Soni & Associates Inc.

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November 5, 2020

Food and Drug Administration
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety (HFS-200)
5100 Campus Drive
College Park, MD 20740

Subject: GRAS Notification for Aqueous Olive Pulp Extract (Hidrox®)

Dear Sir/Madam:

In accordance with 21 CFR part 170, subpart E, Oliphenol LLC, through Soni & Associates Inc. as its agent, hereby submits the enclosed notice of a claim that the food ingredient Aqueous Olive Pulp Extract (Hidrox®) described in the enclosed notification dossier is exempt from the premarket approval requirement of the Federal Food, Drug, and Cosmetic Act because it has been determined to be Generally Recognized As Safe (GRAS), based on scientific procedures.

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

Sincerely,

[redacted]

Madhu G. Soni, Ph.D.

Enclosure: Three copies

EVALUATION OF THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF

AQUEOUS OLIVE PULP EXTRACT (HIDROX®)

AS A FOOD INGREDIENT

Prepared for:

Oliphenol LLC.
26225 Eden Landing Road, Suite C
Hayward, CA 94545

Prepared by:

Soni & Associates Inc.
749 46th Square
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Panel Members

Robert L. Martin., Ph.D.
John A. Thomas, Ph.D., F.A.C.T., F.A.T.S.
Madhusudan G. Soni, Ph.D., F.A.C.N., F.A.T.S.

October, 2020

EVALUATION OF THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF AQUEOUS OLIVE PULP EXTRACT (HIDROX®) AS A FOOD INGREDIENT

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Part I- SIGNED STATEMENT AND CERTIFICATION

1.1. Basis of Conclusion

This GRAS conclusion for the use of Aqueous Olive Pulp Extract (Hidrox®) as a food ingredient has been reached in accordance with requirements as defined in 21 CFR 170.220.

1.2. Name and address of organization:

Oliphenol LLC.
26225 Eden Landing Road, Suite C
Hayward, CA 94545
USA

Phone: +1-833-654-7436
email: info@Oliphenol.com

1.3. Name of substance:

The name of the substance of this GRAS assessment is Aqueous Olive Pulp Extract (Hidrox®).

1.4. Intended conditions of use of Aqueous Olive Pulp Extract (Hidrox®):

Aqueous Olive Pulp Extract (Hidrox®) is intended to be used as a food ingredient and as an antioxidant in selected conventional food categories, such as: bakery products; beverages; dairy products and substitutes; desserts; fats and oils; fruit juices and nectars; dry seasoning mixes for meat, poultry and fish; chewing gum; sauces, dips, gravies and condiments; snacks; and vegetable juices to deliver 5 to 10 mg of hydroxytyrosol *per* serving (reference amounts customarily consumed, 21 CFR 101.12). The actual amount of Aqueous Olive Pulp Extract (Hidrox®) added (approximately 150 to 300 mg/serving) to the food categories will be adjusted such that the resulting addition corresponds to the proposed use levels of hydroxytyrosol. The intended use of Aqueous Olive Pulp Extract is in the same food products and at the identical levels mentioned in the GRN 726[1] (Phenolic preparation from olive fruit). It is recognized that there are Standard of Identity requirements for some of the specified foods and these foods will not be referred to by their commonly recognized names.

1.5. Statutory Basis for GRAS conclusion:

This GRAS conclusion is based on scientific procedures in accordance with 21 CFR 170.30(a) and 170.30(b).

1.6. Exemption from Premarket approval requirements:

Oliphenol LLC (Oliphenol) has concluded that Aqueous Olive Pulp Extract (Hidrox®) is not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on our conclusion that Aqueous Olive Pulp Extract (Hidrox®), meeting the specifications cited herein, and when used as a food ingredient and as an antioxidant, is GRAS and is therefore exempt from the premarket approval requirements.

It is also our opinion that other qualified and competent scientists reviewing the same publicly available toxicological and safety information would reach the same conclusion. Therefore, we

[1] Accessible at: https://www.fda.gov/media/111692/download, and at: https://www.fda.gov/media/111827/download

have also concluded that Aqueous Olive Pulp Extract (Hidrox®), when used as described in this dossier, is GRAS based on scientific procedures.

1.7. Availability of data and information:

The data and information that are the basis for this GRAS conclusion will be made available to FDA upon request by contacting Dr. Crea at the below address. The data and information will be made available to the FDA in a form in accordance with that requested under 21 CFR 170.225(c)(7)(ii)(A) or 21 CFR 170.225(c)(7)(ii)(B).

Dr. Roberto Crea
Chief Executive Officer
Oliphenol LLC.
26225 Eden Landing Road, Suite C
Hayward, CA 94545
USA

Phone: +1 833-654-7436
Email: robertocrea@oliphenol.com

1.8. Data exempt from Disclosure:

Part I through Part VII of this GRAS assessment does not contain any privileged or confidential information such as trade secrets and/or commercial or financial information and can be made publicly available.

1.9. Certification:

Oliphenol certifies that, to the best of its knowledge, this GRAS conclusion is based on a complete, representative, and balanced dossier that includes all relevant information, available and obtainable by Oliphenol, including any favorable or unfavorable information, and pertinent to the evaluation of the safety and GRAS status of the use of Aqueous Olive Pulp Extract (Hidrox®). Oliphenol accepts responsibility for the GRAS conclusion that has been made for Aqueous Olive Pulp Extract (Hidrox®) as described in this dossier.

1.10. Name, position/title of responsible person who signs dossier and signature:

Dr. Roberto Crea
Chief Executive Officer
Oliphenol LLC.
26225 Eden Landing Road, Suite C
Hayward, CA 94545

Signature: 

1.11. FSIS/USDA – Use in Meat and/or Poultry:

Oliphenol does not intend to add Aqueous Olive Pulp Extract (Hidrox®) to any meat and/or poultry products that come under USDA jurisdiction. Therefore, 21 CFR 170.270 does not apply.

Part II- IDENTITY AND TECHNICAL INFORMATION

2.1. Description

The subject of this GRAS assessment, Aqueous Olive Pulp Extract (Hidrox®) is a standardized powder and liquid derived from the juice of olives (*Olea europaea* L.). The preparations (powder and liquid) have an odor of processed olives and a characteristic aromatic sour/olive flavor. The powder is composed of 98-99% dry solids. The extract is prepared by water extraction of the juice recovered during the milling of olive oil. General descriptive characteristics of Aqueous Olive Pulp Extract (Hidrox®) are summarized in Table 1. The biologically active constituents of Hidrox® are polyphenols. Among the phenolics, the major constituent of the pulp extract is hydroxytyrosol (50-70%), while other polyphenols present include oleuropein (5-10%), tyrosol (0.3%), oleuropein aglycone and gallic acid. All of the polyphenols present in the extract are also found in olive oil and are thus commonly consumed.

Table 1. General Descriptive Characteristics of Aqueous Olive Pulp Extract (Hidrox®)

Parameter	Description (Oliphenol)*
Plant Source	*Olea europaea*
Other names	Olive tree; Oliver; Olivenbaum; Oliva; Olea europea oil; Olea europea extract
Part used	Fruit
Starting material	Juice recovered during the milling of olive oil
Active constituents	Phenolic compounds, including hydroxytyrosol
Appearance	Dried powder or liquid
Color	Golden brown powder; Dark brown, semi-viscous liquid
Odor	Processed olives
Flavor	Characteristic aromatic sour/olive
Storage	Preserve in tight containers and prevent exposure to excessive heat
Shelf life	2 years

*Based on information provided by Oliphenol (2020)

2.2. Botanical identification

The hierarchical classification of the source material, *Olea europaea,* is presented in Table 2. The plant is famous for its olive's fruits or "olive," which are the source for a commonly consumed oil, known as olive oil, around the world and one of the core ingredients in Mediterranean cuisine. There are thousands of cultivars of the olive. In Italy alone at least three hundred cultivars have been enumerated, while only a few are grown to a large extent. The olive tree is an evergreen tree or shrub native to Mediterranean Europe, Asia, and Africa. It is short and squat, and rarely exceeds 8-15 m in height. The trunk is typically gnarled and twisted. The leaves are silvery green, oblong, measuring 4-10 cm long and 1-3 cm wide. The small white flowers, with four-cleft calyx and corolla, two stamens and bifid stigma, are borne generally on the last year's wood, in racemes springing from the axils of the leaves. The fruit is a small drupe 1-2.5 cm long, thinner-fleshed and smaller in wild plants than in cultivars. A typical picture of olive branch with fruits is presented in Figure 1.

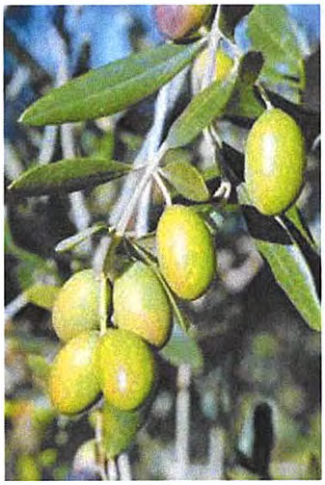


Figure 1. Typical Picture of *Olea europaea* Plant with Fruits

Table 2. Taxonomical Classification of *Olea europaea*

Rank	Scientific Name and Common Name
Kingdom	Plantae – Plants
Subkingdom	Tracheobionta – Vascular plants
Superdivision	Spermatophyta – Seed plants
Division	Magnoliophyta – Flowering plants
Class	Magnoliopsida – Dicotyledons
Subclass	Asteridae
Order	Scrophulariales
Family	Oleaceae – Olive family
Genus	*Olea* L. – olive
Species	*Olea europaea* L. – olive

Source: USDA, Natural Resources Conservation Service

2.3. Specifications

Food grade specifications of Aqueous Olive Pulp Extract (Hidrox®) powder and liquid by Oliphenol are summarized in Table 3. The polyphenol content of powder is >12%, while that of liquid is >7.5%. The hydroxytyrosol (Figure 2) content for powder is >3.5% and for liquid is >3%. Aqueous Olive Pulp Extract is soluble in water (>95%). Oliphenol intends to market two different products derived from olives. The comparison of specifications with certificate of analysis from three non-consecutive lots for Hidrox® Freeze-Dried Powder 12% (Table 4) and for three lots of Hidrox® Liquid 10X (Table 5) demonstrate that these products are produced consistently and meets the food grade specifications.

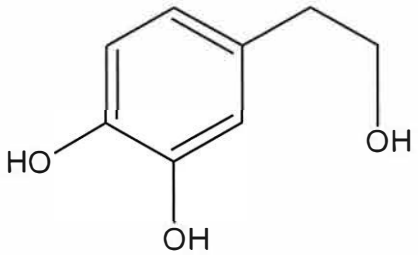


Figure 2. Chemical Structure of Hydroxytyrosol

2.3.1. Hydroxytyrosol

The available information suggest that hydroxytyrosol is a major biologically active constituent of the phenolic fraction of olive extract and olive oil. It is also known as 3,4-dihydroxytyrosol or 3,4-dihydroxyphenylethanol (CAS No.: 10597-60-1; Figure 2). The chemical formula of hydroxytyrosol is $C_8H_{10}O_3$ and the molecular weight is 154. In addition to olives, the presence of hydroxytyrosol has also been identified and quantified in wines (Di Tommaso et al., 1998). In olive oil, hydroxytyrosol is found either as the simple phenol or esterified with elenolic acid to form oleuropein aglycone. Hydroxytyrosol in pure form is a clear, colorless, tasteless liquid and can be soluble in water or lipid. Although hydroxytyrosol represents a minor constituent of the aqueous olive pulp extract, as well as of the olive oil phenolic fraction, it is considered the most potent (as measured by ORAC) phenolic antioxidant of Aqueous Olive Pulp Extract and of olive oil. The presence of hydroxytyrosol in olive oil prevents its autoxidation and, thus significantly contributes to its shelf life.

Table 3. Specifications of Aqueous Olive Pulp Extract

Description	Characteristic/specification		Method
	HIDROX® Freeze-Dried Powder 12%	HIDROX® Liquid 10X	
Appearance	Golden brown to purplish powder	Brown to dark purplish	Visual
Flavor	Sour/Olives	Sour/Olives	Organoleptic
Odor	Processed olives	Processed olives	Organoleptic
pH (in 1 g/10 mL water)	3-4	3-4	AOAC 981.12
Protein (% w/w)	3-8	3-6	AOAC 925.09
Ash (% wt/wt)	12-20	12-20	AOAC 942.05
Solubility in water	>95	>98	In House
Fat (% w/w)	8-18	8-15	AOAC 954.02
Carbohydrates (%w/w)	45-74	30-45	CFR 21-calc.
Moisture (% w/w)	<5	34	AOAC 925.09
Simple & Polyphenols (%)	>12	>7.5	Folin-Ciocalteu (UV)
Hydroxytyrosol	>3.5	>3.0	JAOCS 75/11 (1998) (modified)
Heavy metals			
Lead (mg/kg)	<0.1	<0.1	AOAC 993.14 Mod.
Arsenic (mg/kg)	<0.1	<0.1	AOAC 993.14 Mod.
Cadmium (mg/kg)	<0.1	<0.1	AOAC 993.14 Mod.
Mercury (mg/kg)	<0.01	<0.01	AOAC 993.14 Mod.
Microbial standards			
Coliforms and *E.coli* (MPN/g)	<3	<3	USP Chapter 61
Total Aerobic Plate Count (CFU/g)	<1000	<1000	USP Chapter 62
Staphylococcus aureus (MPN/g)	<3	<3	USP Chapter 62
Salmonella – (ELFA) presumptive	Negative	Negative	USP Chapter 62
Mold (CFU/g)	<10	<10	USP Chapter 62
Yeast (CFU/g)	<20	<20	USP Chapter 62

MPN = Most probable number; CFU = Colony forming units

Table 4. Specification and Certificate of Analysis for HIDROX® Freeze-Dried Powder 12%

Parameter	Specifications	Lot Number		
		12-120928-000	12-130114-000	12-140108-001
Appearance	Golden brown to purplish powder	Conforms	Conforms	Conforms
Flavor	Sour/Olives	Conforms	Conforms	Conforms
Odor	Processed olives	Conforms	Conforms	Conforms
pH (in 1g/10mL water)	3-4	3.5	3.4	3.7
Protein (% w/w)	3-8%	3.52%	3.2%	3.86%
Ash (% wt/wt)	12-20%	13%	12.3%	12.78%
Solubility in water	>95%	Conforms	Conforms	Conforms
Fat (% w/w)	8-18%	10.85%	9.00%	8.36%
Carbohydrates (%w/w)	45-74%	72%	75%	74.59%
Moisture (% w/w)	<5%	3.52%	3.2%	3.86%
Simple & Polyphenols	>12%	13.78%	12.78%	15.93%
Hydroxytyrosol	≥3.5%	3.71%	3.55%	4.34%
Oxygen Radical Absorbance Capacity (ORAC) - µmoles Trolox equivalence (TE)/g	≥2500	3444	2978	2574
Microbial standards				
Coliforms and *E.coli* (MPN/g)	<3	<3	<3	<3
Total Aerobic Plate Count (CFU/g)	<1000	<10	<10	<10
Staphylococcus aureus (MPN/g)	<3	<3 MPN/g	<3 MPN/g	<3 MPN/g
Salmonella – (ELFA) presumptive (per 25 g)	Negative	Negative	Negative	Negative
Mold (CFU/g)	<100	<10	<100	<10
Yeast (CFU/g)	<200	<10	<100	<10
Heavy metals*				
Arsenic (ppm)	<0.1	0.042	0.033	0.133
Cadmium (ppm)	<0.1	<0.005	<0.007	<0.009
Lead (ppm)	<0.1	0.033	0.032	0.154
Mercury (ppm)	<0.01	<0.007	<0.007	<0.009

*Heavy metal analysis was performed from different lots (Lot #12-170623-000; Lot #12-181206-001; Lot #12-190301-000)

Table 5. Certificate of Analysis of HIDROX® Liquid 10X

Parameter	Specifications	Lot Number		
		10x-121119-000	10x-121119-001	10x-121119-002
Appearance	Dark brown, semi-viscous liquid	Conforms	Conforms	Conforms
Flavor	Sour/Olives	Conforms	Conforms	Conforms
Odor	Processed olives	Conforms	Conforms	Conforms
pH (in 1g/10mL water)	3-4	3.1	3.1	3.1
Hydroxytyrosol (g/kg)	≥30	34	30	30
Coliforms and *E.coli* (MPN/g)	<3	<3	<3	<3
Total Aerobic Plate Count (CFU/g)	<1000	<10	<10	<10
Staphylococcus aureus (MPN/g)	<3	<3	<3	<3
Salmonella – (ELFA) presumptive (per 25 g)	Negative	Negative	Negative	Negative
Mold (CFU/g)	<1000	<10	<10	<10
Yeast (CFU/g)	<1000	<10	<10	<10
Heavy Metals				
Lead (mg/kg)	<1	0.04	0.036	0.037
Arsenic (mg/kg)	<1	0.081	0.073	0.075
Cadmium (mg/kg)	<0.5	0.003	0.003	0.003
Mercury (mg/kg)	<0.1	0.01	0.01	0.01

2.4. Manufacturing Process

The production process for Aqueous Olive Pulp Extract (Hidrox®) is illustrated in Figure 3. Aqueous Olive Pulp Extract is manufactured according to current good manufacturing practices (GMPs). The extract is manufactured at Specialty Concentrates, LLC, 9505 Rd. 301/2, Madera, CA. 93638, U.S.A. The FDA food facility registration number for the manufacturing facility is prl 144.

Aqueous Olive Pulp Extract (Hidrox®) is manufactured from byproducts of olive oil processing. Olives are transported to the process plant in large bins. The olives are transferred into a receiving hopper and then to a vibrating tray dry cleaner to remove olive leaves, stones, mud, and dirt. This is followed by passing the olives over a de-stemmer to remove any remaining stems. Following this step, the olives pass through a rotary washer, vibratory tray, and de-watering screen for removal of any small remaining debris. The de-watering screen also removes most of the water from the surface of the olives. Subsequently, the olives are transferred to the stoner, which presses the olives into a minced pulp, and separates the olive pit from the pulp without crushing the pit. Alternatively, olives go directly from the washer to the crushing devise (stone or hammer) where a mix (slurry) of biomass, oil, emulsified water (juice) and wood from pits is generated. Although the manufacturing process hereby described is related to pitted olives, similar results in producing Aqueous Olive Pulp Extract (Hidrox®) can be expected with olives that are not pitted.

