, TA94, TA98, TA100, TA1535 and TA1537 at concentrations up to 5 mg/plate. Mutagenicity was not enhanced by rat liver S-9. It did not induce chromosomal aberrations (CA) in a Chinese hamster fibroblast cell line at concentrations up to 3 mg/ml (Ishidate et al., 1984).

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 the public domain and studies that are published in scientific journals. Several specifically designed studies conducted with the subject

of this GRAS are reviewed and summarized in section 2.2 are published in the peer reviewed journal (Miraglia et al., 2016). These studies conducted with chondroitin sulfate sodium derived from a microorganism source include subchrnic toxicity study in rats, *in vitro* genotoxicity studies and human bioavailability. Additionally, several human clinical studies reviewed for this safety assessment have been published in the scientific literature as reported in Section 2.2. In a comprehensive review of the safety of chondroitin sulfate, Hathkock and Shao (2007) critically reviewed and summarized the safety data from the published literature. Additionally, the Panel recognizes that the safety of chondroitin sulfate is supported by its extensive use as a dietary supplement. Also, many scientific studies have been conducted and published.

4. SUMMARY

Gnosis S.p.A. has developed a process to manufacture standardized chondroitin sulfate sodium from non-animal sources. The chondroitin sulfate manufactured by Gnosis S.p.A. has been shown to be identical to the chondroitin sulfate from animal sources. Chondroitin sulfate sodium is a white to off-white amorphous powder freely soluble in water. The product contains 95 to 105% chondroitin sulfate and meets or exceeds the USP specifications. The production of chondroitin sulfate sodium is achieved by fermentation of a particular strain of *E. coli* [O5:K4:H4 strain U1-41 (ATCC23502)] to produce the K4 polysaccharide that is further chemically transformed to chondroitin sulfate sodium. It is manufactured according cGMP. The *E. coli* strain is fully characterized and is non-pathogenic. Additional steps used in manufacturing, such as downstream processes, are likely to minimize the presence of any endotoxin. At each step of these processes the endotoxins are quantified to make sure that the final product is safe. The production process is fully characterized and standardized.

Gnosis S. p. A. intends to use chondroitin sulfate sodium at levels ranging from 50 to 200 mg/serving (reference amounts customarily consumed, 21 CFR 101.12) in Beverage and beverage bases, Milk and milk products, and Chewing gum. The intended use of chondroitin sulfate sodium in the above mentioned food categories will result in a mean and 90th percentile estimated daily intake of 595 and 1187 mg/person/day, respectively. For safety assessment purposes, chondroitin sulfate sodium intake of 1200 mg/person/day is considered.

Chondroitin sulfate is an amino sugar polymer, made up of glucuronic acid and galactosamine. It is an important structural component of the human body and is found in extracellular matrix. Thus it is an endogenous component of the body. There are no major dietary sources of chondroitin. Given its potential to help support cartilage and joint function, chondroitin sulfate has been extensively used as a dietary supplement. The commonly used dose of chondroitin sulfate as a dietary supplement is 1200 mg/day.

In multiple well designed human clinical trials, the effects, including safety, of chondroitin sulfate has been extensively investigated. Additionally, in several animal and *in vitro* pre-clinical experimental studies there has been considerable effort to understand the mode of action of chondroitin sulfate. Given its endogenous presence, there have been limited, well designed, safety studies investigating its toxic potentials. The available published studies support the safety-in-use of chondroitin sulfate. In human clinical studies, use levels of chondroitin sulfate ranged from 800 to 1200 mg/day. In the published literature, several double-blind, placebo-controlled trials were found. Such trials are considered the least likely to result in bias and they provide an opportunity to assess the safety and tolerability in a diverse population. Collectively, these studies appear to be of sufficient quality and consistency and demonstrate that

chondroitin sulfate did not cause adverse effects and was well tolerated. In a critical risk assessment based on published clinical studies, Hathcock and Shao (2007) determined that use of chondroitin sulfate at levels of 1200 mg/day is safe.

As the source of chondroitin sulfate sodium, the subject of this GRAS assessment, is different from the commonly used animal source, Gnosis has undertaken toxicity studies to support the safety of chondroitin sulfate from this different source. In a series of specifically designed studies that followed standard regulatory guidelines, the potential subchronic toxicity and mutagenicity of chondroitin sulfate sodium was investigated (Miraglia et al., 2016). In the subchronic toxicity study, administration (gavage) of chondroitin sulfate sodium at dose levels of 0, 250, 500 and 1000 mg/kg bw/day for 90 consecutive days did not result in any treatment-related clinical signs of toxicity, mortality or changes in body weights, body weight gain or feed consumption. No toxicologically significant treatment-related changes in hematological, clinical chemistry, urine analysis parameters, and organ weights were noted. No treatment-related macroscopic and microscopic abnormalities were noted at the end of treatment period. The results of mutagenicity studies including the Ames assay, *in vitro* chromosomal aberration and mutation in mouse lymphoma cell assay did not reveal any genotoxicity of chondroitin sulfate sodium. Based on the subchronic study, the no-observed-adverse-effect level (NOAEL) for chondroitin sulfate sodium was determined to be 1000 mg/kg bw/day, the highest dose tested. Additionally, the results of a human bioavailability study suggest that chondroitin sulfate sodium, the subject of this GRAS assessment, is similar to chondroitin sulfate from animal sources. These studies support the safety of chondroitin sulfate sodium.

There is sufficient qualitative and quantitative scientific evidence, including human and animal data, to determine safety-in-use for chondroitin sulfate sodium. The present safety determination of chondroitin sulfate sodium is based on the totality of available evidence, particularly a variety of human clinical trials, endogenous presence in the human body, as well as specific subchronic toxicity, *in vitro* mutagenicity and human bioavailability studies further corroborating the safety. In human clinical trials no adverse effects of chondroitin sulfate sodium were noted at doses of up to 1200 mg/day. Results from a subchronic study in rats show that chondroitin sulfate sodium salt had no adverse effects at dose levels up to 1000 mg/kg bw/day which equates to 60000 mg/day for a 60 kg person. The estimated maximum daily intake of 1200 mg chondroitin sulfate sodium salt/person from its intended uses is approximately 50-fold lower than the demonstrated safe levels of intake in animal studies. The estimated daily intake, if ingested daily over a lifetime, is considered safe.

