



GRAS Notice (GRN) No. 459

<http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/default.htm>

ORIGINAL SUBMISSION

000001

December 14<sup>th</sup>, 2012

**Center for Food Safety and Applied Nutrition  
Food and Drug Administration  
5100 Paint Branch Parkway  
College Park, MD 20740**

K. Att. Mr. Sylvester Mosley, Ph.D., Consumer Safety Officer at FDA  
K. Att. Ms. Lillian Shepherd, Program Analyst, FDA  
K. Att. Ms. Moraima Ramos, Consumer Safety Officer, FDA

***Amendments to Notification of GRAS determination for Olive  
Pulp Extract (OPE) Phenolea®Complex for use in foods as  
antioxidant. GRAS exemption Claim***

*With reference to the conference call held on December 12<sup>th</sup>, 2012*

*We are forwarding herewith the amendments concerning paragraph "declaration of intent" (page 5) and paragraph "Phenolea®Complex Intended Use In Food" (page 11) of our GRAS notification dtd 12/11/2012.*

*Hard copy will be dispatched by registered mail.*

*We remain at your disposal for any further info you may need.*

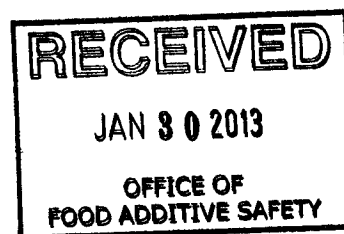
*Best regards,*

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Stefano Germany  
Phenofarm CEO

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**AMENDMENT OF PARAGRAPH “declaration of intent” (pag 5 of GRAS notification dated 12/11/2012)**

***Declaration of intent***

Phenofarm company wishes to market OPE, denominated Phenolea® Complex as antioxidant in food generally, except infant formula and meat and poultry products, and principally in: baked products, dressing/seasonings, vegetables, canned product (see below, table 5), as natural extract with protective efficacy against food oxidation phenomena. Is commonly recognized that polyphenols are effective as antioxidant added to food in order to retard deterioration derived from oxidation processes.

Polyphenols antioxidant activity may also provide a nutritional benefit, in scavenging reactive oxygen species (ROS) and free radical species. Further discussion on biological activities of olive polyphenols is reported in the addendum section of this notice.

Phenolea® Complex could be considered exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act (the act) because it has determined that such use is generally recognized as safe (GRAS).

The purpose of this notification is to summarize the technical, safety, product informations, and scientific data used to support a self-determination by the Phenofarm as to the GRAS status of OPE Phenolea® Complex as antioxidant substance added to food products.

A comprehensive search of the scientific literature for safety and toxicological information on OPE, constituent and related polyphenols was conducted together with an history of safe consumption.

For intended use in food, the maximum dosage of OPE Phenolea® Complex is 3000 ppm (w/w) in the finished food products.

**AMENDMENT OF PARAGRAPH “Phenolea® Complex Intended Use In Food” (pag 11 of GRAS notification dtd 12/11/2012)**

**Phenolea® Complex Intended use In food**

The combination of polyphenolic molecules belonging to *Leccino* and *Carboncella* cultivars confers distinctive characteristics to Phenolea®Complex. Said characteristics make it particularly suitable for food applications.

Phenolea®Complex, is mainly applied in the food production industry, as antioxidant for preventing rancidity, possibly as antimicrobial preservative mainly in baked products but also in sauces and seasonings and generally in food products containing a fat part subjected to oxidation (see table 5).

	FOOD ANTIOXIDANT
FOOD SPECIALTIES	Phenolea® Complex
	Mollis extract
BAKED GOODS	X
BEVERAGES	X
CEREALS	X
SAUCES AND DRESSINGS	X
SEASONINGS	X
SNACKS	X
FUNCTIONAL FOOD	X
SUPPLEMENTS	

**Table 5: Food specialties where Phenolea®Complex can be employed as antioxidant**

**Intended use :** The maximum dosage of Phenolea®Complex for food application is 3000 mg/kg in final preparation.

**As an example**, in table 6 is reported the nutrient contribution of Phenolea®Complex in some food specialties ( cookies and white bread) in comparison with the nutrient intake of an edible portion of olives 20/40 g. edible portions were retrieved from USDA publication "Nutritive value of foods" (Gebhardt and G.T. 2002).