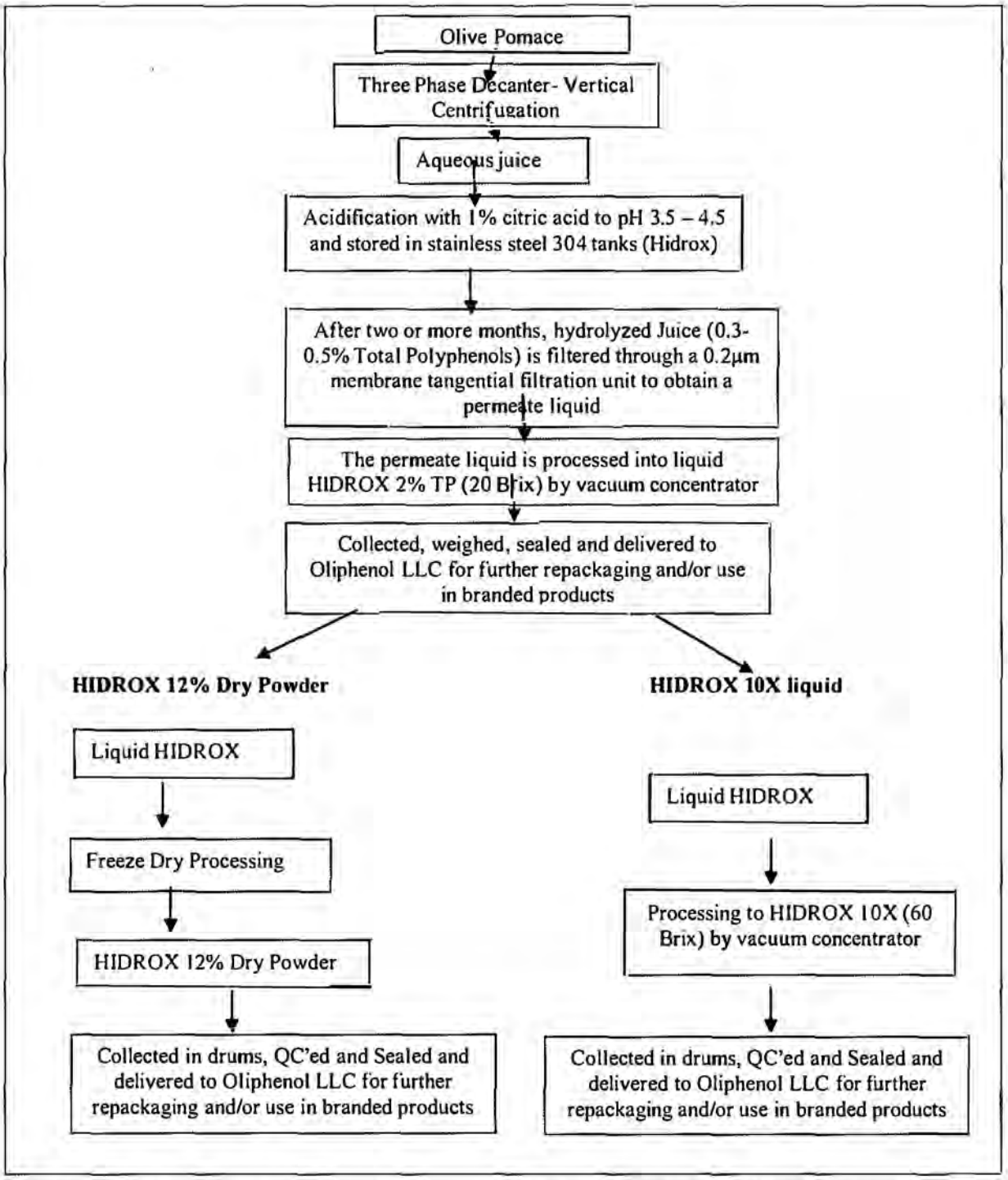


Figure 3. Manufacturing process of Aqueous Olive Pulp Extract (Hidrox 10X and Hidrox 12%)

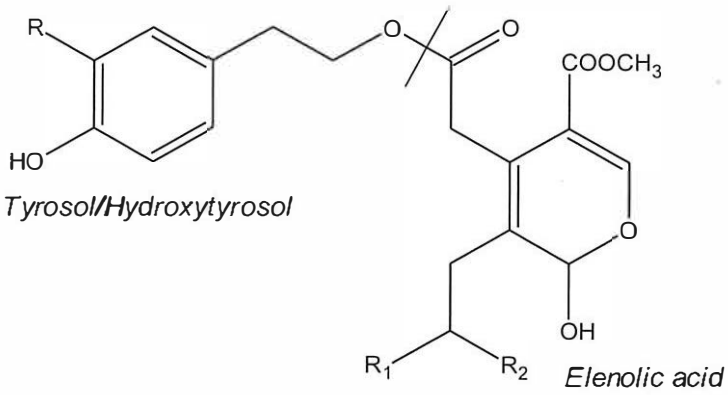
The olive pulp is pumped into one of two double sets of warm water-jacketed cut-and-fold kneaders, where the pulp is gently stirred. The olive pulp is held at a warm temperature (28-30°C) for approximately one hour to facilitate the separation of oil from the aqueous phase. Following this "kneading" process, the mixture is transferred into a horizontal centrifuge (decanter) where three fractions, oil, solid particles (or cake) and vegetation water, are separated.

Vegetation water, generated from the decanter is collected into a separate reservoir and pumped into a vertical centrifuge to separate the residual oil and large particulate solids from the vegetation water. Approximately 90% of the vegetation water is collected into stainless steel tanks and acidified with food grade citric acid to a specific pH range. The acid-treated olive juice is stored at room temperature for a minimum of 2 months (and can be up to 6-8 months), where oleuropein is hydrolyzed to hydroxytyrosol and elenoic acid. This process is monitored by HPLC until a minimum conversion ratio of 5:1 is attained. The liquid is then subjected to filtration through a 0.2 μm tangential membrane filtration unit to remove residual non soluble particulates and microorganisms. The resulting hydrolyzed juice 0.3%-0.5% Total Polyphenols (TP) (called Hidrox® 0.5%) is then concentrated into a semi-viscous liquid using a low-temperature, high-vacuum evaporation process. This produces a convenient concentration of 2% Total Polyphenols (TP) (Hidrox 2% or 20 Brix). This liquid can then be used for producing the high concentration syrup, and/or the production of the freeze-dried powder. In the first case, the concentrated syrup is standardized to 25 g per kg (2.5%) hydroxytyrosol and is called Hidrox® Liquid 10X. The manufacturing flow chart for Hidrox® Liquid10X is provided below.

For the freeze-dried powder of the Aqueous Olive Pulp Extract, the 2.0% TP liquid preparation mentioned above (Hidrox® 2.0%) is used to produce a standardized dry product, called Hidrox 12% TP. This powder product is produced by freeze-drying the Hidrox® 2.0% product, resulting in a powder consisting of 10-12% phenolic compounds (Hidrox 12% TP freeze-dried powder). The manufacturing flow chart is provided in Figure 3.

2.5. Biologically Active Constituents of Olive

Olive fruit is well known for the presence of simple, as well as complex phenolic substances. The phenolic content and composition of phenols in whole olives varies and depend on the altitude where the olive trees are grown, the harvesting time and the processing conditions (Soni et al., 2006). Similarly, the levels of phenolics in olive oil also depend on other factors such as cultivar, climate, ripeness of olives, preparation and storage of the oil. These phenolics are responsible for the stability of the oil from oxidation as well as its organoleptic properties (Visioli and Galli, 2001). Phenolic compounds in olive oil are present at levels up to 1% by weight, both as simple (hydroxytyrosol and tyrosol) and complex chemicals (hydroxytyrosol or tyrosol esterified to elenolic acid, in the form of oleuropein and ligstroside, respectively) (hydroxytyrosol + elenolic acidt→ oleuropein and tyrosol + elenolic acidt→ ligstroside; Figure 4). Oleuropein and ligstroside in intact olives are present in the glycosidic, relatively polar form. During production (crushing) process, hydroxytyrosol and tyrosol, as well as the lipid soluble oleuropein and ligstroside aglycones, are partially released (5-10% of the total in olives) from olives into the oil, while a substantial proportion of these constituents remain in the water phase (vegetation water).



R	R1	R2	
OH	H	H	Oleuropein
H	H	H	Ligstroside

Figure 4. Most relevant phenolics found in olive and olive products. Hydroxytyrosol is formed by cleavage where indicated

The phenolic content of two brined olive drupe types (black and green) has been investigated by Owen et al. (2003) (Table 6). The green olives were found to contain primarily hydroxytyrosol, while the black olives contain tyrosol, hydroxytyrosol, dihydrocaffeic acid, dihydro-*p*-coumaric acid (phloretic acid), acetoside (a disaccharide linked to hydroxytyrosol and caffeic acid), acetoside isomer and the flavonoids apigenin and luteolin. These investigators also reported that consumption of approximately 50 g of black olive pericarp would provide about 400 mg of phenolic substances to the daily dietary intake, while a similar quantity of extra virgin olive oil (produced with conventional methods) provides about 12 mg. In another report on analysis of 48 olive samples, Romero et al. (2004) reported that the 'turning color olives' in brine had the highest concentration of polyphenols (approximately 0.t l 2%).

Table 6. Basic Characteristics and Phenolics in Black *vs.* Green-brined olives

Component	Olive type	
	Black	Green
Pericarp		
Total g wet wt	71.78	111.60
Total g dry wt	35.89	29.30
G dry wt. *per* drupe	1.79	1.46
Water (% of wet wt)	50.00	73.70
Phenolics in pericarp		
Mg *per* drupe	29.43	6.56
% of wet wt	0.82	0.12
% of dry wt.	1.64	0.458
Oil		
Total g	5.52	18.22
% of wet wt.	7.69	16.3
% of dry wt.	15.4	62.2

Values for 20 drupes of each olive type

Olive mill water, also called as vegetation water, is a good source of phenolic antioxidants (1-1.8% w/v), as during the pressing of the drupes approximately 90% of the phenols in olives are transferred to the water phase. Visioli and Galli (2003) reported that approximately 10-20% of the total phenol content from vegetation water can be recovered; the only bioactive catechol recovered is hydroxytyrosol. In another study, Fernandez-Bolanos et al. (2002) reported extraction of 3 kg of hydroxytyrosol (90-95% purity) from 1000 kg of olives during liquid-solid waste of two-phase (conventional) olive oil processing.

Part III- DIETARY EXPOSURE

3.1. Proposed Use Levels and Food Categories

Oliphenol intends to use Aqueous Olive Pulp Extract (Hidrox®) in 11 food categories: bakery products; beverages; dairy products and substitutes; desserts; fats and oils; fruit juices and nectars; dry seasoning mixes for meat, poultry and fish; chewing gum; sauces, dips, gravies and condiments; snacks; and vegetable juices to deliver 5 to 10 mg of hydroxytyrosol *per* serving (reference amounts customarily consumed, 21 CFR 101.12). As the subject of this present GRAS document contains about 3.5% hydroxytyrosol, the per serving use levels of Aqueous Olive Pulp Extract (Hidrox®) will be approximately 150 to 300 mg/serving. The food serving size to which the extract will be added corresponds to the gram weight or mL volume of food as specified by References Amounts Customarily Consumed (RACCs) for food labeling based on FDA's final rule, effective July 26, 2016, with the compliance date of July 26, 2018 (Federal Register, 2016). Table 7 lists the 11 food categories to which Aqueous Olive Pulp Extract is proposed for use, descriptions of the types of foods within the category that was included in the assessment, the serving size associated with each food type, and the maximum use level of the extract.

Aqueous Olive Pulp Extract (Hidrox®) is intended for use in the same foods and at identical levels of addition to those described in the GRAS notification on "Phenolic preparation from olive" (GRN 726) by DSM Nutritional Products, LLC's (DSM, 2017). The Aqueous Olive Pulp Extract (Hidrox®) will not be used in any foods for which food standards would preclude its use. Foods that are intended for infants and toddlers, such as infant formulas or foods formulated for babies or toddlers, and meat and poultry products that come under USDA jurisdiction are excluded from the list of intended food uses of Aqueous Olive Pulp Extract (Hidrox®).

3.2. Estimated Daily Intake

As mentioned above, Aqueous Olive Pulp Extract (Hidrox®) will be used in bakery products; beverages; dairy products and substitutes; desserts; fats and oils; fruit juices and nectars; dry seasoning mixes for meat, poultry and fish; chewing gum; sauces, dips, gravies and condiments; snacks; and vegetable juices to deliver 5 to 10 mg of hydroxytyrosol *per* serving. The intended use of Aqueous Olive Pulp Extract (Hidrox®) is in the same foods and at identical levels of addition to those described in GRN 726 by DSM (2017). There are no new food uses proposed for Aqueous Olive Pulp Extract. The application of Aqueous Olive Pulp Extract (Hidrox®) to the same foods and at the same levels (5-10 mg hydroxytyrosol/serving) as those described in GRN 726 is not expected to noticeably affect the intake of hydroxytyrosol in the diet of the public from introduction into the market by another supplier who will have to compete in essentially the same market and in same foods.

In GRN 726, the intake analysis for the US population's consumption of hydroxytyrosol, from existing and proposed uses was based on food consumption records collected in the What We Eat in America (WWEIA) component of the National Health and Nutrition Examination Surveys (NHANES) conducted in 2007-2008 and 2009-2010 (DSM, 2017). This continuous survey is a complex multistage probability sample designed to be representative of the civilian US population. The NHANES datasets provide nationally representative nutrition and health data and prevalence estimates for nutrition and health status measures in the US. In GRN 726 submitted to the FDA, DSM (2017) reported that the cumulative dietary exposure to

hydroxytyrosol for the total users only US population (2 years and older) is 30 mg/person/day (0.5 mg/kg bw/day) at the mean and 52 mg/person/day (0.9 mg/kg bw/day) at the 90th percentile. In a response letter of February 28, 2018 to GRN 726, FDA (2018) did not question the intake estimate analysis performed and presented by DSM (2017).

Table 7. Proposed Uses of Aqueous Olive Pulp Extract (Hidrox®) in Foods*

Food Category	Use Level (mg/serving)		RACC[b] (g/serving)
	Hydroxytyrosol	Hidrox®[a]	
Bakery Products			
Crackers that are usually used as snacks	5	150	30
Croutons	5	150	7
Grain-based bars with or without filling or coating (e.g., breakfast bars, granola bars, rice cereal bars)	10	300	40
Protein based, meal replacement and energy bars	10	300	40
Beverages			
Sport drinks, energy drinks, milk-based meal replacements, flavored waters and fruit-flavored drinks	5	150	240
Dairy Products and Substitutes			
Yogurt	10	300	225
Desserts			
Frozen yogurt	10	300	120
Fats and Oils			
Butter, margarine, oil and shortening	5	150	15
Dressing for salads	5	150	30
Mayonnaise, sandwich spreads, mayonnaise-type dressings	5	150	15
Fruit and Fruit Juices			
Fruit juices and fruit nectars	5	150	240
Miscellaneous			
Meat, poultry, and fish coating mixes, dry; seasoning mixes, dry (e.g., chili seasoning mixes, pasta salad seasoning mixes)[c]	5	150	4.5
Chewing gum	10	300	3
Sauces, Dips, Gravies, Condiments			
Major main entree sauces (e.g., spaghetti sauce)	5	150	125
Minor main entree sauces (e.g., pizza sauce, pesto sauce), other sauces used as toppings (e.g. gravy, white sauce, cheese sauce), cocktail sauce	5	150	60
Major condiments: catsup only	5	150	15
Barbecue sauce, hollandaise sauce, tartar sauce, other sauces for dipping (e.g., mustard sauce, sweet and sour sauce), all dips (e.g., bean dips, dairy-based dips, salsa)	5	150	30
Snacks			
All varieties, chips, pretzels, popcorns, extruded snacks, fruit-based snacks (e.g., fruit chips), grain-based snack mixes	5	150	30
Vegetable Juices			
Vegetable juice	5	150	240

*Adapted from GRN 726

[a] Hidrox contains approximately 3.5% hydroxytyrosol

[b] RACC= Reference Amounts Customarily Consumed per eating occasion – 21 CFR §101.12 (CFR, 2014). 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.

[c] The estimated RACC for dry seasoning mixes was estimated to be 4.5 g dry spice rub (i.e., 2 teaspoons per serving) based upon publicly available food recipes for mixed dishes containing dry seasonings and rubs from McCormick Spices (http://www.mccormick.com/Grill-Mates/Recipes). This is the lowest value, which would provide a worst-case scenario for estimating exposure to a food additive in dry seasonings and rubs.

As reported in GRN 726, the cumulative estimated daily intake of hydroxytyrosol from existing dietary sources and the proposed uses described in GRN 726 (to deliver 5 to 10 mg/serving of hydroxytyrosol in 11 food categories) the highest 90th percentile per user cumulative estimated dietary intake of hydroxytyrosol was determined as 55.1 mg/day among teenagers ages 13 to 18 years (0.9 mg/kg-bw/day). The 90th percentile per user cumulative estimated daily intake for the US population 2 years and older was determined as 52.4 mg/day (0.9 mg/kg-bw/day). The resulting 90th percentile intake of Aqueous Olive Pulp Extract (Hidrox®) will be approximately 1500 mg/person/day. For safety assessment purposes, the highest intake for Aqueous Olive Pulp Extract of 1500 mg/person/day (25 mg/kg bw/day) is considered.

3.3. Exposure from Presence in Food

The olive fruits and its oil have been used for centuries and are considered as an important part of the Mediterranean diet. Given their organoleptic characteristics, olives require processing prior to consumption (Brenes-Balbuena et al., 1992a; Brenes-Balbuena et al., 1992b; Brenes-Balbuena et al., 1995; Goupy et al., 1991; Robards et al., 1999; Sciancalepore and Longone, 1984). The phenolics in fruits or oil constitute a complex mixture. However, there are some notable differences in composition between the two that are attributed to a series of chemical or enzymatic alterations of some phenols during oil extraction. These changes include hydrolysis of glycosides by glucosidases (Montedoro et al., 1993; Angerosa et al., 1996), oxidation of phenolic compounds by polyphenol oxidases and, the polymerization of free phenols (Ryan et al., 1999).

The quality of virgin oil is affected by the presence of phenolic compounds in olive fruits, as these compounds are partly responsible for the stability and sensory characteristics. Given the antioxidant properties, olive polyphenols, including hydroxytyrosol, have been the subject of several clinical and preclinical investigations. Among the phenolic compounds present in raw olive flesh, hydroxytyrosol and tyrosol are the most abundant and second most abundant phenolics, predominantly occurring as esters. Hydroxytyrosol and tyrosol are structurally similar, hydroxytyrosol possessing an extra hydroxy group in the meta-position. Both also occur as esters, a notable example being the glycoside oleuropein. Oleuropein is an ester consisting of hydroxytyrosol and elenolic acid.