In summary, on the basis of scientific procedures[7], the consumption of chondroitin sulfate sodium, derived from non-animal source, as an added food ingredient is considered safe at the 90th percentile estimated daily intake of 1200 mg/person/day (20 mg/kg bw/day). The intended uses are compatible with current regulations, *i.e.,* chondroitin sulfate sodium will be used in Beverage and beverage bases, Milk and milk products, and Chewing gum at use levels ranging from 50 to 200 mg chondroitin sulfate sodium *per* serving when not otherwise precluded by a Standard of Identity, and it is produced according to current good manufacturing practices (cGMP).

[7] 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.

5. 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 chondroitin sulfate sodium derived from non-animal sources, meeting the specifications cited herein, and when used as a food ingredient in selected food products (Beverage and beverage bases, Milk and milk products, and Chewing gum) at use levels ranging from 50 to 200 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 estimated maximum daily 90th percentile consumption of 1200 mg/day is Generally Recognized As Safe (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 chondroitin sulfate sodium salt, when used as described, is safe and GRAS based on scientific procedures.

Signatures

(b) (6)

Rober L. Martin, Ph.D. — August 8, 2016 — Date

(b) (6)

John A. Thomas, Ph.D., F.A.T.S., D.A.T.S. — Aug. 11, 2016 — Date

(b) (6)

Madhusudan G. Soni, Ph.D., F.A.C.N., F.A.T.S. — Aug. 13, 2016 — Date

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7. APPENDIX I

Analytical data from three manufacturing lots of chondroitin sulfate sodium (information provided by Gnosis S.p.A.)

Tests	Limits	GN47 150710	GN47 C3S6001	GN47 C3S6003
Appearance	White to off-white powder	Complies	Complies	Complies
Identification: - IR - Sodium	 Positive Positive	 Positive Positive	 Positive Positive	 Positive Positive
Clarity and Color of solution (5% aqueous solution, absorbance at 420 nm)	≤ 0.35	0.27	0.21	0.23
Specific Optical Rotation (3% aqueous solution)	-7.0° to -19.0° d.b.	-7.3	-7.1	-9.0
pH (1% aqueous solution)	5.5 to 7.5	6.2	6.7	6.4
Loss on drying (105 °C for 4 hours)	≤ 10.0 %	4.6	5.1	5.3
Residue on ignition	20% to 30 % d.b.	27.5	26.3	26.8
Chloride	≤ 0.50 %	Complies	Complies	Complies
Sulfate	≤ 0.24 %	Complies	Complies	Complies
Heavy metals	≤ 0.002 %	< 0.002	< 0.002	< 0.002
Lead	≤ 0.5 ppm	< 0.05	0.3	0.09
Cadmium	≤ 0.5 ppm	< 0.05	< 0.05	< 0.05
Mercury	≤ 0.1 ppm	< 0.05	0.07	< 0.05
Arsenic	≤ 1.5 ppm	0.6	0.2	< 0.05
Electrophoretic purity: - any individual impurity	 ≤ 2 %	 Complies	 Complies	 Complies
Microbial enumeration: - Total bacterial count - Total combined yeasts/molds - Salmonella sp. - Escherichia coli	 $\leq 10^3$ CFU/g $\leq 10^2$ CFU/g Absent / 10 g Absent / 10 g	 8.5×10^2 0 Absent Absent	 2.5 x 10 1.25 x 10 Absent Absent	 2.4×10^2 100 Absent Absent
Limit of protein	≤ 0.5 % d.b.	Complies	Complies	Complies
Content of chondroitin sulfate sodium	95% to 105% d.b.	100.5	100	99.1

8. APPENDIX II

Appendix II A. Summary of Clinical Trials with Chondroitin Sulfate

Author	Eligibility criteria; Demographics (Age, gender, race)	Study type; Interventions (dose, duration)	Run-in/Washout period; Allowed other medications/interventions	Other population characteristics (Diagnosis, etc)	Number screened/Eligible/Enrolled	Number withdrawn/Lost to follow up/analyzed
Kahan, 2009	Male and female outpatients 45–80 years, primary knee OA of the medial tibiofemoral compartment diagnosed according to ACR. Chondroitin Sulfate: Age: 62.9 ± 0.5; Female: 70%; Race: NR Placebo: Age: 61.8 ± 0.5; Female: 67%; Race: NR	RCT A. Chondroitin sulfates 4&6 800 mg sachet daily, every evening with glass of water B. Placebo sachet daily, every evening with glass of water 2 years	24 hours for acetaminophen, 5 days for NSAIDs prior to symptom assessments Acetaminophen in 500 mg tablets (max dosage 4 gm/day) NSAIDs in cases of acute pain	Duration of knee OA: Left knee: 6.1 ± 0.3 vs. 6.5 ± 0.4; Right knee: 6.6 ± 0.4 vs. 6.3 ± 0.4 KL grade 1: 17.4% vs. 19.7%; KL grade 2: 26.2% vs. 21.6%; KL grade 3: 56.4 vs. 58.7% Minimum JSW, mm: 3.73 ± 0.08 vs. 3.81 ± 0.07 Pain score, 100 mm VAS: 57.2 ± 0.9	1052/NR/622	103 vs. 96 withdrawals/18 vs. 18 lost to followup/ITT analysis 622

				vs. 57.3 ± 1.0 WOMAC score, normalized 100mm scales: Total: 40.5 ±1.2 vs. 41.6 ± 1.2; Pain: 40.0±1.2 vs. 40.5±1.2; Function: 39.2±1.3 vs. 39.0±1.2; Stiffness: 42.3±1.5 vs. 43.5±1.5		
Mazieres, 2007	Male and female outpatients 50–80 years with medial OA, defined according to ACR criteria. Patients with symptomatic knee OA that had lasted for >6 months, with pain during daily activity ≥ 40 mm on a 0–100 mm visual analogue scale, a Lequesne's Index Score of between 6 and 12, and Kellgren/Lawrence grade 2 or 3 on an anterior-posterior view in an extended standing position taken within the previous 6 months. Exclusions: secondary knee OA, isolated patella-femoral OA and those requiring	RCT A. Chondroitin sulfate 500 mg, twice daily by oral route B. Placebo twice daily by oral route 24 weeks	NR; Start with paracetamol (up to 4 gm/day). NSAIDS allowed if paracetamol was not effective. NSAIDs not allowed 2 days and paracetamol not allowed 12 hours prior to evaluation visits.	Duration of disease: 6.2 (6.8) vs. 6.6 (7.6) VAS pain during activity: 62 (13) vs. 61 (12) VAS pain at rest: 40 (20) vs. 40 (22) Lequesne's Index: 9.5 (2) vs. 9.4 (2) KL stages	322/NR/307 (153 CS, 154 Placebo)	14 vs. 14 withdrawals during treatment period, 12 vs. 11 withdrawals during washout period. 307 ITT population

