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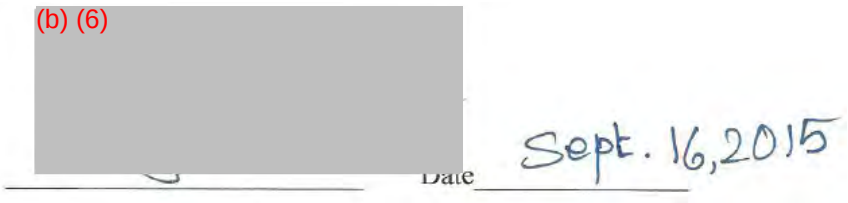
GRAS NOTIFICATION

I. Claim of GRAS Status

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

Seprox Biotech, Spain has determined that hydroxytyrosol (> 99% pure) is Generally Recognized As Safe, consistent with Section 201(s) of the *Federal Food, Drug, and Cosmetic Act*. This determination is based on scientific procedures as described in the following sections, under the conditions of its intended use as a food ingredient. Therefore, the use of hydroxytyrosol is exempt from the requirement of premarket approval.

Signed,



Madhu G. Soni, Ph.D., FATS

Agent for:

Seprox Biotech S.L.
Spain

B. Name and Address of Notifier:

Eduardo Gómez-Acebo, Ph.D.
CEO
Seprox Biotech S.L.
Calle Conde de Aranda, 16, 1º D.
28001 Madrid
Spain

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C. Common or Usual Name of the Notified Substance:

The common name of the substance of this notification is hydroxytyrosol.

D. Conditions of Intended Use in Food

Hydroxytyrosol is intended for use as an antioxidant [21 CFR 170.3(o)(3)][1] and antimicrobial agent [21 CFR 170.3(o)(3)][2] in conventional foods such as beverages (non-alcoholic), fats and oils, fresh and processed fruits/vegetables and juices, and gravy and sauces at use levels of 5.0 mg/serving (reference amounts customarily consumed, 21 CFR 101.12). Hydroxytyrosol is not proposed for uses in foods that are intended for infants and toddlers, such as infant formulas or foods formulated for babies or toddlers, as well as it is not intended for use in meat and poultry products that come under USDA jurisdictions. The intended use of hydroxytyrosol in the above mentioned food categories, is estimated to result in a maximum daily intake of 51.06 mg /person (0.85 mg/kg body weight/day for an individual weighing 60 kg).

E. Basis for GRAS Determination:

In accordance with 21 CFR 170.30, the intended use of hydroxytyrosol has been determined to be Generally Recognized As Safe (GRAS) based on scientific procedures. The determination is supported by the opinion of the Expert Panel. A comprehensive search of the scientific literature was also utilized for this determination. There exists sufficient qualitative and quantitative scientific evidence, including animal and human data to determine safety-in-use for hydroxytyrosol. As hydroxytyrosol is found in olive oil, table olives, red wine, etc, it is commonly consumed from diet. The safety determination of hydroxytyrosol is based on the totality of available evidence.

The safety of hydroxytyrosol is supported by multiple animal and human studies that have been performed with hydroxytyrosol, olive oil, table olives, and olive extract enriched with hydroxytyrosol. Several experimental studies, including subchronic toxicity, reproduction and developmental toxicity, *in vitro* and *in vivo* genotoxicity and human clinical safety data

[1] *Antioxidants*: Substances used to preserve food by retarding deterioration, rancidity, or discoloration due to oxidation.

[2] *Antimicrobial agents* : Substances used to preserve food by preventing growth of microorganisms and subsequent spoilage, including fungistats, mold and rope inhibitors, and the effects listed by the National Academy of Sciences/National Research Council under "preservatives."

support the safety in use of hydroxytyrosol at the intended use levels. Additionally, the safety of hydroxytyrosol is well established in the literature based on the dietary consumption of foods such as olive oil and table olives. Furthermore, European Food Safety Authority (EFSA) has permitted health claims in relation to dietary consumption of hydroxytyrosol and related polyphenol compounds from olive fruit and 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. On the basis of scientific procedures[3], Seprox Biotech considers the consumption of hydroxytyrosol, as a food ingredient to be safe at levels up to 51.06 mg/person/day.

F. Availability of Information:

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

Madhu G. Soni, PhD, FATS
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Vero Beach, FL 32068

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

Hydroxytyrosol is a standardized slightly yellow viscous liquid obtained by chemical synthesis according to a well established process protocol. The product is chemically pure and contains >99% hydroxytyrosol.

A. Chemical name:

Hydroxytyrosol; 4-(2-hydroxyethyl)-benzene-1,2-diol; 3-Hydroxytyrosol; 3,4-dihydroxyphenylethanol (DOPET); Dihydroxyphenylethanol
2-(3,4-Di-hydroxyphenyl)-ethanol (DHPE); 3,4-dihydroxyphenolethanol (3,4-DHPEA)

B. Chemical Abstract Registry and other Number:

Hydroxytyrosol: 10597-60-1

C. Chemical Formula:

The empirical formula of hydroxytyrosol is $C_8H_{10}O_3$

D. Structure:

[3] 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.

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

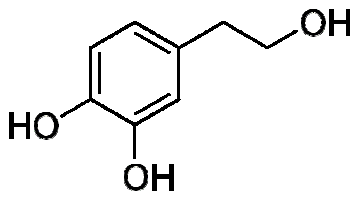


Figure II-D. Chemical Structure of Hydroxytyrosol

E. Molecular Weight

The molecular weight of hydroxytyrosol is 154.16 kDa

F. Physical Characteristics

Hydroxytyrosol is a slightly yellow viscous liquid with a characteristic pungent odor and bitter taste.

G. Identity and Specifications

Food grade specifications of hydroxytyrosol are presented in Tables II-G.1. The product is highly pure and contains > 99% hydroxytyrosol with four residual organic by-products identified by HPLC MS/MS. These by-products include: homovanillic alcohol; iso-homovanillic alcohol; 3-methoxy-4-hydroxyphenylglycol; and hydroxytyrosol acetate. Analytical data from six manufacturing lots are presented in Appendix I.

Table II.G.1. Specifications of Hydroxytyrosol

Parameter		**Assay method**
Description	Slightly yellow viscous liquid	Visual
Odor	Characteristics	Organoleptic
Taste	Slightly bitter	Organoleptic
Solubility (water)	Miscible in water	In house
Moisture	< 4%	Halogen Moisture Analyzer
pH	3.5 – 4.5	1 M water solution
Chemical assay		
Hydroxytyrosol	> 99.0%	In house- HPLC
Hydroxytyrosol acetate	< 0.4%	HPLC
Others	< 0.1%	HPLC
Heavy metals		
Lead	< 0.02 ppm	ICP-MS
Cadmium	< 0.01 ppm	ICP-MS
Mercury	< 0.01 ppm	ICP-MS
Residual solvents		
Ethyl acetate	< 25.00 ppm	Head Space/GC/MS
Isopropanol	< 3.00 ppm	Head Space/GC/MS
Methanol	< 0.01 ppm	Head Space/GC/MS
Tetrahydrofuran	< 0.01 ppm	Head Space/GC/MS

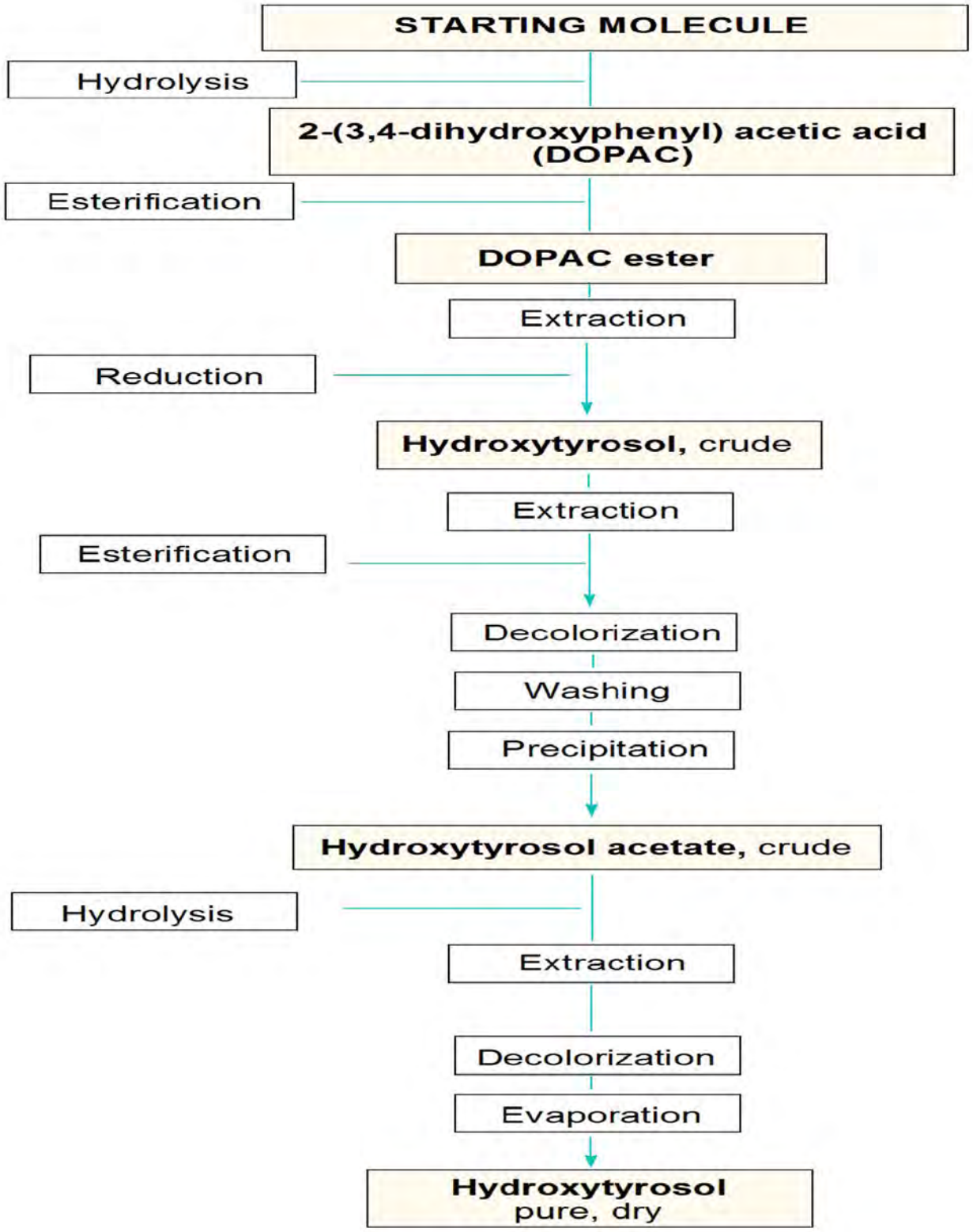
H. Manufacturing process

Hydroxytyrosol is manufactured according to a well established process protocol at Seprox Biotech, Spain. The production process is schematically presented in Figure I.1. It is manufactured via chemical syntheses using reactions that are familiar to those with training in the art of organic chemistry. The synthetic processes involve hydrolysis, esterification, reduction, and purification steps to produce the final product, hydroxytyrosol, in high yields and purity. The synthesis of hydroxytyrosol is based on obtaining an accessible source of an already naturally occurring precursor of dihydroxyphenyl acetic acid. The precursor is esterified and the resulting ester is purified and concentrated. The ester is dissolved and reduced; the excess reducing agent used is eliminated. The crude hydroxytyrosol is extracted. The hydroxytyrosol thus obtained is chemically transformed to hydroxytyrosol acetate, which is then washed with water to eliminate the inorganic salts, hydrolyzed, decolorized and further purified to obtain pure hydroxytyrosol.

The processing agents and starting materials used in the production of hydroxytyrosol are high grade pure chemicals. Seprox Biotech has established quality control measures to make sure that the individual reactions in the production process are optimized; the solvents and reactants are monitored to ensure the product complies with the appropriate food regulations. All of the reagents used in the production of hydroxytyrosol are approved food grade solvents or are purity suitable for the intended use. The preparation procedure assures a consistent and high-quality product. The quality control measures are summarized in Appendix II.

I. Manufacturing process diagram

Figure I.1. Process Flow Scheme for Manufacturing of Hydroxytyrosol



J. Intended Technical Effects

Hydroxytyrosol is intended for addition to selected foods as an antioxidant [21 CFR 170.3(o)(3)] and antimicrobial agent [21 CFR 170.3(o)(3)]. The use of hydroxytyrosol is intended for the general population at the levels identified in this document for addition to the following food categories: beverages (non-alcoholic), fats and oils, fresh and processed fruits/vegetables and juices, and gravy and sauces. It is recognized that there are Standard of Identity requirements for some of the foods, and as such, Seprox Biotech does not intend to refer them by the commonly recognized names.

III. Summary of the Basis for the Notifier's Determination that Hydroxytyrosol is GRAS

The determination that hydroxytyrosol is GRAS is based on scientific procedures. A comprehensive search of the scientific literature for safety and toxicity information on hydroxytyrosol, its natural sources such as olive oil, table olives, olive extract was conducted through August 2015 and was utilized for this assessment. Based on a critical evaluation of the pertinent data and information summarized here and employing scientific procedures, it is determined that the addition of hydroxytyrosol to the selected foods described in this notice and at use levels of 5 mg/serving (in accordance with established reference amounts customarily consumed, 21 CFR 101.12) meeting the specification cited above and manufactured according to current Good Manufacturing Practice, is GRAS under the conditions of intended use as specified herein.

In coming to this decision that hydroxytyrosol is GRAS, Seprox Biotech relied upon the conclusions that neither hydroxytyrosol nor any of its degradation products pose any toxicological hazards or safety concerns at the intended use levels, as well as on published toxicology studies and other articles relating to the safety of the product. Other qualified and competent scientists, reviewing the same publicly available toxicological and safety information, would reach the same conclusion.