Blekas et al. (2002) reported hydroxytyrosol (unbound) content of table olives as 250-750 mg/kg (approximately 0.5 mg/g) in two cultivars. Marsilio et al. (2001) reported that consumption of 50 g black olive pericarp provides approximately 400 mg of phenolic substances, while a similar quantity of extra virgin olive oil provides about 12 mg. As reported in GRN 726, the levels of hydroxytyrosol in raw olives, including that present in conjugated forms, are of the order of up to 1,800 mg/kg (DSM, 2017). However, raw olives are not edible and need to undergo extensive processing to produce the forms such as table olives and olive oil that are most commonly consumed. Depending on the source and specific type of treatment, hydroxytyrosol levels in table olives vary and can range from 400 to 1000 mg/kg. Processing is also known to reduce hydroxytyrosol levels in olive oil and it ranges from 15 to 20 mg/kg.

In some Mediterranean countries, the average consumption of table olives is over 10 g/day, while individual consumption could be as high as 30 g/day. Similarly, in these countries, consumption of olive oil on average is about 70 g/day and could be as high as 200 g/day for high level consumers. Combining these occurrence levels and consumption data results in estimates of

average intakes of hydroxytyrosol in some Mediterranean countries of 12 mg/day, with the potential for high level intakes to exceed 30 mg/day (DSM, 2017). For an individual weighing 60 kg, the combined hydroxytyrosol average and high intake in some Mediterranean countries can range from 0.2 to 0.5 mg/kg bw/day.

Part IV- SELF LIMITING LEVELS OF USE

Oliphenol is unaware of any specific physical or technically impractical effects for Aqueous Olive Pulp Extract (Hidrox®). Given the characteristic taste of Aqueous Olive Pulp Extract (Hidrox®), it is unlikely that excessive amounts of this product are likely to be added to food products.

Part V- EXPERIENCE BASED ON COMMON USE IN FOOD BEFORE 1958

The statutory basis for the conclusion of the GRAS status of Aqueous Olive Pulp Extract (Hidrox®) in this document is not based on common use in food before 1958. The GRAS assessment is based on scientific procedures. Notwithstanding this, the source material of the extractt– olives – has been commonly present in food prior to 1958, as described below.

Part VI- NARRATIVE

6.1. Traditional and Current Uses

The available information indicate that the olive tree was cultivated as early as 3500 BC in Crete (Greek Islands). The tree has always represented a symbol of abundance, glory and peace, and its leafy branches were used to crown the victorious in friendly games and war. It has also been used to anoint the noblest of heads throughout history. During 1300 and 1200 BC, Egyptian ruler, Ramses II, used olive oil for nearly every ailment. Shortly after the Iron Age began (1100 - 750 BC), Greece became a large producer of olives/olive oil, spurred in the sixth century BC by the prohibition of Solon (an Athenian lawmaker) of export of any agricultural produce other than olive oil. Furthermore, throughout the Roman Empire, olive oil became a popular staple in the diet. At present, approximately 90% of the world's olives are used in the production of oil, with Spain, Italy, Greece and Portugal representing the main producers (Kiple and Ornelas, 2000).

Olives cannot be eaten directly from the tree, as the fruit contain a bitter alkaloid. In contrast to olives picked for oil production, olives selected for human consumption are picked green and unripe. Because the raw green olives have a naturally bitter taste, the fruit cannot be consumed unless further prepared/processed or cured. Edible olives preparation involves pickling in a solution of lye to remove the bitter taste (rendered by oleuropein). This practice of picking olives has been in use since Roman times. The traditional way of processing olives, which is still a standard practice, involves three steps: blanching, salting and drying of mature olives (Borzillo et al., 2000). The black color of table olives has nothing to do with ripeness, but it is the result of exposure to air after lye extraction of olives. Black olives, used in the extraction of oil, are allowed to ripen on the tree until after the time of the first frost.

This information suggests that the olive and its oil is consumed as a food without any reported adverse effects.

6.2. Data Pertaining to Safety

In the following section, relevant toxicological and other studies on the olive and its preparations are summarized in the order of their importance and in support of the conclusions drawn in this assessment. This information is provided in the following sequence: published pivotal studies, secondary published studies, corroborative unpublished studies and regulatory agency assessments. Efforts have been made to present both the data supporting the safety as well as any data on the adverse effects of olive fruit and its preparations.

6.2.1. Pivotal Studies of Aqueous Olive Pulp Extract

In a series of toxicity studies, conducted as per current accepted guidelines, the effects of Aqueous Olive Pulp Extract (Hidrox®) has been investigated in animals and *in vitro* experimental systems. All studies were conducted in strict compliance with Good Laboratory Practices (GLPs), as defined by the FDA (FDA, GLP, Final rule, 1987). Toxicological procedures in the acute studies reflect those described in the Redbook 2000. The overall findings from all these studies with the subject of present GRAS, Hidrox®, are published in the journal *Drug and Chemical Toxicology* by Christian et al. (2004). Additionally, these specific studies and other available information on the olive and its preparations is extensively summarized in a review article published in *Food and Chemical Toxicology* (Soni et al., 2006). A summary of the

published pivotal toxicity studies of Aqueous Olive Pulp Extract (Hidrox®) is provided in Table 8.

Table 8. Summary of Pivotal Toxicity Studies of Olive Pulp Extract (Hidrox®)

Study type	Dose, Form and Route	Duration	Animal model	Summary	Reference
Subchronic	Hidrox® (0, 1000, 1500 or 2000 mg/kg bw) per day by oral gavage. (0, 60, 90 & 120 mg phenolics/kg bw/day)	90 days	Crl:CD Sprague-Dawley rats	No effects on body weight, body weight gain, feed consumption	Christian et al., 2004
Reproductive and Developmental Toxicity	Hidrox® (0, 1000, 1500 or 2000 mg/kg bw) per day by oral gavage. (0, 60, 90 & 120 mg phenolics/kg bw/day)	14 days	8/sex/ group Crl:CD Sprague-Dawley rats	Estrous cycle of females, mating & reproductive performance of males & females not affected	Christian et al., 2004
Developmental Toxicity Designed in accordance with FDA Redbook 2000	Hidrox® (0, 1000, 1500 or 2000 mg/kg bw) on day 6-20 of gestation by oral gavage. Phenolic content of extract was 6%.	Entire length of gestation in rat	25 female Crl:CD Sprague-Dawley rats per group	No adverse clinical observations of significant changes in maternal body weights, gravid uterine weights, or feed consumption. No malformations occurred in pups. Maternal & develop-mental no observed adverse effect level (NOAEL) of extract > 2000 mg/kg/day	Christian et al., 2004
Short-term	Hidrox® (5000 mg/kg bw) by oral gavage. (300 mg phenolics/kg bw/day)	29 days	Crl:CD Sprague-Dawley rats	Tolerated after single and repeat dosing	Christian et al., 2004
Acute	Single dose, Hidrox® (aqueous olive pulp extract) (500, 1000, or 2000 mg/kg bw) by oral gavage. (0, 60, 90 & 120 mg phenolics/kg bw day)	Acute study	CD1 mice	No mortality, LD_{50} of extract > 2000 mg/kg	Christian et al., 2004
Acute	Single dose, Hidrox® (0, 1000, 1500 or 2000 mg/kg bw) by oral gavage. (0, 60, 90 & 120 mg phenolics/kg bw/day)	Acute study	Crl:CD Sprague-Dawley rats	No adverse effects except soft or liquid feces	Christian et al., 2004

Mutagenicity and Genotoxicity Studies

Study type	System	Test sample	Results	Conclusion	Reference
In vitro	*Salmonella* and *E. coli* reverse mutation assay	Each gram of test article contains ~24 mg hydroxytyrosol (HT) & 60 mg phenolics.	For *Salmonella*, concentrations of 1000 µg/plate or more showed mutagenic activity in the presence of S9 activation. For *E. coli*, twofold increase occurred in mean number of revertants at concentration of 2500 µg/plate, in	Positive at higher concentrations in certain strains.	Christian et al., 2004

			the absence of S9.		
In vitro	Chromosome aberration in Chinese Hamster Ovary (CHO) cells	In each gram of test article, approximately 24 mg hydroxytyrosol (HT) & 60 mg phenolics.	Extract showed significant increase in percentage of aberrant cells at 1000 µg/mL in presence of S9. Slight increases in numbers of polyploidy & /or endoreduplicated cells noted at 1000 µg/mL.	Extract was positive for induction of chromosome aberrations.	Christian et al., 2004
In vivo	Micronucleus test in rats	In each gram of test article, approximately 24 mg hydroxytyrosol (HT) & 60 mg phenolics.	No increase in micronucleated polymorphic erythrocytes for any dose / time period in study. Hidrox® did not produce adverse clinical or necropsy observations or affect feed consumption values in any of animals. Body weight gains for male & female rats receiving 5000 mg/kg bw reduced at third & fourth weeks of dosing.	Negative	Christian et al., 2004

6.2.1.1 Subchronic Toxicity Study of Aqueous Olive Pulp Extract

In a subchronic study, Crl:CD® Sprague Dawley male and female rats (20/group/sex) were administered (*via* gavage) a daily dose of Aqueous Olive Pulp Extract (Hidrox®) (dissolved in aqueous 0.5% methylcellulose) at levels of 0 (vehicle), 1000, 1500 and 2000 mg/kg bw/day (0, 60, 90 and 120 mg/kg bw/day of phenolics) for 90 days (Christian et al., 2004) (Table 8). The selection of the gavage route was based on the fact that (1) gavage administration most simulates the method of intake in humans, consumed over a relatively short period of time; and (2) high doses of the extract are not palatable. All standard parameters were studied. Physical and ophthalmic examinations were conducted on all rats before and near the end of the study. The following investigative parameters were recorded: daily clinical signs; weekly body weights and feed consumption; hematology and serum chemistry determinations at the time of necropsy; gross necropsy observations and histopathology of selected tissues at termination of the study. Three satellite groups (described later) were also attached to the main study.

Morbidity and mortality observations did not reveal any unusual findings. Excess salivation and the presence of perioral substance noted in the treatment group were probably associated with technical difficulties in administering the relatively thick granular suspensions of the extract. All other clinical observations were considered unrelated to the test article. Administration of the extract did not affect body weights, body weight gains or feed consumption. A significant decrease in body weight gain noted for male rats in the 1000 mg/kg/day group on days 71-78 was considered unrelated to the test article because the value was not dose-related. HIDROX® administration did not affect the organ weights. Ophthalmologic observations did not reveal any treatment-related lesions (Christian et al., 2004).

Hematological investigations [WBC, RBC, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), platelet counts, prothrombin time and total serum protein] did not reveal any treatment-related differences between the groups, except some incidental findings. Although not significant at two lower doses (1000 and 1500 mg/kg bw/day), a dose-related increasing trend in the number of RBC was noted in female rats. The increase was significant in the 2000 mg/kg bw/day dose group. However, the

values were within the range of historical control values. Increases in RBCs in female rats, in the absence of changes in MCV, MCH, and MCHC were interpreted as a slight erythropoietic stimulation of the bone marrow without any toxicological consequences. All other hematological parameters in the male and female rats were unaffected (Christian et al., 2004).

Serum chemistry analysis for liver function tests such as aspartate aminotransferase (AST), alanine aminotransferase (ALT) and sorbitol dehydrogenase (SDH) showed a negative trend. Reduction in these enzymes may be related to decreases in cholesterol levels. Levels of ALT were significantly reduced in both male and female rats in all the extract treated groups. A significant reduction in SDH levels was noted in female rats treated with 1500 and 2000 mg/kg bw/day doses. Increases in plasma levels of ALT, AST and SDH are considered as markers of hepatotoxicity. Decreases in these plasma enzymes (ALT, AST and SDH), observed in most of the extract treated groups of male and female rats, were not considered to be a toxic manifestation. The reasons for decreased activity could not be determined, although it may be associated with the large quantities of olive polyphenols that have to be excreted in the bile. The biliary excretion of large doses of the extract may also account for the slightly decreased serum cholesterol levels in both male and female rats (significantly decreased in females at 2000 mg/kg bw/day), because primary bile acids are synthesized by the liver from cholesterol (Tennant, 1999). Although negative trends in plasma levels of ALT, AST and SDH were noted, all values were within historical control value ranges. The changes were minor and do not appear to be a toxicologically adverse response. Secondly, other biomarkers of liver function (serum bilirubin, alkaline phosphatase, protein levels and histopathology) were unaffected by the extract. In addition to the above-described changes, some incidental findings were noted. These incidental findings were considered insignificant, as these changes were either not dose-related, of small magnitude or not consistent between sexes (Christian et al., 2004).

Histopathological examinations revealed minimal or mild focal hyperplasia of the mucosal squamous epithelium of the limiting ridge of the forestomach of male and female rats in the high dose (2000 mg/kg bw/day) group. Similar changes with a very low incidence of hyperplasia were noted in animals treated with mid dose (1500 mg/kg bw/day) of the extract and in the control group. All other microscopic changes that were noted in the various organs and tissues examined were considered spontaneous in origin, incidental to treatment, and not associated with any systemic toxicity of the extract. Although focal, minimal or mild hyperplasia of the mucosal squamous epithelium of the limiting ridge of the forestomach was noted, this type of change is often associated with irritation of this area of the gastric mucosa and was considered to be due to local irritation by the large intubated volume of viscous, granular formulation. Based on the findings from this study, the no observed adverse effect level (NOAEL) is established as 2000 mg/kg bw/day, the highest dose tested (Christian et al., 2004). The results of this study suggest that the resulting all user maximum intake of 25 mg/kg bw/day from the proposed uses of Aqueous Olive Pulp Extract (Hidrox®), the NOAEL is 80-fold lower.

6.2.1.2 Developmental and Reproductive Toxicity of Aqueous Olive Pulp Extract

In a study designed to investigate potential reproductive and developmental toxicities, Aqueous Olive Pulp Extract (Hidrox®) was administered by oral gavage to Crl:CD® Sprague Dawley rats (Christian et al., 2004) (Table 8). In this study, rats (8/sex/group) were assigned to five dose groups received the extract at dose levels of 0, 500, 1000, 1500 and 2000 mg/kg bw/day once daily for 14 days before cohabitation and continued until the day before necropsy (males were euthanized after being administered a total of 49 daily doses of the extract; females

were euthanized after completion of the 22-day post-partum period). Clinical observations were recorded daily, while body weights of the male and female rats were recorded on days 1 through 7 and 14, and then weekly for the males and daily for the females during gestation and on days 0, 4, 7, 10 and 14 of lactation. Female rats were also observed for estrous cycling (two weeks each pre-treatment and treatment), adverse clinical signs during parturition, maternal behavior, duration of gestation, litter sizes and pup viability at birth and maternal behavior. Pups in each litter were counted and clinical observations were recorded once daily during the one-week postpartum period (birth= day zero postpartum).

In this study, on day 21 postpartum, all F_1 generation pups were weaned. Two male and two female F_1 generation pups *per* litter (*i.e.*, a total of 160 rats, 80 rats *per* sex) were selected for a week of daily gavage treatment and recording of clinical signs, body weights and viability before being euthanized and necropsied on post-partum day 28. All other pups were subjected to gross necropsy on post-partum day 21. On day 28 postpartum, the retained pups were euthanized and examined for gross lesions at necropsy. Male rats in the main study were killed after completion of the cohabitation period. Surviving female rats in the main study were killed after completion of the 22-day postpartum period. All main study rats were subjected to a gross necropsy of the thoracic, abdominal and pelvic viscera (Christian et al., 2004).

In the F0 generation, all male rats survived to the scheduled euthanasia. Occasional instances of excess salivation and non-dose-related increases in body weight gains were the only findings associated with the Aqueous Olive Pulp Extract administration. Absolute and relative feed consumption values for the entire dose period were not affected. Mating and fertility parameters for the male rats were comparable among the five dose groups. All necropsy observations were considered unrelated to the test article, as also were the terminal body weights, and the weights of the paired epididymides and testes. The ratios of the male reproductive organ weights to the terminal body weights were comparable among the five dose groups (Christian et al., 2004).

Except for one female, all female rats in the F0 generation survived until the scheduled euthanasia. The one dam in the 1000 mg/kg bw/day group was found dead on the first day of lactation. The death was attributed to a torsion of the right uterine horn, a finding unrelated to the test article. Incidental observations of excess salivation were noted in the female rats in the 1000, 1500 and 2000 mg/kg bw/day dose groups during the gestation period and in one or two female rats in all extract treated groups during the precohabitation and lactation periods. A tendency for increased body weight gains for the entire precohabitation period was also noted for these groups. Body weight gains for the entire gestation period were comparable among the groups, although the body weight gains for the entire lactation period were reduced in the 1500 and 2000 mg/kg bw/day dose groups, as compared with the control. Absolute and relative feed consumption values for the precohabitation, gestation and lactation periods for the female rats were comparable among the five dose groups, again indicating that the increased weight gains were probably associated with the nutritional value of the extract or increased water retention. Estrous cycling, mating and reproductive performance of the female rats were not affected by the extract treatment. Small reductions (<10%) in pup body weights on lactation days 7, 14 and 21 in the 1000, 1500 and 2000 mg/kg bw/day dose groups were noted. All other delivery and litter observations were comparable among the five dose groups (Christian et al., 2004).

Based on the findings from this study, Aqueous Olive Pulp Extract does not appear to be a reproductive or developmental toxicant. As no toxic effects were noted in the parental rats

during the first two weeks of treatment, similar doses (0, 1000, 1500 and 2000 mg/kg bw/day) of Aqueous Olive Pulp Extract (Hidrox®) were recommended for use in the developmental toxicity (see below) study and 90-day toxicity (described above in section 6.2.1.1.) study in rats. The high dose of 2000 mg/kg bw/day is generally considered the highest dose necessary for studies of this type (Christian et al., 2004).

In a follow-up to the above described study, Christian et al. (2004) evaluated the developmental toxicity (embryo-fetal toxicity and teratogenic potential) of Aqueous Olive Pulp Extract (Hidrox®) in Crl:CD® Sprague-Dawley rats (Table 8). This study was designed as per FDA Redbook 2000 recommendations (FDA, 2004). For these investigations, time-mated female rats (25/group) were randomly divided into four groups. On days 6 through 20 of presumed gestation, the extract or the vehicle (0.5% w/v methylcellulose) was administered *via* gavage once daily at dose levels of 0, 1000, 1500 and 2000 mg/kg bw/day. The phenolic content of the extract was 6% (60 mg/g).