	knee surgery in the coming year, know hypersensitivities to CS or paracetamol, NSAID use for >50% of the time during the previous 2 months, NSAID use within 48 hours before inclusion or SYSADOA, steroid by any route, intra-articular hyaluronic acid or arthroscopic debridement within 6 months before inclusion CS: Age: 66 (8.8); Female 71%; Race: NR Placebo: Age: 66 (7.7); Female: 69%; Race: NR			2–3: 69% vs. 59%		
Messier, 2007	Males and females ≥ 50 years with radiographic evidence of mild to moderate knee OA, Kellgren-Lawrence grade II–III; radiographic classification criteria or confirmation of mild to moderate radiographic evidence of knee OA from a personal physician; not participating in any other intervention study Mean Age Overall NR GH/CS: 70.0 ± 1.28 Placebo: 74.1 ± 1.32, p0.03 Female: GH/CS: 75.6% Placebo: 65.9%	RCT with run-in/washout period, Phase 1 treatment. Phase 2 treatment plus exercise. A. Glucosamine hydrochloride 1500 mg/day and chondroitiin sulfate 1200 mg/day taken either once or three times per day B. Placebo taken either once or three times per day 1 year treatment period	2-week discontinuation of all over-the-counter or prescription medications. Rescue medication with acetaminophen up to 4g per day and any other necessary medications unrelated to OA were permitted. Rescue medication of acetaminophen up to 4g/day	--	865 screened/435 not interested/341 ineligible 89 randomized	17 withdrawn/89 analyzed using ITT last observation carried forward

	Race, GH/CS vs. Placebo: Caucasian: 68.9% vs. 77.3% African American: 20% vs. 11.4% Asian/Pacific Islander: 6.7% vs. 2.3%; Native American: 4.4% vs. 6.8%					
Michel, 2005	Male and female patients 40–85 years with clinically symptomatic knee OA (knee pain while standing, walking, and/or on motion for at least 25 of the 30 days prior to study entry) diagnosed according to the ACR clinical and radiographic criteria for OA of the knee. Exclusion criteria: Kellgren/Lawrence grade 4, any causes of secondary OA, traumatic knee lesions, severe comorbidity (severe renal, heart, lung, or neurologic disease), previous joint surgery, intraarticular medications, including corticosteroids into the last month, and the foreseeable prospect of major surgery during the 2- year study period. Chondroitin Group: Mean age: 62.5 ± 9.1 Female: 51% Race: NR	RCT A. Chondroitin sulfate 4&6 800 mg tablet daily B. Placebo 2 years	3 month washout required for potentially longer acting substances such as Chondroitin Sulfate and Glucosamine Acetaminophen in 500-mg tablets at a maximum dose of 3 gm/day. Secondary rescue with NSAIDs were allowed up to a maximum 5 consecutive days if the primary rescue analgesia with acetaminophen was insufficient. Physical therapy was limited to application of warmth and strengthening exercises No other interventions allowed	ITT Group: Minimum JSW, mm: 2.41 ± 0.14 vs. 2.35 ± 0.14 Mean JSW, mm: 3.04 ± 0.14 vs. 3.00 ± 0.15 WOMAC score, range 0–10: Total: 2.3 ± 1.6 vs. 2.6 ± 1.7 Pain: 2.5 ± 1.6 vs. 2.7 ± 1.8 Function: 2.1 ± 1.6 vs. 2.5 ±1.8 Stiffness: 3.0 ± 2.3 vs. 3.5 ± 2.5	341/300/300	40 vs. 41 withdrawals during treatment 300 ITT analysis

	Placebo Group: Mean age: 63.1 ± 10.7 Female: 52% Race: NR					
Moller, 2010	Males and females ≥ 40 years of age, with OA of the knee as defined by criteria of the American College of Rheumatology, with pain in the affected knee scoring ≥ 30 on Huskisson's VAS, and a confirmatory knee X-ray diagnosis, Kellgren-Lawrence grades I–III, associated to cutaneous plaque-type psoriasis with a Psoriasis Area and Severity Index score of >5. Exclusion criteria were Kellgren-Lawrence grade IV, VAS ≥ 30 due to pain of any cause in other sites, non- plaque type psoriasis forms, concurrent arthritic conditions that could confound evaluation of the index joint, presence of any clinically significant cutaneous disease that may interfere with the assessment of lesions during the study and presence of any medical condition judged by the investigator to preclude the patient's inclusion in the study. Age (mean ± SD): Overall: 59.8±10.8 CS: 58.6±11.4	Randomized controlled trial A. Chondroitin sulfate 800 mg daily B. Placebo 3 months duration	Washout periods: 6 months for intra-articular hyaluronic acid; 3 months for intra-articular corticosteroids and SYSADOAs; 1 month for oral corticosteroids, 1 week for oral NSAIDs; 1 month for high-potency topical corticosteroids, psoralen photochemotherapy and systemic treatment for psoriasis; 2 weeks for ultraviolet and topical treatment for psoriasis. Acetaminophen allowed for osteoarthritic symptoms. Syndet soap and moisturizing body milk for daily skin care were provided by the study.	Kellgren-Lawrence grade I: A: 6.7%, B: 7.1% Kellgren-Lawrence grade II: A: 78.3%, B: 75.0% Kellgren-Lawrence grade III: A: 15.0%, B: 21.4% Pain intensity, VAS score, mm, mean ± SD: A: 58.1 ± 16.7, B: 56.8 ± 16.0	181 screened, 129 randomized	A. 4- No data for at least one follow-up 2-Non compliance 60 – ITT population analyzed 58 – per protocol population analyzed B. 5 – no data for at least one follow-up 3 – No fulfillment of inclusion criteria 3 – Non-compliance 2 – use of medication other than study drug or paracetomo 56 – ITT population analyzed 51 – per protocol