IV. Basis for a Conclusion that Hydroxytyrosol is GRAS for its Intended Use.

An independent panel of recognized experts, qualified by their scientific training and relevant national and international experience to evaluate the safety of food and food ingredients, was convened to determine the safety of hydroxytyrosol used as a food ingredient to provide consumers with a source of hydroxytyrosol in their diets. Based on a critical evaluation of the pertinent data and information summarized herein, the Expert Panel members have individually and collectively determined by scientific procedures that the addition of hydroxytyrosol in beverages (non-alcoholic), fats and oils, fresh and processed fruits/vegetables and juices, and gravy and sauces at use levels up to 5 mg hydroxytyrosol/serving (reference amounts customarily consumed, 21CFR 101.12) when not otherwise precluded by a Standard of Identity as described here and resulting in the 90th percentile all-user estimated intake of 51.06 mg hydroxytyrosol/person/day is GRAS. It is also their opinion that other qualified and competent scientists, reviewing the same publicly available toxicological and safety information, would reach the same conclusion (see attached Expert Panel Statement).

EXPERT PANEL STATEMENT

DETERMINATION OF THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF HYDROXYTYROSOL AS A FOOD INGREDIENT

Prepared for:

Seprox Biotech S.L.
Calle Conde de Aranda, 16, 1º D.
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Prepared by:
Soni & Associates Inc.
749 46th Square
Vero Beach, FL 32968
USA

Potential Panel Members

Francesco Visioli, Ph.D.
Robert L. Martin, Ph.D.
Madhusudan G. Soni, Ph.D., F.A.T.S.

CONFIDENTIAL

September, 2015

EXPERT PANEL STATEMENT

DETERMINATION OF THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF HYDROXYTYROSOL AS A FOOD INGREDIENT

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DETERMINATION OF THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF HYDROXYTYROSOL AS A FOOD INGREDIENT

1. INTRODUCTION

The undersigned, an independent panel of recognized experts (hereinafter referred to as the Expert Panel)[1], qualified by their scientific training and relevant national and international experience to evaluate the safety of food and food ingredients, was convened by Soni & Associates Incorporated, at the request of Seprox Biotech S.L., Spain, to determine the Generally Recognized As Safe (GRAS) status of hydroxytyrosol as an antioxidant [21 CFR 170.3(o)(3)][2] and antimicrobial agent [21 CFR 170.3(o)(3)][3] in conventional foods such as beverages (non-alcoholic), fats and oils, fresh and processed fruits/vegetables and juices, and gravy and sauces at use levels of 5.0 mg/serving (reference amounts customarily consumed, 21 CFR 101.12). A comprehensive search of the scientific literature for safety and toxicity information on hydroxytyrosol was conducted through April 2015 and made available to the Expert Panel. The Expert Panel independently and critically evaluated materials submitted by Seprox Biotech and other information deemed appropriate or necessary. Seprox Biotech accepts responsibility for the GRAS determination that has been made for hydroxytyrosol as described herein. Following an independent, critical evaluation, the Expert Panel conferred on September 02, 2015 and unanimously agreed to the decision described herein.

1.1. Background

Polyphenols are natural plant substances that have antioxidant properties in humans. Polyphenols are present in a variety of fruits and vegetables, but the concentration is typically higher in fruits than in vegetables (Bernini et al., 2013). Among the food products containing high levels of phenolic compounds are olives and extra virgin olive oil in addition to its high proportion of oleic acid. In addition to its fatty acid profile, the purported health benefits of extra virgin olive oil are also attributed to its phenolic compounds (Visioli, 2012). Among the polyphenols present in olive oil is a biophenol named hydroxytyrosol which has recently received attention for its potential health benefits (Bernini et al., 2013). It is the most investigated molecule among olive polyphenols, and it represents the biochemical target in the majority of bioavailability studies performed in humans and animals. Because of their antioxidant activity, olive polyphenols, including hydroxytyrosol, have been the subject of extensive clinical and preclinical investigations addressing their claimed benefits. Over 20 human clinical trials have been undertaken that indicate the superiority of phenol-rich olive oil as compared to other vegetable oils or sources of fat (Visioli and Bernardini, 2011). This notion has been reinforced by the recent European Food Safety Authority (EFSA, 2011) on the substantiation of health claims related to hydroxytyrosol.

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

[2]*Antioxidants*: Substances used to preserve food by retarding deterioration, rancidity, or discoloration due to oxidation.

[3]*Antimicrobial agents* : Substances used to preserve food by preventing growth of microorganisms and subsequent spoilage, including fungistats, mold and rope inhibitors, and the effects listed by the National Academy of Sciences/National Research Council under "preservatives."

Hydroxytyrosol is currently being actively marketed as a potential supplement or preservative in the nutraceutical, cosmeceutical, and food industries (Visioli and Bernardini, 2011). Hydroxytyrosol has been proposed as a cardioprotective (Visioli, 2012), neuroprotective (Schaffer et al., 2007), and chemopreventive (Visioli et al., 2004) agent. Given the potential health benefits of phenolic compounds, particularly hydroxytyrosol, Seprox Biotech intends to market hydroxytyrosol for use as a food ingredient in selected foods as described in this dossier.

1.2. Description

Hydroxytyrosol, a phenylethanoid [2-(3,4- dihydroxyphenyl)ethanol] (Figure 1), in its pure form is a white solid. Hydroxytyrosol produced by Seprox Biotech and the subject of this GRAS assessment is a slightly hydrated (approximately 4%) yellow viscous liquid with a pungent odor and bitter taste. The general descriptive parameters and properties of hydroxytyrosol are summarized in Table 1. Hydroxytyrosol is present in olive oil, as well as in table olives, and is thus commonly consumed from diet by humans.

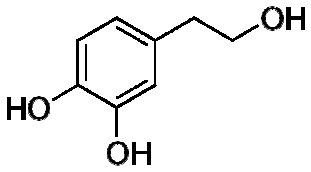


Figure 1. Chemical structure of Hydroxytyrosol

Table 1. General Descriptive Characteristics of Hydroxytyrosol

Parameter	Description (Seprox Biotech, 2013)*
Common name	Hydroxytyrosol
CAS No.	10597-60-1
Synonyms	4-(2-hydroxyethyl)-benzene-1,2-diol; 3-Hydroxytyrosol 3,4-dihydroxyphenylethanol (DOPET); Dihydroxyphenylethanol 2-(3,4-Di-hydroxyphenyl)-ethanol (DHPE); 3,4-dihydroxyphenolethanol (3,4-DHPEA)
Appearance	Slightly yellow viscous liquid
Solubility	Soluble in water in all proportions; Highly soluble in polar organic solvents
Color	Slightly yellow
Odor	Pungent
Taste	Bitter
Molecular weight	154.16
Chemical formula	$C_8H_{10}O_3$
Melting point	56ºC
Boiling point	265ºC
Storage	Store in the dark at 4ºC; protect from oxidizing atmospheres
Stability	At least three years under recommended storage conditions

*Based on information provided by Seprox Biotech

1.3. Manufacturing Process

Hydroxytyrosol is manufactured according to a well established process protocol at Seprox Biotech, Spain. The production process is schematically presented in Figures 2 and 3. It is manufactured via chemical syntheses using reactions that are familiar to those with training in the art of organic chemistry. The synthetic processes involve hydrolysis, esterification, reduction, and purification steps to produce the final product, hydroxytyrosol, in high yields and purity. The synthesis of hydroxytyrosol is based on obtaining an accessible source of an already naturally occurring precursor of dihydroxyphenyl acetic acid. The precursor is esterified and the resulting ester is purified and concentrated. The ester is dissolved and reduced; the excess reducing agent used is eliminated. The crude hydroxytyrosol is extracted. The hydroxytyrosol thus obtained is chemically transformed to hydroxytyrosol acetate, which is then washed with water to eliminate the inorganic salts, hydrolyzed, decolorized and further purified to obtain pure hydroxytyrosol.

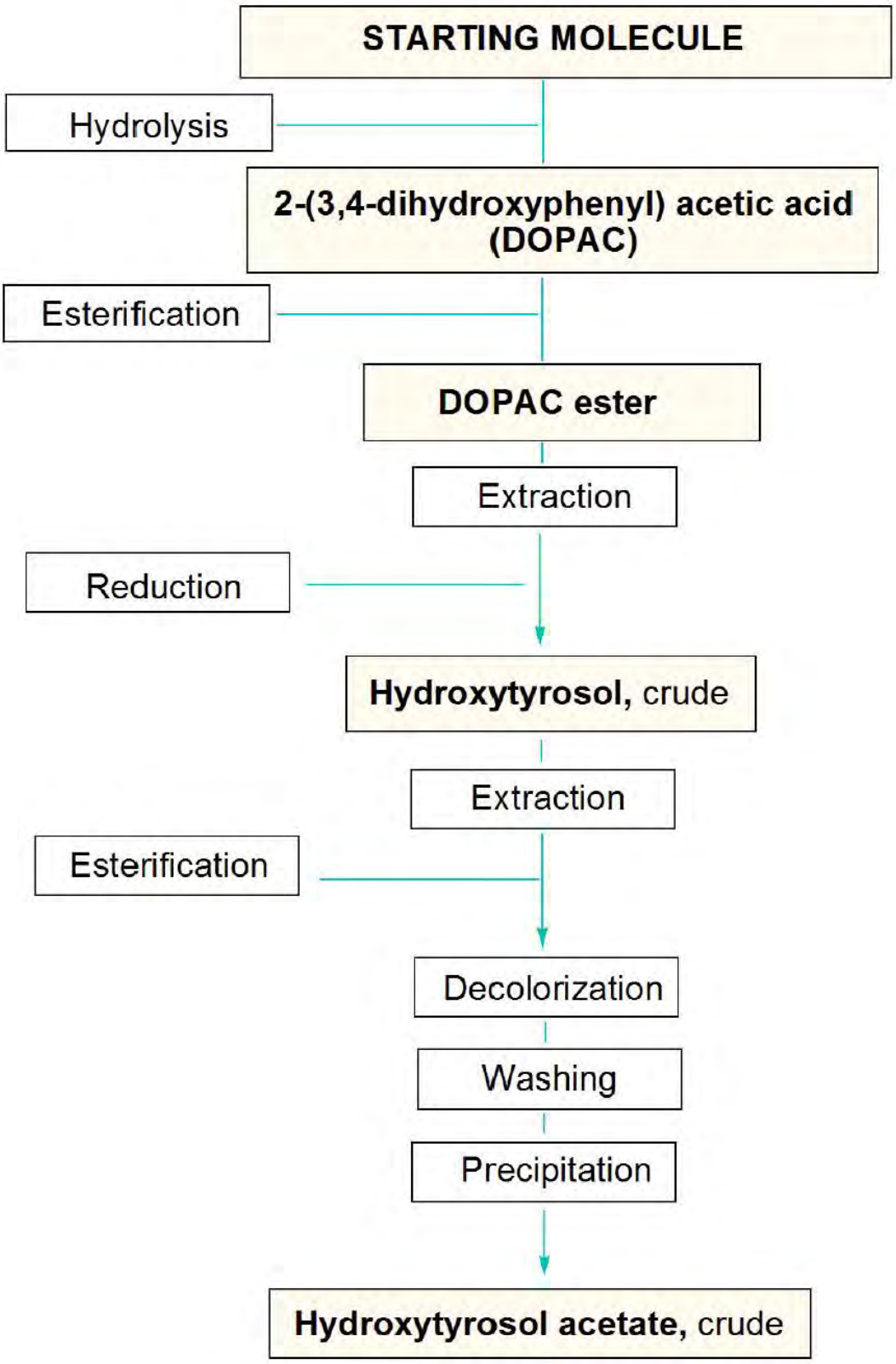


Figure 2: Process Flow Scheme for Manufacturing of Hydroxytyrosol Acetate

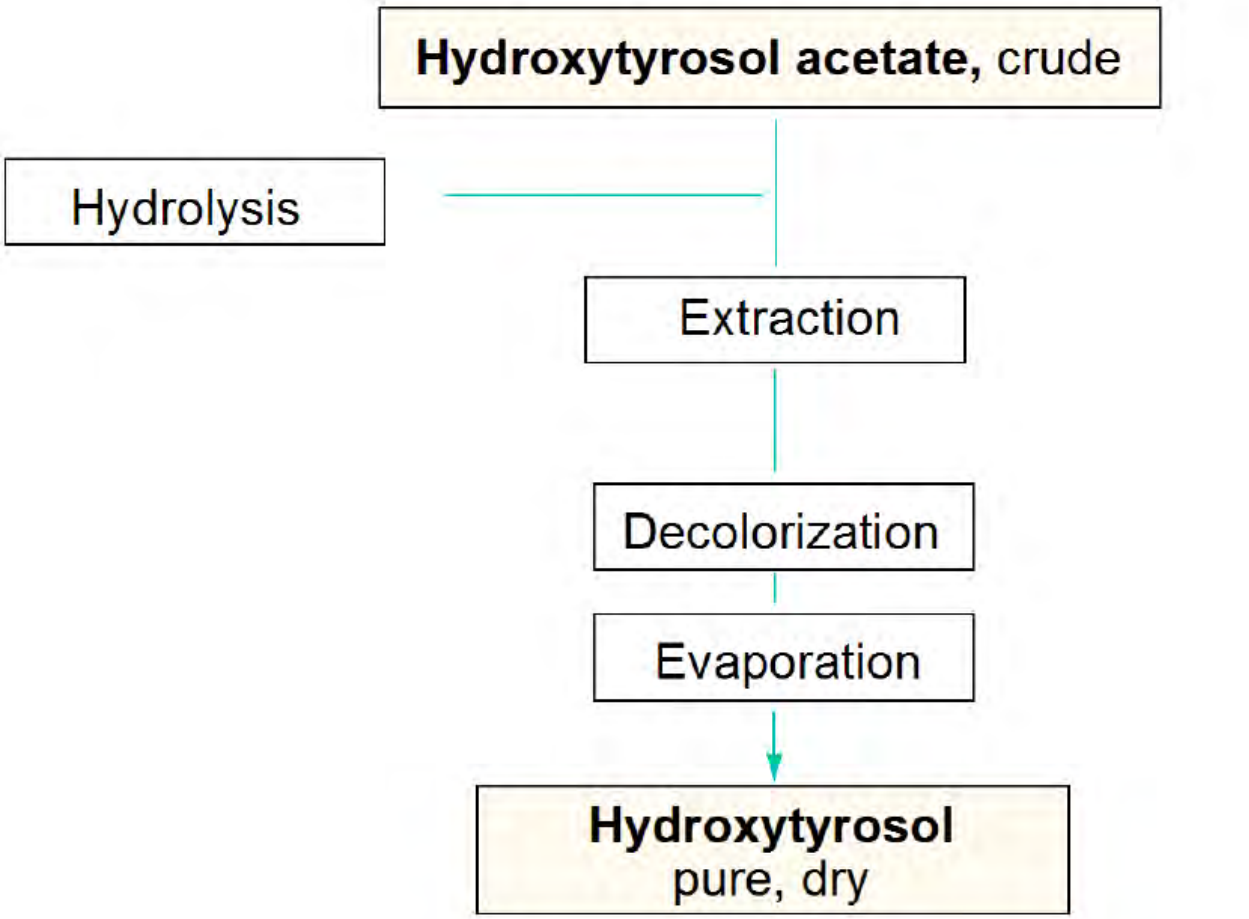


Figure 3. Process Flow Scheme for Enzymatic Hydrolysis of Hydroxytyrosol Acetate to Hydroxytyrosol

The processing agents and starting materials used in the production of hydroxytyrosol are high grade pure chemicals. Seprox Biotech has established quality control measures to make sure that the individual reactions in the production process are optimized; the solvents and reactants are monitored to ensure the product complies with the appropriate food regulations. All of the reagents used in the production of hydroxytyrosol are approved food grade solvents or are purity suitable for the intended use. The preparation procedure assures a consistent and high-quality product. The quality control measures are summarized in Appendix II.

