ORIGINAL SUBMISSION

HISUN
海正药业
浙江海正药业股份有限公司
www.hisunpharm.com

February 25, 2016

Dr. Paulette Gaynor
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Dear Dr. Gaynor:

Re: GRAS Exemption Claim - Pyrroloquinoline Quinone (PQQ) Disodium Salt

In accordance with proposed 21 CFR §170.36 [Notice of a claim for exemption based on a Generally Recognized as Safe (GRAS) determination] published in the *Federal Register* [62 FR 18938 (17 April 1997)], I am submitting one hard copy and one electronic copy (on CD), as the notifier [Zhejiang Hisun Pharmaceutical Co. Ltd., 46 Waisha Road, Jiaojiang District Taizhou City, Zhejiang Province, P.R. China], a Notice of the determination, on the basis of scientific procedures, that pyrroloquinoline quinone (PQQ) disodium salt, produced by Hisun, as defined in the enclosed documents, is GRAS under specific conditions of use in specified beverage products, and therefore, is exempt from the premarket approval requirements of the *Federal, Food, Drug and Cosmetic Act*. Information setting forth the basis for the GRAS determination, which includes detailed information on the notified substance and a summary of the basis for the GRAS determination, as well as a consensus opinion of an independent panel of experts in support of the safety of PQQ under the intended conditions of use, also are enclosed for review by the agency.

The enclosed electronic files for the Notice entitled, "GRAS Exemption Claim Pyrroloquinoline Quinone (PQQ) Disodium Salt" were scanned for viruses prior to submission and is thus certified as being virus-free using McAfee VirusScan 8.8.

Should you have any questions or concerns regarding this GRAS Notice, please do not hesitate to contact me at any point during the review process so that we may provide a response in a timely manner.

Sincerely,

(b) (6)

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GRAS Exemption Claim for Pyrroloquinoline Quinone (PQQ) Disodium Salt

Submitted to:

Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition (CFSAN)
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD
USA 20740-3835

Submitted by:

Zhejiang Hisun Pharmaceutical Co. Ltd.
46 Waisha Road, Jiaojiang District
Taizhou City
Zhejiang Province
318000
P.R. China

February 2016

GRAS Exemption Claim for Pyrroloquinoline Quinone (PQQ) Disodium Salt

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I. GRAS EXEMPTION CLAIM

I.A Claim of Exemption From the Requirement for Premarket Approval Pursuant to Proposed 21 CFR §170.36(c)(1) [62 FR 18938 (17 April 1997)] (U.S. FDA, 1997)

Zhejiang Hisun Pharmaceutical Co. Ltd. (Hisun) hereby claims that the use of pyrroloquinoline quinone (PQQ) disodium salt in energy, sport, and electrolyte drinks and enhanced and fortified water beverages at a maximum use levels of up to 5 and 20 mg/serving respectively in these product types as described in Table I.D-1, is exempt from the premarket approval requirements of the *Federal Food, Drug, and Cosmetic Act* because we have determined that such uses are Generally Recognized as Safe (GRAS).

Signed,

Chen Zhengjie, Vice President

2016.02.25

Date

I.B Name and Address of Notifier

Chen Zhengjie, Vice President
Zhejiang Hisun Pharmaceutical Co. Ltd.
46 Waisha Road, Jiaojiang District
Taizhou City
Zhejiang Province
318000
P.R. China

I.C Common Name(s) of the Notified Substance

Pyrroloquinoline quinone (PQQ) disodium salt; PQQ

I.D Conditions of Intended Use

Pyrroloquinoline quinone (PQQ) disodium salt is intended for use in energy, sport, and electrolyte drinks and enhanced and fortified water beverages at a maximum use levels of up to 5 and 20 mg/serving respectively in these product types as described in Table I.D-1.

Table I.D-1 Summary of the Individual Proposed Food-Uses and Use-Levels for Pyrroloquinoline Quinone (PQQ) in the U.S.				
Food Category	**Food-Uses**	**Serving Size (RACC)[1]**	**Proposed Use Level**	
			(mg/serving)	**(%)**
Beverages and Beverage Bases	Energy, Sport, and Electrolyte Drinks	240 mL	5	0.002
	Enhanced and Fortified Water Beverages	240 mL	20	0.008

[1] RACC refers to Reference Amounts Customarily Consumed per eating occasion – 21 CFR §101.12 (U.S. FDA, 2015). When a range of values is reported for a particular food-use, particular foods within that food-use may differ with respect to their RACC.

I.E Basis for the GRAS Determination

Pursuant to 21 CFR §170.30 of the *Code of Federal Regulations* (CFR) (U.S. FDA, 2015), PQQ disodium salt has been determined by Hisun to be GRAS through scientific procedures.

I.F Availability of Information

The data and information that serve as the basis for this GRAS Notification will be sent to the United States (U.S.) Food and Drug Administration (FDA) upon request, or will be available for review and copying at reasonable times at the offices of:

Zhejiang Hisun Pharmaceutical Co. Ltd.
46 Waisha Road, Jiaojiang District
Taizhou City
Zhejiang Province
318000
P.R. China

Should the FDA have any questions or additional information requests regarding this notification, Hisun will supply these data and information.

previously been determined to be GRAS. A description of the raw materials and processing aids used in the production of PQQ disodium salt is provided in Table II.B.1-1.

Table II.B.1-1 Raw Materials and Processing Aids Used in the Production of PQQ Disodium Salt and Applicable U.S. Regulatory Provisions Relevant to Use in Foods[1]

Materials	Function	Regulatory Status
Fermentation-Aid		
Ammonium sulfate ($(NH_4)_2SO_4$)	Processing-aid (nitrogen source for fermentation)	§184 – Direct food substances affirmed as generally recognized as safe Permitted for use in foods as a dough strengthener, firming agent, and processing aid in accordance to cGMP (21 CFR §184.1143) (U.S. FDA, 2015)
Magnesium sulfate ($MgSO_4•7H_2O$)	Processing-aid (Fermentation nutrient)	21 CFR §184 – Direct food substances affirmed as generally recognized as safe Permitted for use in foods as a flavor enhancer, nutrient supplement, or processing aid in accordance to cGMP (21 CFR §184.1443) (U.S. FDA, 2015)
Calcium chloride ($CaCl_2•2H_2O$)	Processing-aid (Fermentation nutrient)	21 CFR §184 - Direct food substances affirmed as generally recognized as safe Permitted for use in foods as an anti-caking agent, antimicrobial agent, curing or pickling agent, firming agent, flavor enhancer, humectant, nutrient supplement, pH control agent, processing aid, stabilizer and thickener, surface-active agent, synergist, and texturizer not to exceed cGMP (21 CFR §184.1193) (U.S. FDA, 2015)
Agar	Processing-aid (preparation of working inoculum)	FCC 9th ed.
Methanol	Processing-aid (carbon source for fermentation)	Methanol is permitted for use in foods as a GRAS substance when used in accordance with cGMP (21 CFR §182.1) (U.S. FDA, 2015)
Polydimethylsiloxane	Anti-foaming	Anti-foaming agent is permitted for use in the processing of foods (21 CFR §173.340) (U.S. FDA, 2015)
Sodium phosphate dibasic ($Na_2HPO4•12H_2O$)	Processing-aid (Fermentation nutrient)	This substance is generally recognized as safe when used in accordance with good manufacturing practice (21CFR§182.1778)
Potassium phosphate monobasic	Processing-aid (Fermentation nutrient)	Yeast food – FCC 9th ed.
Ammonia water	Processing-aid (nitrogen source for fermentation)	pH control – FCC 9th ed.
Purification-Aids		
NaCl	Crystallization	Sodium chloride is a GRAS substance when used in accordance with cGMP (21 CFR §182.1) (U.S. FDA, 2015)
strongly basic anion-exchange resin with quarternary ammonium functional group	Purification	Ion-exchange resin permitted for use in the treatment of food under 21 CFR §173.25 (U.S. FDA, 2015)

Table II.B.1-1 Raw Materials and Processing Aids Used in the Production of PQQ Disodium Salt and Applicable U.S. Regulatory Provisions Relevant to Use in Foods[1]		
Materials	**Function**	**Regulatory Status**
Ethanol	Crystallization	Ethanol is permitted for use in foods as a GRAS substance when used in accordance with cGMP (21 CFR §182.1) (U.S. FDA, 2015)
Sodium phosphate monobasic (NaH_2PO_4•$2H_2O$)	Buffer	This substance is generally recognized as safe when used in accordance with good manufacturing practice (21 CFR §182.1778)
Sodium phosphate dibasic (Na_2HPO4•$12H_2O$)	Buffer	This substance is generally recognized as safe when used in accordance with good manufacturing practice (21CFR§182.1778)
NaOH	pH	GRAS substance and permitted for use in accordance with cGMP (21 CFR §184.1763) (U.S. FDA, 2015)
HCl	pH	GRAS for use in foods as a buffer and neutralizing agent in accordance with cGMP (21 CFR §182.1057) (U.S. FDA, 2015).

CFR = United States Code of Federal Regulations; cGMP = current Good Manufacturing Processes; GRAS = Generally Recognized as Safe; PQQ = pyrroloquinoline quinone; U.S. FDA = United States Food and Drug Administration.

[1] In accordance to the U.S. Code of Federal Regulations (CFR) Title 21 – Food and Drugs

II.B.1.1 Source Organism, Hyphomicrobium sp.

Hyphomicrobium are facultatively methylotrophic, non-spore forming, gram-negative, rod-shaped bacteria with a unique Q-9 ubiquinone system (Urakami and Komagata, 1979, 1986, 1987). *Hyphomicrobium* are found ubiquitously in a variety of environments and are able to utilize single carbon compounds as an exclusive source of energy and carbon in the presence of nitrate under both aerobic and anaerobic conditions (Sperl and Hoare, 1971; Attwood and Harder, 1972).

The taxonomic classification of the production organism is presented in Table II.B.1.1-1. Molecular identification *via* 16S ribosomal DNA (rDNA) sequence analysis demonstrates that the source organism of PQQ has a 100% sequence identity to *Hyphomicrobium denitrificans* American Type Culture Collection (ATCC) 51888. Further morphological and biochemical analyses demonstrates that *H. denitrificans* is a gram-negative bacterium that forms milky colonies and is positive for nitrate reduction. *Hyphomicrobium denitrificans is not a genetically modified organism (GMO).*

Table II.B.1.1-1 Taxonomic Classification of *Hyphomicrobium denitrificans*	
Class	**Scientific Classification**
Kingdom	*Prokaryota*
Division	*Bacteria*
Subdivision	*Proteobacteria*
Class	*Alphaproteobacteria*
Order	*Rhizobiales*
Family	*Hyphomicrobiaceae*
Genus	*Hyphomicrobium*
Species	*Hyphomicrobium denitrificans*

The *Hyphomicrobium* used for the production of PQQ disodium salt is maintained in-house by Hisun and is subject to strict quality control for compliance with established internal specifications and is free of microbial contamination. The specifications for the fermentation organism, *Hyphomicrobium sp.,* are detailed in Table II.B.1.1-2.

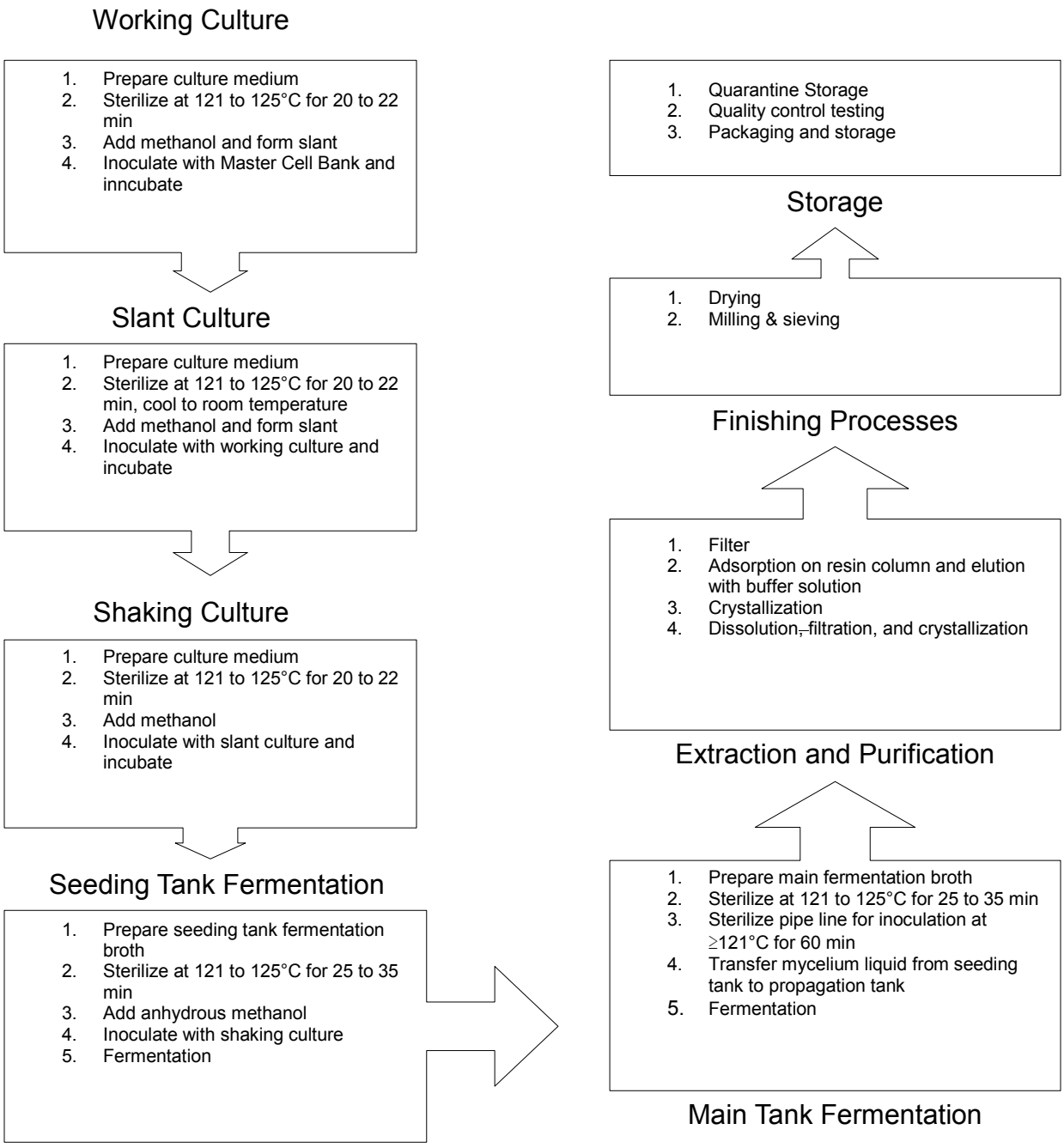
Table II.B.1.1-2 Specifications for the *Hyphomicrobium* Used in the Production of Hisun's PQQ Disodium Salt	
Parameter	**Specification**
Appearance	Beige colony, wet and abundant
Growth Characteristics	Thick and strong, reticular, and deep staining mycelium, no contamination
Colony Survival Number	≥1x10^9/mL
Survival Ratio	≥95%
Fermentation Potency	≥120 μg/mL
Microbial Contamination	Negative for foreign bacteria under microscope

PQQ = pyrroloquinoline quinone

II.B.2 Manufacturing Process

The production of Hisun's PQQ disodium salt is a modification of the production of PQQ by bacterial fermentation reported by several other investigators (Ameyama *et al*., 1984; Urakami *et al*., 1992; Noji *et al.,* 2007). Specifically, Urakami *et al.* (1992) describe a method wherein *Hyphomicrobium* sp. strain TK0441 was utilized in a fermentation process to produce PQQ. A general schematic overview of the manufacturing process of Hisun's PQQ product is outlined in Figure II.B.2-1. Hisun's PQQ ingredient is manufactured consistent with the principles of Hazard Analysis and Critical Control Points (HACCP).

Figure II.B.2-1 Schematic Overview of the Production Process of PQQ Disodium Salt



Preparation of Working Cell Bank (WCB) and Slant Culture

The WCB slant culture medium is prepared by dissolving food grade minerals and agar with purified water in a beaker. Sodium hydroxide solution is then used to adjust to pH. The medium is heat sterilized at approximately 121 to 125°C for 20 to 22 minutes. Once the medium is cooled, methanol is added and a slant is formed. Subsequently, the WCB is produced by culturing the Master Cell Bank suspension containing the source organism on the blank slant and incubation. The colonies are then isolated from the slant and recovered using glycerol solution prior to quality control testing to ensure that they conform to the internal specifications established for *Hyphomicrobium*.