	Placebo: 61.0±10.4 Gender (% female): Overall: 52.6% CS: 48.3% Placebo: 57.1% Race: NR					population analyzed
Sawitzke, 2008	Males and females ≥ 40 years of age, had knee pain for at least 6 months occurring on the majority of days in the month preceding their enrollment in GAIT, and had Kellgren/Lawrence gade 2 or 3 knee OA determined on a screening AP radiograph of the knee in a weight bearing position. Exclusion: Minimum baseline medial tibiofemoral JSW of <2mm, predominant lateral compartment OA on any film of the MTP joints, history of significant trauma or surgery to the knee Age (mean ± SD years): Glucosamine: 56.7± 10.4 CS: 56.4± 9.2 Glucosamine + CS: 56.5± 9.9 Celecoxib: 58.3± 10.7 Placebo: 56.6± 8.4 Female (%): Glucosamine: 61.0 CS: 71.8 Glucosamine + CS: 55.9	Prospective observational study of GAIT enrollees; ancillary study to assess structural changes in knee OA A. Glucosamine 500 mg 3 times daily B. Chondroitin sulfate (400 mg 3 times daily) C. Combination of glucosamine and chondroitin D. Celecoxib 200 mg daily E. Placebo 24 months	NR-check other GAIT pubs	Kellgren/Lawrence Grade 2, %: 80.5 vs. 81.0 vs. 69.2 vs. 72.6 vs. 80.5 Kellgren/Lawrence Grade 3, %: 19.5 vs. 19.0 vs. 30.9 vs. 27.4 vs. 19.5	662 GAIT participants consented to this study	A. (177 initial): 33/NR/77 B. (123 initial): 30/NR/71 C. (128 initial): 40/NR/59 D. (143 initial): 32/NR/80 E. (134 initial): 36/NR/70

	Celecoxib: 63.8 Placebo: 64.3 Race: NR					
Sawitzke, 2010	Males and females ≥ 40 years of age, had knee pain for at least 6 months occuring on the majority of days in the month preceding their enrollment in GAIT, and had Kellgren/Lawrence gade 2 or 3 knee OA determined on a screening AP radiograph of the knee in a weight bearing position. Exclusion: Minimum baseline medial tibifemoral JSW of <2mm, predominant lateral compartment OA on any film of the MTP joints, history of signicant trauma or surgery to the knee Age (mean ± SD years): Glucosamine: 56.7± 10.5 CS: 56.3± 8.8 Glucosamine + CS: 56.7± 10.7 Celecoxib: 57.6± 10.6 Placebo:56.9± 9.8 Female (%): Glucosamine: 68.7 CS: 73.0 Glucosamine + CS: 65.1 Celecoxib: 65.5 Placebo: 65.7 Race: NR	Prospective observational study of GAIT enrollees; ancillary study to assess structural changes in knee OA A. Glucosamine 500 mg 3 times daily B. Chondroitin sulfate (400 mg 3 times daily) C. Combination of glucosamine and chondroitin D. Celecoxib 200 mg daily E. Placebo 24 months	NR	Kellgren/Lawrence Grade 2, %: 59.7 vs. 66.7 vs. 51.9 vs. 62.0 vs. 61.1, p=0.19 Duration of OA symptoms, mean years (SD): 9.7 (10.3) vs. 9.0 (9.0) vs. 10.0 (9.4) vs. 10.2 (9.2) vs. 10.1 (9.4)	662 GAIT participants consented to this study	See Sawitzke, 2008

ACR = American College of Rheumatology; AE = adverse event; BMI = body mass index; CI= confidence interval; CS = chondroitin sulfate; EQ-VAS = EuroQol visual analogue scale; GAIT = Glucosamine/chondroitin Arthritis Intervention Trial; GH = glucosamine hydrochloride; GS = glucosamine sulfate; GUIDE = Glucosamine Unum-in-Die (Once a Day) Efficacy trial; ITT = intention to treat; JSN = joint space narrowing; JSW = joint space width; KL = Kellgren-Lawrence scale; LBP = low back pain; MCII = minimal clinically important improvement; MRI = magnetic resonance imaging; MTP = metatarsophalangeal; NR = not reported; NRS = nonrandomized study; NS = not significant; NSAID = nonsteroidal anti-inflammatory drug; OA = osteoarthritis; OARSI = Osteoarthritis Research Society International; OMERACT-OARSI = Outcomes Measures in Arthritis Clinical Trials-Osteoarthritis Research Society International; PASS = Patient Acceptable Symptom Scale; QOL = quality of life; RCT = randomized controlled trial; RMDQ = Roland Morris Disability Questionnaire; SD = standard deviation; SE = standard error; SYSADOA = Symptomatic Slow Acting Drugs in Osteoarthritis; UTI = urinary tract infection; VAS = visual analogue scale; WOMAC = Western Ontario and McMaster Universities; Adapted from PubMed Health: Analgesics for Osteoarthritis: An Update of the 2006 Comparative Effectiveness Review. Available at: http://www.ncbi.nlm.nih.gov/pubmedhealth/PMH0016482/

Appendix II B. Summary of Reviews

Author, Year	Aims	Time period covered	Eligibility criteria	Number of patients	Characteristics of identified articles: study designs	Characteristics of identified articles: populations	Characteristics of identified articles: interventions	Main results	Subgroups
Bjordal, 2007	To determine the short-term pain-relieving effects of seven pharmacological agents for OA knee pain	MEDLINE, Embase, PedRo, Cochrane Controlled Trials Register 1996 through November 2005	Diagnosis: Knee OA verified by clinical exam and/or by x ray. If less than 4 trials available for an intervention, trials also including hip OA were considered, if more than 2/3 of their patients had knee OA; Symptom duration: 3 months; Trial designs: Blinded, placebo-controlled parallel groups RCTs; Outcome measures: Pain intensity within 4 weeks of treatment start on WOMAC or on a 100mm VAS for global or walking pain. Pain intensity at 8–12 weeks follow-up; Intervention	14,060 patients for all included drugs. 9964 patients received Oral NSAIDs including coxibs, 749 received topical NSAIDs, 401 received glucosamine sulfate, 362 received chondroitin sulfate	64 RCTs total. 25 RCTs of oral NSAIDs (including coxibs), 9 topical NSAIDs, 7 glucosamine sulfate, 6 chondroitin sulfate	Mean Age: Oral NSAIDs: 62.6 years Topical NSAIDs: 64.2 years Glucosamine sulfate: 58.6 years Chondroitin sulfate: 63.0 years Mean baseline pain on 100mm VAS: Oral NSAIDs: 64.3 Topical NSAIDs: 54.7 Glucosamine sulfate: 57.8 Chondroitin sulfate: 50.7	Trials of included Oral NSAIDs: 6 celecoxib studies; 2 naproxen studies; 2 diclofenac studies; 3 etodolac studies; 1 diflunisal study; 1 meloxicam study; 2 nabumetone studies; 1 oxaprozin study Trials of included Topical NSAIDs: 7 diclofenac, 2 eltenac, 1 ibuprofen Trials of glucosamine: 7 Trials of chondroitin: 6	Best mean difference of change over placebo (100mm VAS): Glucosamine: 4.7 (95% CI 0.3 to 9.1) Chondroitin: 3.7 (95% CI 0.3 to 7.0) Glucosamine and chondroitin did not have effect size or 95% CI exceeding the mean threshold for minimal clinical important improvement, slight improvement, or minimal perceptible improvement	NR