1.4. Specifications and Identity

Food grade specifications of hydroxytyrosol have been established by Seprox Biotech and are summarized in Table 2. The product is highly pure and contains > 99% hydroxytyrosol with four residual organic by-products identified by HPLC MS/MS. These by-products include: homovanillic alcohol; iso-homovanillic alcohol; 3-methoxy-4-hydroxyphenylglycol; and hydroxytyrosol acetate. The molecular weight of Seprox Biotech's hydroxytyrosol was confirmed by LC-MS and MS/MS analysis of E011 017 07 in negative mode with that existing in MELTIN database. Analytical results from six non-consecutive lots (Appendix I) demonstrate that hydroxytyrosol is consistently manufactured to meet these specifications. In addition to general specification parameters, Appendix I also provide details of residual solvents and

elemental analysis (minerals, anions) investigated from individual batches. The microbiological specifications of the product have not been established as hydroxytyrosol is known for its antimicrobial activity.

The solvent, ethyl acetate, employed in the manufacturing and extraction of hydroxytyrosol meets the specifications listed in the Food Chemicals Codex (7th Edition). It is permitted under the regulation, 21 CFR 172.859, in the preparation of sucrose esters. According to this regulation, the residual level for ethyl acetate in sucrose esters is established at < 350 ppm. Additionally, as per 21 CFR 172.372 the residual level of ethyl acetate (< 500 ppm) is permitted as a processing aid in the nutrient N-acetyl-L-methionine. Ethyl acetate is also permitted as a secondary direct food additive in accordance with current good manufacturing practice (cGMP) as a solvent in the decaffeination of coffee and tea (21 CFR 173.228). Ethyl acetate is GRAS for use as a flavoring agent under 21 CFR 182.60. The residual levels of ethyl acetate in hydroxytyrosol from three manufacturing lots are below 20 ppm (Appendix I). The residual solvent levels for isopropanol (<3 ppm) used in the manufacturing of hydroxytyrosol is below the JECFA and Code of Federal Register (CFR) regulations cited limits for this solvent. Similarly, the residual solvent levels for other solvents such as methanol (≤0.01 ppm) and tetrahydrofuran (≤0.01 ppm) (Appendix I) are very low and considered as safe.

Table 2. Specifications of Hydroxytyrosol

Parameter		**Assay method**
Description	Slightly yellow viscous liquid	Visual
Odor	Characteristics	Organoleptic
Taste	Slightly bitter	Organoleptic
Solubility (water)	Miscible in water	In house
Moisture	< 4%	Halogen Moisture Analyzer
pH	3.5 – 4.5	1 M water solution
Chemical assay		
Hydroxytyrosol	> 99.0%	In house- HPLC
Hydroxytyrosol acetate	< 0.4%	HPLC
Others	< 0.1%	HPLC
Heavy metals		
Lead	< 0.02 ppm	ICP-MS
Cadmium	< 0.01 ppm	ICP-MS
Mercury	< 0.01 ppm	ICP-MS
Residual solvents		
Ethyl acetate	< 25.00 ppm	Head Space/GC/MS
Isopropanol	< 3.00 ppm	Head Space/GC/MS
Methanol	< 0.01 ppm	Head Space/GC/MS
Tetrahydrofuran	< 0.01 ppm	Head Space/GC/MS

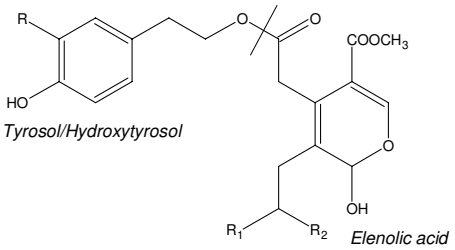
1.5. Chemistry

Also known as 3,4-dihydroxytyrosol or 3,4-dihydroxyphenylethanol (CAS No.: 10597-60-1; Figure 1), hydroxytyrosol is the main active constituent of the phenolic fraction of olive extract and olive oil. The chemical formula of hydroxytyrosol is $C_8H_{10}O_3$ and the molecular weight is 154 (Table 1). It is present in olive oil either as the simple phenol or esterified with elenolic acid to form oleuropein aglycone. Hydroxytyrosol in its pure form is a white solid with slightly bitter taste. Quantitatively, hydroxytyrosol is a minor constituent of the aqueous olive pulp extract, as well as of the olive oil phenolic fraction. However, it is considered the most potent phenolic antioxidant of the olive oil.

1.6. Natural Occurrence

As indicated earlier, hydroxytyrosol is naturally present in olives and olive oil along with other polyphenols. The quality of olive oil is defined by the phenolic compounds of the fruit from which it is derived. Hydroxytyrosol has been also found in both red and white wine in considerable amounts (Di Tommaso et al., 1998; Fernandez-Mara et al., 2012). Simple, as well as complex phenolic substances have been reported from olive fruit. Generally in olive oil, phenols (Figure 4) are found both as simple (hydroxytyrosol and tyrosol) and complex compounds (hydroxytyrosol or tyrosol esterified to elenolic acid, in the form of oleuropein and ligstroside, respectively). In Figure 4, the structures are represented as hydroxytyrosol + elenolic acid → oleuropein and tyrosol + elenolic acid → ligstroside. The levels of phenols in the oil are up to 1% by weight. During the extraction of the oil or processing of the olives, 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. Phenolics in olive oil have been reported to be responsible for the stability of the oil from oxidation and for the organoleptic characteristics (Papadopoulos and Boskou, 1991; Visioli and Galli, 2001).

Approximately 90% of the phenols present in the olive are transferred to the vegetation water during the olive processing (pressing of the drupes) for extraction of oil. Visioli and Galli (2003) reported that approximately 10-20% of the total phenol content from the vegetation water can be recovered. Fernandez-Bolanos et al. (2002) reported that from 1000 kg of olives during liquid-solid waste of two-phase (conventional) olive oil processing can result in extraction of 3 kg of hydroxytyrosol (90-95% purity).



R	R1	R2	
OH	H	H	Oleuropein
H	H	H	Ligstroside

Figure 4. Phenolics found in olive and olive products. Hydroxytyrosol is formed by cleavage where indicated (adapted from Visioli and Galli, 2003)

1.7. Exposure to Hydroxytyrosol

Zoidou et al (2010) analyzed the content of polyphenols, including hydroxytyrosol, from nine commercial types of Greek table olives. The highest levels of hydroxytyrosol (1.8-2.0 mg/fruit) was found in Kalamata olives and Green 'tsakistes' of the variety Megaritiki followed by Greek-style 'chondrolies' (1.0 mg/fruit). These investigators suggested that assuming a usual consumption of 20 olive fruits per day, the daily intake of hydroxytyrosol is likely to range from 20 to 40 mg/day. This also depends on the weight of olive that may range from 2 to 5 g/fruit depending on the variety. Owen et al. (2003) analyzed the phenolic content of brined green and black olive drupe. Hydroxytyrosol was predominantly found in green olives, while the black olives contained 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. The phenolics content in black and green olives was reported as 0.082 and 0.118%, respectively, on a percent wet weight basis. These authors suggested that daily dietary consumption of approximately 50 g of black olive pericarp would provide about 400 mg of phenolic substances. A similar quantity of extra virgin olive oil (produced with conventional methods) consumption will provide about 12 mg of phenolic substances. In an analysis of 48 olive samples (Romero et al., 2004), the 'turning color olives' in brine were reported to contain the highest levels of polyphenols (~ 0.12%).

In yet another study, Marsilio et al. (2001) investigated changes in phenolic compounds during the darkening process (sodium hydroxide and air-oxidation) in California style ripe olives. The tyrosol and hydroxytyrosol content of fresh olives was found to be 40 and 57 mg/100 g dry weight, while the presence of these phenolics in brine stored olives was 63 and 395 mg/ 100 g dry weight, respectively. The lye-treated and air-oxidized olives were found to contain high levels of these phenolics, i.e., 152 mg tyrosol and 1030 mg hydroxytyrosol/100 g of olives. The oleuropein content of fresh and brine stored olives was reported as 1650 and 10 mg/100 g, respectively, while in lye-treated and air-oxidized olives this substance was undetectable. The results of this study suggest that the processing method affects the phenolic composition of the olives. Blekas et al. (2002) analyzed commercially available table olives and reported hydroxytyrosol (unbound) content as 250-750 mg/kg (~0.5 mg/g) in two cultivars. The available information, described above, suggests that consumers are routinely exposed to hydroxytyrosol from food.

In an extensive database specifically focused on foods and more precisely on polyphenols, the average content of hydroxytyrosol based on separate publications for black and green olives was reported as 65.93±81.22 and 55.57±31.15 mg/100 g of olives, respectively (Neveu et al., 2010). In these publications, the maximum reported level of hydroxytyrosol was 413.30 and 116.00 mg/100 g black and green olives, respectively. For black and green olives, values were calculated by aggregating data from 17 and 31 different samples from 5 and 4 unique publications, respectively.

1.8. Intended Use Levels and Food Categories

Seprox Biotech intends to use hydroxytyrosol as an antioxidant [21 CFR 170.3(o)(3)] or antimicrobial agent [21 CFR 170.3(o)(3)] at levels up to 5 mg of hydroxytyrosol *per* serving (reference amounts customarily consumed, 21 CFR 101.12) in beverages (non-alcoholic), fats and oils, fresh and processed fruits/vegetables and juices, and gravy and sauces. Foods that are

intended for infants and toddlers, such as infant formulas or foods formulated for babies or toddlers, and meat and poultry products are excluded from the list of intended food uses of the subject hydroxytyrosol preparation. It is recognized that there are Standard of Identity requirements for some of the above specified foods and these foods will not be referred by their commonly recognized names such as milk, chocolate or yogurt. The proposed use levels of hydroxytyrosol in the various food categories are summarized in Table 3.

1.8.1. Estimated Daily Intake from the Intended Uses

1.8.1.1. Use of USDA Data

The daily intake of hydroxytyrosol is estimated using "maximum" intended use levels and mean consumption estimates of designated food categories based on United States Department of Agriculture (USDA) Continuing Survey of Food Intakes by Individuals (CSFII) mean amount of food consumed and USDA mean portion size of general food categories. Based on USDA CSFII surveys (USDA, 1999; Smiciklas-Wright et al., 2002) for quantities of foods consumed daily, the mean and high (90^{th} percentile) consumption of hydroxytyrosol from the proposed uses in beverages (non-alcoholic- fruit drinks and aids; carbonated soft drinks), fats and oils, processed fruits/vegetables and juices, and gravy and sauces was determined. In order to estimate the 90^{th} percentile consumption of hydroxytyrosol, the corresponding mean total intake value from all food categories was multiplied by two on the grounds that the 90^{th} percentile consumption is unlikely to exceed the mean by more than a factor of two (FDA, 2006). Using USDA estimated mean intakes of the food categories (beverages; fats and oils; fresh and processed fruits/vegetables and juices, gravy and sauces) for which hydroxytyrosol is proposed to be added, the possible mean and maximum daily intake of hydroxytyrosol from each of the categories is summarized in Table 3. The intended use of hydroxytyrosol at levels of 5.0 mg *per* serving will result in mean and 90^{th} percentile intake of 25.53 and 51.06 mg/person/day (0.42 and 0.85 mg/kg bw/day for an individual weighing 60 kg), respectively.

Table 4. Intended Use Levels and Possible Daily Intake of Hydroxytyrosol Based on USDA Data[1]

Food Category	Use Level mg per serving (serving size)	USDA Mean Grams of Food Consumed	Mean Additive Consumed (mg/day)	Mean x 2 Additive Consumed (mg/day)	Data Table, Page Number**
Beverages	5.0 mg (240 ml) = 21 ppm	430	8.96	17.92	32/1
Fats and Oils	5.0 mg (15 g) = 333 ppm	14	4.67	9.34	31/1
Fresh Fruits and juices	5.0 mg (240) = 21 ppm	169	3.52	7.04	28/1
Gravy and sauces	5.0 mg (60 ml) = 83 ppm	80	6.66	13.32	208/2
Processed vegetable/juices	5.0 mg (240) = 21 ppm	82.8	1.72	3.44	34/2
Total amount of hydroxytyrosol/person/day (mg)			**25.53**	**51.06**	
mg/kg bw/day (for 60 kg individual)			**0.42**	**0.85**	

[1]The daily intake calculations are based on USDA data (CSFII) and mean portion size; RACC – Reference Amounts Customarily Consumed Per Eating Occasion (21 CFR §101.12). *Considered to include imitation dairy. **Data source- 1 DATA TABLES: Results from the USDA (1999); 2. Smiciklas-Wright et al. (2002).