Following this, the slant culture is produced by inoculating the WCB onto another blank slant using the same culture medium and incubation conditions as the production of the WCB.

Preparation of the Shaking Culture

The shaking culture medium is prepared by dissolving food grade salts in a beaker. The medium is adjusted using sodium hydroxide solution and subject to heat sterilization at a temperature of approximately 121 to 125°C. Methanol is added to the medium and then cooled.

The colony on the slant culture is isolated and then inoculated into a shaking flask containing the shaking culture medium under aseptic conditions. The culture is incubated and upon completion of the incubation period the morphology of the culture is assessed.

Fermentation

The seeding tank fermentation broth is prepared by adding food grade minerals, and an antifoaming agent into a potable within the seeding tank. The pH is adjusted using sodium hydroxide. The fermentation broth is then heat sterilized at 121 to 125°C for 25 to 35 minutes. The medium is cooled prior to the addition of methanol. The seed tank fermentation is initiated upon addition of the shaking culture under aseptic procedures. Fermentation occurs under tightly controlled conditions (*e.g.*, temperatures, pressure, agitation speed). Fermentation is terminated when mycelium concentration reaches a defined OD and pH range.

The main fermentation broth is produced in a preparation pool and then transferred into a propagation tank using the same raw materials and in a similar manner to the seed tank fermentation broth. Along with heat sterilization of the fermentation broth, the pipe line for inoculation is also sterilized at a pressure of 0.35 to 0.45 MPa and temperature of ≥121°C for 60 minutes. The mycelium solution from the seeding tank fermentation is inoculated into the propagation tank and fermentation occurs under controlled conditions. Through this process, the pH, mycelium concentration, and amino-nitrogen are tested at 24 hours post- inoculation and the potency is tested daily during fermentation. The pH is maintained by the addition of ammonia water. Methanol solution is continuously added to maintain its levels consistent with

the amount within the culture medium. The fermentation is terminated when the mycelia decline, tinting strength is weak, increase of the fermentation potency is slower, pH increased slightly, and the potency is reduced.

Extraction and Purification

Upon completion of the fermentation process, PQQ is isolated and purified through a series of filtration steps through a ceramic membrane followed by resin adsorption and elution (with a sodium phosphate buffer solution). The source organism is removed by ceramic membrane filtration with a pore diameter of <200 nm. Hydrochloric acid is used to adjust the pH prior to the addition of sodium chloride with stirring. Crystallization then occurs over several hours. The crude product is recovered by filtration prior to dissolving in water using sodium hydroxide to facilitate dissolution. The solution undergoes membrane filtration prior to addition to a crystallizing tank. Ethanol is added and the pH is adjusted using hydrochloric acid with stirring. A second crystallization step is then initiated. The mixture is then filtered to obtain the wet substance. This is followed by vacuum drying, milling, sieving. The finished PQQ is then transferred to quarantine storage for quality control testing prior to packaging in 2-ply polyethylene bags and 1 aluminum foil bag and storage at temperatures not to exceed 30°C.

II.B.3 Process Controls

Hisun's PQQ ingredient is manufactured consistent with the principles of Hazard Analysis and Critical Control Points (HACCP). Throughout the production process, all culture media and fermentation broth are tested for sterility prior to use. Additional process controls used during the production of PQQ are listed in Table II.B.3-1.

Table II.B.3-1 Process Controls in the Production of PQQ

Process	Control
Preparation of Working Cell Bank	Microbiological contamination, appearance, growth characteristics, colony survival number, survival ratio, fermentation potency
Preparation of Slant Culture	Microbiological contamination, appearance
Shaking Culture	Microbiological contamination, morphological characteristics
Seed Tank Fermentation	Microbiological contamination, appearance of seeding liquid, mycelium content, pH
Main Fermentation	Mycelium content, amino-nitrogen, pH, fermentation potency

II.C Specifications and Batch Analyses

II.C.1 Specifications

The product specifications for PQQ disodium salt are detailed in Table II.C.1-1. All methods of analyses are nationally or internationally recognized or have been validated by Hisun. The ingredient is ≥85% pure on a weight to weight basis, and the high performance liquid chromatography (HPLC) purity of PQQ disodium salt is ≥99%. Appropriate limits for heavy metals and microbial impurities have been established. Residual ethanol concentrations are limited by specification to 5,000 ppm.

Table II.C.1-1 Chemical and Physical Specifications for PQQ Disodium Salt

Parameter	Specification	Method of Analysis
Identity		
Appearance	Powder, henna color	Visual inspection
Identification	IR spectrum correspond to reference standard	USP<197K>
	A233/A259 = 0.90±0.09 A322/A259 = 0.56±0.03	USP<197U>
PQQ (as-is basis)	≥85%	USP<621>
Purity (chromatography)	≥99%	USP<621>
Water content	≤12%	USP<921>
Ethanol	≤5,000 ppm	Gas chromatography (validated Hisun method)
Heavy Metals		
Lead	≤1 ppm	ICP-MS
Arsenic	≤1.5 ppm	ICP-MS
Cadmium	≤0.3 ppm	ICP-MS
Mercury	≤0.2 ppm	ICP-MS
Microbiological Analysis		
Total Aerobic Count	≤10,000 CFU/g	USP<61>
Total Mold and Yeast	≤1,000 CFU/g	USP<61>
Enterobacteriaceae	≤100 CFU/g	USP<62>
Escherichia coli	Negative/10 g	USP<62>
Staphylococcus aureus	Negative/10 g	USP<62>
Salmonella	Negative/10 g	USP<62>

CFU = colony forming units; ICP-MS = inductively-coupled plasma mass spectroscopy; IR = infrared; ppm = parts per million; PQQ = pyrroloquinoline quinone; USP = United States Pharmacopeia

II.C.2 Product Analysis

Analysis of 3 non-consecutive batches of Hisun's PQQ disodium salt demonstrated that the manufacturing process produces a consistent product that is in compliance with the established specifications. A summary of the results of the product analysis are shown in Table II.C.2-1.

Table II.C.2-1 Product Analysis for 3 Non-Consecutive Batches of PQQ Disodium Salt

Parameter	Specification	Batch No./Date of Manufacture		
		9089-151201 Dec.16, 2015	9089-151202 Dec. 20, 2015	9089-151203 Dec. 24, 2015
Identity				
Appearance	Powder, henna color	Conforms	Conforms	Conforms
Identification	IR spectrum correspond to reference standard	Conforms	Conforms	Conforms
	A233/A259 = 0.90±0.09 A322/A259 = 0.56±0.03	0.85 0.55	0.85 0.56	0.85 0.55
PQQ (as-is basis) (%)	≥85	92.5	92.6	94.5
Purity (chromatography) (%)	≥99	99.5	99.6	99.7
Water content (%)	≤12	6.0	5.1	4.6
Ethanol (ppm)	≤5,000	<118	<118	<118
Heavy Metals				
Lead (ppm)	≤1	<0.2	0.78	0.35
Arsenic (ppm)	≤ 1.5	<0.1	<0.1	<0.1
Cadmium (ppm)	≤0.3	<0.06	<0.06	<0.06
Mercury (ppm)	≤0.2	<0.04	<0.04	<0.04
Microbiological Analysis				
Total Viable Aerobic Count (CFU/g)	≤10,000	50	550	150
Total Mold and Yeast (CFU/g)	≤1,000	50	<50	<50
Enterobacteriaceae (CFU/g)	≤100	<10	<10	<10
Escherichia coli (in 10 g)	Negative	ND	ND	ND
Staphylococcus aureus (in 10 g)	Negative	ND	ND	ND
Salmonella (in 10 g)	Negative	ND	ND	ND

CFU = colony forming units; IR = infrared; ND = not detected; ppm = parts per million; PQQ = pyrroloquinoline quinone

II.C.3 Other Qualitative Analyses

II.C.3.1 Contaminants Derived from the Source Organism

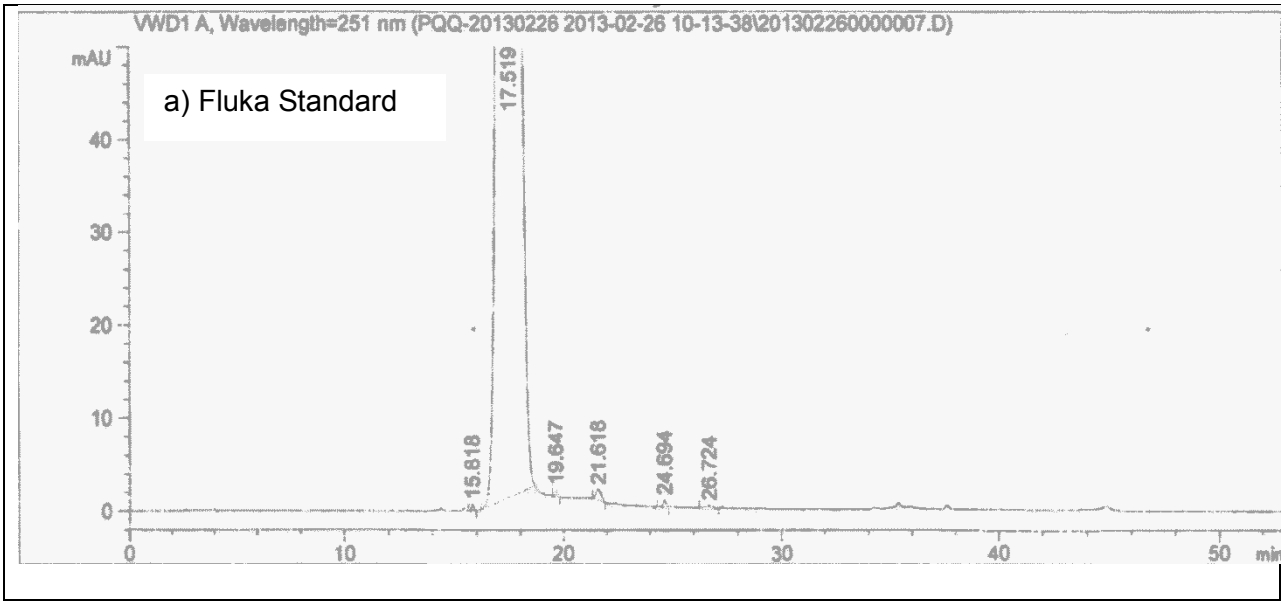
Residual levels of protein in the final PQQ product were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with detection *via* the Argentation method. No detectable levels of protein were observed in 3 consecutive batches of PQQ (batch nos. M130101, M130102, M130103) at a limit of detection of 0.0357% protein.

Hisun's PQQ disodium salt was also analyzed for the presence of the fermentation organism, *Hyphomicrobium* by testing the ability of the final PQQ disodium salt product to induce microbial growth on agar plates. No growth of *Hyphomicrobium* was observed upon testing of 3 non-consecutive lots of PQQ disodium salt (batch nos. M131001, M131101, M131202) indicating that the source organism is not detectable in the final PQQ product.

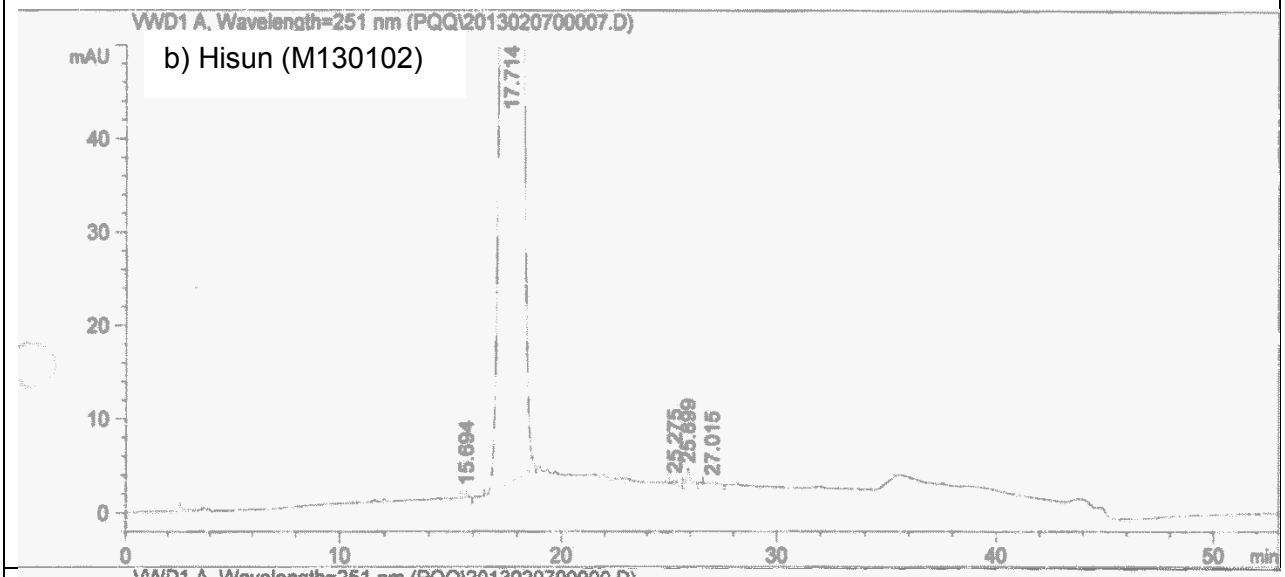
II.C.3.2 Impurities

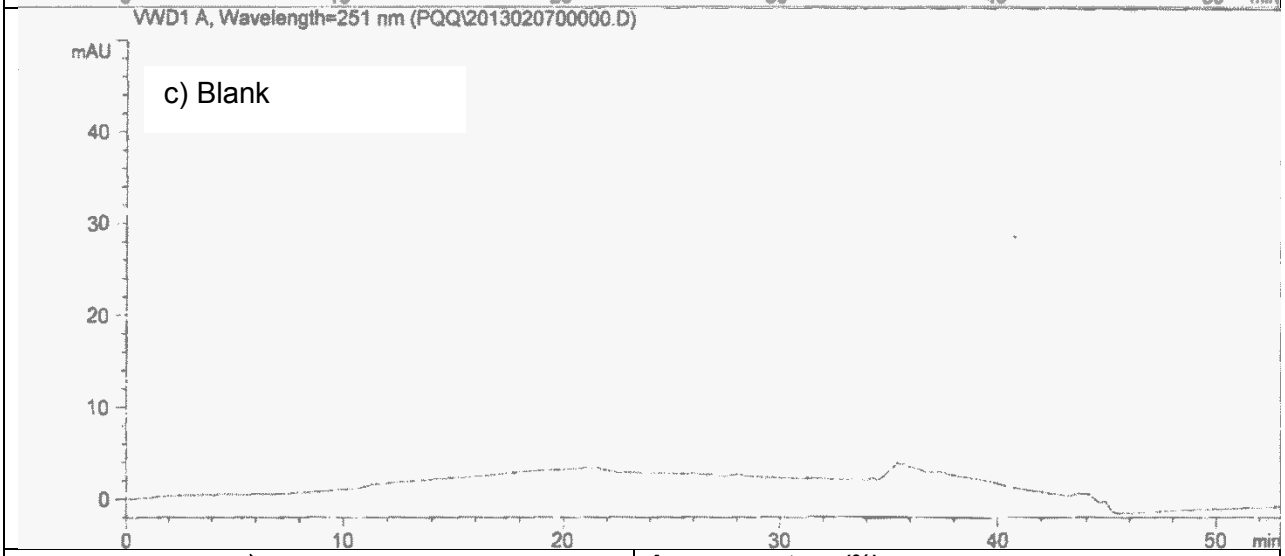
Hisun's PQQ disodium salt is of high purity (≥99%). Chromatographs comparing Hisun's PQQ product (lot no. M130102) to a reference standard of PQQ were obtained using high performance liquid chromatography (HPLC) with VWD[1]. PQQ (lot no. M130102) manufactured by Hisun was 99.83% pure (Figure II.C.3.2-1). Based on the purity profile, Hisun considers the ingredient to meet or exceed food grade quality standards.

Figure II.C.3.2-1 HPLC-VWD Purity Analyses: (a) Fluka Reference Standard; (b) Hisun's PQQ Product; (c) blank



[1] HPLC-DAD analysis confirming purity also conducted during in house stress testing.