			groups: Identical placebo drug and adequate daily defined drug dosage equal to or exceeding set dosages per drug: paracetamol 4g, diclofenac 100mg, etodolac 400mg, ibuprofen 2400 mg, nabumetone 1500mg, naproxen 1000mg, oxaprozin 1200mg, tiaprofenic acid 600mg, valdecoxib 10mg, celecoxib 200mg, meloxicam 7.5mg, etoricoxib 30mg, lumiracoxib 200mg, rofecoxib 12.5mg, topical diclofenac, piroxicam or meloxicam 1%, ibuprofen gel 3%, triamcinolone 20mg, methylprenisolone 40mg, cortivazol 3.75mg, glucosamine sulfate 1500mg, chondroitin sulfate 800mg,							

			codeine 50mg, oxymorphone 20mg, oxycodone 20mg, morphine sulfate 30mg, tramadol 100mg						
Hochberg, 2010	To update the 2008 systematic review and meta-analysis with results of an updated meta-analysis that includes data from two recently published studies and limits the pooling to studies of 2-year duration	1996–October 2007	RCTs of 2-year duration that compared orally administered chondroitin sulfate to placebo and reported structural outcomes in the form of change in minimum joint space. No language restriction was applied.	1179	Randomized controlled trials	Michel et al., 2005: mean age 63, 52% women Sawitzke et al., 2008: mean age 57, 68% women Kahan et al., 2009: mean age 62, 68% women	Michel et al., 2005: 800 mg chondroitin sulfate once daily, 24 month duration Sawitzke et al., 2008: 400 mg chondroitin sulfate three times daily, 24 month duration Kahan et al., 2009: 800 mg chondroitin sulfate once daily, 24 month duration	Joint space narrowing (mm ± SD): Michel et al., 2005: CS: −0.045 ± 0.48, PBO: 0.07 ± 0.56; Mean difference (mm (95% CI)): 0.12 (0.00 to 0.23); Effect size (95 % CI): 0.22 (0.01 to 0.45) Sawitzke et al., 2008: CS: 0.107 ± 0.68, PBO: 0.166 ± 0.68; Mean difference (mm (95% CI)): 0.06 (−0.17 to 0.28); Effect size (95% CI): 0.09 (−0.24 to 0.42)	NR

								Kahan et al., 2009: CS: 0.07 ± 0.03, PBO: 0.31 ± 0.04; Mean difference (mm (95% CI)): 0.14 (0.06 to 0.21); Effect size (95% CI): 0.26 (0.11 to 0.42) Pooled analysis: Mean difference (mm (95% CI)): 0.13 (0.06 to 0.19); Effect size (95% CI): 0.23 (0.11 to 0.35)	
Lee, 2010	To assess the structural efficacies of daily glucosamine sulfate and chondroitin sulfate in patients with knee OA	Through July 2008	English language RCTs that compared glucosamine sulfate or chondroitin sulfate with a placebo in patients with OA, and utilized JSN as an outcome variable after	749	Randomized controlled trials	Pavelka et al, 2002: mean age patients: 61.2, controls: 63.5; 79% female patients, 76% female controls Reginster et al, 2001: mean age patients: 66.0, controls: 65.5;	Pavelka et al, 2002: GS 1,500 mg qd Reginster et al, 2001: GS 1,500 mg qd Kahan et al, 2006: CS 800 mg qd	Glucosamine Sulfate: std diff in means (95% ci): Follow-up for 1 year (2 studies): 0.078 (−0.116 to 0.273), p=0.429 Follow- up	NR

			treatment commencement. Studies were excluded if they did not contain a placebo group, if the OA site was not the knee joint, they did not contain adequate data, or if they were cross-sectional.			75% female patients, 78% female controls Kahan et al, 2006: mean age not available; 68% female patients, 68% female controls Michel et al, 2005: mean age patients: 62.5, controls: 63.1; 51% female patients, 52% female controls Uebelhart et al, 2004: mean age patients: 63.2, controls: 63.7; 79.6% female patients, 82.1% female controls Uebelhart et al, 1998: mean age patients: 60.13, controls: 57.11; 47.8% female patients, 56.5% female controls	Michel et al, 2005: CS 800 mg qd Uebelhart et al, 2004: CS 800 mg 2 periods of 3 months during 1 year Uebelhart et al, 1998: CS 400 bid	for 3 years (2 studies): 0.432 (0.235 to 0.628), p=0.000 JSN > 0.5 mm (2 studies): OR 0.361 (0.204–0.640), p=0.000 Chondroitin Sulfate: std diff in means (95% CI): Minimum JSW (3 studies): 0.317 (0.136–0.497), p=0.001 Mean JSW (4 studies): 0.236 (0.148–0.386), p=0.000 Follow- up for 1 year (2 studies): 0.295 (0.000–0.590), p=0.050 Follow- up for 2 years (2 studies): 0.261 (0.131–0.392), p=0.000	