1.8.1.2. Exposure Based on Daily Servings

According to the USDA Nutrition Insights (Basiotis et al., 2000), a publication of the USDA Center for Nutrition Policy and Promotion, males aged 51 or older consume the greatest servings of food per day. This population consumes 18.2 servings of food per day from the following food categories: grains (6.2 servings), fruits (1.3), vegetables (2.7), milk (1.1), meat (2.4) and other (fats, oils, sweets; 4.5). Hydroxytyrosol is intended to be added to foods such as fruits, vegetables, and other (fats oils, sweets). The total serving of these food products in a day consumed are 8.5. As the intended use levels of hydroxytyrosol is 5 mg/serving, the resulting intake from the proposed uses in the above food categories will be 42.5 mg/person/day. Based on this data, the maximum likely intake of hydroxytyrosol is determined as 42.5 mg/person/day (0.71 mg/kg bw/day for an individual weighing 60 kg).

1.8.2. Consumption Summary

The estimated intake of hydroxytyrosol from its natural presence in table olives ranged from 20-40 mg/day. It is likely that the intake of hydroxytyrosol is expected to be higher in Mediterranean countries where olives in the form of table olives and olive oil are commonly consumed. Based on the USDA CSFII database, the intended use of hydroxytyrosol at use levels of 5 mg/serving in food categories such as beverages, fats and oils, fresh and processed fruits/vegetables and juices, and gravy and sauces will result in mean and high (90^{th} percentile) intakes of 25.53 and 51.06 mg/person/day, respectively. On body weight basis, the mean and high (90^{th} percentile) intake based on USDA data is estimated as 0.42 and 0.85 mg hydroxytyrosol/kg bw/day for an individual weighing 60 kg. Based on the greatest number serving of the food consumed to which hydroxytyrosol is proposed to be added, the intended use at 5 mg/serving will result in 42.5 mg/person/day or 0.71 mg/kg bw/day. For safety assessment purposes, the maximum intake of 51.06 mg hydroxytyrosol/person/day (0.85 mg/kg bw/day) is considered as appropriate.

2. SAFETY RELATED DATA

In several published studies, phenolics found in olive and olive oils have been extensively investigated. In recent years, hydroxytyrosol, also found at low levels in olive oil, has been investigated for its efficacy as an antioxidant. One obvious reason for the lack of safety studies of olives or its constituents, such as hydroxytyrosol, is because of the fact that table olives and olive oil are widely consumed as food products at high levels in Mediterranean countries and elsewhere. The safety assessment of hydroxytyrosol is based on the totality of available evidence, including animal experimental studies and human clinical observations. Efforts have been made to present both the data supporting the safety of hydroxytyrosol as well as any data on potential adverse effects. The assessment of efficacy studies is limited to a review of the results related to safety and tolerability. Relevant biological and toxicological studies on hydroxytyrosol and phenolics present in olive oils are included in the following section in the order of their relevance to provide support for the conclusions reached in this determination.

2.1. Toxicology

2.1.1. Specific Studies with Hydroxytyrosol

In a series of toxicity studies sponsored by Seprox Biotech and conducted as per European Commission Directives and OECD guidelines, subchronic toxicity and potential

genotoxic effects of hydroxytyrosol (the subject of present GRAS determination) were investigated (Auñon-Calles et al., 2013a; 2013b). For the subchronic toxicity study and mutagenicity studies, hydroxytyrosol used in these studies was produced by Seprox Biotech, Spain. These studies are described in the following sections.

2.1.1.1. Subchronic Study

In a dose-response study, Auñon-Calles et al. (2013a) investigated the potential toxicity of hydroxytyrosol in rats. In addition to this publication, a complete animal study report provided by Seprox was utilized for the assessment of findings from this toxicity study. In this study, Wistar Hannover rats (10/sex/group; 6-11 week old) were gavaged daily with hydroxytyrosol at dose levels of 0 (control- Group 1), 5 (low dose- Group 2), 50 (mid dose- Group 3), or 500 (high dose- Group 4) mg/kg body weight (bw)/day for 90 consecutive days. An additional, five males and five females were allocated to the control and high dose recovery groups. The study was conducted following internationally accepted guidelines and recommendations by the European Commission Directives and OECD. The study was performed in compliance of Principles of Good Laboratory Practice for the testing of Chemicals OECD [C(97)/186- Final)] and as per OECD Guideline for the Testing of Chemicals, Guideline 408 Repeated Dose 90-Day Oral Toxicity Study in Rodents, 21 September 1998. Throughout the study period the animals were observed for clinical signs of toxicity and mortality/morbidity (daily), detailed clinical examination, body weight and feed consumption (weekly), functional observation tests during week 13, ophthalmoscopy at pretest and in week 13, hematological, clinical chemistry (at termination), urinalysis, gross pathology and organ weight (at termination). Over 40 tissues and organs were harvested at the necropsy and fixed in 10% buffered neutral formalin. Histopathological examination was carried out on the full set of tissues collected from the high dose and control groups. All gross lesions from all rats irrespective of group were also evaluated for histological changes. All animals in recovery groups were euthanized, necropsied and examined post mortem after a 4-week treatment-free recovery period (Auñon-Calles et al., 2013a).

During the course of the study, no mortality was noted in any group. No relevant treatment-related clinical signs were recorded. During the treatment period, salivation was recorded before and/or after the administration in all animals from group 4 and occasionally in groups 2 and 3 (Auñon-Calles et al., 2013a). This phenomenon was attributed to the bitter taste of hydroxytyrosol and/or the physical characteristics of the formulation (slightly oily and dense). As discussed below, in an earlier study with olive extract containing hydroxytyrosol, increase in salivation was noted (Christian et al., 2004). The other clinical signs recorded are not considered test-article related. No treatment-related differences were recorded in the evaluation of reflex behavior in any of the groups. Ophthalmoscopic examinations did not reveal any ocular changes. 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 a recent study. In a study by Heilman et al. (2015), a similar decrease (17%) in male rat body weight following hydroxytyrosol at dose levels of 500 mg/kg bw/day was noted. Based on the historical data provided by the conducting laboratory, the decrease in body weight was well within the historical control data of the laboratory for Wistar rat (age 20-24 weeks). In females from group 4, the percent body weight gain was lower during the entire treatment period. The differences were statistically significant on Days 8, 15, 25 and 29 in females. However, subsequently, the tendency for lower values in females at 500 mg/kg

bw/day was no longer apparent and there was no obvious dosage-related pattern. These minor changes in body weight and percent body weight gain were not considered as treatment-related. The mean feed consumption (g/rat/day) was comparable in all the dose groups of both sexes (Auñon-Calles et al., 2013a).

Urinalysis did not reveal any significant changes between the groups. As regards hematology, although statistically significant differences compared with the control group were observed in some parameters, such as lower red blood cell distribution width in males from the 500 mg/kg group, higher relative monocyte values in males from the 50 mg/kg group, and higher relative reticulocyte values in males from the 5 mg/kg group, these changes were not considered as toxicologically relevant in the absence of a dose-effect relationship and taking into account that they were within the common range for these parameters. At the end of the recovery period, hemoglobin concentration distribution width (HDW) values in males were lower compared to the Control group. Some other statistically significant differences such as higher mean cell volume (MCV) and mean cell hemoglobin (MCH) in females from mid- and high-dose groups and higher reticulocytes with high fluorescence (HFR) and white blood cells (WBC) in females from high-dose group were observed (Auñon-Calles et al., 2013a). As these changes were minor and noted only in females, they were not considered as treatment-related.

The clinical chemistry parameters revealed significantly lower glucose and creatinine and higher albumin values in males from the 500 mg/kg group with respect to the control group. Higher calcium values were observed in males from the 50 and 500 mg/kg groups. Higher aspartate aminotransferase (ASAT) values were observed in males from all treated groups (significant in low- and mid-group but not in high-dose group) compared with the control group. Statistically significant differences were recorded in potassium in males from the low dose group but they cannot be considered of toxicological relevance in the absence of a dose-effect relationship. No relevant differences were observed in females. At the end of the recovery period, the ASAT values in males from the 500 mg/kg group were still higher compared with the control group. The above noted significant changes in hematology and clinical chemistry parameters following the administration of hydroxytyrosol were not observed in both sexes, lacked correlating changes in other clinical parameters, were of small magnitude, were not noted in a dose-related manner, or were not associated with microscopic changes in the related organs and hence they were considered as incidental changes/biological variations and not treatment-related adverse effects (Auñon-Calles et al., 2013a).

Macroscopic findings recorded at the end of the treatment or recovery periods did not reveal any remarkable alteration and were compatible with those of rats of this strain and age. As regards organ weights, as compared to control group no changes in absolute weights of any of the tissues/organs were noted in any treatment group. Some changes in relative organs weights as determined based on organ to body weight or organ to brain weight were noted. For example higher relative kidneys weights were observed in males and females from the 500 mg/kg group. The differences in relative kidney weights were statistically significant in females as related to brain weight and in males and females as related to body weight. These changes were within the historical data range of relative kidney weight as related to brain weight or body weight for the conducting laboratory. Some other statistically significant differences in organ weights relative to body weight were observed in animals from the 500 mg/kg group compared with controls, including higher mandibular salivary gland weights in males and females, higher brain and epididymes weights in males and higher heart and liver weights in females. In males from the 50

and 500 mg/kg groups, compared with controls, higher heart weights with respect to body weight were observed. Higher testes weight with respect to the control group was recorded in all treated groups. At the end of the recovery period, higher absolute and relative testes weights in males, and higher absolute and relative liver and kidney weights in females, were observed compared with the control group. These changes in organ weight were considered as incidental due to lack of dose-dependency and lack of correlating changes in clinical chemistry and histopathology.

It should be also noted that some of the above reported changes in relative organ weights as related to body weight also may be due to lower body weights of animals particularly in the high dose groups. Additionally, these changes were within the historical data range of relative organs weight as related to body weight or brain weight for the conducting laboratory. Furthermore, the histopathological examinations did not reveal any morphological alteration in any of the organs or tissues examined. In a recent 90-day toxicity study (further described below), lack of an effect on kidney weight at dose levels of 500 mg hydroxytyrosol/kg bw/day was reported (Heilman et al., 2015). The findings from this recently published study indicate that hydroxytyrosol at dose levels up to 500 mg/kg bw/day is unlikely to affect body organs.

Microscopic observations did not reveal any morphological alteration in any of the organs or tissues examined. There were no differences between controls and hydroxytyrosol treated animals. All the gross and histopathological changes observed were considered as spontaneous and incidental to rats of this particular strain and age. The results of this study, indicate that hydroxytyrosol at dose level up to 500 mg/kg bw/day is unlikely to cause any adverse effects (Auñon-Calles et al., 2013a).

2.1.1.2. Mutagenicity studies

Auñon-Calles et al. (2013b) also investigated the genotoxic potentials of hydroxytyrosol by employing *in vitro* reverse mutation (Ames assay) and human lymphocyte chromosomal aberration assay. The Ames assay was performed in accordance with OECD Guideline 471 for the Testing of Chemicals (Bacterial Reverse Mutation Test. Adopted 21st July 1997) and the test Method B13/B14 of Commission Directive 2000/32/EC. In this study bacterial strains [*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and *Escherichia coli* WP2(pKM101)] were exposed to 5 concentrations of hydroxytyrosol (0.06, 0.19, 0.56, 0.167 and 5 µL/plate) with and without S9 under the direct incorporation (main study) and the pre-incubation (confirmatory study) procedures. The assay was conducted as per the standard procedure. Cytotoxicity evaluation of HT was performed in the *S. typhimurium* TA 100 strain by the direct incorporation procedure with 5 concentrations prepared by 1:3 serial dilutions starting. Cytotoxicity evaluation of hydroxytyrosol at 50.0 µL/mL up to 0.6 µL/mL in *S. typhimurium* TA 100 strain was based on the decrease in the number of revertant colonies, or a clearing or diminution of the background lawn. No cytotoxic activity was observed in the bacterial system at the highest concentration. None of the concentrations assayed for hydroxytyrosol showed an increase in the R value either with or without S9 metabolic activation regardless of the procedure. No dose response for HT was observed in any of the tested bacterial strains. The results of this investigation suggest that hydroxytyrosol was non-mutagenic as evaluated by Ames assay (Auñon-Calles et al., 2013b).

For the *in vitro* chromosomal aberration assay, hydroxytyrosol was assessed for its potential to induce aberrations in human lymphocytes in the absence and presence of metabolic activation by S9 (phenobarbital/β-naphthoflavone-induced rat liver) mix (Auñon-Calles et al.,

2013b). For these experiments, blood samples were collected in heparinized tubes from a male donor (29 years old). A preliminary cytotoxicity test was performed to determine the concentrations to be used in the assay. Blood cultures were set up in bulk - within 24 hours of collection in cell culture flasks using standard procedures for this type of assay. About 72 h after seeding, two blood cultures (10 mL each) were set up in parallel for each test group. The culture medium was replaced with serum-free medium containing the test item. After four hours, the cells were centrifuged, the supernatant with the dissolved test item was discarded and the cells were re-suspended. The washing procedure was repeated once as described. After washing, the cells were re-suspended in complete culture medium and cultured until preparation. All cultures were incubated at 37 °C in a humidified atmosphere with 5.5% CO_2 and 94.5% air. Ethylmethane sulfonate and cyclophosphamide were used as positive controls. Three hours before harvesting, colcemid was added to the cultures. The cultures were harvested by centrifugation 22 hours after beginning of treatment and the slides were prepared for and metaphase cells were analyzed as described by Preston et al. (1987). At least 100 well-spread metaphases per culture were scored for cytogenetic damage on coded slides.