Retention Time (min)	Area percentage (%)
15.694	0.0268
17.714 (major peak)	99.8312
25.275	0.0200
25.899	0.0856
27.015	0.0365

I.C.3.3 Qualitative Analyses

The structural identity of a representative sample of Hisun's PQQ product (lot no. M130103) was evaluated based on a number of analyses including infrared (IR) spectroscopy, nuclear magnetic resonance (NMR), ultraviolet (UV) absorbance, and mass spectroscopy (MS) in comparison to a commercially available PQQ product (Fluka; lot no. 1203925 51009082) as a reference standard. A summary of the resultant data from the IR, NMR, and MS analyses are shown in Tables II.C.3.3-1 through II.C.3.3-3 and Figure II.C.3.3-1, respectively. The data

obtained from these analyses demonstrate that Hisun's PQQ product is chemically equivalent to the reference standard; consistent with the presence of carboxyl, keto, and benzene moieties that are characteristic of PQQ, and conforms to the structure of PQQ disodium salt in regards to its molecular weight. Representative MS spectra for Hisun's PQQ product identified a primary fragmentation peak with a m/z ratio of 328.80 $[M-2Na^{+}H]^{-}$ which corresponds to the loss of 2 molecules of sodium from the parent PQQ compound (molecular weight of 374 g/mol). This is comparable to the fragmentation pattern observed with the commercial lot of PQQ disodium salt. Additionally, the UV absorbance of PQQ, measured in 3 different solvents (water, hydrochloric acid and sodium hydroxide), as shown in Table II.C.3.3-3, demonstrates that this compound contains an unsaturated conjugated system which is consistent with the structure of PQQ.

Table II.C.3.3-1 Summary of the Analytical Data for the IR Spectroscopy Analysis of PQQ

Wavelength (cm^{-1})		Vibration Type	Group	Strength	Assignment
Hisun	Fluka				
3446	3419	v	COOH	s	O-H stretching vibration of carboxyl
1716	1716	v	COOH	s	C=O stretching vibration of carboxyl
1679	1674	v	C=O	s	C=O stretching vibration of keto group
1610	1612	v	COO-	s	C=O asymmetry stretching vibration of carboxylic ion
1548	1546	v	C=C	m	Stretching vibration of benzene ring
1500	1501	v	C=C	m	Stretching vibration of benzene ring
1354	1355	v	COO-	s	C=O symmetry stretching vibration of carboxylic ion
1241	1238	v	C-O	s	C-O stretching vibration

IR = infrared; PQQ = pyrroloquinoline quinone

Table II.C.3.3-2 Summary of the Analytical Data for the ^{13}C-NMR, DEPT90°, DEPT135°, HMBC and HSQC Analysis of PQQ

Carbon No.	Chemical Shift (ppm)		DEPT	HSQC Relative H	HMBC Relative H
	Hisun	Fluka			
4	94.73	94.85	C	-	-
5	112.54	112.57	CH	6.74	-
10.7	119.26	119.28	CH,C	7.22	6.74
8	120.21	120.23	C	-	7.22
9	142.41	142.43	C	-	7.22
12	144.09	144.11	C	-	-
6	145.82	145.83	C	-	6.74
11	148.74	148.76	C	-	-
13	161.78	161.78	C	-	-
3	172.76	172.76	C	-	7.22
1	173.70	173.71	C	-	-
2	178.46	178.46	C	-	7.22
14	195.25	195.22	C	-	-

DEPT = distortionless enhancement by polarization transfer; HMBC = heteronuclear multiple-bond correlation spectroscopy; HSQC = heteronuclear single-quantum correlation spectroscopy; NMR = nuclear magnetic resonance; PQQ = pyrroloquinoline quinone

Table II.C.3.3-3 Summary of the Analytical Data for the UV Absorbance Analysis of PQQ

Solvent	λ max (nm)	E ($mol^{-1}cm^{-1}L$)	Assignment
Water	248.8	23083	K absorption band of conjugated system
	333.6	10801	K absorption band of conjugated system
0.1N HCl	199.8	19642	E absorption band of unsaturated double bond
	252.6	22724	K absorption band of conjugated system
	352.2	11994	K absorption band of conjugated system
0.1 N NaOH	248.8	22361	K absorption band of conjugated system
	354.8	11744	K absorption band of conjugated system

λ = wavelength; E = energy; PQQ = pyrroloquinoline quinone; UV = ultraviolet

II.C.3.4 Elemental Analysis

Elemental analyses by high resolution MS was performed on a representative sample of PQQ disodium salt. The elemental composition was consistent with the molecular formula of PQQ disodium salt ($C_{14}H_5N_2Na_2O_8$) (Table II.C.3.4-1).

Table II.C.3.4-1 Elemental Analysis of PQQ Disodium Salt

Elemental Composition	Theoretical Value	Test Result	Deviation (mDa)	Precision (ppm)
$C_{14}H_5N_2Na_2O_8$	374.9836	374.9850	1.4	3.8

PQQ = pyrroloquinoline quinone

Ion chromatography was used to determine the levels of sodium, phosphate, and chloride on the same 3 non-consecutive lots. The sodium content was consistent with the theoretical value of 12.3% sodium for PQQ disodium salt. Only trace levels of phosphate and chloride were reported (<0.02 and 0.01 to 0.07%, respectively) (Table II.C.3.4-2).

Table II.C.3.4-2 Compositional Analysis (Sodium, Phosphate, and Chloride Content) of PQQ Disodium Salt

Component	Theoretical Value	Lot No.		
		M131001	**M131101**	**M131202**
Sodium (%)	12.3	11.7	12.1	12.2
Phosphate (%)	NA	<0.02	<0.02	<0.02
Chloride (%)	NA	0.07	0.02	0.01

NA = not applicable; PQQ = pyrroloquinoline quinone

II.D Stability

II.D.1 Bulk Stability

The bulk stability of 3 consecutive lots of PQQ (lot no. M130101, M130102, M130103) was assessed under ambient and accelerated storage conditions. These studies were conducted in accordance to testing conditions established by the International Conference on Harmonization (ICH) Q1A guidance (ICH, 2003). At 25±2°C room temperature and 60±5% relative humidity, the PQQ disodium salt was stable for at least 24 months. Under accelerated conditions of 40±2°C and 75±5% relative humidity, PQQ was reported to be stable for a period of 6 months. A summary of these findings are presented in Table II.D.1-1.

Table II.D.1-1 Summary of the Stability of PQQ Disodium Salt Under Ambient and Accelerated Conditions				
Parameter	**Specification Limit**	**Lot No.**		
		M130101	**M130102**	**M130103**
Ambient Conditions (25±2°C, 60±5% relative humidity) at 24 months				
Appearance	Henna powder	Conforms	Conforms	Conforms
IR Identification	Corresponds to reference standard	ND	ND	ND
UV Identification	A233/A259 = 0.90±0.09 A322/A259 = 0.56±0.03	0.90 0.57	0.90 0.57	0.90 0.57
Chromographic Purity (%)	≥99.0	99.8	99.8	99.8
Water content (%)	≤12.0	5.7	6.7	6.7
Accelerated Conditions (40±2°C, 75±5% relative humidity) at 6 months				
Appearance	Henna powder	Conforms	Conforms	Conforms
IR Identification	Corresponds to reference standard	Conforms	Conforms	Conforms
UV Identification	A233/A259 = 0.9±0.09 A322/A259 = 0.56±0.03	0.88 0.56	0.89 0.56	0.88 0.56
Chromographic Purity (%)	≥99.0	99.8	99.8	99.7
Water content (%)	≤12.0	9.6	10.1	10.0

IR = infrared; ND = not determined; PQQ = pyrroloquinoline quinone; UV = ultraviolet

III. SELF-LIMITING LEVELS OF USE

No known self-limiting levels of use are associated with food uses of PQQ.

IV. DETAILED SUMMARY OF THE BASIS FOR HISUN'S GRAS DETERMINATION

Hisun's determination that PQQ is GRAS under the conditions of intended use in foods and beverages as described herein is based on scientific procedures. To obtain necessary information relevant to the safety of PQQ, comprehensive searches of the published scientific literature were conducted through to January 2016 using the search program Proquest to identify published studies relevant to the safety of PQQ disodium salt and the source organism, *Hyphomicrobium*. The search was conducted on databases including Adis Clinical Trials Insight, AGRICOLA, AGRIS, Allied & Complementary Medicine™, BIOSIS® Toxicology, CAB ABSTRACTS, Embase®, Foodline®: SCIENCE, FSTA®, MEDLINE®, and Toxfile®. The results of these findings are summarized in the following sections.

PQQ is present naturally in a variety of common foods at levels in the ppb range. Higher levels are reportedly present in food originating from plants relative to that originating from animals. Considering that there is no recognized biological function for PQQ and no mammalian

biosynthetic pathway has been identified for its production, trace levels of endogenous PQQ identified in various human tissues are derived primarily from dietary exposure. However, as estimates of the daily consumption of PQQ and its derivatives range from 0.1 to 2 mg/day, which includes both the intake of the parent PQQ compound and its IPQ metabolites, background intakes of PQQ are minimal and not expected to contribute significantly to the cumulative exposure to PQQ under the intended conditions of use. Under the intended conditions of use of PQQ disodium salt in specified beverage products, the all-user total population consumption of PQQ was estimated to result in mean intakes of 12.8 mg/person/day (0.21 mg/kg body weight/day) and a 90th percentile intake of 27.8 mg/person/day (0.48 mg/kg body weight/day) for the total population. On a body weight basis, children were observed to have the highest all-user intakes (*i.e.*, mean and 90th percentile intakes of 0.32 and 0.77 mg/kg body weight/day, respectively); however, as Hisun does not intend to market their PQQ product for use in products likely to be consumed by children, the actual exposure to PQQ in these population groups is expected to be minimal.

While the nutritional role of dietary PQQ remains controversial, IPQ derivatives, products formed through the condensation of PQQ with amino acids, have been identified in human milk (Mitchell *et al.,* 1999; Kumazawa *et al.,* 2000). In mice, absorption of ^{14}C-radiolabeled PQQ readily occurred in the lower intestine and excretion was primarily *via* urinary elimination (Smidt *et al.*, 1991). Accumulation of radioactivity occurred in the kidney and skin but was not detected in the expired carbon dioxide of mice. There is also some evidence of fecal excretion, as clinical observations in rodent studies have reported the presence of discolored stools which have been attributed to the color of the test compound (Wang *et al.*, 2012; Zhejiang Hisun Pharmaceutical Co., Ltd., 2012; Nakano *et al.*, 2014).

PQQ appears to be poorly bioavailable in mice. Studies in mice administered radiolabeled PQQ have not identified significant radioactivity in the liver or kidneys, suggesting that the limited bioavailability of PQQ may be due to poor absorption of the compound by animals. In humans, peak serum levels of 3.4 ng PQQ/mL occurred at 2 hours following the ingestion of a single dose of 0.2 mg PQQ/kg body weight, and approximately 0.1% of the free PQQ was recovered in the urine upon repeated consumption of PQQ at graduated doses up to 0.3 mg/kg body weight/day over 21 days (Harris *et al.*, 2013).

PQQ disodium salt is of low oral toxicity. In general, acute rodent studies using high doses of PQQ disodium salt (>500 and 1,000 mg/kg body weight in rats and mice, respectively) have demonstrated a dose-dependent increase in soft stools and/or diarrhea, along with discoloration of the stools and organs and tissues; however, the oral acute toxicity is low, as median lethal dose (LD_{50}) values were reported to be as high as 5.84 g/kg body weight/day in female mice and 2 g/kg body weight/day in male rats (Wang *et al.*, 2012; Zhejiang Hisun Pharmaceutical Co., Ltd., 2012; Nakano *et al.*, 2014). The toxicity of PQQ disodium salt (BioPQQ™, >99.1% purity; Mitsubishi Gas Chemical Co., Ltd.) has been evaluated in a series of repeated-dose

studies conducted in rats by Nakano *et al*. (2014). The kidneys appear to be a target organ of PQQ toxicity at high doses. In the 14-day study, female rats were reported to have a significant increase in relative kidney weight upon consumption of PQQ at dose levels of 768 mg/kg body weight/day, which corresponded to focal basophilic changes and atrophy in the renal tubules of minimal to moderate severity. These effects are consistent with the reported histopathological changes in the kidneys of rats administered PQQ by intraperitoneal injection at a dose of 11.5 mg/kg body weight/day for 4 days (Watanabe *et al.*, 1989). In contrast, while significant changes in urinary volume and specific gravity were reported in female rats administered 200 mg PQQ/kg body weight/day in the 28-day study, these changes were not test article-related as no corresponding differences in clinical chemistry or histopathology were reported, as relevant to renal function or damage, and the effects were modest and transient.

In a 90-day study of PQQ in rats, the NOAEL was determined to be 100 mg/kg body weight/day (the highest dose tested) (Nakano *et al.*, 2014). Wang *et al.* (2012) also conducted a 90-day study of PQQ in rats and determined the NOAEL to be 15 mg/kg body weight/day (the highest dose tested). More recently, a 90-day oral toxicity study of PQQ disodium salt conducted under Good Laboratory Practices (GLP) was well-tolerated in rats and no toxicologically relevant changes were noted; the NOAEL was determined to be 400 mg/kg body weight/day (highest dose tested) (Liang *et al.*, 2014). Although urinary measures of kidney function were not reported by the authors, no test article related effects on the kidneys were observed.

PQQ disodium salt has been evaluated by multiple investigators in a standard battery of *in vitro* and *in vivo* mutagenicity/genotoxicity studies including the Ames assay, *in vitro* chromosomal aberration study and *in vivo* micronucleus assay (Wang *et al.*, 2012; Zhejiang Hisun Pharmaceutical Co., Ltd., 2012; Nakano *et al.*, 2013). PQQ was consistently negative for mutagenicity/genotoxicity in these studies. There is no evidence to suggest that PQQ represents a mutagenic/genotoxic risk.

Short-term consumption of PQQ in a fruit-flavored drink at a single dose of 0.2 mg/kg body weight or a repeated dose of 0.3 mg/kg body weight/day for 3 days did not induce any remarkable changes in clinical indices in comparison to baseline values (Harris *et al.*, 2013). Repeated consumption of 20 mg PQQ per day for up to 24 weeks also has been reported to be well-tolerated in healthy adult subjects (Nakano *et al.*, 2009, 2012; Koikeda *et al.*, 2011). Additionally, a double blind study evaluating the administration of PQQ to 10 healthy subjects for 4 weeks at doses of 20 or 60 mg/day did not induce any toxicologically relevant changes in standard clinical tests or markers of liver toxicity (as reviewed in Rucker *et al.*, 2009).

Hyphomicrobium denitrificans is a gram-negative bacterium that utilizes one carbon compounds, such as methanol, as an exclusive source of carbon and energy and is positive for nitrate reduction. There is limited published information on the species or genus. A comprehensive search of the literature did not identify data or information to suggest that the *H. denitrificans* or related species are pathogenic to animals or produce known toxins. PQQ

produced by *Hyphomicrobium sp*. has been previously notified to the FDA as a New Dietary Ingredient for use in dietary supplement products without objection from the Agency. Through the use of appropriate manufacturing controls, which include filter and heat sterilization steps, and chromatographic purification, crystallization, and wash procedures, transfer of the fermentation organism or residues of the fermentation broth into the finished product are prevented. PQQ manufactured by Hisun is a high purity crystalline product that is free of protein and is tested for assurance that viable counts of the source organism are not transferred to the finished product. No safety concerns are anticipated from the use of *H. denitrificans* in the production of PQQ disodium salt.

Hisun has therefore concluded, using scientific procedures, that PQQ, meeting appropriate food-grade specifications and manufactured according to cGMP, is safe and suitable, and is generally recognized as safe for its intended food uses as discussed herein. A summary of all generally available information pertinent to the safety of PQQ is presented below.

IV.A Estimated Dietary Exposure

IV.A.1 Estimated Daily Intake of PQQ from its Intended Food Uses

Estimates for the intake of PQQ disodium salt were based on the proposed food-uses and use levels for PQQ disodium salt in conjunction with food consumption data included in the U.S. National Center for Health Statistics' (NCHS) National Health and Nutrition Examination Surveys (NHANES) 2011-2012 (CDC, 2014; USDA, 2014).

Calculations for the mean and 90th percentile all-person and all-user intakes were performed for each of the individual proposed food-uses of PQQ disodium salt and the percentage of consumers were determined. Similar calculations were used to estimate the total intake of PQQ disodium salt resulting from all proposed food-uses of PQQ disodium salt combined. In both cases, the per person and per kilogram body weight intakes were reported for the following population groups:

- Children (ages 3 to 11)
- Male and female teenagers (ages 12 to 19)
- Male and female adults (ages 20 and up)
- Total population (all age and gender groups combined).