Wandel, 2010	To determine the clinical effect of glucosamine, chondroitin, or the two in combination on joint pain and on radiological progression of disease in OA of the hip or knee	MEDLINE, EMBASE, CINAHL, and Cochrane Controlled Trials Register through June 2010.	Randomized trials with an average of at least 100 patients with knee or hip osteoarthritis per arm. Comparisons included chondroitin sulfate, glucosamine sulfate, glucosamine hydrochloride, or the combination of any two with placebo or head to head. Excluded trial arms with sub-therapeutic doses (<800mg/day of chondroitin, <1500mg/day glucosamine.	3803 to the interventions or placebo. Glucosamine sulfate vs. Placebo: 5 trials, 1104 randomized patients; Glucosamine sulfate or hydrochloride vs. Placebo: 1 trial, 205 patients; Chondroitin sulfate vs. Placebo: 3 trials 1229 patients; Glucosamine hydrochloride, chondroitin sulfate, and their combination vs. placebo: 1 trial, 1265 patients	10 RCTs: designs not specified	8 trials with knee OA only, one trial with hip or knee OA, one trial with hip OA only. Mean age: 58–66 years % Female: 27–86 (median = 68%) Average duration of symptoms: 6 months–10 years	6 glucosamine vs. placebo 3 chondroitin vs. placebo 1 glucosamine, chondroitin, combination vs. placebo	Pain Intensity (10cm VAS): Glucosamine vs. Placebo: −0.4 cm (−0.7 to −0.1) Chondroitin vs. Placebo: −0.3 cm (−0.7 to 0.0) Glucosamine and Chondroitin vs. Placebo: −0.5 cm (−0.9 to 0.0) Radiological joint space difference (negative number favors intervention): Glucosamine vs. Placebo: −0.2 mm (−0.3 to 0.0) Chondroitin vs. Placebo: −0.1mm (−0.3 to 0.1) Glucosamine and Chondroitin vs. Placebo: 0.00 mm (−0.2 to 0.2)	Estimated differences in pain intensity between supplements and placebo were on average 0.5 cm (0.1 to 0.9) higher in industry sponsored trials (p=0.02 for interaction)

								Adverse Events, OR (95% CI): Glucosamine vs. Placebo: 0.94 (0.59 to 1.47) Chondroitin vs. Placebo: 0.99 (0.49 to 2.00) Glucosamine and Chondroitin vs. Placebo: no data Withdrawals due to AE, OR (95% CI) Glucosamine vs. Placebo: 0.99 (0.61 to 1.50) Chondroitin vs. Placebo: 0.92 (0.56 to 1.51) Glucosamine and Chondroitin vs. Placebo: 0.90 (0.43 to 1.85)	

AE = adverse event; CI = confidence interval; CS = chondroitin sulfate; ITT = intention to treat; GS = glucosamine sulfate; NSAID = nonsteroidal anti-inflammatory drug; OA = osteoarthritis; OR = odds ratio; RCT = randomized controlled trial; VAS = visual analogue scale; WOMAC = Western Ontario and McMaster Universities Osteoarthritis Index; * Characteristics of oral NSAID trials of included drugs for the current systematic review. Adapted from PubMed Health: Analgesics for Osteoarthritis: Adapted from: An Update of the 2006 Comparative Effectiveness Review. Available at: http://www.ncbi.nlm.nih.gov/pubmedhealth/PMH0016482/

Appendix III

Microorganism Characteristics:

Detailed Descriptions of Morphological, Biochemical and Genotypic Analysis for the strain

(attached separately)

Appendix III
Morphological, biochemical (API test) and genotypic characterization of strain GN2284

Colonies' morphology

- In 80 mL of demineralized water dissolve LB broth ingredients:
  - tryptone,
  - yeast extract,
  - NaCl.
- Adjust pH to 7.0 ± 0.2 (at 25°C) with NaOH 5N.
- Add 1.5 % Agar Bacteriological (OXOID LP0011).
- Adjust volume to 100 mL with demineralized water and sterilize at 121°C for 20 minutes.
- Cool by immersion in a water bath to 65°C, dispense 25 mL per Petri dish, under a laminar flow hood and wait 30 min. to allow solidification.
- Starting from a vial of GN2284 RCB, prepare ten-fold serial dilutions to 10^{-8} in NaCl 0.9 % solution.
- Spread plates with 0.1 ml of 10^{-8} dilution and incubate at 37 °C for 16 – 18 h.
- At the end of incubation, colonies appear circular shaped with 1-2 mm diameter, white and smooth.

API 20E Assay

- Prepare an incubation box and distribute about 5 mL of demineralized water into the honey-combed wells of the tray to create a humid atmosphere.
- Record the strain reference on the elongated flap of the tray.
- Remove the strip from its packing and place it in the incubation box.
- Open an ampoule of API NaCl 0.85% solution (5 mL).
- Pick carefully an isolated colony from LB agar plate (recommended young culture 18 – 24 h) and inoculate API NaCl solution.
- Carefully emulsify to àchieve a homogenous bacterial suspension (use the bacterial suspension immediately after preparation).

- Keep the strip slightly forward to facilitate the distribution of the prepared bacterial suspension into the tubes and distribute about 120 µL in each tube.
- For CIT, VP and GEL tests, fill both tube and cupola.
- For ADH, LDC, ODC, H2S and URE tests, create anaerobic conditions by overlaying the bacterial suspension in the tube with paraffin oil.
- Close the incubation box and incubate at 36 ± 2 °C for 18 – 24 h.

Biochemical profile of strain GN2284 is shown in the following tables:

Strains	ONPG	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP	GEL
Reference Strain *Escherichia Coli ATCC 25922*	+	-	+	+	-	-	-	-	+	-	-
Negative Control *Lactobacillus acidophilus*	-	-	-	-	-	-	-	-	-	-	-
Negative Control *Bacillus subtilis (natto)*	-	-	-	-	-	-	-	-	-	-	-
Escherichia coli O5:K4:H4 *(CCUG 11307)*	+	-	+	+	-	-	-	-	+	-	-

Strains	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	OX
Reference Strain *Escherichia Coli ATCC 25922*	+	+	-	+	+	-	+	-	+	-
Negative Control *Lactobacillus acidophilus*	-	-	-	-	-	-	-	-	-	-
Negative Control *Bacillus subtilis (natto)*	-	-	-	-	-	-	-	-	-	-
Escherichia coli O5:K4:H4 *(CCUG 11307)*	+	+	-	+	+	-	+	-	+	-

API-20E legend:

ONPG: β-galattosidase
ADH: arginine dihydrolase
LDC: lysine decarboxylase
ODC: ornithine decarboxylase
CIT: use of citrate
H_2S: production of H_2S
URE: urease
TDA: tryptophan deaminase
IND: production of indole
VP: production of acetoin
GEL: gelatinase
GLU: glucose ferm/oss
MAN: mannose ferm/oss
INO: inositol ferm/oss
SOR: sorbitol ferm/oss
RHA: rhamnose ferm/oss
SAC: sucrose ferm/oss
MEL: melibiose ferm/oss
AMY: amygdalin ferm/oss
ARA: arabinose ferm/oss
OX: oxidase

Amplification and sequence analysis of *kfoC* gene

In order to obtain a genotypic characterization of the strain GN2284 both Polymerase Chain Reaction (PCR) and the sequence analysis of *kfoC* gene were performed according to the following procedure.