According to the OECD Guidelines only one experiment was performed, since the test item was considered to be clastogenic after the first experiment. The exposure period was 4 hours with and without S9 mix. The chromosomes were prepared 22 hours after the start of treatment with the test item. 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 cytogenetic tests. No visible precipitation of the test item in the culture medium was noted. No relevant influence on osmolarity or pH value was observed. In the absence and presence of S9 mix concentrations showing clear cytotoxicity were not evaluable for cytogenetic damage. 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. Positive controls showed distinct increases in cells with structural chromosome aberrations. The investigators concluded that, at physiologically-feasible concentrations, hydroxytyrosol is non-genotoxic and non-mutagenic (Auñon-Calles et al., 2013b).

Additionally, in the above described 13-weeks rats study with hydroxytyrosol bone marrow of animals were examined without any significant finding even at the highest dose (500 mg/kg bw/day) (Auñon-Calles et al., 2013a). In another *in vitro study* of chromosome aberrations in Chinese hamster ovary cells, Christian et al. (2004) reported that olive pulp extract containing hydroxytyrosol elicited a significant increase in the percentage of aberrant cells in the presence of S9 and a slight increases in the numbers of polyploid and/or endoreduplicated cells (numerical chromosome changes) at a concentration of 1000 µg/mL. Instability of certain antioxidants, such as polyphenols, in cultured media is well documented and thus genotoxicity could be due more to hydrogen peroxide or oxidizing quinones generated by instability in these media than proper hydroxytyrosol effects (Halliwell, 2008; Long et al., 2010). However, in the *in vivo* micronucleus assay in mice following administration of olive pulp extract containing hydroxytyrosol, Christian et al. (2004) did not notice any genotoxic effects at 24 hours after 28 daily doses of olive pulp

extract at the highest dose level of 5000 mg/kg bw/day (120 mg hydroxytyrosol/kg bw/day). The conclusion is that the available evidence suggests that hydroxytyrosol is unlikely to be genotoxic.

2.1.2. Other Studies

2.1.2.1. Acute Toxicity Studies

In a single dose toxicity study, D'Angelo et al. (2001) investigated 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.

In another study, Christian et al. (2004) investigated the acute effects of a standardized aqueous olive pulp extract containing 6% phenolics, 60% of which was hydroxytyrosol. In this study, mice were treated orally (gavage) or by dermal application with a single dose of aqueous olive pulp extract at levels of 500, 1000 or 2000 mg/kg bw. The resulting dose of hydroxytyrosol was estimated as 16, 36, and 72 mg/kg bw, respectively. Clinical observations, body weight, body weight changes or gross pathology did not reveal any adverse effects. No mortality was noted in any of the treatment groups. In another study by these investigators, oral administration of a single gavage dose of solid olive pulp extract at levels of 0, 1000, 1500 or 2000 mg/kg to Sprague Dawley rats (5/sex/group) did not produce any adverse effects except soft or liquid feces (Christian et al., 2004). The results of both these acute studies suggest the LD_{50} of olive pulp extract was greater than 2000 mg/kg (72 mg hydroxytyrosol/kg bw).

2.1.2.2. Subchronic Study

In a recent study 90-day toxicity study, Heilman et al. (2015) investigated safety of olive extract H35 containing 35% hydroxytyrosol following oral gavage study to Wistar rats. In this study H35 was administered at dose levels of 0, 345, 691 and 1381 mg/kg bw/day, equivalent to 0, 125, 250 and 500 mg hydroxytyrosol/kg bw/day. 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 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, sperm parameters or estrus cycle were noted. Reproductive parameters investigated, including estrous cycle assessment and sperm analysis did not result in the observation of any statistically significant changes between treated animals and control. 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) as 500 mg hydroxytyrosol/kg bw/day. The investigators reported that conservatively based solely on the reduction in body weight and body weight gain in the high dose males, it is concluded that the NOAEL of hydroxytyrosol is 250 mg/kg bw/day.

In a 90-day repeat dose toxicity study, Christian et al. (2004) investigated adverse effects of aqueous olive pulp extract. In this study, Sprague Dawley rats (20/group/sex) were orally (gavage) administered daily dose of aqueous olive pulp extract at levels of 0, 1000, 1500 and 2000 mg/kg bw/day (0, 60, 90 and 120 mg/kg bw/day of phenolics; 0, 36, 54 and 72 mg hydroxytyrosol/kg bw/day) for 90 days. Morbidity and mortality observations did not reveal any unusual findings. No treatment related biologically significant effects on body weights, body weight gains, feed consumption, or organ weights were noted. Except for some incidental findings, there were no adverse hematological, clinical chemistry, or gross necropsy effects. The incidental findings were not considered as treatment related by the authors. Focal, minimal or mild hyperplasia of the mucosal squamous epithelium of the limiting ridge of the forestomach occurred in some rats at 2000 mg/kg bw/day dose. This change was attributed to local irritation by repeated intubation of large volumes of the viscous, granular dosing suspension. The results of this study suggest a no-observed adverse effect level (NOAEL) of 2000 mg/kg bw/day, the highest dose administered. As aqueous olive pulp extract used in the study was reported to contain 6% phenolics of which 60% was reported as hydroxytyrosol, the corresponding NOAEL for hydroxytyrosol in rats will be 72 mg/kg bw/day (Christian et al., 2004).

2.1.2.3. Reproductive Toxicity

In a reproductive toxicity study in rats, Christian et al. (2004) evaluated the potential adverse effects of olive pulp extract containing 24 mg hydroxytyrosol/g of the extract. Sprague Dawley rats (8/sex/group) were administered once daily with the extract at a dose level of 0, 500, 1000, 1500 and 2000 mg/kg bw/day for 14 days before cohabitation. The equivalent dose of hydroxytyrosol for each group was 0, 12, 24, 36 and 48 mg/kg bw/day, respectively. The treatment was 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). All F_1 generation pups were weaned on day 21 post-partum. Two male and two female from the F_1 generation pups/litter 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 F_o generation 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 treatment. 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 treatment, 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 all the groups. The F_o generation female rats also did not reveal any unusual findings that can be related to treatment, except incidental observations of excess salivation. Estrous cycling, mating and reproductive performance of the female rats were not affected by the extract treatment. The results of this study suggest that aqueous olive pulp extract containing hydroxytyrosol as its major component is unlikely to be a reproductive toxicant.

2.1.3. Teratogenicity

In a teratogenicity study conducted as per the FDA Redbook guidelines, Christian et al. (2004) investigated potential embryo-fetal toxicity of aqueous olive pulp extract in Sprague-Dawley rats. In this study, time-mated female rats were gavaged from day 6 through 20 of gestation with the extract at a dose of 0, 1000, 1500 and 2000 mg/kg bw/day. The equivalent

dose of hydroxytyrosol for each group was 0, 24, 36 and 48 mg/kg bw/day, respectively. 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 noted. A significantly increased mean number of corpora lutea of the 2000 mg/kg dose was well within the historical range of 14.5-20.1 per litter and was attributed to two females that had 27 or 30 corpora lutea. The maternal and developmental NOAEL of the extract was determined as 2000 mg/kg bw/day (48 mg hydroxytyrosol/kg bw/day), the highest dose administered.

2.1.4. Genotoxicity studies

2.1.4.1. Ames Assay

Christian et al. (2004) investigated mutagenicity of olive pulp extract in a bacterial reverse mutation assay (Ames test). For this study, *Salmonella typhimurium* strains TA97, TA98, TA100 and TA1535 and *Escherichia coli* strain WP2 *uvr*A were used and the assay was conducted in the presence and absence of metabolic activation (S9). The extract containing hydroxytyrosol at levels of 24 mg/g was tested at concentrations of 0, 5, 10, 50, 100, 500, 1000, 2500 and 5000 μg/plate. Concentrations of 50, 100, 500, 1000 and 2500 μg/plate were used in the confirmatory preincubation test. At concentrations of 100 μg/plate or above of the extract, precipitates were observed and 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 for both the strains). No mutagenicity was noted at any of the concentrations tested in *E. coli*, 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 preincubation test, but only with metabolic activation. The results revealed some inconsistencies between the regular and repeat trials. The investigators noted that antibacterial properties of the test article, and observation of positive findings only at one or two concentrations, where precipitates and toxicity occurred, complicated the interpretation of the mutagenic findings. The authors concluded that under the conditions of the study, equivocal evidence of mutagenic activity of the extract was detected in *S. typhimurium* strains TA98 and TA100 (Christian et al., 2004).

2.1.4.2. *In vitro* Chromosomal Aberration

In another *in vitro* assay, Christian et al. (2004) investigated the effects of olive pulp extract on chromosome aberrations in Chinese hamster ovary cells, in the presence and absence of metabolic activation (S9). Following a standard protocol, the cell cultures were treated with 0, 10, 50, 100, 300, 600 and 1000 μg of the extract/ml as well as with positive and negative (vehicle, dimethyl sulfoxide) controls. The 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). No clear evidence of test article-associated 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 results of this study, Christian et al. (2004) concluded that the extract was positive for the induction of chromosome aberrations.

2.1.4.3. *In vivo* Micronucleus Assay

As the above described assays indicated some genotoxic potentials of olive pulp extract, Christian et al. (2004) further conducted a more affirmative assay of genotoxicity, i.e., *in vivo* micronucleus assay. In this study, adult Sprague Dawley male and female rats were administered 0, 1000, 1500, 2000 or 5000 mg/kg bw/day olive pulp extract via gavage for 28 days. The rats were euthanized on day 29 and bone marrow samples from the femur were collected for further analysis. In addition to this, experiments were also performed with single doses of the extract at 1000, 1500 or 2000 mg/kg. Following single administration, the rats were euthanized at 24 or 48 hours and bone marrow samples were collected. The extract did not produce adverse clinical or necropsy observations or affect absolute or relative feed consumption values. Compared to the control group, the numbers of micronucleated polymorphic erythrocytes were not significantly increased in any of the extract treated groups. Similarly, the ratio of polychromatic erythrocytes to normochromatic erythrocytes was not affected by the administration of the olive pulp extract. The results of this study suggest that the extract 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. These results also show that administration of hydroxytyrosol to rats at a dose level of 120 mg/kg bw/day for 28 days did not cause genotoxic effects as evaluated by micronucleus assay.

In a recent study, Kirkland et al. (2015) further investigated the potential genotoxic effects of hydroxytyrosol and olive extract containing hydroxytyrosol. 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. Another extract (H40) containing 40% hydroxytyrosol also induced micronuclei *in vitro*, probably via the same mechanism. However, both extracts were negative in robust Ames tests. The 15% hydroxytyrosol formulated extract did not induce micronuclei in rat bone marrow after 4 weeks of dosing up to 561 mg hydroxytyrosol/kg/day. H40 produced increased rat bone marrow micronucleus frequencies at 250 and 500 mg hydroxytyrosol/kg bw/day in a 90-day toxicity study. 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 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.

In yet another recent study, Dolan et al. (2014) studied the potential clastogenic effects of pure hydroxytyrosol in a bone marrow chromosome aberration study in rats. 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 five males and five females. The oral limit dose of 2000 mg/kg (bw) was evaluated. 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. The

oral limit dose of 2000 mg/kg hydroxytyrosol 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/kg hydroxytyrosol does not induce chromosome aberrations in bone marrow cells of the rat. This suggest that hydroxytyrosol is not a clastogen *in vivo*.

2.2. Human Studies

Polyphenols, including hydroxytyrosol, as components of olive oil or olive leaf extract has been investigated for their potential benefits in multiple clinical studies. The clinical evidence related to hydroxytyrosol efficacy or safety is primarily based on the human studies with olive oil. In an extensive review article, Raederstorff (2009) summarized human studies of olive polyphenols. 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. These studies, along with some other human trials of olive phenolics, are summarized in Table 5. The data from these studies indicate that a dietary intake of approximately 10 mg olive phenols/day may show antioxidant effects on low-density lipoprotein oxidation.