A summary of the estimated daily intake of PQQ disodium salt from all proposed food-uses is provided in Table IV.A.1-1 on an absolute basis (mg/person/day) and in Table IV.A.1-2 on a body weight basis (mg/kg body weight/day).

Within the NHANES survey data, the percentage of users of beverage products to which PQQ may be added was low among the total population (12.1%). The highest prevalence of potential consumers within a particular age group was estimated for male teenagers at 25.8%.

Table IV.A.1-1 Summary of the Estimated Daily Intake of PQQ Disodium Salt from Proposed Food-Uses in the U.S. by Population Group (2011-2012 NHANES Data)

Population Group	Age Group (Years)	All-Person Consumption (mg/day)		All-Users Consumption (mg/day)			
		Mean	90th Percentile	% Users	n	Mean	90th Percentile
Children	3 to 11	1.1	2.5	12.8	175	8.3	19.3
Female Teenagers	12 to 19	2.2	6.3	16.6	71	13.2	24.7*
Male Teenagers	12 to 19	2.3	9.2	25.8	102	8.7	14.0
Female Adults	20 and up	1.1	Not available	8.1	138	13.3	30.8
Male Adults	20 and up	2.2	5.1	14.0	256	15.3	30.5
Total Population	All Ages	1.6	3.2	12.1	777	12.8	27.8

* Indicates an intake estimate that may not be statistically reliable, as the sample size does not meet the minimum reporting requirements.

Table IV.A.1-2 Summary of the Estimated Daily Per Kilogram Body Weight Intake of Pyrroloquinoline Quinone (PQQ) from Proposed Food-Uses in the United States by Population Group (NHANES 2011-2012 Data)

Population Group	Age Group (Years)	All-Person Consumption (mg/kg bw/day)		All-Users Consumption (mg/kg bw/day)			
		Mean	90th Percentile	% Users	n	Mean	90th Percentile
Children	3 to 11	0.04	0.08	12.8	175	0.32	0.77
Female Teenagers	12 to 19	0.04	0.10	16.5	67	0.24	0.48*
Male Teenagers	12 to 19	0.03	0.12	25.9	102	0.13	0.29
Female Adults	20 and up	0.02	Not available	8.0	135	0.20	0.47
Male Adults	20 and up	0.03	0.06	14.1	253	0.18	0.41
Total Population	All Ages	0.03	0.05	12.1	767	0.21	0.48

* Indicates an intake estimate that may not be statistically reliable, as the sample size does not meet the minimum reporting requirements.

IV.A.2 Background Dietary Intake of PQQ from Natural Occurrence in Food Supply

PQQ has been detected in a variety of foods; however, background concentrations of PQQ in foods are low, typically in the ppb range (Kumazawa *et al.,* 1993, 1995; Noji *et al.,* 2007). In general, foods originating from plants (*i.e.,* parsley, green pepper, spinach, *etc*.) and fermented food products (*i.e.,* miso, natto, tofu, *etc*.) appear to contain greater levels of PQQ relative to foods originating from animals. Using a redox cycling method, Paz *et al*. (1988, 1989) reported

levels of 574 to 16,500 ng PQQ/mL in eggs and skim milk. However, Kumazawa *et al*. (1993) using a gas chromatography method reported levels of PQQ in eggs and milk to be substantially lower, 4.1 to 28.3 and 3.4 ng/mL, respectively. A summary of PQQ concentrations in various foodstuffs is presented in Table IV.A.2-1 below.

Table IV.A.2-1 Levels of PQQ in Common Foods

Food Item	PQQ Content (ng/g wet weight or ng/mL)	Food Item	PQQ Content (ng/g wet weight or ng/mL)
Broad bean	17.8	Green soybeans	9.26
Potato	16.6	Sweet potato	13.3
Parsley	34.2	Cabbage	16.3
Carrot	16.8	Celery	6.33
Green pepper	2.12 to 28.2	Spinach	7.0 to 21.9
Tomato	9.24	Apple	6.09
Banana	12.6	Kiwi fruit	27.4
Orange	6.83	Papaya	26.7
Field mustard	5.54	Broccoli sprout	1.55
Japanese radish	0.70	Rape blossom	5.44
Green tea	0.16 to 29.6	Miso (bean paste)	16.7
Coke	20.1	Fermented soybeans (natto)	61.0
Wine	5.79	Fermented soybeans	1.42
Oolong (tea)	27.7	Tofu (bean curd)	24.4
Whiskey	7.93	Skim milk (lyphosilisate)	2.5[1]
Sake	3.65	Milk	3.4
Beer	1.66	Egg yolk[2]	7.0 to 19.3
Bread	9.14	Egg white[2]	4.1 to 28.8

PQQ = pyrroloquinoline quinone
Adapted from Kumazawa *et al*. (1993, 1995); Noji *et al*. (2007).
[1] Units for skim milk lyphosilisate are ng/g dry weight.
[2] Eggs were obtained from domestic fowl (*Gallus gallus*) and duck (*Cairina moschata*)

Trace levels of PQQ have been reported in human tissue obtained from cadavers during forensic autopsies at levels up to 5.9 ng/g wet tissue, the highest levels reported were in the spleen, pancreas, lung, and kidney; none was reported in the brain or heart (Kumazawa *et al*., 1992). The levels of PQQ in human milk were reported to range from 140 to 180 ng/mL (Mitchell *et al.,* 1999); however, this has primarily been attributed to the combined detection of PQQ and its corresponding imidazolopyrroloquinoline (IPQ) derivatives rather than the detection of free PQQ alone.

No mammalian biosynthetic pathway has been reported for PQQ; endogenous tissue levels of PQQ in humans are derived exclusively from dietary exposure[2]. Harris *et al.* (2013) reported

[2] Production of PQQ from common intestinal microbiota such as *Escherichia coli* has been reported to be negligible in the overall contribution to PQQ exposure in humans (Matsushita et al., 1997).

the daily consumption of PQQ and its derivatives in humans to be 0.1 to 1.0 mg/day on the basis of compositional data in food (Kumazawa *et al.*, 1992, 1995; Mitchell *et al.*, 1999). Rucker *et al.* (2009) suggest a value of 1 to 2 mg/day based on total PQQ derived from the parent compound and IPQ metabolites. In light of these estimates, background levels are not expected to add appreciably to the total dietary exposure to PQQ following the introduction of PQQ disodium salt to the food supply from GRAS food uses.

IV.B Biological Role of PQQ

PQQ is an aromatic heterocyclic orthoquinone that was initially identified as a bacterial cofactor for primary alcohol dehydrogenases (Hauge, 1964; Anthony and Zatman, 1967; Salisbury *et al*., 1979; Duine and Jongejan, 1989) and is commonly produced in gram-negative, methanol-utilizing bacteria (Urakami *et al*., 1992). In *Hyphomicrobium X*, PQQ is biosynthesized from L-tyrosine and L-glutamic acid precursors (van Kleef and Duine, 1988) and similar amino acid requirements have been observed for PQQ production in *Methylobacterium* AM1 (Houck *et al.*, 1988).

Despite the natural presence of PQQ within the background dietary intake of humans and detection in human tissues (Kumazawa *et al.*, 1992), the significance of PQQ in mammalian systems remains controversial. Deprivation studies in rodents fed chemically-defined diets have reported its importance to reproductive performance and neonatal growth (Killgore *et al.*, 1989; Steinberg *et al.*, 1994, 2003). PQQ may have beneficial effects in terms of neuroprotection (Yamaguchi *et al*., 1993; Bishop *et al*., 1998; Rucker *et al*., 2009) and mitochondrial function (Bauerly *et al.*, 2006; Stites *et al*., 2006). Furthermore, in light of the ability of PQQ to act as a redox cycling agent through a semiquinone intermediate (Paz *et al*., 1990, 1996; Stites *et al.*, 2000), PQQ has been investigated as an antioxidant under conditions of oxidative stress (Bishop *et al.,* 1998; Rucker *et al.*, 2009; Misra *et al.*, 2012). Although some investigators suggested that PQQ may be an essential vitamin, identification of a mammalian enzyme that requires PQQ as an essential cofactor remains controversial (Kasahara and Kato, 2003; Felton and Anthony, 2005; Rucker *et al.*, 2005; Matsumura *et al.*, 2014).

IV.C Metabolic Fate

There is limited information on the pharmacokinetics and metabolic fate of PQQ. Smidt *et al.* (1991) reported a pharmacokinetic study in which male Swiss-Webster mice (n=5/time point) were gavaged with 28 µg of ^{14}C-radiolabeled PQQ that was biosynthetically derived from the incorporation of ^{14}C-tyrosine by PQQ-producing *Methylobacterium extorquens* AM1. Tissue and urine samples were extracted 6 and 24 hours after administration of PQQ. Samples were solubilized and decolorized prior to the measurement of radioactivity using a scintillation spectrometer. Absorption of PQQ was reported to occur primarily in the lower intestine and approximately 62% of the given dose of radioactivity was absorbed within 24 hours. Of the

amount absorbed, approximately 81% was excreted *via* the urine, while 1.5, 1.2, and 1.3% remained in the liver, red blood cells, and skin, respectively. Consistent with the urinary route of excretion, 11% of the absorbed PQQ was observed in the kidney within 24 hours of PQQ administration. PQQ accumulated primarily in the kidney and skin on the basis of a 3- and 4-fold increase in radioactivity, respectively, from 6 to 24 hours post-PQQ administration. However, it should be noted that the detection of PQQ *via* radioactivity does not qualitatively distinguish between the contributions from the parent PQQ compound *versus* any of its metabolites. No ^{14}C-radioactivity was detected in the expired carbon dioxide.

There is indirect evidence to suggest that PQQ is excreted *via* the feces. Rodent feeding studies have consistently reported findings of discolored stools attributed to the dark color of the test compound (PQQ is a brown henna colored powder with a characteristic green fluorescence). In particular, dark black/green-colored contents in the cecum and greenish stools have been observed in rats fed PQQ at dose levels ≥100 mg/kg body weight/day (Nakano *et al.*, 2014). Based on the apparent slow absorption of PQQ over 24 hours and limited radioactivity localized in the liver of mice administered radiolabeled PQQ, the fecal coloring is likely attributed to poor absorption of PQQ rather than biliary secretion of absorbed PQQ. An explanation for the green coloration is unknown as PQQ is a reddish-brown henna colored powder; however the material is reported to display a green fluorescence. Quantitative estimates of PQQ absorption and elimination *via* urine and fecal routes are not available.

The kinetics of PQQ were evaluated in 10 healthy subjects (5 male and 5 female, mean age of 28.1 years) (Harris *et al.*, 2013). Subjects were administered a single dose of PQQ (Mitsubishi Gas Chemical Co.; purity not specified) formulated in an orange drink at a dose providing 0.2 mg PQQ/kg body weight (equivalent to 14 mg PQQ for a 70 kg individual). Blood samples were obtained at 0, 2, 4, 8, 24, or 48 h post-administration and PQQ was measured using a glucose dehydrogenase-based assay system. Serum levels of PQQ peaked at 2 hours. Maximum serum concentrations of 9 nM (~3.4 ng/mL) were reported. Furthermore, in healthy subjects (5/sex) administered PQQ orally in a fruit-flavored drink at graduating dose levels of 0.075, 0.15, and 0.3 mg/kg body weight/day (corresponding to approximately 5.25, 10.5, and 14 mg PQQ/day for a 70 kg individual) over 3 consecutive 7-day periods (total study period of 21 days), approximately 0.1% of the PQQ administered was recovered in the urine as non-derivatized (free) PQQ (Harris *et al*., 2013). Analysis of serum and urine samples for PQQ metabolites was not conducted.

No studies were identified on the metabolic fate of exogenously consumed PQQ *per se*; however, PQQ has been reported to undergo condensation with amino acids to form IPQ derivatives (Ishida *et al*., 1995; Urakami *et al.,* 1995-1996), which have been identified endogenously in human milk (Mitchell *et al.,* 1999; Kumazawa *et al.,* 2000). Furthermore, considering the ability of PQQ to undergo repeated oxidation and reduction as a redox cycling

IV.D Toxicological Studies

Toxicity studies conducted with PQQ manufactured by Hisun are limited to acute toxicity evaluations in ICR mice and to *in vitro*/*in vivo* genotoxicity studies; however, the acute and repeated-dose toxicity (up to 90 days) of a high purity PQQ disodium salt ingredient (BioPQQ >99%; Mitsubishi Gas Chemical Co., Inc.), produced by *Hyphomicrobium* fermentation, has been evaluated by Nakano *et al*. (2013). The test articles used in these studies are of similar purity to PQQ manufactured by Hisun, and use a similar production process, fermentation by *Hyphomicrobium* species. The results of these studies were considered applicable to the safety assessment of Hisun's PQQ ingredient on the basis of the compositional similarities. High purity PQQ ingredients manufactured by Shanghai Med Co. and Shanghai Rixin Biotechnology Co. Ltd. have also been the subject of toxicity testing. Although details of the manufacturing processes of the PQQ ingredients used in these studies have not been identified, these studies were evaluated within the safety assessment as corroborative studies. A comparison of the PQQ products reviewed below is presented in Table IV.D-1.

Table IV.D-1 Comparison of the PQQ Test Articles Used in the Animal Studies

Specification Parameter	Source			
	Hisun	**Mitsubishi Gas Chemical Co., Inc.**	**Shanghai Med Co., Ltd.**	**Shanghai Rixin Biotechnology Co. Ltd.**
Manufacturing Method	Fermentation by *Hyphomicrobium sp.*	Fermentation by *Hyphomicrobium sp.*[1]	Not specified	Not specified
Appearance	Henna powder	Reddish brown crystalline powder	Reddish brown crystalline powder	Orange red to dark red crystalline powder
Purity	>99%	>99%	>98%	Not specified

PQQ = pyrroloquinoline quinine
[1] Urakami *et al*. (1992); Urakami (1994 - Patent US5344768)

IV.D.1 Acute Toxicity Studies

PQQ disodium salt is of low oral toxicity in mice and rats (Table IV.D.1-1). The LD_{50} of PQQ disodium salt in male and female ICR mice was reported to be 4.22 and 5.84 g/kg body weight (Zhejiang Hisun Pharmaceutical Co., Ltd., 2012). In another study, the oral LD_{50} of PQQ was reported be 2.71 and 3.69 g/kg body weight for male and female mice, respectively (Wang *et al.*, 2012). In male and female Sprague-Dawley rats, the oral LD_{50} of PQQ disodium salt was reported to range between 1 to 2 g/kg body weight for males and 0.5 to 1 g/kg body weight for females (Nakano *et al.*, 2014). Loose stools and diarrhea (dose-dependent) were observed shortly after PQQ administration and a dose-dependent discoloration (greenness) was consistently observed in intestinal contents and stools upon necropsy of animals that had received the PQQ disodium salt (Zhejiang Hisun Pharmaceutical Co., Ltd., 2012; Nakano *et al.*, 2014). Body weight gain was suppressed in rats given doses of 1,000 or 2,000 mg PQQ/kg

body weight and whitish or enlarged kidneys were reported upon necropsy of rats from the same 2 dose groups (Nakano *et al.*, 2014).

PQQ has been reported to be toxic to the kidneys when administered *via* the intravenous or intraperitoneal route. For example, as reported by Watanabe *et al*. (1989) intraperitoneal injection of PQQ to male Wistar rats (360 to 430 g) daily for 4 days resulted in necrotic and degenerative changes of the proximal tubular epithelium and hematuria and elevation in serum creatinine levels. Kumar *et al*. (2015) reported that intraperitoneal administration of PQQ at a dose of 15 mg/kg for 15 days was protective against renal tubular necrosis in streptozotocin induced diabetes model using male Swiss albino mice (30±2 g). These corroborate the available absorption, distribution, metabolism, and excretion (ADME) information on PQQ indicating that the compound, a charged acidic molecule, is poorly absorbed in rodents as no histopathological evidence of kidney toxicity has been observed in when PQQ is administered *via* the oral route during subchronic toxicity evaluations at does up to 400 mg/kg body weight per day (Liang *et al*., 2014; Nakano *et al*., 2014).