One dam in the high dose (2000 mg/kg bw/day) group was euthanized, on day 21 of gestation, because of premature delivery of litter before scheduled Caesarean-sectioning. No abnormal findings were noted for this dam or its litter. All other rats survived until scheduled Caesarean sectioning. No adverse clinical or necropsy observations or significant differences in maternal body weights, body weight gains, gravid uterine weights, corrected maternal body weights or body weight gains or absolute or relative feed consumption values were noted between the groups. Caesarean-sectioning observations were based on 23, 22, 22 and 24 pregnant rats with one or more live fetuses in the four respective groups. The extract treatment did not affect litter parameters at any of the doses. No treatment-related increases in gross external, soft tissue and skeletal fetal alterations (malformations or variations) were observed. A significantly increased mean number of corpora lutea of the 2000 mg/kg be/day dose group was well within the historical range of 14.5-20.1 *per* litter of the laboratory and was attributed to two females that had 27 or 30 corpora lutea. Based on the findings from this study, the maternal and developmental NOAEL of Aqueous Olive Pulp Extract (Hidrox®) was 2000 mg/kg bw/day, the highest dose tested (Christian et al., 2004).

6.2.1.3 Genotoxicity Studies of Aqueous Olive Pulp Extract

In order to investigate genotoxic potentials, Aqueous Olive Pulp Extract (Hidrox®) was subjected to three mutagenicity assays: a bacterial reverse mutation assay, a chromosome aberration assay and a rat micronucleus assay (Christian et al., 2004; Soni et al., 2006) (Table 8).

6.2.1.3.1 Bacterial Reverse Mutation Assay (Ames test)

In an *in vitro* study, Aqueous Olive Pulp Extract (Hidrox®) was tested for potential mutagenicity in a bacterial reverse mutation assay employing *Salmonella typhimurium* strains TA97, TA98, TA100 and TA1535 and *Escherichia coli* strain WP2 *uvr*A (328), in the presence and absence of S9 (Table 8). The test was performed at different concentrations of the extract (0, 5, 10, 50, 100, 500, 1000, 2500 and 5000 µg/plate) and concentrations of 50, 100, 500, 1000 and 2500 µg/plate were used in the more sensitive confirmatory preincubation assay. A result was classified as positive (*i.e.*, mutagenic) if the average number of revertants in any strain at any test article concentration was at least two times greater than the average number of revertants in the concurrent vehicle control and/or there was a concentration-related increase in the mean revertants/plate in that same strain. In the Ames test, at concentrations of 100 µg/plate or above of the extract, precipitates were observed. As determined by a concentration-related

reduction in the mean number of revertants/plate and/or the reduction of the microcolony background lawns, toxicity was noted at concentrations of 500 µg/plate or above. Evidence of mutagenic activity was only detected in strains TA98 and TA100 at doses of 1000 and 2500 µg/plate (in the presence of S9 activation for both the strains). The number of revertants *per* plate at concentrations of 0, 1000 and 2500 µg/plate for TA 98 were reported as 23, 52 and 133, respectively. Similarly, the number of revertants *per* plate for the strain TA100 was reported as 157, 372 and 1051, respectively (Christian et al., 2004).

In experiments with *E. coli,* no mutagenicity was noted at any of the concentrations tested, except for a two-fold increase in mean number of revertants at concentration of 2500 µg/plate, in the absence of S9. The positive results were confirmed in the more sensitive preincubation test, but only with metabolic activation. Some inconsistencies between the regular and repeat trials were noticed. The investigators stated that the antibacterial properties of the test article, and observation of positive findings only at one or two concentrations, where precipitates and toxicity occurred, tended to complicate the interpretation of these mutagenic findings. The investigators concluded that under the conditions of the study, equivocal evidence of mutagenic activity of Aqueous Olive Pulp Extract (Hidrox®) was detected in *S. typhimurium* strains TA98 and TA100 (Christian et al., 2004).

6.2.1.3.2 Chromosomal Aberrations Assay

In another *in vitro* genotoxicity assay, the effects of Aqueous Olive Pulp Extract (Hidrox®) on chromosome aberrations in Chinese Hamster Ovary (CHO) cells were investigated, in the presence and absence of metabolic activation system (S9) (Christian et al., 2004; Soni et al., 2006) (Table 8). The cell cultures were treated with 0, 10, 50, 100, 300, 600 and 1000 µg of the extract/ml; positive and negative (vehicle, dimethyl sulfoxide) controls were also included. Cultures were incubated with the extract for approximately three hours, after which the treatment medium was washed and replaced with a fresh culture medium. Cells were sampled at a time approximately 20 hours from the beginning of treatment. Approximately two hours prior to harvest, Colcemid® was added to arrest cells in metaphase. Test article concentrations of 100, 300 and 1000 µg/ml were assessed for effects on mitotic index, polypoid cells and aberrations (chromatid and chromosome breaks/exchanges).

In this study, no clear evidence of test article-related toxicity, as evidenced by the confluence rate or mitotic index, was observed at any concentration level of the extract. The extract elicited a significant increase in the *percentage* of aberrant cells at 1000 µg/ml in the presence of S9. At this concentration, slight increases in the numbers of polyploid and/or endoreduplicated cells (numerical chromosome changes) were also noted. The positive response was associated with the presence of test article precipitate during treatment. Based on the findings of this study, the investigators concluded that Aqueous Olive Pulp Extract (Hidrox®) was positive for the induction of chromosome aberrations in CHO cells (Christian et al., 2004).

6.2.1.3.3 Micronucleus Assay

As described above, both *in vitro* assays (bacterial reverse mutation and CHO chromosome aberration) exhibited evidence of mutagenic activity of Aqueous Olive Pulp Extract (Hidrox®) (Table 8). However, the positive results in both these assays were only observed at one or two of the highest concentrations of Aqueous Olive Pulp Extract (Hidrox®), where slight amounts of precipitation were noted, and were confirmed only in the presence of

S9 metabolic activation. The effect of the precipitate on the outcome of the results could not be determined. Given the confounding factors present in the bacterial and tissue culture assays, a study in live animals, *i.e.,* a micronucleus assay in rats for Aqueous Olive Pulp Extract (Hidrox®) was undertaken (Christian et al., 2004).

In the micronucleus assay, adult male and female Crl:CD® Sprague Dawley IGS BR rats were used. Single and 28 consecutive daily doses of 1000, 1500 and 2000 mg/kg bw/day, and 29 consecutive days of 5000 mg/kg bw/day, of Aqueous Olive Pulp Extract (Hidrox®) were gavaged. The number of rats included in each group were as follows: vehicle or negative control (10/sex); 1000 mg/kg bw/day dose (5/sex); 1500 mg/kg bw/day dose (5/sex); 2000 mg/kg bw/day dose (14/sex); 5000 mg/kg bw/day dose (5/sex; additional group of rats tested after 29 consecutive days of treatment); and positive control dose (5/sex- treated with cyclophosphamide). In addition to euthanization at 24 hours from all groups, rats in negative control dose (5/sex) and receiving 2000 mg/kg bw/day (7/sex) were also euthanized at 48 hours. Following euthanization of the rat at approximately 24 hours after the last dose, bone marrow samples from the femur of each rat were collected for further analysis (Christian et al., 2004).

A minimum of 2000 polychromatic erythrocytes (PCEs) was scored for micronuclei. The number of PCEs among 500 total erythrocytes (expressed as PCEs fraction) was determined for each animal. The number of micronucleated normochromatic erythrocytes (NCEs) was also recorded. The extract did not produce adverse clinical or necropsy observations or affect absolute or relative feed consumption values. Body weight gains for the male and female rats in the highest dose group were reduced at the third and fourth weeks of daily dose, as compared with previous weeks. The numbers of micronucleated PCEs were not significantly increased in any of the extract treated groups, as compared to the control. The ratio of PCEs to NCEs was not affected by the administration of the extract. The positive control, on the other hand, elicited clear increases in micronucleated-PCEs in both sexes, without causing excessive toxicity. The micronucleus assays after repeated dosages of the extract confirmed the results achieved after single dosages, and it was concluded that the extract was not mutagenic. The results of this study demonstrate that Aqueous Olive Pulp Extract (Hidrox®) was negative in the micronucleus assay at 24 and 48 hours after a single dose of 1000, 1500 or 2000 mg/kg and also at 24 hours after 28 daily doses of 0, 1000, 1500, 2000 or 5000 mg/kg (Christian et al., 2004).

6.2.1.4 Acute and Short-term Toxicity Studies of Aqueous Olive Pulp Extract

In an acute toxicity study in CRL:CD1 ICR (BR) mice, oral gavage administration or dermal application of a single dose of Aqueous Olive Pulp Extract (Hidrox®) at levels of 500, 1000 or 2000 mg/kg failed to produce any adverse effects on clinical observations, body weight, body weight changes or gross pathology (Table 8). No mortality was noted in any of the treatment groups, suggesting that the LD_{50} of the extract is greater than 2000 mg/kg in mice. The extract was well tolerated in mice (Christian et al., 2004). In another study, oral administration of a single gavage dose of Aqueous Olive Pulp Extract (Hidrox®) (Christian et al., 2004) at levels of 0, 1000, 1500 or 2000 mg/kg to Crl:CD® Sprague Dawley rats (5/sex/group) did not cause any adverse effects except soft or liquid feces (Christian et al., 2004).

In a short term study that was conducted as part of a micronucleus assay, Crl:CD® Sprague Dawley rats (5/sex) were administered (gavage) a single dose of 5000 mg olive pulp

extract/kg, and the rats were observed for six days, after which the 5000 mg/kg dose was given for 29 consecutive days (Christian et al., 2004) (Table 8). No mortality or clinical signs of toxicity were noted. Both male and female rats continued to gain weight, although at a reduced rate as compared to the control rats. Absolute and relative feed intake were similar to that in the control animals. The findings from this study show that the LD_{50} of Aqueous Olive Pulp Extract (Hidrox®) is greater than 5000 mg/kg and suggests that Hidrox® is practically nontoxic (Christian et al., 2004).

6.2.1.5 Absorption Study of Aqueous Olive Pulp Extract

As part of the above described subchronic toxicity study, Christian et al. (2004) also investigated the absorption of hydroxytyrosol from exposure to Aqueous Olive Pulp Extract (Hidrox®). For this assessment, Sprague Dawley rats (6/sex/group) were administered Aqueous Olive Pulp Extract (Hidrox®) at dose levels of 1000, 1500 and 2000 mg/kg bw/day (corresponding to hydroxytyrosol at 24, 36 and 48 mg/kg/day, respectively) by oral gavage for 90 days (Christian et al., 2004). Blood samples were collected on Day 90, prior to dosing and at 0.5, 1, 2, 4 and 8 hours post-dose. Pre-dose plasma samples contained no measurable mean concentrations of hydroxytyrosol, suggesting minimal carry-over of hydroxytyrosol from prior doses. The findings from this study suggest that hydroxytyrosol is rapidly absorbed, and mean concentrations were measurable through 1 to 4 hours at dose levels of 1000 and 1500 mg/kg bw/day and through 8 hours at 2000 mg/kg bw/day for 90 consecutive days. This study also indicate that hydroxytyrosol is unlikely to accumulate in the body.

6.2.1.6 Human Study of Aqueous Olive Pulp Extract

In a double-blind, randomized, placebo-controlled trial, Bitler et al. (2007) investigated the effects of a polyphenolic-rich olive extract (freeze-dried olive vegetation water; Hidrox) on a series of parameters in male and female subjects (n=105; age 55-75 years) with osteoarthritis and rheumatoid arthritis. The olive water fraction was reported to contain at least 6% simple phenols and polyphenols. The subjects in the treatment group (n=51) received 400 mg of the freeze-dried extract/day for eight weeks. Of the 105 subjects, 47 in the placebo group and 43 in the treatment group completed the study. Serum samples were analyzed for clinical and biochemical parameters. The rheumatoid arthritis subjects in the extract treatment group showed significant decreases in serum homocysteine levels after eight weeks of treatment.

No significant changes in any other clinical marker, including markers of renal (serum blood urea nitrogen and creatinine) and hepatic function (aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and total bilirubin) were noted at any time during the study (Bitler et al., 2007). These observations support safety of the supplement. Overall, the participants tolerated the placebo and supplement well; only two participants, one from each group (placebo and supplement), complained of heartburn at the two-week visit. This problem was alleviated when the participants took the placebo or supplement with food. The results of this study did not reveal any adverse effects of the olive extract in the arthritis subjects. Although the levels of hydroxytyrosol were not reported in the publication, given the affiliation of the authors of this study, the extract used in this study appears to be the subject of this present GRAS assessment and the resulting intake of hydroxytyrosol appear to be approximately 10 mg/person/day.

6.2.2. Secondary Pivotal Published Studies of Olive Extracts

In addition to the specific studies on Aqueous Olive Pulp Extract (Hidrox®), extensive safety analyses have also been conducted on similar substances derived from olive fruit, including other products such as elaVida™ 40% (the subject GRN 726), a polyphenol preparation made from olive fruits using solvent-free process; elaVida™ 15% (contains 15% hydroxytyrosol). Moreover, published studies with chemically pure hydroxytyrosol (subject of GRN 876 and GRN 600) have also been considered in establishing the safety of Aqueous Olive Pulp Extract (Hidrox®). While these substances are not identical in composition, findings from all these studies contribute to the total weight of safety evidence for the present GRAS assessment. The current safety assessment is focused on the safety evaluation of Aqueous Olive Pulp Extract (Hidrox®) and the main phenolic component hydroxytyrosol and the supportive data contained in this dossier.

6.2.2.1. Subchronic Toxicity Studies

In a 13-week rat study, Heilman et al. (2015) investigated the safety of olive extract H35 containing 35% hydroxytyrosol (Table 9). In this study, olive extract H35 was administered orally (gavage) to Wistar rats for 13 weeks, followed by a 4-week treatment-free period, at doses of 0, 345,t691 and 1381 mg/kg bw/day. The doses were equivalent to hydroxytyrosol content of 0, 125, 250 and 500 mg/kg bw/day. The study was performed following OECD guideline 408 and GLP with inclusion of additional elements. These included neurobehavioral observations, seminology, estrous cycling and a MNT genotoxicity element. Also, blood samples were collected 30 minutes after dosing one day in weeks 4, 8, and 13 for hydroxytyrosol analysis.

No mortality or morbidity was observed during the study period (Heilman et al., 2015). At termination, reductions in body weight of 9%, and a statistically significant reduction in body weight gain of approximately 17% ($p< 0.05$) at week 13 were noted in high dose males (500 mg hydroxytyrosol/kg bw/day). In addition to this, a statistically significant increase in relative weights of the liver, heart, and kidneys of high dose males and females were noted. These changes were not accompanied by pathological or clinical observations and a trend towards reversal was observed in the recovery phase. The results of this study show that olive extract H35 was well-tolerated and no toxicologically significant treatment-related changes were observed in condition and appearance of rats, neurobehavioral outcomes, motor activity assessments, functional observational battery (FOB), food intake, ophthalmoscopic examinations, hematology, clinical chemistry, urinalysis, necropsy findings. Additional parameters studied such as reproductive effects (including estrous cycle assessment and sperm analysis) did not reveal observation of any statistically significant changes between treated animals and control (Heilman et al., 2015).

Based on statistically significant reductions in body weight gain and decreased absolute body weight in males, the investigators determined the lowest observed adverse effect level (LOAEL) of olive extract H35 as 1381 mg /kg bw/day (500 mg hydroxytyrosol/kg bw/day). The investigators reported that, based solely on the reduction in body weight and body weight gain in the high dose males, it is conservatively concluded that the NOAEL of olive extract H35 is 691 mg/kg bw/day (250 mg hydroxytyrosol/kg bw/day) (Heilman et al., 2015).

Aunon-Calles et al. (2013a) conducted a subchronic toxicity study with pure hydroxytyrosol in accordance with OECD Guideline 408, and GLP (Table 9). In this study, hydroxytyrosol was administered orally daily by gavage to groups of Wistar rats (10/sex/group) at dose levels of 0, 5, 50, and 500 mg/kg bw/day for 90 consecutive days. Additional animals (5 rats/sex) in groups 1

and 4 were included for a four-week recovery period. During the course of the study, no mortality was noted in any group. No relevant treatment-related clinical signs were recorded. Salivation was recorded in all animals treated at the high dose and sporadically in some animals from groups treated at the intermediate- and low-doses. This phenomenon was attributed to the bitter taste of hydroxytyrosol and/or the physical characteristics of the formulation (slightly oily and dense). In the high dose treated group (500 mg/kg bw/day), slightly but significant, lower body weight (14%) in males and body weight gain in males and females were observed. The decrease in male body weight is supported by the Heilman et al. (2015) study where a similar decrease (17%) in male rat body weight following administration of hydroxytyrosol at dose levels of 500 mg/kg bw/day was noted.

Table 9. Summary of Secondary Pivotal Published Subchronic Toxicity Studies

Test substance; Study type; Route; Duration	Animals (sex/group); Doses (mg/kg bw/day)	Results NOAEL	Reference
Olive extract H35; Rat sub-chronic; Gavage; 90 days plus 28-day treatment-free period	10/sex/group, plus recovery animals; 0, 345, 691 and 1381 olive extract H35; 0, 125, 250 and 500 as hydroxytyrosol	Based on results excluding MNT phase: 691 mg/kg bw/day (250 mg hydroxytyrosol) (slight reduction in male weight at 500 mg/kg bw/day)	Heilman et al., 2015
Hydroxytyrosol; Rat sub-chronic; Gavage; 90 days	10/sex/group, plus recovery animals; 0, 5, 50, and 500 pure hydroxytyrosol	500 mg/kg bw/day (minor changes observed were considered not adverse)	Aunon-Calles et al., 2013a

The hematological and clinical chemistry changes revealed higher MCV and MCH in females treated at the high- and intermediate-doses; higher HFR and WBC values in females treated at the high dose; lower creatinine and higher albumin values in males treated at the high dose; and higher calcium values in males treated at intermediate- and high-doses (Aunon-Calles et al., 2013a). As these significant changes in hematology and clinical chemistry parameters were not observed in both sexes, were of small magnitude, lacked correlating changes in other clinical parameters and were not noted in a dose-related manner, or were not associated with microscopic changes in the related organs, they were considered as incidental changes/biological variations and not treatment-related adverse effects (Aunon-Calles et al., 2013a). Among the organ weights, higher kidney weights were observed in males and females from the 500 mg/kg group. However, no alterations in this organ were observed on histopathological examination and this finding was not considered to be toxicologically relevant. Microscopic observations did not reveal any morphological alteration in any of the organs or tissues examined. Based on the results obtained, daily oral administration of hydroxytyrosol to rats for a period of 13 weeks did not induce effects that can be considered of toxicological relevance. Hence, the investigators proposed the dose of 500 mg/kg bw/day as the NOAEL.