- Thaw a vial of GN2284 RCB by immersion in a water bath at 20°C and mix gently.
- Prepare 10-fold dilutions of the suspension to 10^{-7} in NaCl 0.9 % solution,
- Spread 0.1 mL of the 10^{-7} dilution on a plate of LB agar and incubate for 24 h at 37°C.
- Pick a single colony and extract DNA by using FastDNA® SPIN Kit (MP Biomedicals) according to manufacturer instructions.
- Use the following primers pair and prepare the PCR Master Mix as described:
  - **kfoCf** (forward) 5'-tactacaatcaacccagcttcg-3'
  - **kfoCr** (reverse) 5'-tcgcgtattgcatggtgaaaa -3'

PCR Master Mix (50 µL):

DNA	5 µL
KfoCf primer	1.5 µL
KfoCr primer	1.5 µL
PCR Mix solution (10x)	5 µL
H_2O	36.6 µL
Accuprime Pfx DNA-pol (Invitrogen)	0.4 µL

- Set the Thermal Cycler with the following PCR conditions:

94°C for 5 min.	1 cycle
94 °C for 30 sec. 58 °C for 30 sec. 68 °C for 1 min.	35 cycles
68 °C for 10 min.	1 cycle
4 °C ∞	

- Prepare a 0.8% agarose gel (400 mg of agarose, 50 mL of Tris Acetate EDTA (TAE) Buffer 1x, 2.5 µL of Ethidium Bromide).
- Load 10 µL of sample (2 µL of PCR Mix + 1 µL of gel loading buffer (Orange G) + 7 µL of H2O) and 10 µL of 1Kb DNA ladder Molecular Marker (New England Biolabs).
- Set conditions for gel electrophoresis (BioRad Applied Biosystems) at 80V for 35 min.

- Check results under UV light and cut from the gel the expected DNA band corresponding to KfoC amplicon (1813 bp).
- Purify KfoC DNA by using the extraction kit "PerfectPrep Gel Cleanup" (Eppendorf), following the manufacturer instructions.
- Use the forward kfoCf primer to perform a DNA sequencing of purified kfoC DNA and then perform a nucleotide BLAST1 alignment (blastn suite) against the nucleotide collection database (nr/nt).

KfoC sequence identity between GN2284 RCB and *Escherichia coli* O5:K4:H4 U1-41 (ATCC 23502) deposited at the National Center for Biotechnology Information (NCBI), U.S. National Library of Medicine, Bethesda MD.

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AB079602 Escherichia coli kpsS, kfoG, kfoF, kfoE, kfoD, kfoIS, kfoC, kfoB,
kfoA, kpsT genes-complete cds.

Length = 14483
Score = 3349 bits (1813), Expect = 0.0
Identities = 1813/1813 (100%), Gaps = 0/1813 (0%)
Strand = Plus/Plus

Query  1     TTTTCACCATGCAATACGCGATTATAACAAATTTTGTTTATATGCTTGAACGGTCCAACT  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  8797  TTTTCACCATGCAATACGCGATTATAACAAATTTTGTTTATATGCTTGAACGGTCCAACT  8856

Query  61    TCACTAAGTTTTAAATACATATCGTAATCAACTGCGTTGCTGATCGATTCGTTGAAACCT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  8857  TCACTAAGTTTTAAATACATATCGTAATCAACTGCGTTGCTGATCGATTCGTTGAAACCT  8916

Query  121   TCAGTTAGGTTCCATGCTCTTGCTGTGAACATCCTGAAATGATGACATATCATTGCACTA  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  8917  TCAGTTAGGTTCCATGCTCTTGCTGTGAACATCCTGAAATGATGACATATCATTGCACTA  8976

Query  181   GTAAGTTTTTCTCGCGAATAAATGGGCCAATTATAGCCATTTGATATCAAATTACCTTCA  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  8977  GTAAGTTTTTCTCGCGAATAAATGGGCCAATTATAGCCATTTGATATCAAATTACCTTCA  9036

Query  241   CGATCTATATTACGGTTAGTTGTATAAACACATGCCAATGATAGATCTTTTCTAAATTCA  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  9037  CGATCTATATTACGGTTAGTTGTATAAACACATGCCAATGATAGATCTTTTCTAAATTCA  9096

Query  301   TCTAGACATAGTTCAACAGCATCTGGTTCAAGAAAGTCATCAGAGTCTAACTGACCTATA  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  9097  TCTAGACATAGTTCAACAGCATCTGGTTCAAGAAAGTCATCAGAGTCTAACTGACCTATA  9156

Query  361   TAGAATCCCCGACACAATCTAACTGCTGTATTAGATGCTGAACCAATTCCTTTGTTTTTT  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  9157  TAGAATCCCCGACACAATCTAACTGCTGTATTAGATGCTGAACCAATTCCTTTGTTTTTT  9216
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Query  421    TGTGAAATAAAACGAACTCGAGGATGGTTTGCATAATGCTCCTGAAGAATCCGCAATGTA  480
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  9217   TGTGAAATAAAACGAACTCGAGGATGGTTTGCATAATGCTCCTGAAGAATCCGCAATGTA  9276

Query  481    TCATCTGTGGAACCATCATCGCATATGCATACTTCTAAGTCAGTTATTGTCTGATTAAGG  540
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  9277   TCATCTGTGGAACCATCATCGCATATGCATACTTCTAAGTCAGTTATTGTCTGATTAAGG  9336

Query  541    GCGCTTTCAACACAACGAACAATATATTTAGAGCAGTTATAGGCGGGAATATATATAGAT  600
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  9337   GCGCTTTCAACACAACGAACAATATATTTAGAGCAGTTATAGGCGGGAATATATATAGAT  9396