Table IV.D.1-1 Summary of Acute Toxicity Studies on PQQ Disodium Salt

Species/Strain	Compound	Dose/Route of Administration	LD_{50}	Reference
Mice				
ICR 5/sex/group	PQQ disodium salt[1]	1.0 to 21.5 g/kg bw Oral gavage	♂: 4.22 g/kg bw ♀ : 5.84 g/kg bw	Zhejiang Hisun Pharmaceutical Co., Ltd. (2012)
ICR Number/group NR	PQQ[2]	Dose ranges NR Oral	♂: 2.71 g/kg bw ♀: 3.69 g/kg bw	Wang *et al.* (2012)
Rats				
Sprague-Dawley [Crl:CD(SD)] 10/sex/group	PQQ disodium salt[3]	0, 500, 1,000, or 2,000 mg/kg bw Oral gavage	♂: 1,000 to 2,000 mg/kg bw ♀: 500 to 1,000 mg/kg bw	Nakano *et al.* (2014)

♂ = male; ♀ = female; bw = body weight; CDC = Center for Disease Control and Prevention; LD_{50} = median lethal dose; NR = not reported; PQQ = pyrroloquinoline quinone.
[1] Manufactured by Hisun, purity NR.
[2] Provided by Shanghai Rixin Biotechnology Co. Ltd., purity NR.
[3] Manufactured by Mitsubishi Gas Chemical Co., Inc. as BioPQQ™, >99.1% purity.

IV.D.2 Repeated-Dose Studies

The repeated dose toxicity of high purity crystalline PQQ (Mitsubishi Gas Chemical Co.; BioPQQ™; >99.1% purity), produced by *Hyphomicrobium* sp., was evaluated in 14-day, 28-day, and 90-day studies performed in consistent with GLP and the guidelines established by the Japanese Ministry of Health and Welfare for single and repeated-dose toxicity study of drugs (MHLW, 1993, 1997). These studies also were conducted in accordance with the Organization for Economic Co-Operation and Development (OECD) test guidelines 407 and 408 (OECD, 1998, 2008).

In the initial 14-day dose-range finding study, no remarkable clinical observations or mortalities were reported in 5-week-old Sprague-Dawley rats (6/sex/group) administered PQQ at doses of 0, 3, 12, 48, 192, or 768 mg/kg body weight/day by gavage (Nakano *et al.*, 2014). No differences were reported in body weight or food consumption. Green-colored feces were observed in some rats receiving 192 mg/kg body weight/day (1/6 male) or 768 mg/kg body weight/day (2/6 rats of each sex); however, feces were normal-shaped. Although some changes were reported in hematology and clinical chemistry parameters in the PQQ treated rats, they were within the normal historical range and deemed unrelated to the treatment. A significant increase in urinary sodium levels in the high-dose PQQ group (768 mg/kg body weight/day; both sexes), was reported; there were no other changes. Green-colored cecal contents were reported in the highest dose group (4 males, 5 females). A significant increase in relative kidney weight (approximately 14%, $p<0.05$) in female rats administered PQQ at the highest dose was reported, along with focal basophilic changes and atrophy in the renal tubules of minimal to moderate severity. The renal effects were limited to females. No other toxicologically relevant histopathological changes were reported.

In light of these results, a 28-day follow-up study was conducted in female Sprague-Dawley rats (12/group) to investigate the potential renal-toxic effects of PQQ at doses of 0, 200, or 700 mg/kg body weight/day (Nakano *et al.*, 2014). No mortalities or remarkable clinical observations, body weight, or food and water consumption were reported. Black-colored feces observed in the high-dose group were again attributed to the coloration of the test article and not considered a toxicological effect. A few apparent non-treatment-related changes were reported, including elevations in urinary protein and urinary crystals of slight to moderate severity in all PQQ treatment groups. There were no corresponding changes in clinical chemistry or histopathological lesions observed with regard to renal function or damage. Although a significant decrease in urinary volume and a significant increase in urine specific gravity were observed in the low dose PQQ group, these changes were deemed to be unrelated to the test-compound since they were transient, did not follow a dose-response relationship, and were within the range of 2 standard deviations from the mean value of the control group.

Subsequently, in the 90-day oral toxicity study no toxicologically significant differences were reported in hematology, clinical biochemistry, urinalysis, or gross necropsy and histopathology when Sprague-Dawley rats (10/sex/group) were administered PQQ at dosages of 0, 3, 20, or 100 mg/kg body weight/day by gavage; with the exception of green-colored feces, which were observed in male and female rats in the highest dose group. The no-observed-adverse-effect level (NOAEL) for PQQ was determined to be 100 mg/kg body weight/day.

No consistent treatment-related, dose-dependent adverse effects were reported in another 90-day study conducted consistent with the Technical Standards for Testing & Assessment of Health Food (2003) in Sprague-Dawley rats (12/sex/group) that received PQQ (Shanghai Rixin Biotechnology Co. Ltd.; purity not reported) by gavage at doses of 0, 3.8, 7.5, or 15 mg/kg body

weight/day[3] (Wang *et al.*, 2012). Although some differences were reported in hematology and blood biochemistry parameters, the changes were sporadic and within the range of historical controls, did not have a dose-response relationship, and were considered to be not toxicologically relevant. Similarly, some histopathological changes were observed in the liver, spleen, and gastric mucosa; however, these changes were not deemed to be compound-related effects as the incidence was low and the occurrence was similar to that of the control group. Based on these findings, the NOAEL of PQQ was determined to be 15 mg/kg body weight/day (the highest dose tested) in male and female rats.

In another 90-day study conducted in Sprague-Dawley rats (10/sex/group), PQQ disodium salt was administered by gavage at doses of 0 (control), 100, 200, or 400 mg/kg body weight/day (Liang *et al.*, 2014). PQQ disodium salt was obtained from Shanghai Med Co., Ltd. (China) and was characterized by a molecular weight of 372.17 g/mol and purity of >98%. This study was conducted in accordance with GLP and the FDA Guidance for Industry and Other Stakeholders (U.S. FDA, 2007). Through the duration of the study, clinical observations were monitored daily while body weight and food consumption were assessed weekly. Blood was collected for evaluation of hematological and clinical chemistry parameters at the mid-point (Day 46) and upon termination of the treatment period (Day 91). Rats were then euthanized for necropsy and histopathological assessment. All rats survived to the end of the study period.

PQQ disodium salt was well-tolerated and no adverse clinical reactions were reported. No significant differences were reported in body weight or food consumption. Although some significant hematological and biochemical changes were reported on Day 46 (*i.e.,* an increase in the percentage of granulocytes in mid-dose females and a decrease in blood glucose levels in the low-dose male group) these changes were sporadic and not observed on Day 91. Changes in absolute and relative organ weights were unremarkable. The incidence and severity of histopathological changes were not different from the control groups at any of the treatment doses tested. Urinary parameters were not reported by the authors. Histopathological changes in the kidney were unremarkable; 1 rat in the high-dose group was reported to have deposition of calcium salts in the renal tubule (additional details not specified). The authors established a NOAEL of 400 mg/kg body weight/day, the highest dose tested.

Based on review of the available animal toxicity studies Hisun has concluded that large margins of safety exist between potential dietary exposures to PQQ from the intended food uses and the reported NOAEL values that have determined in rodent studies. NOAEL determinations of 100 mg/kg body weight and 400 mg/kg body weight as reported by Nakano *et al*., (2014) and Liang *et al*., (2014) were considered appropriate for use in the safety assessment of Hisun's PQQ ingredient.

[3] The highest dose in rats was chosen based on recommended literature doses of 3 mg PQQ/day in humans (which corresponds to 0.05 mg/kg body weight/day for a 60 kg individual) and an interspecies safety factor of 300 for humans to rats (*i.e.,* 3 mg PQQ/day/60 kg individual*300-fold interspecies safety factor = 15 mg/kg body weight/day). The underlying rationale for the safety factor was not provided.

IV.D.3 Other Animal Studies

Several studies examining the relationship between PQQ and reproductive parameters in mice have suggested that PQQ is required for proper neonatal development and growth (Killgore *et al.*, 1989; Steinberg *et al.*, 1994, 2003). These studies were designed to determine the effects of PQQ deprivation on reproductive performance and growth by giving mice chemically-defined, nutritionally complete, amino acid-based diets deficient in PQQ[4] and comparing the effects to PQQ-supplemented diets.

Killgore *et al.* (1989) observed impairment of growth in mice (strain not reported) exposed to PQQ-deficient diets that had been born to dams which had also been given PQQ-deficient diets for 2 to 3 weeks prior to mating. A significant reduction in body weight was observed within 5 weeks post-weaning in mice given PQQ-deficient diets, in comparison to mice that had been given PQQ-supplemented diets (800 ng PQQ/g diet) throughout the study. Approximately 20 to 30% of the mice in the PQQ-deficient group were also observed to have clinical signs of toxicity including friable skin, mild alopecia, and a hunched posture that was not observed in mice that had been given the PQQ-supplemented diet. These effects were reported to be abolished upon PQQ supplementation in the diet. By Week 8, a 20% incidence of mortality was reported in the PQQ-deprived group, whereas a 3% incidence of mortality was observed in the PQQ-supplemented group. When female mice were given the PQQ-deficient diet 8 to 9 weeks prior to breeding, no litters were produced or offspring were cannibalized upon birth.

Subsequently, Steinberg *et al.* (1994, 2003) reported that administration of PQQ-deficient diets to weanling female BALB/c mice throughout development and pregnancy, gestation, and lactation significantly impaired fertility, and reduced the litter size and survival of offspring in comparison to groups that were given PQQ-supplemented diets (≥300 ng PQQ/g diet). Surviving offspring from the dams given the PQQ-deficient diet were observed to have transient weight reduction during the neonatal period (up to 10 to 12 weeks after birth) when fed the same PQQ-deficient diets following weaning; however, no changes in body weight were observed between groups by 5 months termination of the study. These data suggest an important role for PQQ in growth and development during the neonatal period.

BALB/c mice born from dams that had been given PQQ-deficient diets for 1 to 2 months before mating, and subsequently weaned to PQQ-deficient diets onwards for 20 weeks after birth, had significantly decreased mitogenic reactivity of splenocytes to concanavalin A and lipopolysaccharide stimulation compared to mice given ≥200 ng PQQ/diet (Steinberg *et al.*, 1994). Interleukin 2 (IL-2), an autocrine and paracrine growth factor important for immune function produced from the splenic T-cells, was similarly decreased in mice fed a PQQ-deficient diet in comparison to mice that had been given PQQ supplementation.

[4] Mice were also given antibiotics to prevent the production of PQQ by gut microflora.

These studies have been the subject of much controversy as they are suggestive that PQQ may be a previously uncharacterized nutrient necessary for proper development in mammals. The debate continues concerning whether PQQ is a mammalian enzyme co-factor (Kasahara and Kato, 2003; Felton and Anthony, 2005). Findings from these studies were not considered relevant to the safety assessment.

PQQ was reported to be protective when evaluated in rat models of oxidative stress-associated pathology including liver injury induced by various hepatotoxic substances such as carbon tetrachloride, D-galactosamine, thioacetamide, and allyl formate (Watanabe *et al.*, 1989; Tsuchida *et al.*, 1993; Urakami *et al.*, 1997), ethanol-induced liver damage (Hobara *et al.*, 1988; Singh *et al.*, 2014), and l-thyroxine-induced hyperthyroidism (Kumar *et al.*, 2014). PQQ was also shown to improve memory retention in a model of oxidative stress induced memory impairment in rats (Ohwada *et al.*, 2008). However, these studies are of limited relevance to the assessment of the safety of PQQ as the measurement of toxicological parameters was limited in the context of disease states.

Samuel and colleagues (2015) evaluated the effects of dietary PQQ on growth performance, carcass yield and antioxidant status of broiler chicks. The study was conducted in 784 one-day-old male Arbor Acres broilers. Birds were randomly allotted into 1 of 7 dietary groups. The negative control group (NC) was fed a basal diet without antibiotics or PQQ. Birds in the positive control/treatment groups were provided a diets containing antibiotics and increasing quantities of PQQ (0, 0.05, 0.10, 0.20, 0.40, or 0.8 mg PQQ/kg diet) for 42 days. Dietary administration of PQQ was not associated with adverse effects in the broilers and overall improvements in growth performance were associated with PQQ administration. The authors concluded that dietary PQQ had the potential to act as a growth promoter comparable to antibiotics in broiler chicks.

Additionally, the effect of PQQ disodium (≥99.5%; Shanghai Medical Life Sciences Research Center Co. Ltd., Shanghai, China) supplementation in broiler chicks was evaluated by Wang *et al*. (2015). Male 1-day-old Arbor Acres broiler chicks (7 replicates of 14 chicks/group) were administered 0, 0.05, 0.1, 0.2, or 0.4 mg PQQ disodium/kg feed[5] for a period of up to 42 days. Through the study, no differences in mortality rate were observed in any of the groups nor were any adverse health effects or differences in carcass quality reported. Feed conversion ratios (*i.e*., feed: gain) were significantly reduced during the overall treatment period in chicks fed up to 0.2 mg/kg PQQ disodium/kg feed (maximum reduction of 7%, p=0.039). Changes in plasma biochemical parameters were minimal; while plasma albumin levels were significantly increased in the 0.2 mg PQQ disodium/kg feed group relative to control, the changes were modest (9.1%, p=0.037) and not dose-responsive. Total protein in the plasma also increased quadratically in a dose-response manner upon PQQ supplementation (p=0.037). A subsequent trial with another

[5] Equivalent to approximately 0, 0.005, 0.01, 0.02, or 0.04 mg/day based on an average daily feed intake of 96 mg/day during the whole test period.

set of 1-day-old male chicks (6 replicates of 10/group) confirmed the reduction in feed conversion ratio in response to the supplementation of 0.2 mg PQQ disodium/kg feed relative to the unsupplemented group. On the basis of these findings, the authors concluded that PQQ disodium induces growth-promoting effects in broiler chicks. Moreover, no growth-inhibiting effects were observed in the unsupplemented diet, which provided basal levels of PQQ of approximately 18 ng/g feed.

The studies in broiler chicks are of limited relevance to the safety assessment; however, considering that broiler chicks are rapidly growing animals, which can be sensitive to dietary manipulations, the findings are consistent with overall observations that PQQ is not toxic or detrimental to animal growth.

IV.D.4 Mutagenicity and Genotoxicity Studies

PQQ has consistently been reported to be negative in the reverse mutation (Ames) assay (Wang *et al.*, 2012; Zhejiang Hisun Pharmaceutical Co., Ltd., 2012; Nakano *et al.*, 2013), the *in vivo* micronucleus assay (Wang *et al.*, 2012; Zhejiang Hisun Pharmaceutical Co., Ltd., 2012; Nakano *et al.*, 2013), and in chromosomal aberration tests (Zhejiang Hisun Pharmaceutical Co., Ltd., 2012; Nakano *et al.*, 2013). These studies are summarized in Table IV.D.4-1. Although a modest but significant increase (0.5 to 4%) in structural chromosomal aberrations was reported in Chinese hamster lung fibroblasts (CHL/IU) when incubated with PQQ at dose levels of 200 µg/mL in the absence of S9 metabolic activation, these changes were below the historical 5% threshold for a positive response and repeat testing showed no significant differences in chromosomal aberrations (Nakano *et al.*, 2013). No differences were reported in the incidence of structural chromosomal aberrations and polyploidy when human peripheral blood lymphocytes were incubated with PQQ in the presence or absence of metabolic activation. No changes in the number of micronucleated polychromatic erythrocytes were reported when male Crlj:CD1 mice (6/group) were administered PQQ by gavage at doses up to 2,000 mg/kg body weight, or when received over 2 doses separated by a 24-hour period in an i*n vivo* micronucleus assay (Nakano *et al.*, 2013). These studies demonstrate that PQQ disodium salt is of low genotoxic concern *in vivo*.