In a recent subchronic toxicity study conducted as per OECD-408 guidelines, Rodriguez-Lara et al. (2019) investigated the effects of an aqueous virgin olive oil (VOO) extract rich in hydroxytyrosol in rats. The extract contained an initial concentration of 15% of hydroxytyrosol. For this study, 80 Wistar SHD rats were divided into four group (10/sex/group) and were administered VOO at levels of 0, 100, 300 and 1000 mg/kg bw/day in the drinking water. The VOO concentration in drinking water was adjusted such that the daily exposure was achieved. All standard parameters, as per OECD guidance, during the course of this study and at termination were measured. No toxic effect of VOO extract rich in hydroxytyrosol were noted after the sub-chronic supplementation during 90 days with 100, 300 and 1000 mg/kg bw/day,

according to the OECD-408 guidelines. Although minor hematological and biochemical differences were observed between the control and the VOO extract-supplemented groups, these changes did not follow a dose-dependent pattern, and all evaluated clinical, hematological, biochemical, and histological parameters were within normal ranges in all of the animals, which indicates no toxicological effect of VOO extract at the length and dosages examined. The investigators concluded VOO extract containing 15% of hydroxytyrosol did not induce effects that can be considered of toxicological relevance, and proposed a NOAEL dose as 1000 mg/kg bw/day of pure hydroxytyrosol.

6.2.2.2. Mutagenicity and Genotoxicity Studies

In an extensive article, Kirkland et al. (2015) reviewed the available studies and also published mutagenicity and genotoxicity related findings from studies conducted by their group. These investigators noted that pure hydroxytyrosol, and an olive extract containing 15% hydroxytyrosol, both induced micronuclei in cultured cells *in vitro*, but show that these responses were either due to high levels of cytotoxicity or to reaction of hydroxytyrosol with culture medium components to produce hydrogen peroxide. However, both extracts (15% and 40%) were negative in robust Ames tests. Another olive extract containing 40% hydroxytyrosol also induced micronuclei *in vitro*, probably via the same mechanism. Kirkland et al. (2015) concluded that the amounts of hydrogen peroxide produced at the concentrations tested may well be sufficient to account for the increased micronucleus frequencies seen with olive extracts in the absence of S9.

A summary of *in vivo* genotoxicity studies summarized in Kirkland et al. (2015) are provided in Table 10. This table also includes pivotal genotoxicity studies of Hidrox® that are described earlier. In the *in vivo* study by Kirkland et al. (2015), an olive extract containing 15% hydroxytyrosol did not induce micronuclei in rat bone marrow after four weeks of dosing at levels up to 561 mg hydroxytyrosol/kg bw/day. In a 90-day repeat-dose study, olive extract containing 35% hydroxytyrosol produced increased rat bone marrow micronucleus frequencies at 250 and 500 mg hydroxytyrosol/kg bw/day, but the results were questionable for various reasons. However, when two different batches of this extract were tested in acute micronucleus studies at doses up to 2000 mg hydroxytyrosol/kg bw, giving plasma exposures that exceeded those in the 90-day study, negative results were obtained. Based on the weight of evidence, these investigators concluded that the olive extracts tested are not genotoxic at high doses *in vivo*, and any genotoxic risks for human consumers are negligible (Kirkland et al., 2015).

As described in GRN 726 (DSM, 2017), the genotoxicity studies of olive extract provide a conclusion of a lack of genotoxic concern on a Weight of Evidence basis. It was also stated in GRN 726 that, based on the overall genotoxicity evaluation, the notifier concluded that for olive extracts in general, the specific olive extract from the process used to make olive extract H40, and for the main olive polyphenol (hydroxytyrosol), that any genotoxic risks for human consumers are negligible. Negligible risk is usually regarded as the lowest level of risk. Subsequent to the Kirkland et al. (2015) publication that went to press in 2014, an *in vivo* chromosome aberration test in rats (Dolan et al., 2014) was published. At the oral limit dose of 2000 mg/kg bw, hydroxytyrosol also did not induce chromosome aberrations in bone marrow cells.

In the study by Dolan et al. (2014), potential clastogenic effects of pure hydroxytyrosol in a bone marrow chromosome aberration study in rats was investigated. The study was conducted

as per OECD Guideline 475 (mammalian bone marrow chromosome aberration test) in rats with the oral limit dose of 2000 mg/kg bw. Hydroxytyrosol, dissolved in distilled water, was administered via gavage to two groups of rats (5/group/sex). Two groups of five animals per sex (negative controls) were dosed with vehicle (distilled water) only. Five male and five female rats served as positive controls and received 40 mg/kg bw cyclophosphamide in saline by intraperitoneal injection. Four hours before scheduled euthanization (24 and 48 hour time points for both treated and negative control animals and 24 hours for the positive control group), the rats received 2 mg/kg colchicine (a metaphase arresting agent) by intraperitoneal injection. At termination, femurs were removed and bone marrow was harvested. The oral limit dose of 2000 mg hydroxytyrosol/kg bw was well tolerated by most rats. However, some rats exhibited clinical signs that abated within 24 hours. Treatment with hydroxytyrosol did not significantly enhance the number of aberrant cells or the mitotic index 24 or 48 hours post-dose. The positive control (cyclophosphamide) induced the expected increase in chromosomal aberrations and a decrease in the mitotic index, confirming the validity of the assay. The investigators concluded that an oral limit dose of 2000 mg hydroxytyrosol/kg does not induce chromosome aberrations in bone marrow cells of the rat. This suggest that hydroxytyrosol is not a clastogen *in vivo*.

Table 10. Summary of *In vivo* Genotoxicity Studies Reviewed by Kirkland et al. (2015)

Reference	Study type; Route; Duration	Animals (sex/group); Doses (mg/kg bw/day)	Results NOAEL for hydroxytyrosol
Studies with Olive Extracts, including H35, H40 and H40 Mild Process Conditions and Hidrox			
Kirkland et al. (2015)	MNT element in rat sub-chronic; Gavage H35; 90 days, 24 hours following last dose	10/sex/group, plus recovery animals; 0, 125, 250, and 500	MNT phase: 125 mg/kg bw/day Positive MN effect at higher dosages
Kirkland et al., 2015	Classic acute MNT in rat; Gavage H40; Single dose 24 and 48 hours post dose	7 males/group; 0, 500, 1000, and 2000	Non-genotoxic
Kirkland et al. (2015)	Classic acute MNT in rat; Gavage H40 MPC; Single dose 24 and 48 hours post dose	7 males/group; 0, 500, 1000, and 2000	Non-genotoxic
Christian et al. (2004)	Acute MNT in rat; Gavage Hidrox; Single dose 24 and 48 hours post dose	Up to 2000 mg/kg bw in terms of extract HT content 2.4%	Non-genotoxic at up to 48 mg/kg bw
Christian et al. (2004)	Rat sub-acute; Gavage Hidrox; 4 weeks, 24 hours after last dose	Up to 5000 mg/kg bw/day in terms of extract hydroxytyrosol content 2.4%	Non-genotoxic at up to 120 mg/kg bw
Studies with Hydroxytyrosol			
Kirkland et al. (2015)	Rat sub-acute; Gavage Hydroxytyrosol 15% SD; 4 weeks, 27 days	10/sex/group, plus recovery animals 0, 62, 187, and 561	Non-genotoxic at ≥561 mg/kg bw/day
Dolan et al. (2014)	Rat bone marrow chromosome aberration; Gavage Hydroxytyrosol; Single dose 24 and 48 hours	2000 mg/kg bw of hydroxytyrosol	Non-clastogenic

MNT=micronucleus test

Aunon-Calles et al. (2013b) investigated the genotoxic and mutagenic potential of hydroxytyrosol, using well-established *in vitro* models, i.e., the chromosomal aberration assay and the Ames test (by using the *S. typhimurium* TA 100, TA98, TA1535, and TA1537 strains and *E. coli* WP2(pKM101)), with and without S9-induced metabolic activation). No dose response for hydroxytyrosol was observed in any of the tested bacterial strains. The investigators

noted that, even though it cannot be ruled out, prolonged exposure to hydroxytyrosol and its metabolites might have untoward effects. However, the results of this study indicate that hydroxytyrosol is non-genotoxic and non-mutagenic at concentrations that far exceed those attainable after intake.

In another *in vitro* study, Aunon Calles et al. (2013b) also investigated the potential of hydroxytyrosol to induce chromosomal aberrations in human lymphocytes in the absence and presence of metabolic activation by S9 mix. The highest treatment concentration in this study, 1542 µg/mL (~10 mM) was chosen based on the molecular weight of the test item and with respect to the OECD Guideline for *in vitro* mammalian cytogenetictests. No visible precipitation of the test item in the culture medium was observed. No relevant influence on osmolality or pH value was observed. In the absence of S9 mix one statistically significant increase in the number of aberrant cells, excluding gaps (9%) was observed after treatment with 503.5 µg/mL. In the presence of S9 mix after treatment with 287.7 and 503.5 µg/mL two statistically significant increases (3.5% and 4.5% aberrant cells, excluding gaps, respectively) were observed. These values exceeded the range of the laboratory historical solvent control data (0.0-3.0% aberrant cells, excluding gaps). No evidence of an increase in polyploid metaphases was noticed after treatment with the test item as compared to the control cultures. The positive controls showed distinct increases in cells with structural chromosome aberrations.

In summary, the available evidence from mutagenicity and genotoxicity studies revealed that in *in vitro* Ames assays olive extracts (15% and 40%) were negative. In *in vitro* micronucleus and chromosomal aberration assay positive results with olive extract or hydroxytyrosol were reported. In the *in vivo* studies olive extract containing 15% hydroxytyrosol did not induce micronuclei in rat bone marrow after four weeks of dosing at levels up to 561 mg hydroxytyrosol/kg bw/day, while in a 90-day repeat-dose study, olive extract containing 35% hydroxytyrosol produced increased rat bone marrow micronucleus frequencies, but the results were questionable for various reasons. In acute micronucleus studies negative results were obtained. A weight of evidence analysis of the *in vitro* and *in vivo* genotoxicity data for olive extracts in general, for the specific olive extract (the subject of present GRAS assessment), and the main olive polyphenol (hydroxytyrosol), demonstrates that any genotoxic risks for human consumers are negligible (Kirkland et al., 2015).

6.2.2.3. Acute Toxicity Study

In addition to the above described specific acute toxicity studies with Hidrox®, in an acute toxicity study, in a single dose toxicity study, D'Angelo et al. (2001) investigated the acute effects of hydroxytyrosol in rats. In this study, Sprague Dawley male rats (n=6) were treated with a single oral (gavage) dose of 2000 mg hydroxytyrosol/kg bw. After the treatment, the rats were observed for clinical signs. On day 14, the rats were euthanized and gross and pathological changes in "main organs" (not specified in the publication) were evaluated. No deaths were noted during the course of the study period. The only clinical sign observed in the rats was piloerection, which started two hours after treatment and disappeared within 48 hours of treatment. The findings from this study suggest that LD_{50} of hydroxytyrosol is >2000 mg/kg bw.

6.2.2.4. Absorption, Distribution, Metabolism and Excretion

In multiple publicly available publications on animal and human studies, the bioavailability and metabolism of hydroxytyrosol (pure) or as a component of olive phenolics present in olive oil or other olive-derived products (fruits, olive extracts, olive cake, etc.) has

been extensively investigated. In several review articles, an overview on these *in vitro* and *in vivo* animal and human studies on absorption, distribution, metabolism and excretion (ADME) of olive phenolic compounds, with primary focus on hydroxytyrosol is provided (Soni et al., 2006; Beck, 2014; EFSA, 2017; Karkovic Markovic et. al., 2019).

The available information related to the bioavailability indicate that hydroxytyrosol as a pure substance or as a component of olive oil or olive extracts is rapidly and dose-dependently absorbed in humans and the rat (Bai et al., 1998; Christian et al., 2004; Visioli et al., 2000, 2001; Miro-Casas et al., 2001, 2003; Tuck and Hayball, 2002; Covas et al., 2006; Soni et al., 2006; Gonzalez-Santiago et al., 2010; Kotronoulas et al., 2013). Following absorption, hydroxytyrosol is rapidly distributed in several tissues in rats, with no preference for a specific organ or tissue. The available information shows a rapid decrease in plasma and tissue levels of hydroxytyrosol, and there is no indication of any accumulation in the body (D'Angelo et al., 2001; Serra et al., 2012).

The metabolism of hydroxytyrosol has been studied in some detail (Tuck et al., 2001, 2002; D'Angelo et al., 2001; Visioli et al., 2000, 2003; Caruso et al., 2001; Vissers et al., 2002; MiroCasas et al., 2001; Rubio et al., 2012a; Karkovic Markovic et. al., 2019). A summary of endogenous and exogenous metabolic pathways of hydroxytyrosol is presented in Figure 5. The available information indicate that only a minor portion (<6%) of unchanged hydroxytyrosol is found in plasma or urine, and the majority of hydroxytyrosol and its metabolites are present in conjugated (glucuronides and sulfate) forms. The enzymes involved in HTyr phase-I metabolism, mostly present in the intestinal wall, are non-microsomal alcohol and aldehyde dehydrogenases (ALDH), both located in the cytosol. In addition to direct phase II conjugation ofthydroxytyrosol, a major metabolic transformation is found to be the methylation (via catechol-o-methyl transferase, COMT) leading to homovanillyl alcohol (HVAlc) as well as to homovanillic acid. The Intestinal phase II conjugation and COMT activity contribute to the high first pass elimination observed. The enzymes involved in hydroxytyrosol phase-II reactions, sulphotransferases (SULT), uridine 5′-diphosphoglucuronosyl transferases (UGT) and catechol-*O*-methyltransferase (COMT), form the main hydroxytyrosol metabolites detected in biological samples.

The bioavailability ofthydroxytyrosol in rats and humans following oral ingestion of olive oil or other olive-derived food products (in humans), or administration of pure hydroxytyrosol (in rats), has been investigated by several investigators. The findings from these studies reveal the presence of hydroxytyrosol and its metabolites in blood and urine. Precursors of hydroxytyrosol such as oleuropein and its aglycones are extensively hydrolyzed to hydroxytyrosol in the gut (Corona et al., 2006; Pereira-Caro et al., 2012; Mosele et al., 2014) and thus contribute to its high absorption (Visioli et al., 2003; Serra et al., 2012; Kendall et al., 2012). Hydroxytyrosol is rapidly absorbed and reaches a plasma maximum within minutes (5-30 min) after intake (Bai et al., 1998; Miro-Casas et al., 2003; Gonzalez-Santiago et al., 2010; Suarez et al., 2011; Rubio et al., 2012b). Because of the high first pass metabolism in the intestine and liver, elimination of hydroxytyrosol from plasma is also rapid. Hydroxytyrosol is primarily excreted via urine and urinary excretion rate (including all metabolites) is highest within the first 8 hours (D'Angelo et al., 2001; Tuck et al., 2001; Visioli et al., 2000, 2001; MiroCasas et al., 2001; Kountouri et al., 2007). These bioavailability estimates, based on recovery of hydroxytyrosol and its metabolites in urine, reach levels >90% in rats (D'Angelo et al., 2001,

Tuck et al., 2001), and range from 30-75% in human studies (Visioli et al., 2000; Vissers et al., 2002; Miro-Casas et al., 2001; Weinbrenner et al., 2004a; 2004b).

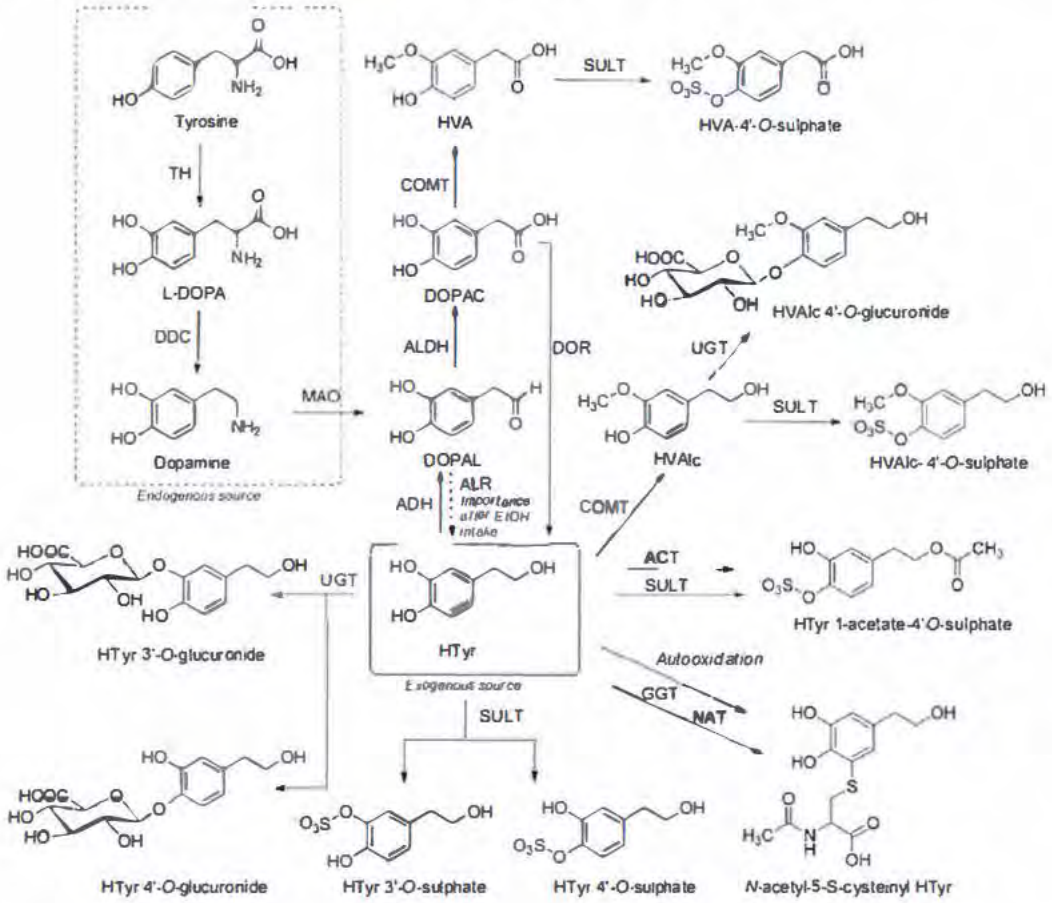


Figure 5. Metabolic Pathways of Endogenous and Exogenous Hydroxytyrosol. HVAlc: homovanillic alcohol; HVA: homovanillic acid; EtOH: ethanol; TH: tyrosine hydroxylase; DDC: dopa decarboxylase; MAO: monoaminoxidase; ALDH: aldehyde dehydrogenase; ALR: aldehyde/aldosa reductase; ADH: alcohol dehydrogenase; DOR: DOPAC reductase; COMT: catechol-O-methyltransferase; UGT: uridine 5'-diphosphoglucuronosyl transferases; SULT: sulphotransferase; ACT: *O*-acetyltransferase; GGT: γ-glutamyl transpeptidase; NAT: *N*-acetyl transferase. (Adapted from Karkovic Markovic et. al., 2019)

6.2.2.5. Human Studies with Olive Preparations

In the published literature, several human clinical studies with olive oil and virgin olive oil have appeared. Olive oil is a functional food that, besides its high content in mono-unsaturated fatty acids (75%), also contains other minor, biologically active, components, such as vitamins, minerals, and polyphenols. Virgin olive oil contain bioactive polyphenols as minor components. Polyphenols, including hydroxytyrosol, as components of olive oil or olive leaf extract has been investigated for their potential benefits in multiple clinical studies. In some available review articles (Raederstorff, 2009; EFSA, 2011; Rigacci and Stefani, 2016; Tsartsou et al., 2019), the available human clinical studies of olive polyphenols have been summarized. A majority of the clinical studies of olive polyphenols are conducted to evaluate the efficacy. These intervention studies suggest that olive polyphenols protects against oxidative damage as evaluated by decreases the levels of oxidized-LDL in plasma. The available information suggest

that it is not feasible to achieve high dosages of olive polyphenols from consumption of olive oil. The European Food Safety Authority (EFSA, 2011) has approved a health claim concerning the effectiveness of the ingestion of olive oil polyphenols (5 mg/day) on protecting LDL from oxidation.