Table 5. Summary of human studies of olive phenolics*

Reference	Test component/ daily dose	Study type	Duration on Intake	Subjects	Primary Outcome
Crespo et al. 2015	Hydroxytyrosol (olive mill water enriched) Placebo; 5 mg; 25 mg	Double-blind, randomized, placebo-controlled	Daily for 7 days	21 healthy control-n=6; 5 mg- n=7 25 mg-n=8	Biochemical parameters including safety parameters did not show any adverse effects. Hydroxytyrosol was well tolerated. No adverse effects reported.
Visioli et al., 2000	Olive oil + olive phenolic extract (24 mg); Olive oil + olive phenolic extract (49 mg); Olive oil + olive phenolic extract (73 mg); Olive oil + olive phenolic extract (97 mg)	Cross-over	Single dose	6 healthy male volunteers	Polyphenolic rich oils dose-dependently decreased urinary isoprostane excretion, a biomarker of *in vivo* lipid peroxidation processes
Vissers et al., 2001	Refined olive oil content (0 mg phenolic) Virgin olive oil (21 mg phenolic)	Randomized, cross-over, controlled	3 weeks	46 healthy volunteers	*Ex vivo* resistance of LDL and HDL to oxidation as well as markers of lipid peroxidation were not affected by treatments.
Oubina et al., 2001	High oleic sunflower oil Virgin olive oil (Diet study no fixed amounts of oils)	Cross-over	4 weeks	14 women	Total lipid peroxides in serum and TXB2 concentrations in platelet-rich plasma were significantly lower in the

Table 5. Summary of human studies of olive phenolics*

Reference	Test component/ daily dose	Study type	Duration on Intake	Subjects	Primary Outcome
					virgin olive oil group as compared to the sunflower oil group
Moschandreas et al., 2002	Olive oil low phenolic content (3 mg) Olive oil high phenolic content (21 mg)	Randomized, cross-over	3 weeks	25 smokers (11 males and 14 females)	No change in markers of plasma antioxidant capacity (MDA, FRAP, lipid hydroperoxides) in smokers.
Marrugat et al., 2004	Olive oil low phenolic content (0 mg) Olive oil medium phenolic content (2 mg) Olive oil high phenolic content (4 mg)	Randomized, cross-over, controlled, double-blind	3 weeks	30 healthy volunteers	Olive polyphenols dose-dependently decreased *in vivo* plasma oxidized LDL and increased *ex vivo* resistance of LDL to oxidation, HDL cholesterol levels and urinary hydroxytyrosol levels.
Weinbrenner et al., 2004	Olive oil low phenolic content (0 mg) Olive oil medium phenolic content (3 mg) Olive oil high phenolic content (12 mg)	Randomized, cross-over, controlled double-blind	4 days	12 healthy male volunteers	Consumption of olive oil rich in polyphenols decreased plasma oxidized LDL (oxLDL), 8-oxo-dG in mitochondrial DNA, and malondialdehyde in urine and increased HDL cholesterol and glutathione peroxidase activity in a dose-dependent manner, related to the phenolic content of the olive oil administered.
Visioli et al., 2005	Refined olive oil (Total HT (free + esterified) 0 mg) Virgin olive oil (Total HT 7 mg)	Randomized, cross-over, controlled	7 weeks	21 mildly hyperlipidemic subjects	Serum TXB2 production decreased and total antioxidant capacity increased after phenolic rich oil intake.
Ruano et al., 2005	Olive oil low phenolic content (3 mg) Olive oil high phenolic content (16 mg)	Randomized, cross-over	Single dose	21 hypercholesterolemic volunteers	Consumption of a meal based on olive oil rich in polyphenolic compounds improved endothelial-dependent vasodilatory response, decreased oxidative stress (lipoperoxides and isoprostanes) and increased the final products of nitric oxide
Fito et al., 2005	Olive oil low phenolic content (1 mg) Olive oil high	Randomized, cross-over, controlled	3 weeks	40 males with stable coronary	Consumption of virgin olive oil rich in polyphenolics decreased *in vivo* oxidized LDL and lipid peroxide

Table 5. Summary of human studies of olive phenolics*

Reference	Test component/ daily dose	Study type	Duration on Intake	Subjects	Primary Outcome
	phenolic content (8 mg)			heart disease	plasma levels and increased glutathione peroxidase activity as compared to refined olive oil consumption. A decrease in systolic blood pressure was also observed with the high phenolic content product.
Leger et al., 2005	Olive phenolic concentrate (first day 25 mg HT and then 12.5 mg HT)	Open study	4 days	5 males with type I diabetes	The olive polyphenolic concentrate had no effect on urine isoprostane excretion but significantly decreased serum thromboxane B2 (TXB2) levels.
Covas et al., 2006a	Olive oil low phenolic content (0 mg) Olive oil medium phenolic content (4 mg) Olive oil high phenolic content (9 mg)	Randomized, cross-over, controlled, double-blind	3 weeks	200 healthy male volunteers	Plasma oxidative stress markers (conjugated dienes, hydroxyl fatty acid, oxidized LDL) and total cholesterol to HDL cholesterol ratio decreased linearly with increasing phenolic content in olive oil
Covas et al., 2006b	Olive oil low phenolic content (0 mg) Olive oil medium phenolic content (6 mg) Olive oil high phenolic content (15 mg)	Randomized, cross-over, controlled, double-blind	Single dose	12 healthy male volunteers	Olive polyphenols dose-dependently decreased *in vivo* oxidized LDL in the postprandial site
Salvini et al., 2006	Olive oil low phenolic content (7 mg) Olive oil high phenolic content (30 mg)	Randomized, cross-over, double-blind	8 weeks	10 post-menopausal women	The high polyphenolic olive oil lowered oxidized DNA damage measured by the comet assay
Gimeno et al., 2007	Olive oil low phenolic content (0 mg) Olive oil medium phenolic content (9 mg) Olive oil high phenolic content (20 mg)	Randomized, cross over, controlled, double-blind	3 weeks	30 healthy volunteers	Olive polyphenols dose dependently decreased *in vivo* oxidized LDL and increased resistance of LDL to oxidation and high-density lipoprotein (HDL) cholesterol
Machowetz et al., 2007	Olive oil low phenolic content (0 mg) Olive oil medium phenolic content (4	Randomized, cross-over, controlled	3 weeks	200 healthy male volunteers	The phenolic content of the oil had no effect on DNA and RNA oxidation (8-oxo-deoxyguanosine, 8-oxo-guanosine)

Table 5. Summary of human studies of olive phenolics*

Reference	Test component/ daily dose	Study type	Duration on Intake	Subjects	Primary Outcome
	mg) Olive oil high phenolic content (9 mg)				

*Adapted from Raederstorff, 2009 and other studies

In a recent study, 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 men. In this double-blinded, placebo-controlled, crossover trial, 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 leaf polyphenols 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 was a flare up of acne. The 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.

Bitler et al. (2007) conducted a double-blind, randomized, placebo-controlled trial to investigate the effects of a polyphenolic-rich olive extract (freeze-dried olive vegetation water) on a series of parameters in male and female subjects (n=105; age 55-75 years) with osteoarthritis and rheumatoid arthritis. The subjects in the treatment group (n=51) received 400 mg of the freeze-dried extract/day for 8 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 tests. The rheumatoid arthritis subjects in the extract treatment group showed significant decreases in serum homocysteine levels after 8 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. These observations support safety of the supplement. Overall, the participants tolerated placebo and supplement well, with only 2 participants, one from each group (placebo and supplement), complaining 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 publications, given the affiliation of the authors of this study, the extract used in this study appears to be the subject of earlier described Christian et al. (2004) safety studies and the resulting intake of hydroxytyrosol appear to be approximately 10 mg/person/day.

In a crossover study, 200 healthy male volunteers (20 to 60 years old) were randomly assigned to three sequences of daily administration of 25 mL of three olive oils with low (2.7 mg/kg of olive oil), medium (164 mg/kg), or high (366 mg/kg) phenolic content but were otherwise similar. Intervention periods were three weeks preceded by two week washout periods (Covas et al., 2006a). A linear increase in HDL cholesterol levels and decrease in total cholesterol was observed for low-, medium-, and high-polyphenol olive oil. Of the 200 participants, 18 (9%) did not complete the study. The dropout rates were 8.9%, 7.4%, and 10.6% in sequences 1, 2, and 3, respectively. None of the adverse effects were related to the olive oil intake. In another randomized, double-blind, crossover trial by the same investigators (Covas et al., 2006b), 12 healthy male volunteers were given 40 mL of similar olive oils, but with high (366 mg/kg), moderate (164 mg/kg), and low (2.7 mg/kg) phenolic content. During the washout period the subjects followed a strict phenolic compound-low diet. Tyrosol and hydroxytyrosol were dose-dependently absorbed. Plasma concentrations of tyrosol, hydroxytyrosol, and 3-O-methyl-hydroxytyrosol directly correlated with changes in the total phenolic compounds content of the LDL after the high phenolic compounds content olive oil ingestion. The investigators concluded that olive oil phenolic content modulates the LDL phenolic content and the postprandial oxidative stress promoted by 40 mL olive oil ingestion in human.

Marrugat et al. (2004) investigated the effects of olive oils, with differences in their phenolic content, on *in vivo* LDL oxidation and urinary tyrosol and hydroxytyrosol levels. In this double-blind, randomized, crossover clinical trial, 30 healthy Spanish non-smoking males were divided into three groups. Olive oils were administered over three periods of three weeks preceded by a two-week washout periods. The study subjects were given three similar olive oils (refined, common, and virgin olive) with increasing phenolic concentration (from 0 to 150 mg/kg). The phenolic compounds were undetectable in refined virgin olive oil. Common olive oil (a mixture of refined and virgin olive oil) contained 68 mg/kg of phenols of which 2% was tyrosol, 9% was hydroxytyrosol, 52% were oleuropein aglycones, and 15% were ligstroside aglycones. Virgin olive oil contained 150 mg/kg of phenols of which 3% was tyrosol, 7% was hydroxytyrosol, 42% were oleuropein aglycones, and 14% were ligstroside aglycones. The results of this study showed increased urinary tyrosol and hydroxytyrosol, decreased *in vivo* plasma oxidized LDL, and increased *ex vivo* resistance of LDL to oxidation with the phenolic content of the olive oil administered. No adverse effects were reported.

2.3. Evaluation by EFSA

The European Foods Safety Authority (EFSA, 2011) has issued a scientific opinion on health claims in relation to dietary consumption 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 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.

2.1. Absorption, Distribution, Metabolism and Excretion

The bioavailability of polyphenolic compounds, including hydroxytyrosol, from olive oil has been extensively reviewed and summarized in a recent EFSA (2011) publication on olive oil health claims. These studies show that the absorption of olive oil phenolics is probably larger than 55-66 mol%, and that the absorption of hydroxytyrosol is dose-dependent, suggesting that olive oil phenolics are absorbed from the intestine, that tyrosol and hydroxytyrosol are incorporated in lipoprotein fractions, and that hydroxytyrosol is excreted in urine as a glucuronide conjugate (Bonanome et al., 2000; de la Torre-Carbot et al., 2010; Edgecombe et al., 2000; Miro-Casas et al., 2003; Visioli et al., 2000, 2001; Vissers et al., 2002). An increase in the dose of phenolics administered increased the proportion of conjugation with glucuronide. The total amount of hydroxytyrosol excreted ranged from 30-60% (Visioli et al., 2000). The available studies indicate that orally administered hydroxytyrosol can be absorbed both in rats (Bai et al., 1998) and in humans (Visioli et al., 2000),

In a pharmacokinetic study, Bai et al. (1998) investigated levels of hydroxytyrosol in rat plasma following administration of pure and chemically synthesized hydroxytyrosol. Following oral administration to rats, hydroxytyrosol rapidly appeared in the blood, with maximal levels in 5-10 minutes and within 180 minutes it was almost completely eliminated and/or metabolized. As compared to the dose administered, hydroxytyrosol levels in plasma/blood were low and greatly fluctuated. In another study, Christian et al. (2004) investigated changes in blood plasma levels of hydroxytyrosol in rats following oral administration of an olive extract product containing hydroxytyrosol to Sprague Dawley rats at dose levels of 24, 36 and 48 mg hydroxytyrosol/kg bw/day for 90 days. Blood samples collected on day 90, prior to dosing did not reveal the presence of hydroxytyrosol suggesting minimal carry-over of hydroxytyrosol from prior daily doses. Blood samples collected at 0.5, 1, 2, 4 and 8 hours post-dose revealed rapid absorption of hydroxytyrosol with mean concentrations measurable through 1 to 4 hours at the dose levels of 24 and 36 mg/kg bw and through 8 hours at 48 mg/kg bw dose levels. These studies suggest a rapid absorption and excretion of hydroxytyrosol. In another study, based on the observations from a single dose administration of phenolic extract from olive cake to Wistar rats, Serra et al. (2011) concluded that olive oil phenolic compounds were absorbed, metabolized and distributed through the blood stream to practically all parts of the body, even across the blood-brain barrier. The Cmax of hydroxytyrosol in plasma (2 h), kidney (4 h) and testicles (2 h) was reported as 5.2, 3.8, 2.7 nmol/g, respectively.

Visioli et al. (2000) investigated the absorption of olive oil phenolics in humans. In this study, 6 male volunteers (ages 27-33) were given 50 ml of four olive oil samples spiked with hydroxytyrosol, and the first 24 hours urine was analyzed. The levels of total phenol, hydroxytyrosol and tyrosol in the four oils were 488/20/36, 975/44/72, 1463/66/110 and 1950/84/140 ppm, respectively. The urinary excretion of tyrosol and hydroxytyrosol for the four individual oils was 21/29, 28/64, 21/35 and 24/40 (% of the administered dose). The investigators reported that the ratio of tyrosol/hydroxytyrosol found in urine was similar to that present in the oil (~1.7). The proportions of total tyrosol and hydroxytyrosol excreted were in the range of 20-22% for tyrosol and 30-60% for hydroxytyrosol. The results of this study suggest that simple olive oil phenols such as tyrosol and hydroxytyrosol are absorbed after administration and are excreted as glucuronide conjugates.

In another study, Tuck et al. (2001) investigated the bioavailability of radiolabeled hydroxytyrosol and tyrosol, in Sprague Dawley male rats following intravenous (in saline) and oral (in oil- and water-based solutions) administration. The oil-based dosing resulted in

significantly greater elimination of the phenolics in urine within 24 hours compared to the oral aqueous dosing method. There was no significant difference in the amount eliminated in urine between the intravenous and the oral oil-based dosing methods for both tyrosol and hydroxytyrosol. The presence of hydroxytyrosol and five metabolites was noted in urine samples. The results of this study revealed the oral bioavailability of hydroxytyrosol in olive oil and aqueous solution as 99 and 75%, respectively, and for tyrosol as 98 and 71%, respectively.

In a review article, de la Torre (2008) reported that the main sources of hydroxytyrosol are oleuropein and its glycoside. Hydroxytyrosol is well absorbed in the gastrointestinal tract but its bioavailability is poor because of an important first pass metabolism both in gut and liver, leading to the formation of sulphate and glucuronide conjugates, to the extent that concentrations in body fluids of its free form are almost undetectable. In a recent study, Rodríguez-Morató at al. (2015) reported that despite its good absorption, hydroxytyrosol bioavailability is poor due to an extensive first pass metabolism. Before entering the portal blood stream, it appears to undergo phase I/II metabolism in the enterocytes, and after having reached the liver through portal circulation, it is subject of additional phase II metabolism. The enzymes implicated in hydroxytyrosol phase II metabolism are uridine 5'-diphosphoglucuronosyl transferases, catecholmethyltransferase, and sulfotransferases. In yet another review article, Perez-Jimenez et al. (2010) assessed the usefulness of polyphenol metabolites excreted in urine as biomarkers of polyphenol intake in humans. For this assessment, 162 controlled intervention studies with polyphenols were reviewed, and mean recovery yield and correlations with the dose ingested were determined for 40 polyphenols, including hydroxytyrosol. Hydroxytyrosol showed both a high recovery yield and a high correlation with the dose indicating its value as biomarkers of intake.