Table IV.D.4-1 Summary of Mutagenicity and Genotoxicity Studies with PQQ					
Assay	**Test System**	**Test Compound/Dose/Route of Administration**	**Metabolic Activation**	**Results**	**Reference**
In vitro					
Reverse Mutation Assay[1]	*Salmonella typhimurium* TA97, TA98, TA100	PQQ disodium salt[2] 1.6, 8, 40, 200, or 1,000 µg/plate	+/- S9	Negative	Zhejiang Hisun Pharmaceutical Co., Ltd. (2012)
	S. typhimurium TA102	PQQ disodium salt[2] 0.16, 0.8, 4, 20, or 100 µg/plate	+/- S9	Negative	
Reverse Mutation Assay[3]	*S. typhimurium* TA97, TA98, TA100, TA102	PQQ[2] 8, 40, 200, 1,000, or 5,000 µg/plate	+/- S9	Negative	Wang *et al.* (2012)
Reverse Mutation Assay[4]	*S. typhimurium* TA98, TA100, TA1535, TA1537 *and E. coli* WP2*uvrA*	BioPQQ™ [5] 0, 10, 20, 39, 78, 156, 313, 625, 1,250, 2,500, or 5,000 µg/plate[1] (-S9) 0, 156, 313, 625, 1,250, 2,500, or 5,000 µg/plate[1] (+S9)	+/- S9	Negative	Nakano *et al.* (2013)
Chromosomal Aberration Test[4]	CHL/IU cells	BioPQQ™ [5] 0, 12.5, 25, 50, 100, 200, or 400 µg/mL[1] (-S9) 0, 117.2, 234.4, 468.8, 937.5, 1875, or 3,750 µg/mL[1] (+S9)	+/- S9	Modest ↑ structural chromosomal aberrations(0.5 to 4%, $p<0.01$) at dose levels of 200 µg/mL (-S9); however, repeat testing was negative	Nakano *et al.* (2013)
	Human PBLs	BioPQQ™ [5] 0, 234.4, 468.8, 937.5, 1,875, or 3,750 µg/mL[1]	+/- S9	Negative	
In vivo					
Micronucleus Assay[1]	ICR mice 5/sex/group	PQQ disodium salt[2] 0, 0.73, 1.46, or 2.92 g/kg bw/day for 2 days Oral	NA	Negative	Zhejiang Hisun Pharmaceutical Co., Ltd. (2012)

Table IV.D.4-1 Summary of Mutagenicity and Genotoxicity Studies with PQQ					
Assay	**Test System**	**Test Compound/Dose/Route of Administration**	**Metabolic Activation**	**Results**	**Reference**
Micronucleus Assay[3]	Mice [ICR 5/sex/ group	PQQ[2] ♂: 0, 338.75, 677.5, or 1,355 mg/kg bw (over 2 doses within 30 h) ♀: 0, 461.25, 922.5, or 1, 845 mg/kg bw (over 2 doses within 30 h) Oral gavage	NA	Negative	Wang *et al.* (2012)
Micronucleus Assay[6]	Crlj:CD1 mice 6♂/group	BioPQQ™ [5] 0, 250, 500, 1,000, or 2,000 mg/kg bw (over 2 doses separated by 24 h interval)[1] Oral gavage	NA	Negative	Nakano *et al.* (2013)
Chromosomal Aberration Test[1]	ICR mice 5/sex/group	PQQ disodium salt[2] ♂: 0, 0.53, 1.05, or 2.11 g/kg bw/day for 4 days ♀: 0, 0.73,1.46, or 2.92 g/kg bw/day for 4 days Oral gavage	NA	Negative	Zhejiang Hisun Pharmaceutical Co., Ltd. (2012)

♂ = male; ♀ = female; bw = body weight; CDC = Center for Disease Control and Prevention; CHL/IU = Chinese hamster lung fibroblasts; NA = not applicable; NR = not reported; PBL = peripheral blood lymphocyte; PQQ = pyrroloquinoline quinone; S9 = metabolic activation.

[1] Conducted in accordance to Ministry of Health's "Test and evaluation technical regulations of health food" (2003).

[2] No other compositional details provided. Purity NR.

[3] Conducted in accordance to Technical Standards for Testing & Assessment of Health Food (2003)

[4] Conducted in accordance to "Guidelines for Genotoxicity Studies of Drugs'' (Notification No. 1604, Evaluation and Licensing Division, Pharmaceutical and Medical Safety Bureau, Ministry of Health and Welfare, Japan, November 1, 1999).

[5] BioPQQ™ as manufactured by Mitsubishi Gas Chemical Co., Inc. is >99.3% purity.

[6] Conducted in accordance to the Law for Partial Amendments to the Law concerning the Protection and Control of Animals (2005) and the research facility's Guideline for Animal Care and Use.

IV.E Human Studies

Human studies have shown that the oral ingestion of PQQ is well-tolerated at doses ranging from 20 to 60 mg/day. As reviewed by Rucker *et al.* (2009), PQQ administered at 20 or 60 mg/day to healthy subjects (10/group; sex not specified) for 4 weeks, in a double-blind study conducted at 2 commercial drug testing facilities (New Drug Clinical Center, Fukuhara Clinic, Eniwa, Hokkaido, Japan and Cronova Co., Ltd., Suminoeku, Osaka, Japan), did not induce any adverse events in standard clinical tests that included glucose, triglycerides, various lipoprotein fractions, as well as markers of liver toxicity such as aspartate aminotransferase and serum glutamic oxaloacetic transaminase. Similarly, in a cross-over study conducted in healthy human subjects (5/sex) in which PQQ was consumed in a fruit-flavored drink as a single dose of 0.2 mg/kg body weight (equivalent to 14 mg for a 70 kg individual) or a daily dose of 0.3 mg/kg body weight (equivalent to 21 mg/kg body weight/day for a 70 kg individual) for 3 days, standard clinical indices including total cholesterol, creatine, glucose, low-density lipoprotein, high-density lipoprotein, triglycerides, uric acid, total protein, and aspartate aminotransferase were within normal ranges compared to baseline values (Harris *et al.,* 2013).

Several additional studies evaluating various efficacy related effects of PQQ also were identified in the literature. These studies were not designed to evaluate the safety of PQQ and did not obtain/report clinical safety measures. These studies were of limited value to the safety assessment of PQQ. A summary of these studies is presented in Table IV.E-1. No specific findings were reported by the authors in these studies that raise concerns about dietary consumption of PQQ at the levels consumed in the trials.

Table IV.E-1 Summary of Human Studies Investigating the Ingestion of PQQ

Study Design/ Objective	Study Population	PQQ Dose/ Duration	Major findings	Reference
Open-label trial to evaluate the effect of PQQ on stress, fatigue, and sleep	Healthy subjects with diagnosed sleep disorder or complaint and fatigue[2] 7♂, 10♀ 38.1±7.4 years of age	20 mg/day [1] (capsule) 8 weeks	• No AE reported. • Transient ↓ SBP at Week 4 that recovered by Week 8, no other effects on BP. • No changes in anthropometry, HR, blood and urine tests.	Nakano *et al.* (2012)
DB, PCT to determine the effect of PQQ on memory and cognitive performance.	Healthy subjects Control: 8♂, 15♀ PQQ: 9♂, 13♀ 45 to 65 years of age	0 or 20 mg/day[1] (capsule) 12 weeks	• No AE reported. • Test values during physical and clinical examinations were normal through study duration.	Nakano *et al*. (2009)

Table IV.E-1 Summary of Human Studies Investigating the Ingestion of PQQ				
Study Design/ Objective	**Study Population**	**PQQ Dose/ Duration**	**Major findings**	**Reference**
DB, PCT to determine the effect of PQQ on cognitive functions in the elderly.	Healthy subjects Control: 7♂, 13♀ PQQ: 7♂, 14♀ Control: 58.4±5.2 years of age PQQ: 58.6±5.1 years of age	0 or 20 mg/day[1] (capsule) 12 weeks	• No AE reported. • No abnormal values in blood or urinary measures[3]. • No adverse internal or physical examination observations.	Itoh *et al.* (2016)
DB, PCT to investigate the effect of PQQ on memory and higher brain function.	Healthy subjects with self-identified forgetfulness Control: 7♂, 15♀ PQQ: 7♂, 15♀ 50 to 70 years of age	0 or 20 mg/day[1] (capsule) 24 weeks	• No AE reported.	Koikeda *et al.* (2011)
Crossover study to evaluate the effect of PQQ on inflammation and mitochondrial-related metabolism	Healthy subjects 5♂, 5♀ 21 to 34 years of age	0.2 mg/kg bw[1] (fruit-flavored drink) 1 day	• No AE were specified in the study report. • Decrease in TBARS over time (~0.2% decrease/hour, p<0.05) • NSD TRAP.	Harris *et al.* (2013)
		0.3 mg/kg bw/day[1] (fruit flavored drink) 3 days	• No AE were specified in the study report. • Reduction in CRP(~50%; p<0.05) and IL-6 (~33%; p<0.05) from baseline	

♂ = male; ♀ = female; AE = adverse events; BP = blood pressure; bw = body weight; CRP = c-reactive protein; DB = double blind; HR = heart rate; NR = not reported; IL-6 = interleukin 6; NSD = no significant difference; PCT = placebo-controlled trial; PQQ = pyrroloquinoline quinone; SBP = systolic blood pressure; TBARS = thiobarbituric acid reactive products; TRAP = total peroxyl radical trapping potential.
[1] Manufactured by Mitsubishi Gas Chemical Co., Inc. as BioPQQ™, purity NR.
[2] Selected based on criteria from the Athens Insomnia Scale (AIS) (>6 points) and the Profile of Mood States-Short Form (POMS-S) (>50 points in T score of fatigue and <50 points in the T score of vigor).
[3] Specific parameters measured in blood and urine samples were not disclosed in the publication.

IV.F Safety of the Source Organism

As previously discussed in Section II.B.1.1, the source organism, *H. denitrificans,* is a gram-negative, non-spore-forming, rod shaped appendaged bacteria that reproduce by budding (Rainey *et al*., 1998). Hyphomicrobia are restricted factultative mythylotrophs that utilize one carbon compounds, such as methanol, monomethylamine, dimethylamine, trimethylamine, pectin, and acetate. *Hyphomicrobium denitrifican* cells do not grow in nutrient broth or peptone broth. Some strains are reported to grow on formate and ethanol. This species cannot ferment sugars, is negative for gelatin and starch hydrolysis, does not produce ammonia, and is oxidase, urease, and catalase positive. The species do not growth at 37 or 42°C and cannot utilize arabinose, xylose, glucose, mannose, glactose, maltose, sucrose, lactose, trehalose, sorbitol, manniitol, inositol, glycerol, soluble starch, propionic acid, isobutyric acid, valeric acid,

lactic acid succinic acid, and oxalic acid. Vitamins and amino acids are not required for growth (Urakami *et al*., 1995). *Hyphomicrobium* sp. are ubiquitous in the environment and are often present in waste-water treatment environments due to their propensity for denitrification and remediation of C_1 compounds (halomethanes, methyl sulphates and methylated phosphates), which many bacteria cannot effectively metabolize (Rainey *et al*., 1998).

The production of PQQ by *Hyphomicrobium* sp. (strain TK0145) was first reported in the literature by Urakami and colleagues through joint research conducted by the Mitsubishi Gas Chemical Co., and the University of Tokyo (Urakami *et al*., 1992). The strain TK0145 was further characterized by Urakami and colleagues in 1995, and a new species *Hyphomicrobium denitrificans* proposed (Urakami *et al*., 1995). *Hyphomicrobium denitrificans* TK 0415 was designated the type strain for the species, and was subsequently deposited in the DSM, NCIMB, and ATCC cell-culture banks as DSM 1869, NCIMB 11706, and ATCC 51888 respectively. The complete genome of *Hyphomicrobium denitrificans* ATCC 51888 was sequenced by Brown and colleagues in 2011 (Brown *et al*., 2011). The nucleotide sequence accession number is deposited in GenBank under NC_014313, and annotation of the genome was conducted using the JGI-Oak Ridge National Library annotation pipeline. A search of the annotated genome *via* the Pathosystems Resource Integration Center (PATRIC) identified 3 homologous (*Brucella melitensis* biovar Abortus 2308) virulence genes encoding the following proteins: Imidazole glycerol phosphate synthase cyclase subunit (EC 4.1.3.-); RNA-binding protein Hfq; Dihydroxy-acid dehydratase (EC 4.2.1.9). Imidazole glycerol phosphate synthase cyclase subunit has biological roles in histidine biosynthesis, pyruvate metabolism, 1- and 2-methylnaphthalene degradation, and dihydroxy-acid dehydratase has established roles in valine, leucine and isoleucine biosynthesis as well as in pantothenate and CoA biosynthesis. None of these proteins encode potential toxins or are involved in biosynthetic pathways that would be predicted to impart undesirable phenotypes to *Hyphomicrobium* sp. The genome did not contain any known antibiotic resistance genes.

A comprehensive search of the publically available literature databases did not identify any data or information suggestive that members of the *Hyphomicrobium* genus are pathogenic or toxic to animals. The organism does have an established history of safe use in the production of PQQ salts (BioPQQ; 99% purity; Mitsubishi Gas Chemical Co., Inc.) that have been marketed globally for use in dietary supplement products. PQQ produced by *Hyphomicrobium* sp. fermentation[6] has been notified to the FDA for use as a new dietary ingredient in dietary supplement preparations without objection from the Agency (U.S. FDA, 1995).

The source organism is excluded from the manufacturing process using filter and heat sterilization steps and the absence of the *Hyphomicrobium denitrificans* in the finished product has been confirmed in 3 non-consecutive batches of the ingredient (see Section II.C.3.1). PQQ manufactured by Hisun is of high purity and also is subjected to extensive purification and

[6] Edahiro *et al*. (2012); Ikemoto and Nakano (2013); Urakami *et al*. (1992, 1995)

washing steps, no residual carry-over products of fermentation are expected in the ingredient. Protein levels in the ingredient are below the detection limit of 0.3% in all lots of material that have been produced. Based on the phenotypic and genotypic properties of the organism, the history of safe use of the organism for production of high purity PQQ salts, and implementation of appropriate controls during manufacturing, no safety concerns were identified for the source organism.

V Conclusions on the Safety of PQQ for Use in Foods

The weight of the available evidence supports the safety of the use of PQQ in foods. The safety of PQQ for its intended uses is supported by the data generally available in the public domain pertaining to the safety of PQQ and is further corroborated by its natural occurrence in the normal human diet.

Hisun's PQQ disodium salt is produced, in compliance with cGMP and according to HACCP principles, by bacterial fermentation using *Hyphomicrobium denitrificans* ATCC 51888. The source organism is not genetically modified. The *Hyphomicrobium* used for the production of PQQ disodium salt is maintained in-house by Hisun and is subject to strict quality control for compliance with established internal specifications. The results of batch analyses indicate that the manufacturing method yields a consistent product that reproducibly meets product specifications.

PQQ disodium salt is of low acute oral toxicity. In a GLP-compliant 90-day oral toxicity study in rats, the NOAEL of PQQ disodium salt (manufactured by Shanghai Med Co., Ltd) was determined to be 400 mg/kg body weight/day (highest dose tested) (Liang *et al.*, 2014), which was further corroborated by another 90-day oral toxicity study in which PQQ disodium salt (BioPQQ™) was determined to be 100 mg/kg body weight/day (the highest dose tested). Based on the results of several *in vitro* and *in vivo* genotoxicity studies, there is no evidence to suggest that PQQ presents a mutagenic/genotoxic risk. Human studies have shown that the oral ingestion of PQQ is well-tolerated at intakes ranging from 20 to 60 mg/day.

Under the intended conditions of use of PQQ disodium in beverages, the all-user total population consumption of PQQ was estimated to result in mean intakes of 12.8 mg/person/day (0.21 mg/kg body weight/day) and a 90th percentile intake of 27.8 mg/person/day (0.48 mg/kg body weight/day) for the total population. Highest intakes of PQQ were estimated for male teenagers at 24.7 mg/person/day (0.29 mg/kg body weight). Thus, a large margin of safety exists between the available NOAELs from rodent toxicity studies of PQQ and the estimated 90th percentile of intakes.

Hyphomicrobium denitrificans is a gram-negative bacterium that utilizes one carbon compounds, such as methanol, as an exclusive source of carbon and energy and is positive for nitrate reduction. A comprehensive search of the literature did not identify data or information to

suggest that the *H. denitrificans* or related species are pathogenic to animals or produce known toxins. PQQ produced by *Hyphomicrobium sp*. has been previously notified to the FDA as a New Dietary Ingredient for use in dietary supplement products without objection from the Agency. Through the use of appropriate manufacturing controls, transfer of the fermentation organism or residues of the fermentation broth into the finished product are prevented. No safety concerns are anticipated from the use of *H. denitrificans* in the production of PQQ disodium salt.

On the basis of the data and information summarized in this dossier, Hisun has concluded that PQQ produced from microbial fermentation by *Hyphomicrobium sp.*, meeting appropriate food-grade specifications and manufactured in accordance with cGMP, as described herein, is GRAS based on scientific procedures under the intended conditions of use as an ingredient in enhanced and fortified water beverages at a maximum use-level of up to 20 mg/serving.

V.A Expert Panel Evaluation

Hisun has determined that PQQ disodium salt is GRAS for use in energy, sport, and electrolyte drinks and enhanced and fortified water beverages as described in Table I.D-1. Hisun's GRAS determination was conducted on the basis of scientific procedures using data generally available in the public domain pertaining to the safety of PQQ, as discussed herein, and on consensus among a panel of experts (the Expert Panel) who are qualified by scientific training and experience to evaluate the safety of infant formula ingredients and food ingredients. The Expert Panel consisted of the following qualified scientific experts: Dr. Joseph F. Borzelleca (Virginia Commonwealth University School of Medicine), Dr. John Doull (University of Kansas Medical Center), and Dr. Stanley M. Tarka Jr. (The Pennsylvania State University).