In a randomized, crossover, controlled trial, Castaner et al. (2012) investigated the effects of olive oil polyphenols on cardiovascular health benefits. In this study, 18 healthy European volunteers who daily received 25 mL olive oil with a low polyphenol content of 2.7 mg/L or a high polyphenol content of 366 mg/L in intervention periods of 3 weeks separated by 2 week washout periods. The high polyphenol content group was associated with increased tyrosol and hydroxytyrosol in urine and showed beneficial biomarker changes. The polyphenol intake from high polyphenol content of olive oil was 9.15 mg/day (366 mg/L x 25 ml/day) for an individual weighing 60 kg. Compliance by participants was reported as good with no mention of adverse effects. The polyphenol intake in this study can be considered as moderate.

In a another double-blinded, placebo-controlled, crossover trial, de Bock et al. (2013) assessed the effects of olive leaf polyphenols (51.1 mg oleuropein, 9.7 mg hydroxytyrosol/day) on insulin action and cardiovascular risk factors in middle-aged overweight male subjects. In this study, 46 participants (aged 46.4±5.5 years and BMI 28.0±2.0 kg/m^2) were randomized to receive capsules with olive leaf extract or placebo for 12 weeks, crossing over to other treatment after a 6-week washout. All participants took >96% of prescribed capsules. The extract supplementation was associated with a 15% improvement in insulin sensitivity compared to placebo. There was also a 28% improvement in pancreatic β-cell responsiveness. The extract supplementation also led to increased fasting interleukin-6, IGFBP-1, and IGFBP-2 concentrations. There were, however, no effects on interleukin-8, TNF-α, ultra-sensitive CRP, lipid profile, ambulatory blood pressure, body composition, carotid intima-media thickness, or liver function. The results of this study revealed that supplementation with olive leaftpolyphenols for 12 weeks significantly improved insulin sensitivity and pancreatic β-cell secretory capacity in overweight middle-aged men at risk of developing the metabolic syndrome. The only adverse event reported by one participant was a flare up of acne. This participant withdrew from the study and un-blinding showed that he was receiving placebo. Liver function tests showed no differences in AST, ALP, ALT, or GGT among participants in supplement vs placebo group.

In addition to the above described human studies with olive and its preparations, in the published literature, some studies with hydroxytyrosol, the active constituent of olives, have appeared. Crespo et al. (2015) tested the effects of hydroxytyrosol on expression of Phase II enzymes in 21 healthy human subjects. In this double-blind, randomized, placebo controlled trial, the effects of hydroxytyrosol were investigated following a Latin square design. After one-week initial washout (i.e., olive-free diet), the subjects were randomly assigned to either the placebo (maltodextrin), 5 mg hydroxytyrosol/day or 25 mg hydroxytyrosol/day groups. Administration of each treatment was carried out for one week, followed by one-week washout after which treatments were switched. In this study, Hytolive®, an olive mill and olive mill waste water extract selectively enriched in hydroxytyrosol was used. Both 5 and 25 mg/day doses were well tolerated and no adverse effects were reported. No differences in anthropometric variables or significant variations in vital signs were noted. Hydroxytyrosol was well tolerated without any significant alterations in Phase II enzyme expression in peripheral blood mononuclear cells.

In another randomized double-blinded, placebo-controlled crossover trial, Colica et al. (2017) determined the effect of hydroxytyrosol. In this study, healthy volunteers received two

gastro-resistant capsules containing 15 mg hydroxytyrosol/day for 3 weeks. Evaluation of the nutritional status, serum metabolites, oxidative stress biomarkers, and gene expression of 9 genes related to oxidative stress, inflammation, and CVDs was performed. This study did not report any adverse effect.

Lopez-Huertas and Fonolla (2017) investigated the effects of pure hydroxytyrosol in humans as a supplement in an aqueous solution. In this study, hydroxytyrosol was administered at a dose level of 45 mg/day for eight weeks to volunteers with mild hyperlipidemia (n=14) and markers of cardiovascular disease risk, enzyme markers of several clinical conditions, hematology, antioxidant parameters, vitamins and minerals were measured at baseline, 4 weeks and 8 weeks. The hydroxytyrosol dose administered was well tolerated, safe, and did not influence markers of cardiovascular disease, blood lipids, inflammatory markers, liver or kidney functions and the electrolyte balance in healthy subjects with borderline high levels of cholesterol. Some minor changes were detected in biochemical parameters analyzed in serum such as a decrease in lactate dehydrogenase or an increase in creatinine phosphokinase enzymes, but their values were within the normal range without any clinical relevance. Serum iron levels remained constant but a significant decrease in ferritin at weeks 4 and 8 was found albeit within the physiological range. Serum folate and red blood cell folate levels were also reduced at weeks 4 and 8, while vitamin C increased by two-fold at weeks 4 and 8 as compared with levels at baseline.

6.2.3. Secondary Unpublished Studies of Olive Extracts

6.2.3.1. Secondary Unpublished Toxicity Studies of Olive Extracts

Several unpublished studies of olive extracts, as well hydroxytyrosol, are described in the GRAS notice (GRN 726) dossier submitted by DSM (2017). These studies provides further support for the safe uses of olive extract and are briefly summarized here for the sake of completeness. A summary of acute and short-term studies is provided in Table 11.

Table 11. Summary of Acute and Short-term Unpublished Toxicity Studies

Study type	Route; Duration; Doses	Results
Acute study with olive extract from process used to make H40 (H35)		
Mouse acute	Oral (gavage); Single dose, 14 days; 5, 50, 300 or 2000 (pilot plant extract)	LD50 > 2000 mg/kg pilot plant extract ≥13 mg/kg hydroxytyrosol
Short-term studies with Olive Extracts (Hydroxytyrosol 15% SD)		
Rat preliminary	Gavage; 2 weeks; 10/sex/group 1500 and 3000 15% hydroxytyrosol formulation in feed and by gavage	450 mg/kg bw/day high dose in feed and by gavage well tolerated
Rat sub-acute	Gavage; 4 weeks; 5/sex/group, plus recovery animals, 0, 0 (placebo), 333, 1000 and 3000, DSM 15% hydroxytyrosol extract (0, 0, 62, 187 and 561 as hydroxytyrosol)	561 mg/kg bw/day

GRN 726 Adapted from

The available unpublished studies of mutagenicity and genotoxicity described in GRN 726 (DSM, 2017) are provided in Table 12.

Table 12. *In vitro* Mutagenicity and Genotoxicity Studies

Test	Test System; Strain(s)/Target cells	Hydroxytyrosol concentration/dose	Results
Other Studies with Hydroxytyrosol from Different Sources			
Ames test with hydroxytyrosol 15% SD	*S. typhimurium* (plate incorporation and pre-incubation methods); TA98, TA100, TA0535, TA1537 and TA102	Both up to 5000 µg/plate in the presence and absence of metabolic activation	Non-mutagenic
Hydroxytyrosol 15% SD MNT screening assay	CHO cells; With/without metabolic activation	Without S9: up to 200 µg/ml With S9: Up to 1000 µg/ml	Positive or borderline in absence of S9
Pure hydroxytyrosol MNT screening assay	CHO cells; With/without metabolic activation	With and without S9: Up to 200 µg/mL	Positive or borderline in absence of S9
Study with H35			
MNT screening assay	CHO cells; With/without metabolic activation	Without S9: 0.002 to 0.200 µg/ml; With S9: 0.039 to 5.000 µg/ml	Positive in absence of S9 Equivocal, in presence of S9

The available unpublished reproduction toxicity study of 15% hydroxytyrosol olive formulation (hydroxytyrosol 15% SD) described in GRN 726 (DSM, 2017) are provided in Table 13.

Table 13. Summary of Reproduction Toxicity Study with Hydroxytyrosol 15% SD

Study type	Route; Duration	Doses (mg/kg bw/day)	Results; NOAEL for hydroxytyrosol
Rat developmental toxicity	Gavage; Day 6 through 20 of gestation	0, 333, 1000, and 3000 hydroxytyrosol 15% SD (0, 56, 168 and 504, hydroxytyrosol)	168 mg/kg bw/day (intermediate dosage)

6.2.3.1. Secondary Unpublished Human Studies of Olive Extracts

In an unpublished human study described in GRN 726 GRAS dossier (DSM, 2017), volunteers were administered Hidrox at 400 mg/day and 800 mg/day, or approximately 8 and 16 mg/day in terms of doses of hydroxytyrosol (total dose split between a.m. and p.m.), over two weeks. The findings showed that supplementation with hydroxytyrosol resulted in a significant increase in plasma total antioxidant capacity, and there was an up regulation of the glutathione defense system in skeletal muscle following strenuous exercise. No adverse effects or side effects attributable to Hidrox were seen. Additional details of the study were not available. It should be noted that this study described in GRN 726 is conducted with the product that is the subject of this present GRAS.

In another unpublished study, also mentioned in GRN 726, the effects of 15% hydroxytyrosol olive formulation (Hydroxytyrosol 15% SD) were investigated in a placebo-controlled, double blind, parallel, cross-over clinical study. This 6-week clinical study used dosages of 50 and 150 mg/day in terms of hydroxytyrosol given orally to 19 to 22 young men per group. There were no serious adverse events. There were four adverse events (out of 43 across all groups) ascribed by the physician to the treatment: Platelet cell decrease in one subject at high dose (already low at baseline); Tightness in chest in one subject at both high dose and low dose; Mood swings in one subject at low dose and; Two further adverse events were a persistent cough possibly linked to a respiratory infection. It is concluded that the study showed no safety concerns for hydroxytyrosol at a dosage of 150 mg/ day orally to young men over six weeks. In

GRN 726, it is stated that this study supports an ADI of 150 mg/day, in terms of hydroxytyrosol, as derived from the 90-day rat safety study with H35. Additional details of the study were not available for independent review.

6.2.4. Corroborative Information

6.2.4.1. FDA GRAS Notices on Olive Phenolic Preparation and Hydroxytyrosol

The FDA received three GRAS notifications on ingredients related to olive, one on phenolic preparation from olive fruit [GRN 726 (DSM, 2017)] and two on purified hydroxytyrosol, an active constituent found in olive fruits [GRN 876 (Nova Mentis, 2019) and GRN 600 (Seprox, 2015)]. In these submissions, extensive data from the published literature on phenolics from olives, including hydroxytyrosol, were presented by the notifiers. The FDA did not question the acceptability and suitability of the available evidence to support the safe use of olive phenolics and hydroxytyrosol as evidenced by the FDA 'no question' letters that were sent to the notifiers. The discussion presented below suggests that the agency is comfortable with the GRAS status of phenolic preparation from olive fruit and its highly pure active ingredient hydroxytyrosol, for uses in selected foods as presented in these GRAS notices. As the subject of this present GRAS, this assessment is substantially similar to the products or active constituent of these FDA notifications, and, therefore, the studies described in these notifications can also be utilized to support the safety in the present GRAS assessment of Aqueous Olive Pulp Extract (Hidrox®). Although there are some differences in the compositional analysis, the available information, particularly from a safety assessment perspective, indicates that the primary active chemical in all these GRAS notices is identical and handled similarly in the body. A summary of product similarities between the FDA notified ingredient and the subject of the present GRAS evaluation is presented in Table 14.

Table 14. Comparison of Aqueous Olive Pulp Extract with Similar Ingredient FDA GRAS Notices

Specifications/ Parameters	Hidrox (present GRAS)	elaVida™ (GRN 726)*	Hydroxytyrosol (GRN 876)*	Hydroxytyrosol (GRN 600)*
Description	Purple brownish powder	Yellow - brown Viscous liquid	Off white powder	Yellow Viscous liquid
Source material	Olive fruits	Olive fruits	Recombinant *E. Coli*	Chemical synthesis
Phenolics	6%	8% (minor polyphenols)	NA	NA
Hydroxytyrosol (HT)	3.5%	40%	99%	99%
Moisture	2.8 (1-3)%	33-37%	<0.5%	<4%
Ash	9.6 (6-10)%	3%	NA	NA
Protein	6.7 (4-7)%	0.7-0.8%	NA	NA
Fat	27.0 (20-30)%	0.8%	NA	NA
Carbohydrate (total)	53.5 (40-55)%	17-18%	NA	NA
Intended uses	Multiple foods	Multiple foods	Multiple foods	Multiple foods
Use levels	5-10 mg HT per serving	5-10 mg HT per serving	5-10 mg HT per serving	5-10 mg HT per serving
EDI- cumulative	52 mg HT/p/day (0.9 mg/kg bw/day)	52 mg HT /p/day (0.9 mg/kg bw/day)	52 mg HT/p/day (0.9 mg/kg bw/day)	51 mg HT/p/day (0.85 mg/kg bw/day)
ADI	Proposed use levels	Proposed use levels	Proposed use levels	Proposed use levels
Safety determination	Totality of available evidence	Totality of available evidence	Totality of available evidence	Totality of available evidence

*Adapted from GRN 726, GRN 876 and GRN 600; HT=Hydroxytyrosol; p=person; NA=Not available; ND =Not detected

6.2.4.1.1. GRN 726- Phenolic Preparation from Olive Fruit

In 2017, DSM Nutritional Products, LLC (DSM) submitted a GRAS notice (GRN 726) on phenolic preparation from olive fruit (PPOF) for use as an ingredient and as an antioxidant in bakery products; beverages; dairy products and substitutes; desserts; fats and oils; fruit juices and nectars; dry seasoning mixes for meat, poultry, and fish; chewing gum; sauces, dips, gravies, and condiments; snacks; and vegetable juices at levels of 5 to 10 mg of hydroxytyrosol per serving of food. PPOF is described as a clear, colorless liquid consisting of≥40% hydroxytyrosol, which is the major phenolic compound found in olives. PPOF was produced either by extraction of olive fruit pomace or isolation from the water inherent in the olives (the vegetation water). The food grade specifications for PPOF included hydroxytyrosol content (≥40%), limits on ash (<3%), minor polyphenols (<8%), and heavy metals and microbial contaminants. The cumulative maximum (90^{th} percentile) dietary exposure to hydroxytyrosol for the total users only U.S. population (2 years and older) was determined as 52 mg/person/day (0.9 mg/kg bw/day).

In this GRAS notification, DSM (2017) extensively summarized and discussed the published safety data (for the period through August 2017) and information pertaining to olive extracts containing up to 35% hydroxytyrosol as well as with pure hydroxytyrosol to support the safety of PPOF. DSM summarized the published rat and human studies on the absorption, distribution, metabolism, and excretion of hydroxytyrosol itself, as a component of olive oil or olive-derived products. Several published acute oral toxicity studies in rodents administered by gavage either pure hydroxytyrosol or olive extracts containing different amounts of hydroxytyrosol were described, concluding that the LD_{50} for hydroxytyrosol is >2000 mg/kg bw, the highest dose tested. DSM stated that no adverse toxicological effects were reported in a published subchronic 90-day oral toxicity study in rats, administered by gavage, olive extract containing 35% hydroxytyrosol at doses up to 691 mg/kg bw/day (equivalent to 250 mg hydroxytyrosol/kg bw/day). Additionally, published reproductive toxicity and teratogenicity studies in rats, administered by gavage, a hydrolyzed aqueous olive pulp extract containing 2.4% hydroxytyrosol from days 6 to 20 of gestation were described. No adverse maternal, reproductive, or developmental effects were reported for olive pulp extract at doses up to 2000 mg/kg bw/day (equivalent to 48 mg hydroxytyrosol/kg bw/day), the highest dose tested.

Furthermore, findings from published human studies with either pure hydroxytyrosol or olive extract containing 15% hydroxytyrosol for durations up to eight weeks were described. Based on these studies, DSM concluded that no adverse effects were noted in any of these human studies. The results of published *in vitro* and *in vivo* genotoxicity studies on hydroxytyrosol and olive extracts tested indicate that any genotoxic risks for human consumers are negligible. Based on the totality of the data and information described in the GRAS notification, DSM concluded that PPOF is GRAS for its intended uses in food. Following the review of the information summarized in GRN 726, as well as other information available to the FDA, the agency provided a "no questions" letter to the notifier regarding the GRAS status of PPOF under the intended conditions of use. Given the similarity of the product described in GRN 726 and the subject of present GRAS, the data and information described in GRN 726 are applicable to the present GRAS.

6.2.4.1.2. GRN 600 and 876- Hydroxytyrosol

In these two GRAS notices [GRN 600 (Seprox, 2015) and GRN 876 (Nova Mentis, 2019)], extensive information on safety ofthydroxytyrosol, as well as olive preparations has been summarized. In the first GRAS notice (GRN 600), Seprox discussed published subchronic toxicological studies in rats fed hydroxytyrosol, olive extract, or an aqueous olive pulp extract containing hydroxytyrosol. In these studies, no adverse toxicological effects were reported at levels up to 250 mg hydroxytyrosol/kg bw/day. In this GRAS notice, Seprox summarized published reproductive toxicity and teratogenicity studies in rats fed aqueous olive pulp extract. No adverse reproductive, maternal, or developmental effects were noted at 48 mg hydroxytyrosol/kg bw/day, the highest level tested. Additionally, Seprox also summarized published human studies ranging from one day to eight weeks duration with olive mill water enriched with hydroxytyrosol, olive oil, high oleic sunflower oil, or olive phenolic concentrate containing hydroxytyrosol. The hydroxytyrosol levels in these studies ranged from 2 to 97 mg/person/day. No adverse toxicological outcomes were noted in any of these human studies. Furthermore, Seprox also discussed published *in vitro* and *in vivo* genotoxicity studies and concluded that hydroxytyrosol is not genotoxic. Following the review of the information summarized in GRN 600, the agency provided a "no questions" letter to the notifier regarding the GRAS status of hydroxytyrosol.