Suarez et al. (2011) evaluated the concentration of phenolic compounds and their metabolites in human plasma (0, 60, 120, 240 and 300 min) from thirteen healthy volunteers (seven men and six women, aged 25 and 69 years) following ingestion of a single dose (30 ml) of either enriched (phenolics) virgin olive oil (961·17 mg/kg oil) or control virgin olive oil (288·89 mg/kg oil). In this cross over study, the levels hydroxytyrosol in control and enriched oils were 0.37 and 6.64 mg/kg oil, while that of tyrosol in these oils were 1.03 and 8.70 mg/kg oil. Compared with virgin olive oil, the enriched oil increased plasma concentration of the phenol metabolites, particularly hydroxytyrosol sulphate and vanillin sulphate. After the consumption of virgin olive oil, the maximum concentration of these metabolite peaks was reached at 60 minutes, while enriched virgin olive oil shifted this maximum to 120 minutes. The wide variability of results indicates that the absorption and metabolism of olive oil phenols are dependent on the individual.

Based on the findings from an intravenous study in rats, D'Angelo et al. (2001) proposed a metabolic pathway for exogenously administered hydroxytyrosol that involves catechol-*o*-methyltransferase, alcohol dehydrogenase, aldehyde dehydrogenase and phenolsulfotransferase. Based on a human study, Caruso et al. (2001) suggested that hydroxytyrosol was metabolized by the enzyme catechol-*o*-methyl transferase resulting in an enhanced excretion of homovanillyl alcohol. Additionally, an increase in homovanillic acid was also noted, indicating oxidation of the ethanolic residue of hydroxytyrosol and/or of homovanillyl alcohol in humans. An increase in hydroxytyrosol in 24-hour urine was noted following both single-dose ingestion (50 ml) and short-term consumption (25 ml/day for a week) of virgin olive oil by seven healthy subjects. Miro-Casas et al. (2003) also reported increases in plasma hydroxytyrosol and 3-*o*-methyl-

hydroxytyrosol following ingestion of virgin olive oil (25 ml) by humans, reaching maximum concentrations at 32 and 53 min, respectively. The estimated hydroxytyrosol elimination half-life was 2.43 hours, while the Cmax was reported as 26 μg/L. Based on the results of this study, approximately 98% of hydroxytyrosol appears to be present in plasma and urine in conjugated forms, mainly glucuronides, suggesting extensive first-pass intestinal/hepatic metabolism of the ingested hydroxytyrosol.

The available studies from animals and humans reveal some differences in the elimination of hydroxytyrosol. The differences noted in human and animal studies as regards the elimination of hydroxytyrosol and tyrosol indicate that these phenolics may be handled differently in humans and rats or may be related to the analytical methods used (Visioli et al., 2000; Tuck et al., 2001). It is noted that as Tuck et al. (2001) employed a more accurate method the presence of numerous labeled conjugates of hydroxytyrosol and tyrosol could have been detected, not just those hydrolyzed from the parent compound in β-glucuronidase-hydrolyzed urine. Based on observations from rat and human investigations, Visioli et al. (2003) suggested that caution should be used in the interpretation of data obtained in rats as the *in vivo* model of absorption and excretion of hydroxytyrosol and related compounds. In rats a high basal excretion of hydroxytyrosol and of its main metabolites was noted, and when given extra virgin olive oil, they appeared to absorb and/or excrete hydroxytyrosol less than do humans. These differences might be due to the absence of a gall bladder in rats, which results in the presentation of lipid-soluble or amphiphilic molecules such as hydroxytyrosol to the intestinal flora.

In a recent study in human subjects, Crespo et al. (2015) tested the effects of hydroxytyrosol on Phase II enzymes' expression. In this double-blind, randomized, placebo-controlled study, effects of two hydroxytyrosol doses, i.e. 5 and 25 mg/day, vs. placebo were tested following a Latin square design. In this study, Hytolive®, an olive mill wastewater extract selectively enriched in hydroxytyrosol, i.e. devoid of oleuropein or other hydroxytyrosol-containing secoiridoids was used. Hydroxytyrosol was well tolerated without any significant alterations in Phase II enzyme expression in peripheral blood mononuclear cells. Additionally, no significant effects on a variety of surrogate markers of cardiovascular disease such as lipid profile and inflammation and oxidation markers were recorded. The investigators indicated that the "hormesis hypothesis" that (poly)phenols activate Phase II enzymes requires solid human confirmation that might be provided by future trials.

In summary, the above described information from the bioavailability studies with olive oil and hydroxytyrosol suggest that hydroxytyrosol is rapidly absorbed from blood, distributed in tissues, metabolized and rapidly eliminated primarily in the urine as glucuronide conjugates. The absorption of hydroxytyrosol differs depending on the vehicle in which it is administered. The absorption, and excretion of hydroxytyrosol and its metabolites in urine differed between rats and humans. The available studies indicate that, as compared to humans, the absorption and elimination of hydroxytyrosol is lower in rats. The bioavailability of olive phenolics is poor in humans, and they are found in biological fluids mainly as conjugated metabolites. Oleuropein, which is also present in olive oil, can be absorbed and hydrolyzed to hydroxytyrosol.

2.2. Extrapolation of Animal Observations to Human

One of the major current issues in toxicology research is one cannot -obviously - use humans to test the noxious effects of drugs and dietary or food supplements. Therefore, one must rely on rodents at least for the first screening. This is often frustrated by the fact that, sometimes

the data obtained from animal studies are not easily extrapolated to data obtained via human studies[4]. In the case of hydroxytyrosol, rats do produce hydroxytyrosol in their body, while humans also produce it but to a lesser extent. This is reflected by the high basal excretion of hydroxytyrosol and one of its main metabolites, i.e. homovanillyl alcohol (Visioli et al., 2003). Also, rats metabolize hydroxytyrosol differently as compared to humans. This is obvious as the urinary excretion of hydroxytyrosol after intake is much higher in humans than in rats. A speculative interpretation of the different metabolic pathways and different excretion of hydroxytyrosol in rats as compared to humans might be based on the lack of gall bladder in the rat, which will result in a metabolic diversion of lipid-soluble or amphiphilic molecules (such as hydroxytyrosol) to the intestinal flora.

Given the above observations, at present there is no published study that shows any evident sign of hydroxytyrosol toxicity when administered to rats, even in high doses. Regrettably, human studies of any nutraceutical rarely take toxicity, i.e. alteration of liver enzymes into account, based on the premise that "if it is natural it is safe". One exception is the Crespo et al. (2015) study where administration of up to 25 mg/day for one week to human healthy volunteers did not modify GOT (glutamyl oxaloacetic transaminase); GPT (glutamic-pyruvatetransaminase); GGT (gamma-glutamyltransferase); or total bilirubin, indicating safety.

Unfortunately, only one human study of pure hydroxytyrosol is available in the literature (González-Santiago et al., 2010), which addressed absorption and disposition and did not inform on biochemical data, including safety. The issue of whether hydroxytyrosol pharmacokinetics/pharmacodynamics, safety, and activity when given as pure compound vs. purified mixtures or extra virgin olive oil remains unresolved in humans. However, the available human studies along with investigations in rat at high doses supports the safety of hydroxytyrosol at intended use levels in foods is safe.

2.3. Biological Effects

It has been suggested that the beneficial effects of olive oil in lowering the incidence of degenerative pathologies could be ascribed to the antioxidant properties of its polyphenols (Soni et al., 2006; Visioli and Bernardini, 2011). Hydroxytyrosol has been shown to prevent *in vitro* LDL oxidation, inhibit platelet aggregation, inhibit 5- and 12-lipooxygenases, effectively counteract the cytotoxic effects of reactive oxygen species in various human cellular systems and, act as a free radical scavenger. Hydroxytyrosol has been also shown to exert an antiproliferative effect, inducing apoptosis in HL-60 cells and in resting and activated peripheral blood lymphocytes. The research involving olive phenols and health, as related to the cardiovascular system, and over 15 human clinical studies with virgin olive oil, indicate the superiority of phenol-rich olive oil to other vegetable oils or sources of fat (Visioli and Bernardini, 2011). The available evidence also suggest that olive oil phenolic compounds accumulate in plasma and urine following olive oil consumption, and the amount of phenolic compounds ingested with the olive oil appear to modulate the oxidative/antioxidative status in the human body (Weinbrenner et al., 2004).

Based on findings from *in vitro* studies, Sabatini (2010) also reported that hydroxytyrosol scavenges free radicals, inhibits human low-density lipoprotein oxidation (a process involved in

[4] Concordance of the Toxicity of Pharmaceuticals in Humans and in Animals. Available at: http://www.gwern.net/docs/dnb/2000-olson.pdf

the pathogenesis of atherosclerosis), inhibits platelet aggregation and acts as an anticancer agent by means of pro-apoptotic mechanisms. Additionally, *in vitro* studies show that hydroxytyrosol acts against both Gram-positive and Gram-negative bacteria, which are involved in many infections of respiratory and intestinal tracts. Based on a critical review of the published studies, Raederstorff (2009) reported that the potent antioxidant activity of olive polyphenols is supported by *in vitro* and animal studies. Approximately 50% of the phenolic compounds contained in olives and virgin olive oil are hydroxytyrosol and derivatives thereof. Human intervention studies suggest that olive polyphenols decreases the levels of oxidized-LDL in plasma and positively affects several biomarkers of oxidative damage. Some of these studies are summarized in Table 5. The antioxidant effects of olive phenols on low-density lipoprotein oxidation can be found at a daily intake of approximately 10 mg of olive phenols.

3. SUMMARY AND DISCUSSION

In recent years, hydroxytyrosol naturally found in olives and its products has gained considerable attention because of its potential health benefits. Given the presence of hydroxytyrosol in olive oil and olives that are commonly consumed, humans are commonly exposed to this ingredient. Seprox Biotech S.L. intends to use hydroxytyrosol (> 99% pure) as an antioxidant or antimicrobial in selected food products. The product is a slightly yellow viscous liquid with a pungent odor and bitter taste. The proposed food categories for the use of hydroxytyrosol at levels up to 5 mg *per* serving are beverages (non-alcoholic), fats and oils, fresh and processed fruits/vegetables and juices, and gravy and sauces. The estimated intake of hydroxytyrosol from its natural presence in table olives has been estimated to range from 20-40 mg/day. In Mediterranean countries, where olives in the form of table olives and olive oil are routinely consumed, the intake of hydroxytyrosol is expected to be higher. The intended use of hydroxytyrosol in the specified food categories will result in mean and high (90^{th} percentile) estimated daily intakes of 25.53 and 51.06 mg/person/day (0.42 and 0.85 mg/kg bw/day for an individual weighing 60 kg).

There is sufficient qualitative and quantitative scientific as well as common dietary exposure evidence to determine the safety-in-use of hydroxytyrosol in the above mentioned food applications. Polyphenolics from olive oil, olive preparations and table olives are considered as the constituents of biological significance. Among the polyphenolics, hydroxytyrosol is the major active constituent; hence, studies related to polyphenolics are also important in determining the safe use of hydroxytyrosol. The safety data on hydroxytyrosol, olive oil, and olive extracts includes several animal toxicity studies in rats, genotoxicity studies, reproduction/developmental studies in rats and human experience. Additionally, the history of consumption of olive oil and table olives provides evidence of safe uses of its constituents, including hydroxytyrosol.

In pharmacokinetic studies in animals and human subjects, urinary excretion of hydroxytyrosol and its glucuronide was found to be closely associated (qualitatively) with the oral hydroxytyrosol intake. Following absorption, hydroxytyrosol is incorporated in lipoprotein fractions and is excreted in urine as a glucuronide conjugate. Absorption of hydroxytyrosol when given in extra virgin olive oil was higher in humans as compared to rats. The estimated elimination half-life of hydroxytyrosol was reported as 2.43 hours. The majority of the hydroxytyrosol found in plasma and urine is in conjugated forms, mainly glucuronides, suggesting extensive first-pass intestinal/hepatic metabolism of the ingested hydroxytyrosol.

Hydroxytyrosol is excreted in urine as the unchanged parent compound, in the form of metabolites or as glucuronide and sulfate conjugates.

Christian et al. (2004) investigated the acute toxicity, subchronic toxicity, genotoxicity, reproductive toxicity and teratogenicity of aqueous olive pulp extract. These studies with aqueous olive pulp extract are applicable to the present GRAS assessment as the product used in these studies contains hydroxytyrosol as an active ingredient. In another similar subchronic study, no toxicity of aqueous pulp extract was noted at doses up to 2000 mg/kg bw/day (72 mg hydroxytyrosol/kg bw/day). In a developmental toxicity and a reproductive study in rats, olive pulp extract did not cause maternal or developmental toxicity or reproductive effects at levels up to 2000 mg/kg bw/day (highest dose tested). Although the results of *in vitro* mutagenicity studies with hydroxytyrosol and aqueous olive pulp extract were equivocal, *in vivo* study in rats with the olive pulp extract did not reveal any genotoxic potentials.