The Expert Panel, convened by Hisun, independently and critically evaluated all data and information presented herein, and also concluded that PQQ was GRAS for use in food and beverage products as described in Section I.D based on scientific procedures. A summary of data and information reviewed by the Expert Panel, and evaluation of such data as it pertains to the proposed GRAS uses of PQQ is presented in Appendix A.

V.B Conclusion

Based on the above data and information presented herein, Hisun has concluded that the intended uses of PQQ in food and beverages, as described in Section I.D, is GRAS based on scientific procedures. General recognition of Hisun's GRAS determination is supported by the unanimous consensus rendered by an independent Panel of Experts, qualified by experience and scientific training, to evaluate the use of PQQ in food, who similarly concluded that the intended use of PQQ in food as described herein is GRAS.

PQQ therefore may be marketed and sold for its intended purpose in the U.S. without the promulgation of a food additive regulation under Title 21, Section 170.3 of the Code of Federal Regulations (U.S. FDA, 2015).

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Part	Section §	Section Title
101—Food labeling	101.12	Reference amounts customarily consumed per eating occasion
170—Food additives	170.3	Definitions
	170.30	Eligibility for classification as generally recognized as safe (GRAS)
173—Secondary direct food additives permitted in food for human consumption	173.25	Ion-exchange resins
	173.340	Defoaming agents
182—Substances generally recognized as safe	182.1	Substances that are generally recognized as safe
	182.1057	Hydrochloric acid
	182.1778	Sodium phosphate
184—Direct food substances affirmed as generally recognized as safe	184.1143	Ammonium sulfate
	184.1193	Calcium chloride
	184.1443	Magnesium sulfate
	184.1763	Sodium hydroxide

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Expert Panel Consensus Statement Concerning the Generally Recognized as Safe (GRAS) Status of Pyrroloquinoline Quinone (PQQ) Disodium Salt for Use as an Ingredient in Food

February 3, 2016

Zhejiang Hisun Pharmaceutical Co., Ltd. (hereafter "Hisun") has determined, using scientific procedures, that pyrroloquinoline quinone (PQQ) disodium salt, produced by *Hyphomicrobium denitrificans* fermentation as described herein, is Generally Recognized as Safe (GRAS) for addition to energy, sport and electrolyte drinks and enhanced and fortified water beverages at respective maximum use levels of up to 5 and 20 mg per serving as described in Table A-1.

Hisun convened an Expert Panel of independent scientists, qualified by their scientific training and relevant national and international experience to evaluate the safety of food ingredients, to conduct a critical and comprehensive evaluation of the available and pertinent data and information on PQQ disodium salt, and to determine whether, under the intended conditions of use as an ingredient in foods and beverages, PQQ disodium salt would be Generally Recognized as Safe (GRAS), based on scientific procedures. The Expert Panel consisted of the below-signed qualified scientific experts: Dr. Joseph F. Borzelleca, Ph.D. (Virginia Commonwealth University School of Medicine), Dr. John Doull (University of Kansas Medical Center), and Dr. Stanley M. Tarka Jr. (The Tarka Group Inc., and The Pennsylvania State University). For purposes of the Expert Panel's evaluation, "safe" or "safety" indicates that there is a reasonable certainty of no harm under the intended conditions of use of the ingredient in foods, as stated in 21 CFR §170.3(i) (U.S. FDA, 2014).

The Expert Panel, independently and collectively, critically evaluated a supporting dossier **[Documentation Supporting the Generally Recognized as Safe (GRAS) Status of Pyrroloquinoline Quinone (PQQ) Disodium Salt for Use as an Ingredient in Food]** submitted by Hisun which included a comprehensive package of data and information pertaining to the method of manufacture, product specifications and analytical data, stability, the conditions of intended use of PQQ disodium salt in specified food and beverage products, dietary consumption estimates for all intended uses, and a comprehensive assessment of the available scientific literature through to January 2015 pertaining to the safety of PQQ disodium salt.

Following an independent and collaborative critical evaluation of the data and information, the Expert Panel convened *via* teleconference on April 28, 2015 and unanimously concluded that the intended uses described herein of PQQ disodium salt, meeting appropriate food-grade specifications and manufactured consistent with current Good Manufacturing Practices (cGMP), are GRAS based on scientific procedures. A summary of the basis for the Expert Panel's conclusion is provided in the following section.

Summary and Basis for GRAS Determination

Hisun's PQQ disodium salt (≥99% purity) is manufactured using cGMP *via* a fermentation process using *Hyphomicrobium denitrificans*. All raw materials and processing aids are safe and suitable food-grade reagents permitted for use in food in the U.S. and/or determined to be GRAS. Master cell banks and working cell banks are used during manufacturing to maintain the phenotypic/genotypic stability of the strain. Frozen vials from the working cell bank are propagated in common culture medium salts and methanol as a carbon source to produce concentrated seed vials, which are then used as inoculation starters for the industrial fermentation process. Fermentation occurs under aseptic conditions by the addition of the starting culture to the seeding tank and proceeds at ambient temperatures for 3 days. Inoculation with the mycelium solution from the seeding tank into the propagation tank occurs in a closed system *via* differential pressure, and the main fermentation proceeds at ambient temperatures for several days. Upon completion of the fermentation process ceramic membrane filtration is used to remove the source organism from the media PQQ is purified through a series of filtration and washing steps including and resin adsorption (sodium phosphate elution buffer), and crystallization (ethanol). The crude product undergoes further dissolution, and filtration prior to undergoing a second ethanol crystallization step. The crystals are filtered and vacuum dried prior to milling and sieving. The final PQQ product is then stored under quarantine for quality control testing prior to packaging and storage at not above 30°C.

PQQ disodium salt is produced consistent with the principles of Hazard Analysis and Critical Control Points (HACCP) and process controls are employed throughout the production process. Quality control testing procedures ensure sterility of the culture media and fermentation broth prior to use. The inoculation cultures produced from the working cell bank undergo quality control testing to ensure conformance with internal specifications for *Hyphomicrobium*. Throughout the fermentation period, the pH, mycelium concentration, and amino-nitrogen are tested at 24 hours after inoculation, and the potency is tested daily after 4 days of fermentation. Fermentation is terminated when the mycelia decline, tinting strength is weak, increase of the fermentation potency is slower, pH increased slightly, and the potency becomes ≥600 μg/mL.

The Expert Panel reviewed batch analyses data from 3 non-consecutive batches of Hisun's PQQ disodium salt, demonstrating that the ingredient is manufactured in a reproducible manner and a consistent product is produced that conforms to the established physical and chemical specifications established by Hisun. Additionally the Panel reviewed data demonstrating that, no undesirable substances were present at levels of toxicological concern in regards to residual protein, heavy metals and microbial contaminants. Analysis by high performance liquid chromatography (HPLC) demonstrates that PQQ disodium salt is of high purity (≥99%). Qualitative analyses *via* infrared (IR) spectroscopy, nuclear magnetic resonance (NMR), and mass spectroscopy (MS) demonstrate that Hisun's PQQ product is chemically equivalent to a commercial PQQ standard (Fluka, Japan). Further analyses by high resolution MS

demonstrates that Hisun's product has an elemental composition consistent with the molecular formula of PQQ disodium salt ($C_{14}H_5N_2Na_2O_8$). Analytical data from stability testing demonstrate that PQQ disodium salt is stable for at least 24 months under ambient conditions (25±2°C, 60±5% relative humidity) and 6 months under accelerated conditions (40±2°C, 75±5% relative humidity).

PQQ is naturally present in a number of common foods including fruits, vegetables, fermented foods, and dairy; PQQ levels are typically in the parts per billion range. Plant-derived foods (*i.e.*, parsley, green pepper, spinach, *etc*.) and fermented foods (*i.e.*, miso, natto, tofu, *etc*.) generally contain higher concentrations of PQQ than animal-derived foods such as milk which contains approximately 2.5 to 3.4 ng/mL (Kumazawa *et al.,* 1993, 1995; Noji *et al.,* 2007). Trace levels of PQQ (≤5.9 ng/g wet tissue) were reported in human cadavers, with the highest levels in the spleen, pancreas, lung, and kidney (Kumazawa *et al*., 1992). PQQ, alone or with its corresponding imidazolopyrroloquinoline (IPQ) derivatives, have been detected in human milk at concentrations of 140 to 180 ng/mL (Mitchell *et al.,* 1999). In consideration of the daily intake of human milk by infants of approximately 750 mL/day, the estimated background exposure to PQQ in infants would be equivalent to 0.135 mg/day. No mammalian biosynthetic pathway for PQQ has been identified. Endogenous tissue levels of PQQ in humans are therefore presumed to be derived primarily from dietary exposure, with a negligible contribution from the intestinal microbiota (Matsushita *et al*., 1997). The consumption of PQQ and its derivatives has been estimated to range from 0.1 to 2.0 mg/day in humans (Kumazawa *et al.*, 1992, 1995; Mitchell *et al.*, 1999; Rucker *et al*., 2009; Harris *et al*., 2013). At the 90th percentile consumption from the proposed uses, the estimated daily intake of PQQ for the total population would be 108 and 217 mg/day at the mean and 90th percentile, respectively.

PQQ disodium salt is commercially available in the U.S. as an ingredient in dietary supplements with recommended intakes of 10 to 20 mg/day. While it is marketed by several companies, PQQ dietary supplements are primarily formulated with PQQ manufactured by Mitsubishi Gas Chemical Co., Inc. (BioPQQ™). In 2008, Mitsubishi Gas Chemical Co., Inc. filed a New Dietary Ingredient (NDI) Notification to the U.S. Food and Drug Administration (FDA) for BioPQQ™ (PQQ disodium salt) for use in dietary supplements, and in Canada PQQ disodium salt is permitted for sale as natural health product for use as an antioxidant in the maintenance of good health (EC, 1997; Health Canada, 2015).

Consumption data pertaining to the individual proposed food-uses of PQQ disodium salt were used to estimate the all-person and all-user intakes for specific demographic groups and for the total U.S. population using data from the 2011-2012 National Health and Nutrition Examination Survey (NHANES). For the total population, the consumption of PQQ disodium salt under the intended conditions of use was estimated to result in a mean intake of 12.8 mg/person/day (0.21 mg/kg body weight/day) and 90th percentile intake of 27.8 mg/person/day (0.48 mg/kg body weight/day) for all-users. On an absolute basis, the individual population group with the

highest estimated intakes resulting from the proposed food uses were male adults, who displayed mean and 90th percentile intake levels of 15.3 and 30.5 mg/day, respectively (corresponding to 0.18 and 0.41 mg/kg body weight/day, respectively). In contrast, children aged 3 to 11 were estimated to have the highest mean and 90th percentile intake on a body weight basis at levels of 0.32 and 0.77 mg/kg body weight/day, respectively. However, as PQQ disodium salt is not intended for addition to foods marketed to children, the intake assessment for this population group is included as a conservative measure due to the inclusion of food-use categories to which exposure in children may occur. Additionally, PQQ will be marketed in 'functional food' type products marketed at a premium price and will be purchased by adult consumers seeking PQQ in the diet. The premium pricing of foods containing PQQ relative to comparator foods not containing PQQ will serve as a deterrent to significant and/or regular consumption by non-target consumers such as children. Historical experience with phytosterols, a similar 'functional food' ingredient targeted to adult consumers, has demonstrated that consumption by non-target users such as children was low, with reported occasional consumption less than 8% and regular use less than 1% for children below the age of 5 (EFSA, 2008). The Expert Panel concluded that the intake modeling of PQQ in children aged 3 to 11 would not occur on a regular basis and would not be representative of actual intake of the ingredient by these individuals.

Data on the pharmacokinetic and metabolic fate of PQQ in rodents and humans is limited. Absorption and tissue distribution of PQQ was evaluated in Swiss Webster mice (n=10) administered a single 0.1 µmole dose (0.42 µCi/µmol) of [^{14}C]PQQ (Smidt *et al.*, 1991) *via* oral gavage. Absorption[1] and tissue distribution of "PQQ-like" substances were evaluated at 6 and 24 hours post-dosing. Only 3.3% of the administered dose was absorbed by the 6-hour time-point, and 62% of the dose was absorbed by 24 hours. Eighty-one percent (81%) of the absorbed dose was excreted in the urine, and consistent with this route of elimination the kidneys were the major site of tissue distribution. Minimal quantities were retained in the remaining tissues (≤1.5% in each of the liver, red blood cell, and skin), and the low concentrations detected in the liver suggests that biliary elimination is not a major excretion route. No radioactivity was detected in the expired carbon dioxide. The Expert Panel noted that the detection method used for quantitation of PQQ was non-qualitative, which complicates interpretation of the data. In contrast to the authors' view that PQQ is readily available, only 3% of the administered radioactivity was absorbed at the 6-hour time-point which suggests that the compound may in-fact be poorly absorbed. This conclusion would be consistent with findings from animal feeding studies where dark/green colored intestinal contents and feces have been detected on multiple occasions (*e.g.*, Nakano *et al*., 2014; Liang *et al*., 2014). Available kinetic data from studies evaluating PQQ consumption in humans also suggest that PQQ is poorly absorbed. In humans, peak serum concentrations of 9 nM (approximately 3.4 ng/mL) were reported at approximately 2 hours following acute ingestion of PQQ disodium at a dose of

[1] Absorption estimated by dividing the total amount of [^{14}C]PQQ administered into the sum of ^{14}C retained in tissues, urine and carbon dioxide, exclusive of the radioactivity in the gastrointestinal tract.

0.2 mg/kg body weight (14 mg PQQ for a 70 kg individual) (Harris *et al*., 2013). Following repeated consumption of PQQ disodium at graduating doses up to 0.3 mg/kg body weight/day (corresponding to 21 mg PQQ/day for a 70 kg individual) for a period of 21 days, only 0.1% of the administered PQQ dose was identified in the urine in the non-derivatized (free) form (Harris *et al*., 2013).

While no studies on the metabolic fate of exogenously consumed PQQ were identified in the published literature, PQQ can reportedly undergo condensation with amino acids to form IPQ derivatives (Ishida *et al*., 1995; Urakami *et al.,* 1995-1996), which have been identified in human milk (Mitchell *et al.,* 1999; Kumazawa *et al.,* 2000). PQQ can undergo oxidation and reduction cycling from a quinone to a quinol (Duine *et al*., 1987; Duine, 1991), and may form stable conjugates with thiols and alcohols (Flückiger *et al*., 1988; van Kleef *et al*., 1989; Park and Churchich, 1992).

The Expert Panel reviewed published studies characterizing the toxicity of PQQ, and included acute, repeated-dose toxicity, and genotoxicity studies conducted with high purity PQQ disodium salt produced by *Hyphomicrobium* sp. fermentation. The test articles used in these studies were high purity PQQ disodium salt (≥98%) ingredients, and were manufactured by fermentation processes using *Hyphomicrobium* sp. as the fermentation organism. Accordingly, data provided by these studies were considered applicable to the safety assessment of Hisun's PQQ disodium salt. The Expert Panel also reviewed available product specific studies on the toxicity of PQQ disodium salt conducted using PQQ manufactured by Hisun, which included an acute toxicity study, an *in vitro* bacterial reversion assay, an *in vitro* chromosomal aberration assay and an *in vivo* micronucleus assay. Findings from these studies were consistent with existing published safety data on PQQ and therefore the Panel considered this information to represent corroborative information in support of the GRAS determination.

In general, animal studies have demonstrated that PQQ disodium salt is of low oral toxicity. The acute toxicity of PQQ disodium salt was evaluated by Nakano *et al*. (2014). Five-week old male and female Sprague-Dawley (SD) rats (n=10/sex/group) were administered PQQ disodium salt (purity = 99.6%; BioPQQ; Mitsubishi Chemical Company) *via* oral gavage at doses (20 mL/kg body weight) providing 0, 500, 1,000, or 2,000 mg PQQ disodium/kg body weight. Consistent with information suggesting that PQQ is poorly absorbed, high acute doses resulted in discoloration of the stools and osmotic effects of unabsorbed PQQ in the colon resulted in transient diarrhea and/or softening of the stools within one hour of dosing. On day 2, 3/10 males and 3/10 females in the 2,000 mg/kg body weight group died. On day 3, 1/10 females in the 1,000 mg/kg body weight group and 3/10 males and 7/10 females in the 2,000 mg/kg group died. One male in the high-dose group died on day 7. The target organ of high-dose PQQ appears to be the kidney as all dead and surviving animals in the 1,000 and 2,000 mg/kg body weight groups had pale and enlarged kidneys. Evidence for renal toxicity from high systemic exposure to PQQ has been observed in rats administered PQQ *via* the intraperitoneal route at

doses of 11.5 mg/kg body weight/day for 4 days (Watanabe *et al*., 1989). Wang *et al*. (2012) reported oral median lethal dose (LD_{50}) values of 3,690 and 2,710 mg PQQ/kg body weight for female and male ICR mice respectively, and studies conducted by Hisun determined an LD_{50} of 4,220 and 5,840 mg/kg body weight following acute oral administration of PQQ disodium salt to male and female ICR mice (Zhejiang Hisun Pharmaceutical Co., Ltd., 2012).