In the second, and more recent, GRAS notice on hydroxytyrosol (GRN 876), Nova Mentis (2019) also extensively reviewed and summarized safety studies of hydroxytyrosol and olive preparations. The subject of this notice is the phenolic compound hydroxytyrosol (>99% pure) produced by fermentation of a culture of non-pathogenic *Escherichia coli* BL21 (DE3) #145 strain. The studies summarized in this GRAS notice to support the safety of hydroxytyrosol were similar to those described in GRN 726 and GRN 600. Nova Mentis (2019) discussed published data and information supporting the safety of hydroxytyrosol based on a scientific literature search conducted through April 2019.

Nova Mentis (2019) summarized the results of three published subchronic studies in rats. In all three studies, the test substance was administered by gavage. In one study, no adverse effects were observed at 50 mg/kg bw/day of hydroxytyrosol. In another study, no adverse effects were reported after the administration of 691 mg olive extract containing 35% hydroxytyrosol, equivalent to 250 mg/kg bw/day of hydroxytyrosol. In a third study, no adverse effects were observed after the administration of 2000 mg/kg bw/day of olive pulp extract (Hidrox®) estimated to provide 48 mg/kg bw/day of hydroxytyrosol. Based on the results of a published reproductive toxicity study and a published developmental toxicity study in rats, Nova Mentis (2019) concluded that olive extract containing hydroxytyrosol as its major component is unlikely to be a reproductive toxicant and that no maternal or developmental toxicity was reported at up to 2000 mg/kg bw/day of the extract (or 48 mg/kg bw/day of hydroxytyrosol). Based on the results of multiple published *in vitro* bacterial reverse mutation assays, *in vitro* chromosomal aberration assays, and *in vivo* micronucleus assays, Nova Mentis (2019) concluded that hydroxytyrosol is unlikely to be genotoxic or clastogenic. Nova Mentis (2019) also stated that the results of human studies with olive oil containing phenolics, including hydroxytyrosol, did not reveal any adverse effects. Following the review of the information summarized in GRN 876, the FDA provided a "no questions" letter to Nova Mentis regarding the GRAS status of hydroxytyrosol.

6.2.4.2. Evaluation by the European Food Safety Authority (EFSA)

The EFSA (2011) has issued a scientific opinion on health claims as it relates to dietary intake of hydroxytyrosol and related polyphenol compounds from olive fruit and oil and protection of blood lipids from oxidative damage. The EFSA panel critically reviewed the available information and concluded that a cause-and-effect relationship has been established between the consumption of hydroxytyrosol and related compounds from olives and olive oil and protection of blood lipids from oxidative damage. The EFSA panel determined that a minimum of 5 mg of hydroxytyrosol and its derivatives in olive oil should be consumed daily to use a cardiovascular health claim. Although, the EFSA panel did not comment on the safety of hydroxytyrosol, it can be assumed that this ingredient is safe for human consumption at the recommended level.

6.7. Expert Panel Review, Summary and Discussion

At the request of Oliphenol LLC, an independent panel of recognized experts (hereinafter referred to as the Expert Panel)[2], qualified by their scientific training and relevant national and international experience to evaluate the safety of food and food ingredients, was convened to evaluate the Generally Recognized As Safe (GRAS) status of Aqueous Olive Pulp Extract (Hidrox®), for use as a food ingredient and as an antioxidant in multiple selected food products, described in this dossier, and at use levels to deliver 5 to 10 mg of hydroxytyrosol *per* serving (reference amounts customarily consumed, 21 CFR 101.12). A comprehensive search of the scientific literature for safety and toxicity information on olive fruit, its preparations, olive phenolics and active constituent such as hydroxytyrosol was conducted through August 2020 and made available to the Expert Panel. The Expert Panel independently and critically evaluated materials submitted by Oliphenol and other information deemed appropriate or necessary. Following an independent, critical evaluation, the Expert Panel conferred on October 16, 2020 and unanimously agreed to the decision described herein.

Oliphenol ensured that all reasonable efforts were made to identify and select a balanced Expert Panel with expertise in food safety, toxicology, and nutrition. The Expert Panel was selected and convened in accordance with the Food and Drug Administration (FDA)'s guidance for industry on "Best Practices for Convening a GRAS Panel"[3]. Efforts were placed on identifying conflicts of interest or relevant "appearance issues" that could potentially bias the outcome of the deliberations of the Expert Panel and no such conflicts of interest or "appearance issues" were identified. The Expert Panel members received a reasonable honorarium as compensation for their time; the honoraria provided to the Expert Panel members were not contingent upon the outcome of their deliberations.

The *Olea europaea* L. plant bear a well-known fruit that is a berry, capsule, drupe (e.g., Olea, olive). The fruit is initially green then red and blue-black when ripe, surrounds a very hard stone (or pit), which contains oblong compact seeds with plentiful endosperm. This fruit has long been recognized as having inherent nutritional and health-enhancing potential. Despite its known nutritional value, raw olives are rarely consumed and the fruit undergoes extensive processing to produce the forms most commonly consumed, i.e., table olives and olive oil. The oil contained in

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

[3] Available at: https://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/ucm583856.htm

olives is normally extracted by a multi-stage process. Preparation of edible olives involves pickling in a solution of lye to remove the bitter taste (rendered by oleuropein), and this practice has been in use since Roman times. The content of phenolic compounds (simple as well as complex) in olives and olive oil that are considered as important constituents depends on the cultivars and the ripeness of the fruit at the time of harvest. Oliphenol has developed and standardized a method to produce Aqueous Olive Pulp Extract (Hidrox®) from byproducts of olive oil processing by he water extraction process. The extract is produced as standardized powder and liquid forms.

The Aqueous Olive Pulp Extract (Hidrox®) is a standardized powder and liquid. The preparations (powder and liquid) have an odor of processed olives and a characteristic aromatic sour/olive flavor. The biologically active constituents of Hidrox® are polyphenols. Among the phenolics, the major constituent of the pulp extract is hydroxytyrosol (50-70%), while other polyphenols present include oleuropein (5-10%), tyrosol (0.3%), oleuropein aglycone and gallic acid. All of the polyphenols present in the extract are also found in olive oil and are thus commonly consumed. The phenolic acid content of the extract is 6%, while the hydroxytyrosol content is approximately 2.0-3.5%. As a food ingredient, Aqueous Olive Pulp Extract (Hidrox®) will be added to bakery products; beverages; dairy products and substitutes; desserts; fats and oils; fruit juices and nectars; dry seasoning mixes for meat, poultry and fish; chewing gum; sauces, dips, gravies and condiments; snacks; and vegetable juices, such that it will deliver 5 to 10 mg of hydroxytyrosol *per* serving (reference amounts customarily consumed, 21 CFR 101.12). Given that the extract contains 3.5% hydroxytyrosol, the per serving use levels of the extract will be approximately 150 to 300 mg/serving. The proposed use of the extract will result in a 90th percentile per user cumulative estimated daily intake of 52.4 mg/person/day (0.9 mg/kg bw/day). The resulting 90th percentile intake of Aqueous Olive Pulp Extract (Hidrox®) will be approximately 1500 mg/person/day (25 mg/kg bw/day).

In a series of specifically designed studies, the potential toxicity of Aqueous Olive Pulp Extract (Hidrox®) has been extensively investigated. These pivotal studies included Subchronic toxicity, Reproductive/Developmental Toxicity, Developmental Toxicity, Short-term toxicity, Acute toxicity and Genotoxicity (Ames assay; *in vitro* chromosomal aberration assay and *in vivo* micronucleus assay). All of these pivotal studies were published in the peer-reviewed scientific literature (Christian et al., 2004; Soni et al., 2006) and thus meet the requirement for the "common knowledge" element of GRAS assessment. In the acute toxicity studies in rats and mice, the LD_{50} of extract was > 2000 mg/kg. In a short term study, oral administration of the extract to rats at dose levels of 5000 mg/kg bw/day for 29 days, no mortality or clinical signs of toxicity were noted, suggesting the LD_{50} to be greater than 5000 mg/kg.

In the subchronic study in rats, the gavage administration of an aqueous pulp extract at doses up to 2000 mg/kg/day for a period of 90 days did not reveal any signs of toxicity. Markers of liver function tests, such as levels of ALT, AST and SDH, trended downward. However, the decreases were within historical control values ranges and do not represent treatment-related toxicity. Histological investigations of the major tissues did not reveal any treatment-related pathological changes except for very low to mild focal hyperplasia of the mucosal squamous epithelium of the limiting ridge of the forestomach, apparently related to gavage administration of the extract and the granularity and high viscosity of the suspended extract. Based on the data from this subchronic toxicity study, the no observed adverse effect level (NOAEL) of the extract for rats is 2000 mg/kg/day, the highest dose tested.

In a developmental toxicity study in rats, Aqueous Olive Pulp Extract (Hidrox®) did not cause maternal or developmental toxicity at levels up to 2000 mg/kg/day (highest dose tested). In an oral dose-range reproduction study in rats, doses of the extract ranging from 500 to 2000 mg/kg/day did not adversely affect any of the parental reproductive performance parameters (estrous cycling, mating, fertility, parturition, lactation, maternal behavior) investigated or the viability, growth or development of the offspring through one week postpartum. In an *in vitro* mutagenicity study, aqueous olive pulp extract was not mutagenic in the presence or absence of metabolic activation in *S. typhimurium* strains TA97 and TA 1535, while the results in strains TA98 and TA100 were equivocal. In genotoxicity assays with *E. coli,* no mutagenicity was noted. In chromosome aberration studies in Chinese hamster ovary cells, the extract elicited increases in aberrant cells at 1000 μg/ml in the presence of metabolic activation. In contrast to these *in vitro* positive results, in an *in vivo* micronucleus assay, exposure of rats to the extract did not induce increases in polychromatic erythrocytes in bone marrow.

The NOAEL in the subchronic (90-day) toxicity, reproductive/developmental toxicity and developmental toxicity studies was the highest level tested, 2000 mg/kg bw/day. As compared to the NOAEL of 2000 mg/kg bw/day determined from the subchronic toxicity study (the highest dose tested), the maximum daily intake of 25 mg/kg bw/day of Aqueous Olive Pulp Extract (Hidrox®) from its proposed food uses is over 80-fold lower. Although this safety margin is lower as compared to the standard 100-fold, the additional studies as described in this dossier, and also mentioned below, further provides support for the safe uses of Aqueous Olive Pulp Extract (Hidrox®) at the proposed use levels.

In addition to the above described pivotal studies of Aqueous Olive Pulp Extract (Hidrox®), the safety of the extract is supported by several secondary published and unpublished studies, as well as by corroborative evidence. These studies include the safety studies with other olive extracts, including higher concentrated preparations (containing 35 and 15% hydroxytyrosol), and studies with pure hydroxytyrosol that are summarized in this dossier. The two published subchronic toxicity studies include one in which no adverse effects were reported after the administration of 691 mg olive extract containing 35% hydroxytyrosol, equivalent to 250 mg/kg bw/day of hydroxytyrosol (Heilman et al., 2015) and the other study in which, no adverse effects were observed at 50 mg/kg bw/day of pure hydroxytyrosol (Aunon-Calles et al., 2013a). Several published acute oral toxicity studies in rodents administered by gavage either pure hydroxytyrosol or olive extracts containing different amounts of hydroxytyrosol suggest the LD_{50} for hydroxytyrosol is >2000 mg/kg bw, the highest dose tested.

A critical analysis of *in vitro* and *in vivo* genotoxicity data for olive extracts and the main olive polyphenol (hydroxytyrosol) suggest that any genotoxic risks for human consumers are negligible (Kirkland et al., 2015). Additionally, published rat and human studies on the absorption, distribution, metabolism, and excretion of hydroxytyrosol itself, as a component of olive oil or olive-derived products, suggest that hydroxytyrosol is unlikely to accumulate in the body. Furthermore, an unpublished study (described in GRN 726) in rats with olive extract (Hydroxytyrosol 15% SD) provided a NOAEL of 168 mg/kg bw/day when expressed in terms of hydroxytyrosol. Based on these secondary studies, application of a 100-fold safety factor to the NOAEL (250 mg hydroxytyrosol/kg bw/day) from the sub-chronic study with olive extract 35% results in an ADI of 150 mg hydroxytyrosol/day (for a 60 kg person). Similarly, applying a 100-fold safety factor to the NOAEL of 168 mg/kg bw/day from an embryo-fetal (developmental) toxicity study gives an intake up to 100 mg/day in terms of hydroxytyrosol for a 60 kg adult. All of these studies suggest that exposure to 52 mg hydroxytyrosol/day from the proposed uses of

Aqueous Olive Pulp Extract (Hidrox®), including background intake, is unlikely to cause any adverse effects and is considered as safe.

In summary, there is sufficient qualitative and quantitative scientific evidence, including animal data, to assess the safety-in-use for Aqueous Olive Pulp Extract (Hidrox®), the subject of this present GRAS assessment. The safety assessment of Aqueous Olive Pulp Extract (Hidrox®) is based on the totality of available evidence, including a variety of specifically designed animal toxicity studies. The totality of the available evidence supports the safety of Aqueous Olive Pulp Extract (Hidrox®) at the maximum (90^{th} percentile) all users intake of 1500 mg/person/day (25 mg/kg bw/day) and its active constituent hydroxytyrosol at maximum cumulative intake of 52 mg/person/day. On the basis of scientific procedures[4], the consumption of Aqueous Olive Pulp Extract (Hidrox®) as an added food ingredient is considered safe at use levels up to 300 mg/serving. The intended uses are compatible with current regulations, *i.e.,* Aqueous Olive Pulp Extract (Hidrox®) is used in specified foods (described in this document) and is produced according to current good manufacturing practices (cGMP).

[4] 21 CFR §170.3 Definitions. (h) Scientific procedures include those human, animal, analytical, and other scientific studies, whether published or unpublished, appropriate to establish the safety of a substance.

6.8. Expert Panel Conclusion

Based on a critical evaluation of the publicly available data, summarized herein, the Expert Panel members whose signatures appear below, have individually and collectively concluded that Aqueous Olive Pulp Extract (Hidrox®), meeting the specifications cited herein, and when used as a food ingredient and as an antioxidant at use levels ranging from 150 to 300 mg/serving (used such that it will deliver 5 to 10 mg of hydroxytyrosol *per* serving) in conventional foods such as bakery products; beverages; dairy products and substitutes; desserts; fats and oils; fruit juices and nectars; dry seasoning mixes for meat, poultry and fish; chewing gum; sauces, dips, gravies and condiments; snacks; and, vegetable juices (when not otherwise precluded by a Standard of Identity) as described in this monograph, and resulting in the maximum (90th percentile) estimated intake of 1500 mg Aqueous Olive Pulp Extract (Hidrox®)/person/day, is safe.

It is also our opinion that other qualified and competent scientists reviewing the same publicly available toxicological and safety information would reach the same conclusion. Therefore, we have also concluded that Aqueous Olive Pulp Extract (Hidrox®), when used as described, is Generally Recognized As Safe (GRAS) based on scientific procedures.

Signatures

[redacted]

Robert L. Martin, Ph.D.

Oct. 19, 2020

Date

[redacted]

A. Thomas, Ph.D., F.A.C.T., F.A.I.C.

Oct. 20, 2020

Date

[redacted]

Madhusudan G. Soni, Ph.D., F.A.C.
F.A.I.C.S. Advisor to Expert Panel

Oct. 21, 2020

Date

Part VII- SUPPORTING DOCUMENTS AND REFERENCES

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Santos, Marissa

From: Madhu Soni <sonim@bellsouth.net>
Sent: Monday, July 12, 2021 3:55 PM
To: Santos, Marissa
Subject: [EXTERNAL] RE: GRN 000978 - Question for the Notifier
Attachments: GRN 978 FDA Query Responses final-1.pdf

Dear Ms. Santos,

Please find attached a pdf file providing a point-by-point response to the agency queries related to our GRAS notification (GRN 000978) .

I hope the information and clarifications, along with some discussion in the response addresses the FDA queries. If you have any questions or need additional explanation, please let me know.

Thank you for the opportunity to provide this explanation.

Best regards

Madhu

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From: Santos, Marissa [mailto:Marissa.Santos@fda.hhs.gov]
Sent: Friday, June 25, 2021 1:55 PM
To: Madhu Soni <sonim@bellsouth.net>
Subject: GRN 000978 - Question for the Notifier

Dear Dr. Soni,

During our review of GRAS Notice No. 000978, we noted several questions that need to be addressed and are attached to this email.

We respectfully request a response wi thin **10 business days**. If you are unable to complete the response within that time frame, please contact me to discuss further options. Please do not include any confidential information in your responses.

If you have any questions or need further clarification, please feel free to reach out to me.

Regards,
Marissa

Marissa Santos, M.S.

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Dear Dr. Santos,

RE: GRN 978 (Hydrolyzed aqueous olive pulp extract)

This responds to your email of June 25, 2021, regarding your queries that need to be addressed for Hydrolyzed aqueous olive pulp extract GRAS Notice (GRN 978) submitted on behalf of Oliphenol LLC. We are providing a point-by-point response to all your queries along with some additional relevant clarifications/discussion.

FDA Query: (1) In Table 3 (page 8) of your notice, you provide the specifications for aqueous olive pulp extract and list the method used to assess water solubility as an "in house" method. Please indicate the concentration of the solutions and the temperature at which the test is conducted. Please also confirm that the method to determine water solubility is validated for the intended purpose of assessing solubility of the two forms of aqueous olive pulp extract.

Response: The "in house" method is based upon centrifugation. A 1% solution of the test compound (HIDROX® powder) is stirred in water for 15 minutes at room temperature. The solution is then centrifuged at 12,000 rpm for 15 minutes and the solid residue at the bottom of the glass tube is separated from the supernatant, dried in an oven at 70°C for 4 hours, and weighed. The solubility is calculated as % difference between the original sample minus the quantity of solid collected after centrifugation. We apply this standard assay to both HIDROX® in powder and HIDROX® in liquid form. We confirm that the method to determine water solubility is validated for the intended purpose of assessing solubility of the two forms of aqueous olive pulp extract.

FDA Query: (2) On pages 10-12 of the notice you describe and provide a flow chart of the manufacturing process for aqueous olive pulp extract. We request that the method of manufacture is clarified.

a) Your narrative states that aqueous olive pulp extract is manufactured from the byproducts of olive oil production and the flow chart indicates that olive pomace is the starting material. Please comment on whether there is a pasteurization step and if the olive enzymes are inactivated during the manufacture of aqueous olive pulp extract.