Query  601    ACTAGTGGTACTCTTTTTAATGTCGCGGATTCTATTTTTTCTTTTTTTCTATAGAAATAA  660
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  9397   ACTAGTGGTACTCTTTTTAATGTCGCGGATTCTATTTTTTCTTTTTTTCTATAGAAATAA  9456

Query  661    GGAACTTTTTGCTGTAACAATTGAACAGTAATATTTTTCCCTGCCGCACGATCCGTCTCG  720
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  9457   GGAACTTTTTGCTGTAACAATTGAACAGTAATATTTTTCCCTGCCGCACGATCCGTCTCG  9516

Query  721    TTTTCTTTCCCGGGTGGTTCTTGATGATATGCCATTGCTCCTTCAACAGACCGAAAGTAA  780
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  9517   TTTTCTTTCCCGGGTGGTTCTTGATGATATGCCATTGCTCCTTCAACAGACCGAAAGTAA  9576

Query  781    CATCCTTCTCTGTAGAGACGATATCCAAACTCATTATCCTCCCCCCCCCAATGCGTAAAC  840
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  9577   CATCCTTCTCTGTAGAGACGATATCCAAACTCATTATCCTCCCCCCCCCAATGCGTAAAC  9636

Query  841    TCTTCATCAAACCATCCTGCACGGAAAAGCCATTTTTTCGCAAAAGCGACATTACCTCCG  900
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  9637   TCTTCATCAAACCATCCTGCACGGAAAAGCCATTTTTTCGCAAAAGCGACATTACCTCCG  9696

Query  901    CTAAAAAATCGAAATGGTGTGTTGCATAATCTTAGATTATCGGTATTTTTGAAATGTTCT  960
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  9697   CTAAAAAATCGAAATGGTGTGTTGCATAATCTTAGATTATCGGTATTTTTGAAATGTTCT  9756

Query  961    ATTCGCCAGTCAACTGATTTGTTTTGCTCAACCTTGCCTGCAACCTGATTATTAGTAATG  1020
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  9757   ATTCGCCAGTCAACTGATTTGTTTTGCTCAACCTTGCCTGCAACCTGATTATTAGTAATG  9816

Query  1021   ATTTCAGGAATTTCATTTATTAGTGATTTTTGGGAAAGGAAATCTAAATATGTATGCTTG  1080
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  9817   ATTTCAGGAATTTCATTTATTAGTGATTTTTGGGAAAGGAAATCTAAATATGTATGCTTG  9876

Query  1081   CTTGTATCTATATATTTTCTAGGGCCAATTAGAGCAACATTATCGTCCACCGCTAATAGT  1140
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  9877   CTTGTATCTATATATTTTCTAGGGCCAATTAGAGCAACATTATCGTCCACCGCTAATAGT  9936

Query  1141   TCCATATATGACTGAACCCATAGTGGGTTCGGAGCCATATCACAATCCAGAATTGCAACA  1200
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  9937   TCCATATATGACTGAACCCATAGTGGGTTCGGAGCCATATCACAATCCAGAATTGCAACA  9996

Query  1201   TAATTATACTTTGCAGCCCTAAGCCCAAGATTTCTAACAGCACACAGTTGATATCCATAA  1260
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  9997   TAATTATACTTTGCAGCCCTAAGCCCAAGATTTCTAACAGCACACAGTTGATATCCATAA  10056
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Query  1261   TCCTTCTGACGTACATATTTTATATTTAATAAACTTTCAAATTCTCTTACTATTTCTTCA  1320
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  10057  TCCTTCTGACGTACATATTTTATATTTAATAAACTTTCAAATTCTCTTACTATTTCTTCA  10116

Query  1321   ATATTTTCTTTACTTCCATCATCGGCAACAATAACTTCATAGTCGTATATGGTCTTTTGG  1380
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  10117  ATATTTTCTTTACTTCCATCATCGGCAACAATAACTTCATAGTCGTATATGGTCTTTTGG  10176

Query  1381   TTACAAAGACAAGCAAGTGTAATTGCAAGTATTTTTGCTCGATTATATGTAGGAATTACA  1440
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  10177  TTACAAAGACAAGCAAGTGTAATTGCAAGTATTTTTGCTCGATTATATGTAGGAATTACA  10236

Query  1441   ATACTAAGCCCGTCAATGATTAACTGTTTTCTTGGATAATCATCAAGCTCTTTTCTTTTC  1500
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  10237  ATACTAAGCCCGTCAATGATTAACTGTTTTCTTGGATAATCATCAAGCTCTTTTCTTTTC  10296

Query  1501   CCCGCCCAAACATAATCGTTTGTGCTCTCAGGTAACGGCGGTAAAGTTAAATCACTAGGC  1560
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  10297  CCCGCCCAAACATAATCGTTTGTGCTCTCAGGTAACGGCGGTAAAGTTAAATCACTAGGC  10356

Query  1561   CAATCTAAAGGAATGGGTTCGACTTCCTTTAACTCCGCCCGTTCTGATTTCTTTGCGGTT  1620
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  10357  CAATCTAAAGGAATGGGTTCGACTTCCTTTAACTCCGCCCGTTCTGATTTCTTTGCGGTT  10416

Query  1621   ATTTCTCGGTATTTGCTTATTATTTCATTTTTTTCAACCTCGTTCAGACTAATTGCTTTG  1680
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  10417  ATTTCTCGGTATTTGCTTATTATTTCATTTTTTTCAACCTCGTTCAGACTAATTGCTTTG  10476

Query  1681   GCGTTAGAACACATTATTTTTGTTGCTGCATCAATATCAATAACAGCTTTACGATTTAAC  1740
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  10477  GCGTTAGAACACATTATTTTTGTTGCTGCATCAATATCAATAACAGCTTTACGATTTAAC  10536

Query  1741   TTATCAACTTCTTCAGAAAGATTGAGTGCGGTTTGGCATAATTTTATATTTGCTTCGACC  1800
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  10537  TTATCAACTTCTTCAGAAAGATTGAGTGCGGTTTGGCATAATTTTATATTTGCTTCGACC  10596

Query  1801   CAACTAACATCAT  1813
              |||||||||||||
Sbjct  10597  CAACTAACATCAT  10609
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SUBMISSION END