The toxicity of PQQ was also evaluated in a series of repeated-dose oral toxicity studies conducted in Sprague-Dawley rats administered PQQ disodium salt (Mitsubishi Gas Chemical Co., Inc.; BioPQQ™; ≥99.1% purity) including a 14-day, 28-day, and 90-day study performed in accordance with Good Laboratory Practices (GLP) (Nakano *et al*., 2014). In the 14-day study dose-ranging study, no adverse effects with respect to clinical observations, body weight, food consumption, hematology, blood biochemistry, or mortalities were observed in rats (6/sex/ group) administered PQQ disodium salt at doses up to 768 mg/kg body weight/day by oral gavage (Nakano *et al.*, 2014). Although green-colored feces and intestinal contents were observed in some rats in the groups given the 2 highest doses, feces were otherwise normal. Kidney weights were increased by 13% ($p<0.05$) relative to controls in females; however, this effect was not dose-responsive and no similar trend was observed in the males. Although slight to moderate focal basophilic changes and atrophy of the renal tubules were reported in the high-dose female group, minimal to slight basophilic atrophy also was observed in all male treatment groups including the control animals. Based on the apparent renal findings observed in the acute and 14-day repeat-dose toxicity studies, a 28-day repeat-dose follow-up study in female SD rats (12/group) was conducted. The doses selected for this study were based on the results of the 14-day dose-range study in which no effects were observed at a dose of 192 mg/kg body weight/day; however, some renal effects were noted at 768 mg/kg body weight/day in female animals only. Therefore, doses of 200 and 700 mg/kg body weight/day were selected as the low- and high-dose levels, respectively. Rodents (12/group) were administered PQQ at doses of 0, 200, or 700 mg PQQ/kg body weight/day by oral gavage for 28 days. At the end of the administration period, 6 animals from each group were exsanguinated and necropsied. The remaining six animals in each group were monitored during a 28-day recovery period. Slight to moderate elevations in urinary protein and crystals were reported in the 200 and 700 mg/kg body weight groups; however, these changes were not dose-responsive and resolved by the end of the 4-week recovery period. No other corresponding changes in in clinical chemistry or histopathology suggestive of kidney toxicity were observed (Nakano *et al*., 2014). In the 90-day study, no toxicologically significant effects were reported with respect to hematology, clinical biochemistry, urinalysis, gross necropsy, or histopathology when rats (10/sex/group) were administered 0, 3, 20, or 100 mg PQQ/kg body weight/day by oral gavage (Nakano *et al*., 2014). Based on the results of these 3 studies, the no-observed-adverse-effect level (NOAEL) for PQQ was determined to be 100 mg/kg body weight/day.

Two additional sub-chronic rodent toxicity studies evaluating the toxicity of PQQ have been reported in the literature (Wang *et al*., 2012; Liang *et al*., 2014). The study by Wang and

colleagues was a 90-day repeated-dose toxicity study conducted in accordance to the Technical Standards for Testing & Assessment of Health Food (2003) using Sprague-Dawley rats (12/sex/group). Animals were administered PQQ (Shanghai Rixin Biotechnology Co. Ltd, China) by oral gavage at dose levels of 0, 3.8, 7.5, or 15 mg/kg body weight/day.[2] A NOAEL of 15 mg/kg body weight/day (the highest dose tested) was determined by the authors. The Expert Panel noted that the PQQ test article used in the study was not adequately characterized and the dose selections were not based upon a maximum tolerated dose level. The study was corroborative of the safety of PQQ, but due to limitations in the study design was not pivotal to the safety assessment.

The study by Liang *et al*. (2014) was a 90-day sub-chronic toxicity study conducted in Sprague-Dawley rats (10/sex/group). Rodents administered PQQ disodium salt (purity >98%; Shanghai Med Co., Ltd., China) by oral gavage at dose levels of 0 (control), 100 (low-dose), 200 (mid-dose), or 400 (high-dose) mg/kg body weight/day. This study was conducted in accordance to GLP and the FDA Guidance for Industry and Other Stakeholders (U.S. FDA, 2007). A NOAEL of 400 mg/kg body weight/day, the highest dose tested, was established by the authors. Urinary analyses data were not reported by the authors, however, this information was not required for the safety assessment as histopathological changes in the kidneys were not observed. Moreover, the apparent slight to moderate elevations in the incidences of urinary crystals and protein in female rats administered PQQ disodium salt at doses of 700 mg/kg body weight over 28 days were unlikely to be of toxicological significance as the findings were not dose-responsive, resolved following the recovery period, and were not associated correlating biochemical or histopathological changes indicative of kidney toxicity (Nakano *et al*., 2014). Therefore the authors' (Liang *et al*., 2014) NOAEL determination of 400 mg/kg body weight, the highest dose tested, was considered appropriate for use as a pivotal study in the GRAS determination.

Results from feeding studies in which mice received PQQ- deficient diets have suggested that PQQ may be a nutrient involved in reproductive performance and is required for proper neonatal development and growth (Killgore *et al.*, 1989; Steinberg *et al.*, 1994, 2003). These studies remain controversial since no biosynthetic pathways for PQQ synthesis have been reported (Kasahara and Kato, 2003; Felton and Anthony, 2005).

Samuel and colleagues evaluated the effects of dietary PQQ on growth performance, carcass yield and antioxidant status of broiler chicks (Samuel *et al*., 2014). The study was conducted in 784 one-day-old male Arbor Acres broilers. Birds were randomly allotted into 1 of 7 dietary groups. The negative control group (NC) was fed a basal diet without antibiotics or PQQ. Birds in the positive control/treatment groups were provided diets containing antibiotics and increasing

[2] The Expert Panel noted that the highest dose selected for the study was not based upon a determination of the maximum tolerated dose but based on recommended literature doses of 3 mg PQQ/day in humans and corresponding margin of safety of >100.

concentrations of PQQ (0, 0.05, 0.10, 0.20, 0.40, or 0.8 mg PQQ/kg diet) for 42 days. No treatment related adverse effects were reported in the broilers. PQQ administration was reported to improve growth. The authors concluded that dietary PQQ "*had the potential to act as a growth promoter comparable to antibiotics in broiler chicks*". Similar findings on growth for PQQ in broiler chicks were reported by Wang *et al.* (2012). The Expert Panel considered these studies to provide strong supportive evidence of safety since broiler chicks are rapidly growing animals that are sensitive to dietary manipulations.

PQQ is neither genotoxic nor mutagenic. In a standard battery of *in vitro* and *in vivo* mutagenicity and genotoxicity studies including the reverse mutation assay, *in vitro* and *in vivo* chromosomal aberration tests, and *in vivo* micronucleus assay, PQQ reportedly demonstrated consistently negative results (Wang *et al.*, 2012; Zhejiang Hisun Pharmaceutical Co., Ltd., 2012; Nakano *et al.*, 2013). Although a modest but significant increase (0.5 to 4%) in structural chromosomal aberrations was reported *in vitro* in Chinese hamster lung fibroblasts incubated with 200 µg BioPQQ™/mL, in the absence of metabolic activation, these increases were below the historical 5% threshold for a positive response, and repeat testing showed no significant effects on chromosomal aberrations (Nakano *et al.*, 2013).

Studies in healthy human subjects have demonstrated that PQQ disodium salt is well-tolerated with no reports of serious adverse events. For example, in a double-blind study in 10 healthy human subjects (age, body weight and sex not specified) who consumed 20 to 60 mg PQQ/day for up to 4 weeks, it was reported that PQQ did not induce any remarkable changes in standard clinical tests including glucose, triglycerides, various lipoprotein fractions or markers of liver toxicity including aspartate aminotransferase and serum glutamic oxaloacetic transaminase (as reviewed by Rucker *et al.*, 2009). Harris and colleagues reported that consumption of PQQ formulated in a fruit-flavored drink at a single dose of 0.2 mg/kg body weight (equivalent to 14 mg for a 70 kg individual) or consumption of repeated doses of 0.3 mg/kg body weight/day (equivalent to 21 mg/day for a 70 kg individual) for 3 days did not induce any adverse changes in clinical indices such as total cholesterol, creatine, glucose, low-density lipoprotein, high-density lipoprotein, triglycerides, uric acid, total protein, and aspartate aminotransferase relative to baseline values (Harris *et al*., 2013). Other investigators also evaluated the effects of PQQ on various biological indices in human subjects. Consumption of 20 mg PQQ/day for up to 24 weeks in healthy adults did not elicit any adverse effects and these studies provide additional supportive evidence of the safety of PQQ in humans (Nakano *et al*., 2009, 2012; Koikeda *et al*., 2011). The Expert Panel noted that the daily intakes of PQQ disodium salt administered in these studies were quantitatively comparable to those expected from the consumption of one to 12 servings of beverage products to which PQQ may be added under the conditions of intended use described in Table A-1.

The Expert Panel reviewed information on the safety of the source organism used for fermentation of PQQ. *Hyphomicrobium* sp. are facultatively methylotrophic, non-spore forming, gram-negative, rod-shaped bacteria with a unique Q-9 ubiquinone system (Urakami and Komagata, 1979, 1986, 1987). *Hyphomicrobium* sp. utilizing one-carbon compounds as exclusive sources of carbon and energy are ubiquitous in the environment and are often present in waste-water treatment environments due to their propensity for denitrification and remediation of C_1 compounds (Rainey *et al*., 1998). *Hyphomicrobium denitrificans* does not grow at 37 or 42°C and cannot utilize nutrient-rich broth for growth as the species is unable to ferment common food sugars. The species identity of Hisun's production strain was determined using 16S rDNA sequence analysis, verifying the identity of the strain as *Hyphomicrobium denitrificans* based on a 100% sequence match to *Hyphomicrobium denitrificans* ATCC 51888^T, the type strain for the species. The complete genome of *Hyphomicrobium denitrificans* ATCC 51888 was sequenced by Brown and colleagues in 2011 (Brown *et al*., 2011). The nucleotide sequence is deposited in GenBank under NC_014313, and annotation of the genome was conducted using the JGI-Oak Ridge National Library annotation pipeline. Analysis of the genome through the Pathosystems Resource Integration Center (PATRIC) database identified 3 homologous[3] virulence genes encoding the following proteins: Imidazole glycerol phosphate synthase cyclase subunit (EC 4.1.3.-); RNA-binding protein Hfq; Dihydroxy-acid dehydratase (EC 4.2.1.9); however, none of these proteins encode potential toxins or are involved in biosynthetic pathways that would be predicted to impart undesirable phenotypes to *Hyphomicrobium* sp. and the genome did not contain any known antibiotic resistance genes. Hisun maintains the source organism used for the production of PQQ disodium salt in-house, which is subject to strict quality control for compliance with established internal specifications and is free of microbial contamination.

A comprehensive search of the published literature did not identify any data or information to suggest that *H. denitrificans* or related species are pathogenic to animals or humans or produce known toxins. The species cannot ferment sugars, and does not grow at 37°C; the inherent phenotypic properties of the species would not be conducive to potential pathogenicity in humans. Data to support the use of *Hyphomicrobium* sp. in the production of foods is minimal. PQQ produced by *Hyphomicrobium* sp. has been notified to the FDA as a NDI for use in dietary supplements without objection from the Agency (U.S. FDA, 2007). PQQ disodium salt is of high purity and is free of any residual protein, the source organism and any microbial contamination. Appropriate process controls including filtration, heat sterilization, chromatographic purification, crystallization and wash parameters also prevent any transfer of the fermentation organism to the final product. The source organism is not expected to pose safety concerns in the final PQQ disodium salt based on the phenotypic and genotypic properties of the organism, the history of safe use of the organism for production of high purity PQQ disodium salt, and implementation of appropriate controls during manufacturing. The source organism also was determined to be

[3]Genes homologous to *Brucella melitensis* biovar Abortus 2308 identified.

safe and suitable for use in the production of PQQ disodium salt intended for food uses based on the use of the Pariza-Johnson decision tree (Pariza and Johnson, 2001). A summary of the Pariza-Johnson decision tree is presented in Attachment B.

CONCLUSION

We, the Expert Panel, have independently and collectively critically evaluated the data and information summarized above, and conclude that PQQ disodium salt as described herein, produced in accordance with current Good Manufacturing Practices (cGMP) and meeting food-grade specifications, is Generally Recognized a Safe (GRAS), on the basis of scientific procedures, for use as an ingredient in energy, sport and electrolyte drinks and enhanced and fortified water beverages at maximum respective use level of 5 and 20 mg/serving as described in Table A-1.

It is our opinion that other qualified experts would concur with these conclusions

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ATTACHMENT A

Proposed Uses and Use-Levels of PQQ Disodium Salt

Table A-1 Summary of the Individual Proposed Food-Uses and Use-Levels for Pyrroloquinoline Quinone (PQQ) in the U.S. (NHANES 2011-2012)

Food Category	Food-Uses	Serving Size[1]	Proposed Use Level	
			(mg/serving)	(%)
Beverages and Beverage Bases	Energy, Sport, and Electrolyte Drinks	240 mL	5	0.002
	Water (Bottled, Enhanced, and Fortified)	240 mL	20	0.008

[1] RACC refers to Reference Amounts Customarily Consumed per eating occasion – 21 CFR §101.12 (U.S. FDA, 2014).

ATTACHMENT B

Pariza-Foster Decision Tree Analysis

This analysis is based on the Decision Tree of MW Pariza and EA Johnson (2001): *Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century*, Regulatory Toxicology and Pharmacology, 33:173-186. Decision points that do not pertain are included for completeness but crossed out.

1. Is the production strain genetically modified?
If yes, go to 2. If no, go to 6. **NO**

~~2. Is the production strain modified using rDNA techniques?~~
~~If yes, go to 3. If no, go to 5.~~

~~3. Issues relating to the introduced DNA are addressed in 3a–3e.~~

~~3a. Do the expressed enzyme product(s) which are encoded by the introduced DNA have a history of safe use in food?~~
~~If yes, go to 3c. If no, go to 3b~~

~~3b. Is the NOAEL for the test article in appropriate short-term oral studies sufficiently high to ensure safety?~~
~~If yes, go to 3c. If no, go to 12.~~

~~3c. Is the test article free of transferable antibiotic resistance gene DNA?~~
~~If yes, go to 3e. If no, go to 3d.~~

~~3d. Does the resistance gene(s) code for resistance to a drug substance used in treatment of disease agents in man or animal? If yes, go to 12. If no, go to 3e.~~

~~3e. Is all other introduced DNA well characterized and free of attributes that would render it unsafe for constructing microorganisms to be used to produce food-grade products?~~
~~If yes, go to 4. If no, go to 12.~~

~~4. Is the introduced DNA randomly integrated into the chromosome?~~
~~If yes, go to 5. If no, go to 6.~~

~~5. Is the production strain sufficiently well characterized so that one may reasonably conclude that unintended pleiotropic effects which may result in the synthesis of toxins or other unsafe metabolites will not arise due to the genetic modification method that was employed?~~
~~If yes, go to 6. If no, go to 7.~~

6. Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure? **YES**
If yes, the test article is ACCEPTED. If no, go to 7.

7. Is the organism nonpathogenic? **YES**
If yes, go to 8. If no, go to 12.

8. Is the test article free of antibiotics? **YES (Species not known to produce antibiotics)**
If yes, go to 9. If no, go to 12.

9. Is the test article free of oral toxins known to be produced by other members of the same species?
If yes, go to 11. If no, go to 10. **YES (Species not a known toxin producer)**

10. Are the amounts of such toxins in the test article below levels of concern?
If yes, go to 11. If no, go to 12.

11. Is the NOAEL for the test article in appropriate oral studies sufficiently high to ensure safety? **YES**
If yes, the test article is ACCEPTED. **TEST ARTICLE ACCEPTED**

~~12. An undesirable trait or substance may be present and the test article is not acceptable for food use. If the genetic potential for producing the undesirable trait or substance can be permanently inactivated or deleted, the test article may be passed through the decision tree again.~~

SUBMISSION END