Response: a) Please note that there is no pasteurization step and or enzyme inactivation step in the production of the olive pomace. The olive pulp is heated to 30ºC to facilitate the separation of the olive oil and olive vegetation water. This temperature does not denature proteins.

b) On page 12 you state the 'manufacturing flow chart for liquid aqueous olive pulp extract is provided below.' However, there is no flow chart specifically for the manufacture of liquid aqueous olive pulp extract after the text. If there is a figure missing or this is a misstatement, please provide the figure or clarify for the record.

Response: b) Thank you for bringing this to our attention and sorry for the misstatement. The Figure 3 flow chart shows the manufacturing flow for both the HIDROX® powder and the HIDROX® liquid. In Figure 3, the "powder" process branches off to the left and the "liquid" branches off to the right on the flow chart.

FDA Query: (3) In Table 1 you provide a general description of the characteristics of aqueous olive pulp extract and list a shelf life of 2 years. Please provide information that substantiates that the food ingredient has the indicated shelf life, including information regarding the test substance (powder or liquid) and the indicator parameters measured.

Response: Aqueous olive pulp extract (HIDROX®) was tested in an accelerated and long-term shelf-stability study on representative lots of HIDROX® 6% Freeze-dried Powder. Accelerated shelf-life samples were stored at 40ºC and 75% relative humidity over a period of six months. Long term shelf-stability testing was also performed on samples and stored at 25ºC and 60% relative humidity over a period of twelve months. These shelf-life stability studies demonstrated that HIDROX® is stable for 12 months when stored at 25ºC and 60% relative humidity, when stored in well-closed containers and protected from light, moisture, and heat. There are no changes in appearance, no substantial changes in the analysis for phenolics, hydroxytyrosol, or ORAC value, all of which are key specifications for guaranteeing antioxidant potential and, therefore, the intended technical function of HIDROX®. There were no changes in total aerobic microbial count, total combined yeast and mold count and after storage for 12 months in well-closed containers, protected from light, moisture, and heat, at a temperature of 25ºC. Besides this study, we have tested production lots that are over three-year old for microbial analysis and the results again show no changes. Based on these results, we determined that a shelf life of two years is supported.

FDA Query: (4) In Table 3 you list the specifications for aqueous olive pulp extract liquid and powder and the methods of analyses.

a) You list AOAC 925.09 as a method to assess the protein content, however this analytical method is used to determine moisture content. Please provide the method used assess protein content in aqueous olive pulp extract.

Response: a) Thank you for bringing this to our attention. The correct Protein Combustion (A) Method Reference is: AOAC 990.03, AOAC 992.15

b) We also note that you cite "21 CFR-calc" as a reference for the method to assess the level of carbohydrates in aqueous olive pulp extract. It is our understanding that this reflects total carbohydrates (by calculation) and includes fiber. Please provide a more complete description of where in 21 CFR the method is located.

Response: b) Thank you for pointing this. Your understanding about the method is correct. The correct 21 CFR method is 21 CFR 101.9 (c)(6). A copy of this section is provided below.

> (6) "Carbohydrate, total" or "Total carbohydrate": A statement of the number of grams of total carbohydrate in a serving expressed to the nearest gram, except that if a serving contains less than 1 gram, the statement "Contains less than 1 gram" or "less than 1 gram" may be used as an alternative, or if the serving contains less than 0.5 gram, the content may be expressed as zero. Total carbohydrate content shall be calculated by subtraction of the sum of the crude protein, total fat, moisture, and ash from the total weight of the food. This calculation method is described in A. L. Merrill and B. K. Watt, "Energy Value of Foods - Basis and Derivation," USDA Handbook 74 (slightly revised 1973) pp. 2 and 3, which is incorporated by reference in accordance with 5 U.S.C. 552(a) and 1 CFR part 51 (the availability of this incorporation by reference is given in paragraph (c)(1)(i)(A) of this section).

c) You indicate a minimum value for total polyphenols and hydroxytyrosol but do not indicate a maximum value. Please provide a range for these components in your ingredient.

Response: c) The minimum and maximum values are as follows: For HIDROX® 12% the polyphenol range is 12-16% (based on our experience over the years it is mostly 12-13%) and the Hydroxytyrosol range is 3.5-4.5%. For HIDROX® 10X the polyphenol range is 7.5-8.5% and the Hydroxytyrosol range is 3.0-4.0%.

FDA Query: (5) In Tables 4 and 5 you provide certificate of analyses data of aqueous olive pulp extract liquid in comparison to aqueous olive extract pulp extract specifications.

a) Please clarify the composition of the ingredients by:

1. listing the major mineral components of ash

Response: For one of the HIDROX® 12% batch, Lot#12-180828-01 has been tested for the following minerals. The available data from one lot is given in the below Table.

HIDROX 12%	Lot #12-180828-001
Iron by ICP	0.0126%
Potassium by ICP	6.52%
Sodium by ICP	0.161%
Calcium by ICP	0.105%

2. indicating whether simple & polyphenols are expressed on a dry weight percent basis or other basis.

Response: Please note the Simple & polyphenols are analyzed by the Folin-Ciocalteu Assay and the results for HIDROX® 12% are expressed as the percentage mg of GAE (Gallic Acid Equivalent) per g of dried sample. Sorry for not mentioning the units in the Table.

b) In Table 4, for lot 12-140108-001, the batch analyses (0.133 mg/kg arsenic; 0.154 mg/kg lead) do not meet stated specifications (both < 0.1 ppm (mg/kg) for these metals). Please provide a statement that your ingredient will meet stated specifications. Further, please revise the product specifications so that the specifications are reflective of your lot analyses.

Response: Thank you very much for bringing this to our notice, we are sorry for the oversight. We confirm that our ingredient (subject of the GRAS) will meet the specifications. To support that our product meets the heavy metal specification, we are providing heavy metal analysis from additional batches (please see Table below). This data from 5 different lots of HIDROX® 12% suggest that the product meets established heavy metal specifications. Please note that if the lot does not meet the established specifications, the lot will not be marketed.

Parameter	**Lot Number**				
	Lot #12-190926-000	Lot #12-190403-000	Lot #12-170623-000	Lot #12-180228-001	Lot #12-181206-001
Arsenic (As) (ppm)	0.0295	0.037	0.042	0.034	0.033
Cadmium (Cd) (ppm)	<0.005	0.00519	<0.005	<0.010	<0.007
Lead (Pb) (ppm)	0.0616	0.0583	0.033	0.037	0.032
Mercury (Hg) (ppm)	<0.005	<0.005	<0.007	<0.010	<0.007
Report Date **Eurofins**	01/14/20	05/20/19	08/23/17	10/05/18	01/18/19

FDA Query: (6) You provide an estimate of exposure of 1500 mg/p/d of the ingredient at the 90th percentile of intake. Please provide an estimate of mean consumption of this ingredient.

Response: Sorry for not mentioning this in the GRAS dossier. The cumulative mean consumption of hydrolyzed aqueous olive pulp extract from the proposed uses is estimated as 843 mg/p/d.

FDA Query: (7) Pages 22 (Table 8) and 28: For the summary of the acute toxicity study in rats, the notifier states that "no adverse effects except soft or liquid feces" (Christian et al., 2004). During the review of the original publication, FDA was unable to find the note regarding soft or liquid feces in the acute study. FDA also notes that the publication by Christian et al. (2004) states that "In rats, an acute oral NOAEL of 1000 mg/kg was established, based on partial reductions in weight gains in both sexes at 5000 mg/kg, and reduced weight gains in female rats at 1500 and 2000 mg/kg." Therefore, please clarify what effects were seen in the acute rat study and based on what effects the NOAEL was established.

Response: We are sorry for our confusion and the oversight. Based on the findings from the acute oral toxicity study in rats, Christian et al. (2004) considered the acute oral NOAEL of Hydrolyzed Aqueous Olive Pulp Extract as 1000 mg/kg bw (Christian et al., 2004). This was determined as the NOAEL based on the observation that at higher doses of 1500 and 2000 mg/kg bw reduced body weight gains noted in female rats, while at the highest dose level of 5000 mg/kg bw, both males and females continued to gain weight, although at a reduced rate as compared to control rats (Christian et al., 2004).

FDA Query: (8) On page 24 the notifier states "The results of this study suggest that the resulting all user maximum intake of 25 mg/kg body weight (bw)/day from the proposed uses of Aqueous Olive Pulp Extract (Hidrox®), the NOAEL is 80-fold lower." This statement, as written, does not make sense. Please concur whether you meant to state that the all user maximum intake of 25 mg/kg bw/day resulting from the proposed uses of Aqueous Olive Pulp Extract (Hidrox®) is 80-fold lower than the NOAEL obtained in the subchronic toxicity study by Christian et al. (2004).

Response: We concur with the above statement and apologize for the confusing statement.

FDA Query: (9) On page 29 regarding the Bitler et al. (2007) study, the notifier states that "Although the levels of hydroxytyrosol were not reported in the publication, given the

affiliation of the authors of this study, the extract used in this study appears to be the subject of this present GRAS assessment and the resulting intake of hydroxytyrosol appear to be approximately 10 mg/person/day."

a) FDA notes that seven of the nine authors of the publication by Bitler et al. (2007) are affiliated with Arizona State University and two are affiliated with CreAgri, Inc. This GRAS notice was submitted on behalf Oliphenol LLC. Please explain how the affiliation of the authors of the Bitler et al. (2007) article makes the extract used in that study to "appear to be" the same as the subject of this notice.

Response: a) Please note that Dr. Roberto Crea, currently CEO of Oliphenol LLC (notifier) and is also the founder President and CEO of CreAgri Inc. The Bitler et al. (2007) study, conducted at Arizona State University, used freeze-dried olive vegetation water that is manufactured similarly to the subject of present GRAS (with some minor differences). However, the levels of hydroxytyrosol were not determined or reported in the publication. The study product was reported to contain at least 6% simple phenols and polyphenols. The subject of this GRAS Notice, hydrolyzed aqueous olive pulp extract powder and liquid form contains >12.0 and >7.5% polyphenols, and >3.5 and >3.0% hydroxytyrosol, respectively. Hence, we made some assumptions to determine the hydroxytyrosol content of study product. If the Bitler et al. (2007) study product contains 6% phenols and polyphenols, it is likely that it may contain around 2.5% hydroxytyrosol and the resulting intake of hydroxytyrosol from a 400 mg/day dose of the extract used in the study will be approximately 10 mg/person/day. Hence, we indicated that the likely intake of hydroxytyrosol in the Bitler et al. (2007) study to be 10 mg/person/day.

b) Appear does not indicate certainty. Is or is not the test article in the Bitler et al. (2007) the same as the subject of this notice?

Response: b) Based on the available information, the subject of the Bitler et al. (2007) study is substantially similar to the subject of present GRAS.

c) We also note that in its GRAS notice the notifier identifies the test article used in this study as "Hidrox", the notified substance, but the article never refers to the test article as such. Please state whether you concur.

Response: c) As the product of Bitler et al. (2007) study is manufactured similarly to the subject of current GRAS, we used the term Hidrox®, although it is not mentioned in the publication. At the time CreAgri marketed a range of products containing different levels of polyphenols under the name Hidrox®. We concur that Hidrox is not mentioned in the article.

d) We also note that the participants received 400 mg/day of the test article. You state that this corresponds to 10 mg hydroxytyrosol (HT)/day. Given that the specifications for the notified substance state that the hydroxytyrosol content of the notified substance is >3.5%,

the test article in the Bitler et al. (2007) study should provide 14 mg hydroxytyrosol/day at a minimum if it was the same as the notified substance. Please explain the discrepancy.

Response: d) As described above in "Response a)", the hydroxytyrosol content was calculated by making some assumptions.

FDA Query: (10) The notifier discusses a published 90-day study in which rats of both sexes were fed 0, 5, 50, or 500 mg of HT/kg bw/day via gavage (Auñon-Calles et al., 2013) and states that "the investigators proposed the dose of 500 mg/kg bw/day as the NOAEL.

FDA notes that at 500 mg/kg bw/day slightly but significantly lower body weight (14%) in males and body weight gains in both sexes were reported despite of the fact that the mean food consumption was comparable in all dose groups for both sexes. Statistically significant higher relative kidney weights were observed in males and females as related to body weight. Some other statistically significant differences in organ weights relative to body weight were observed in animals from the 500 mg/kg bw/day group compared to controls. Moreover, at the end of the recovery period, higher relative and absolute testes weights in males and higher absolute and relative liver and kidney weights in females were observed compared to the control group. No pathological changes were reported in these organs. The authors of this study assigned the No Observed Adverse Effect Level (NOAEL) to 500 mg/kg bw/day. On the basis of significantly lower body weight in males, body weight gains in both sexes, and some other statistically significant differences in organ weights, the Food and Drug Administration assigns the NOAEL to 50 mg/kg bw/day for this study. Please state whether you concur with FDA's assignment of 50 mg/kg bw/day as the NOAEL, if not, explain why not.

Response: We concur with FDA's assigned NOAEL of 50 mg/kg/bw/day.

FDA Query: (11) Pages 31-32: The notifier states that rats "were administered VOO" (i.e., aqueous virgin olive oil) "at levels of 0, 100, 300, and 1,000 mg/kg bw/day in the drinking water" (page 31). At the end of the study summary, the notifier states "The investigators concluded VOO extract containing 15% of hydroxytyrosol did not induce effects that can be considered of toxicological relevance, and proposed a NOAEL dose as 1,000 mg/kg bw/day of pure hydroxytyrosol."

FDA notes that these statements are contradictory as if the top dose was 1,000 mg VOO containing 15% HT, the top HT level would be 150 mg/kg bw/day and not 1,000. FDA's review of the original publication by Rodrigues-Lara et al. (2019) revealed that "The VOO extract was dissolved and administered in the drinking water in order to minimize the manipulation of the animals. After calculating the median daily amount of water intake, dilutions of the extract were prepared in milli Q water to achieve final different doses of

hydroxytyrosol: 100 mg/kg (low dose), 300 mg/kg (intermediate dose), and 1000 mg/kg (high dose)." Therefore, the animals were administered 0, 100, 300, or 1,000 mg HT/kg bw/day and not VOO. Please state whether you concur.

Response: We concur with FDA. We apologize for not clarifying this. Apparently, the confusion arose from the abstract of the publication that states, "The sub-chronic study included 60 rats distributed in three groups (n = 20: 10 males and 10 females) receiving daily different three doses of the VOO extract in the drinking water during 90 days: (1) 100 mg/kg, (2) 300 mg/kg, and (3) 1000 mg/kg"

We trust that the above information and clarification addresses your queries. If you have any questions or need additional explanation, please let me know.

Thank you for the opportunity to provide this explanation to your questions.

Best regards,

Madhu Soni

Santos, Marissa

From: Madhu Soni <sonim@bellsouth.net>
Sent: Monday, October 11, 2021 4:03 PM
To: Santos, Marissa
Subject: RE: [EXTERNAL] RE: GRN 000978 - Question for the Notifier

Dear Ms. Santos,

I have received the responses from the notifier (Oliphenol) of GRN 000978 for the below described three queries. Please see below responses included next to the query:

1. Please confirm that all materials used in the manufacturing process of hydrolyzed aqueous olive pulp extract (HAOPE) are used in accordance with U.S. regulations.

Response: We confirm that all materials used in the manufacturing of HAOPE are in accordance with U.S. regulation.

2. On page 8 (Table 3), the specification for moisture in the liquid form of HAOPE is listed as 34% indicating that there is no acceptable range for the moisture content. Please clarify whether there is a range of moisture content that would be acceptable for the liquid form of HAOPE.

Response: We are sorry for not providing the range. Please note that the moisture content of liquid product ranges from 30-45%.

3. On page 8 (Table 3), please clarify the acronym "ELFA" under the *Salmonella* specification.

Response: We are sorry for the confusion, please note that *Salmonella* is routinely measured as per USP Chapter 62 method. By oversight ELFA (Enzyme-Linked Fluorescent Assay) got inserted as sometime this method has also been used.

Hope the above responses are satisfactory. Thank you for the opportunity to provide these clarifications. If you have any questions, please let me know.

Best regards

Madhu

From: Santos, Marissa [mailto:Marissa.Santos@fda.hhs.gov]
Sent: Thursday, October 7, 2021 1:25 PM
To: Madhu Soni <sonim@bellsouth.net>
Subject: RE: [EXTERNAL] RE: GRN 000978 - Question for the Notifier

Dear Dr. Soni,

During our review of GRAS Notice No. 000978, we noted additional questions that need to be addressed and are included here:

1. Please confirm that all materials used in the manufacturing process of hydrolyzed aqueous olive pulp extract (HAOPE) are used in accordance with U.S. regulations.

2. On page 8 (Table 3), the specification for moisture in the liquid form of HAOPE is listed as 34% indicating that there is no acceptable range for the moisture content. Please clarify whether there is a range of moisture content that would be acceptable for the liquid form of HAOPE.
3. On page 8 (Table 3), please clarify the acronym "ELFA" under the *Salmonella* specification.

We respectfully request a response within **10 business days**. If you are unable to complete the response within that time frame, please contact me to discuss further options. Please do not include any confidential information in your responses.

If you have any questions or need further clarification, please feel free to reach out to me.

Regards,
Marissa

Marissa Santos, M.S.
Regulatory Review Scientist
Division of Food Ingredients
Office of Food Additive Safety
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From: Madhu Soni <sonim@bellsouth.net>
Sent: Monday, July 12, 2021 3:55 PM
To: Santos, Marissa <Marissa.Santos@fda.hhs.gov>
Subject: [EXTERNAL] RE: GRN 000978 - Question for the Notifier

Dear Ms. Santos,

Please find attached a pdf file providing a point-by-point response to the agency queries related to our GRAS notification (GRN 000978) .

I hope the information and clarifications, along with some discussion in the response addresses the FDA queries. If you have any questions or need additional explanation, please let me know.

Thank you for the opportunity to provide this explanation.

Best regards

Madhu

Madhu Soni, *PhD, FACN, FATS*
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From: Santos, Marissa [mailto:Marissa.Santos@fda.hhs.gov]
Sent: Friday, June 25, 2021 1:55 PM
To: Madhu Soni <sonim@bellsouth.net>
Subject: GRN 000978 - Question for the Notifier

Dear Dr. Soni,

During our review of GRAS Notice No. 000978, we noted several questions that need to be addressed and are attached to this email.

We respectfully request a response wi thin **10 business days**. If you are unable to complete the response within that time frame, please contact me to discuss further options. Please do not include any confidential information in your responses.

If you have any questions or need further clarification, please feel free to reach out to me.

Regards,
Marissa

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