The result of an acute oral toxicity study indicates that the oral LD_{50} of hydroxytyrosol is greater than 2000 mg/kg. In a recent 90-day dose-response study, safety of olive extract H35 containing 35% hydroxytyrosol revealed statistically significant reductions in body weight gain (9%) and decreased absolute body weight (17%) in males was noted. No other adverse effects were noted. Based on these observations, the investigators determined the lowest observed adverse effect level (LOAEL) as 500 mg hydroxytyrosol/kg bw/day. The NOAEL of hydroxytyrosol was determined as 250 mg/kg bw/day. In a series of well designed, specific safety studies, the potential subchronic toxicity and genotoxicity of hydroxytyrosol, the subject of this GRAS assessment, was investigated. In the subchronic study in rats, the gavage administration of hydroxytyrosol (the subject of this GRAS determination) to rats at dose level of 5, 50 and 500 mg/kg bw/day for 90 days did not show significant toxic effects. In this study although no changes in absolute organ weight were noted, some changes in relative organs weights as determined based on organ to body weight or organ to brain weight were noted. Conservatively based on these changes a low-observed adverse effect level (LOAEL) for this study is considered as 500 mg/kg bw/day. In several human efficacy studies, effects of hydroxytyrosol from ingestion of olive oil were investigated. The results of human studies with olive oil containing phenolics, including hydroxytyrosol, did not reveal any adverse effects. Based on the findings from above described toxicity studies, the NOAEL of hydroxytyrosol can be considered as 250 mg/kg bw/day.

The resulting maximum (90^{th} percentile) intake of hydroxytyrosol from the proposed food uses is estimated as 0.85 mg/kg bw/day. The subchronic toxicity study of hydroxytyrosol suggests a NOAEL of 250 mg/kg bw/day. Additionally, the available studies with aqueous olive pulp extract in rats suggest a NOAEL of 72 mg /kg bw/day for hydroxytyrosol. Based on the results of the subchronic toxicity study there is a safety margin of 294-fold between the estimated daily intake of hydroxytyrosol and the safe dose noted in the animal study. Additionally, the human experience with olive oil and table olive consumption also supports the safety of hydroxytyrosol. The available evidence from animal studies, as well as evidence from human dietary exposure to table olives and olive oil suggests that a daily intake of hydroxytyrosol at levels up to 51.06 mg/day is unlikely to cause any adverse effects.

In summary, on the basis of scientific procedures[5], history of exposure and use, consumption of hydroxytyrosol as an antioxidant or antimicrobial agent at use levels of up to 5 mg/serving in certain specified foods is considered safe. The proposed uses are compatible with current regulations, *i.e*., hydroxytyrosol as an antioxidant [21 CFR 170.3(o)(3)] or antimicrobial agent [21 CFR 170.3(o)(3)] in beverages (non-alcoholic), fats and oils, fresh and processed fruits/vegetables and juices, and gravy and sauces when not otherwise precluded by a Standard of Identity, and is produced as described in this document.

[5] 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.

4. 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 hydroxytyrosol, meeting the specifications cited herein, and when used as an antioxidant [21 CFR 170.3(o)(3)] or antimicrobial agent [21 CFR 170.3(o)(3)] in specific foods such as beverages (non-alcoholic), fats and oils, fresh and processed fruits/vegetables and juices, and gravy and sauces at use levels up to 5.00 mg/serving (reference amounts customarily consumed, 21 CFR 101.12) when not otherwise precluded by a Standard of Identity as described in this monograph and resulting in the 90th percentile all-user estimated intake of 51.06 mg hydroxytyrosol/person/day is safe and GRAS.

It is also our opinion that other qualified and competent scientists reviewing the same publicly available toxicological and safety information would reach the same conclusion. Therefore, we have also concluded that hydroxytyrosol, when used as described, is GRAS based on scientific procedures.

Signatures

(b) (6)

Robert L. Martin, Ph.D.

Sept. 10, 2015
Date

(b) (6)

Francesco Visioli, Ph.D.

Sept 4, 2015
Date

(b) (6)

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

Sept. 14, 2015
Date

5. CONCLUSION

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6. APPENDIX I

Analytical data from non-consecutive manufacturing lots of hydroxytyrosol*

Batch Number		E012 182016	E012 030521	E012 16222524	E012 161726	E012 12131829	E012 071634	Average	Std Dev.	ppb per 5 mg of HT ingested	Legal limits in foods mg/Kg (ppm)
Manufacturing date	**dd/mm/yy**	**28/02/12**	**20/03/12**	**11/04/12**	**23/04/12**	**07/05/12**	**14/06/12**				
Chromatographic purity	**HPLC-280 nm**	99.51	99.42	99.43	99.41	99.43	99.34	**99.42**	0.05		
Hydroxytyrosyl acetate	**HPLC-280 nm**	0.18	0.20	0.25	0.13	0.10	0.26	**0.19**	0.06		
Hydroxytyrosol content	**Calc.****	97.67	95.51	95.45	95.90	95.17	95.41	**95.85**	0.92		
Moisture %	**%**	1.56	3.88	3.95	3.71	4.07	3.87	**3.50**	0.96		
Acetic acid	**%**	0.32	0.30	0.25	0.11	0.41	0.28	**0.28**	0.10	<0.01	
Metals											
Boron	**ppm**	n.a.	12.7	4.3	6.5	5.0	8.7	**7.44**	3.39	0.04	
Sodium	**ppm**	413.0	376.8	378.4	491.5	514.0	425.1	**433.13**	57.59	2.16	n.a.
Potasium	**ppm**	811.6	175.0	202.3	168.3	753.1	480.0	**431.72**	295.92	2.16	n.a.
Calcium	**ppm**	77.5	65.3	139.4	58.5	173.4	202.6	**119.45**	61.04	0.60	n.a.
Cadmium	**ppm**	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	**-**	-	-	0.05-3.00[a)]
Chromium	**ppm**	0.02	0.16	0.07	0.13	0.04	<0.01	**0.08**	0.06	<0.01	0.25[e)]
Iron	**ppm**	1.07	4.1	0.91	3.3	0.81	2.09	**2.05**	1.38	0.01	n.a.[d)]
Mercury	**ppm**	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	**-**	-		0.10-1.00[a)]
Nickel	**ppm**	0.08	0.08	0.02	0.03	0.02	0.02	**0.04**	0.03	<0.01	0.08-0.900[c)]
Lead	**ppm**	0.03	0.03	0.02	0.02	<0.01	<0.01	**0.03**	0.01	<0.01	0.02-3.00[a)]
Anions											
Chloride	**ppm**	955.3	344.3	161.0	441.8	820.5	325.9	**508.13**	310.64	2.54	n.a.
Nitrate	**ppm**	12.9	22.7	1.2	24.0	1.4	1.8	**10.7**	10.8	0.05	200-4500[a)]
Nitrite	**ppm**	7.6	<1.0	0.14	<1.0	0.25	0.2	**2.05**	3.70	0.01	50-250[b)]
Sulphate	**ppm**	431.6	107.9	141.6	169.8	273.2	192.0	**219.35**	117.99	1.10	n.a.
TOTAL INORGANICS	**ppm**	2710.7	1109.07	1029.36	1363.88	2541.72	1638.41	**1732.19**	726.57	8.66	
	%	0.27	0.11	0.1	0.14	0.25	0.17	**0.17**	0.07	<0.01	
Acetic acid	**%**	0.32	0.30	0.25	0.11	0.41	0.28	**0.28**	0.10	<0.01	
Residual organic solvents											

Ethyl acetate	**ppm**	22.65	7.51	17.71	8.09	0.91	0.91	**9.63**	8.87	0.05	
Acetone	**ppm**	<0.01	1.85	<0.01	<0.01	<0.01	<0.01	**1.85**	-	0.01	
Isopropyl Alcohol	**ppm**	1.8	0.28	1.10	2.27	0.92	0.65	**1.17**	0.74	<0.01	
Methanol	**ppm**	<0.01	<0.01	<0.01	1.93	<0.01	<0.01	**1.93**	-	0.01	
Tetrahydrofuran	**ppm**	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	**-**	-	-	0.154[f)]
TOTAL ORGANIC SOLVENTS	**ppm**	24.45	9.64	18.81	12.29	1.83	1.56	**11.43**	9.14		
	%	0.0024	0.0010	0.0019	0.0012	0.0002	0.0002	**0.0012**	0.0009		

**Based on analytical data reports in Spanish; translation provided by Seprox Biotech

*Calculated: 100 – (HT acetate) – (Moisture Karl Fisher) – (Inorganics) – (Acetic acid) – (Residual organic solvents)

n.a.=not applicable

[a)]Regulation CE 1881/2006

[b)]Regulation CE 52/2006

[c)]Average intake levels 80-150 µg/person/day that may reach 900 µg/person/day. The EFSA Journal (2005) 146, 1-21

[d)]RDA=18 mg; [e)]World Health Organization 1996; [f)]US EPA 7.8 mg/day

[e)]World Health Organization 1996

[f)]NOAEL in drinking water, which is estimated as 20% of total acceptable exposure to THF to allow for potential exposure contribution from other sources. Environtment Fact Sheet ARD-EHP-23.

7. APPENDIX II

Quality Control Measures utilized in the Production of Hydroxytyrosol

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Department of R&D

QUALITY CONTROL OF PRODUCTION OF HYDROXYTYROSOL

How will end points of individual reactions be determined in production?

The end points of individual reactions are determined in production by time with the exception of enzymatic hydrolysis.

All reaction times were carefully studied at laboratory scale following reaction progress by HPLC-UV. After that, times were extrapolated to pilot scale making necessary adjustments and always checking the goodness of extrapolation by HPLC-UV.

In the case of enzymatic hydrolysis the pH of the media indicates the finish of the reaction although the final result is equally checked by HPLC-UV.

How will remaining solvents and reactants be monitored to make sure the product complies with the food law?

The process has been designed in such form that sequence of physical processes minimizes the amount of contaminants organic and inorganic.

With regard to solvents the evaporation steps preceding to the final products are done always in water which is the highest boiling point solvent of all used so lower boiling point solvents used before or azeotropic mixtures of them were eliminated at least to the solubility point in water in distillation conditions.

In the case of inorganic or organic salts all reaction are followed by successive extraction processes to ensure that this changes in solvents eliminates the maximum possible of residues. In the case of boron residues after reduction reaction there are three changes of solvent from water to organic solvent and a filtration with active carbon before precipitation which is done too in aqueous media, and before to obtain final hydroxytyrosol there are

another extraction process from water to organic solvent and a filtration with activated carbon so boron residues if any must be under law limits in final product.

Anyway final product is analyzed for inorganic residues and solvent determination.

Check points are indicated below within scheme process.

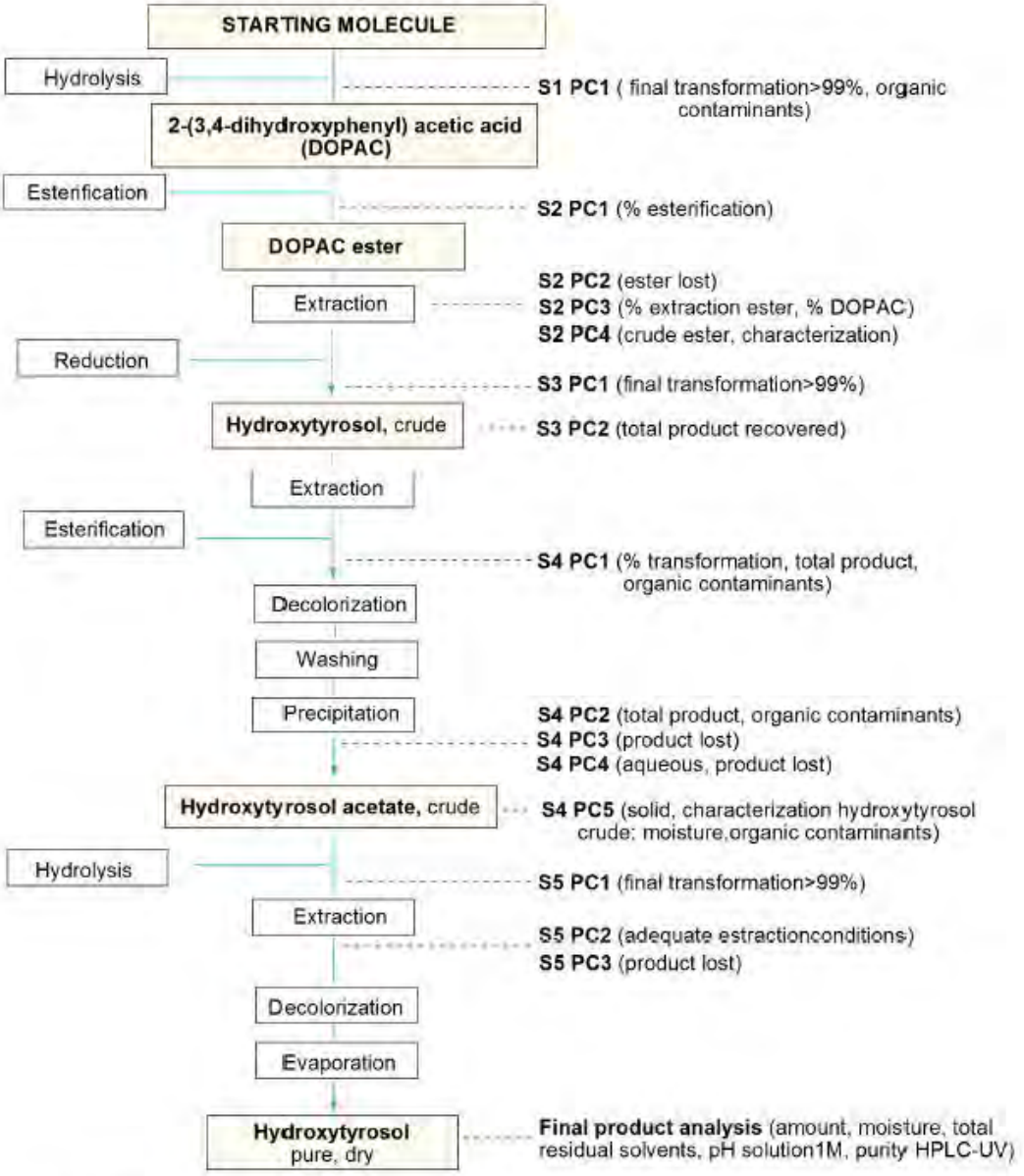

Figure 1. Check points: manufacturing of hydroxytyrosol

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(b) (6)