	<b>Table olive 20/40 g (edible portion min/max )<sup>1</sup></b>	<b>cookie+ Phenolea®Complex (edible port. 5g, 1 piece)</b>	<b>White bread + Phenolea®Complex (edible portion,1 slice 25 g)</b>
	mg	mg	mg
<b>Proteins</b>	tr	0.45	2.28
<b>Carbohydrates</b>	700/1400	10.86	54.33
<b>Fats</b>	2000/4000	0.03	0.09
<b>Fibers</b>	1000/2000	0.36	1.77
<b>Ashes</b>		1.08	5.37
<b>Sodium</b>	192/384	0,001	0.03
<b>Polyphenols</b>	200/400*	0.75	3.75
<b>Hydroxytyrosol</b>	10/20	0.375	1.87

**Table 6. comparison of nutrient intake cookies and white bread related with the addition of Phenolea®Complex at the maximum dosage of 3000 mg/kg. (edible portions are obtained from USDA publication "Nutritive value of foods" (Gebhardt and G.T. 2002))**

The addition of Phenolea®Complex in the referred foods (cookies, white bread) at the maximum dosage (3000 mg/kg) provide a nutrient intake well below the standard portion of olives normally consumed 20/40g.

self limiting levels individuated for phenolea®Complex are related to toxicological data reported in the paper of Christian et al 2004 (Christian, Sharper et al. 2004) (see below under section preclinical and toxicological) where acute studies of toxicity were conducted with a maximum dosage of 5000 mg/kg.

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Phenofarm S.r.l.  
Stefano Germani  
CEO (b) (6)

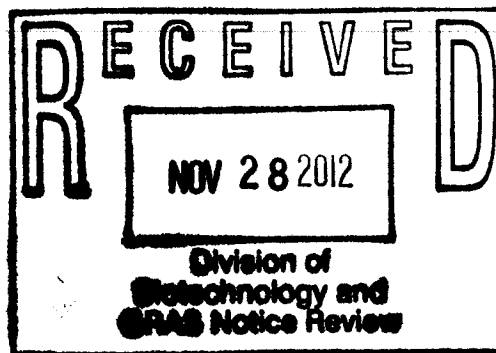
December 14<sup>th</sup>, 2012



November 12<sup>th</sup>, 2012

Center for Food Safety and Applied Nutrition  
Food and Drug Administration  
5100 Paint Branch Parkway  
College Park, MD 20740

K. Att. Dr. Paulette Gaynor



**Notification of GRAS determination for Olive Pulp Extract  
(OPE) Phenolea®Complex for use in foods as antioxidant.  
GRAS exemption Claim**

Dear Madam,

Pursuant to FDA's policy described at 63 fed. Reg. 18938, 18969 (8 april 17 1997)

**Phenofarm s.r.l.** hereby notifies to the Food and Drug Administration (FDA) that it has determined that the use of **Phenolea®Complex** olive pulp extract (OPE), food ingredient with antioxidant capacities, is "generally recognized as safe" (GRAS) and is therefore exempt from the premarket approval requirements of the Federal Food, Drug and Cosmetic Act.

A detailed summary of the basis of the GRAS determination is attached to this exemption claim. The following information is provided under proposed 21 C.F.R. § 170.36 (c)(1)

NOTIFIER :	Phenofarm s.r.l.(Via Domenico Chelini 5, 00197, Rome. ITALY)
GRAS SUBSTANCE:	Aqueous olive pulp extract (OPE) named Phenolea®Complex rich in polyphenols compounds a food grade ingredient with antioxidant properties
INTENDED USE:	Phenolea®Complex is employed in foods as: baked products, meat, dressing/seasonings, vegetables, canned product, etc. as natural ingredient with antioxidant activities effective against oxidation phenomena occurring in foods. The maximum dosage for intended use is 3000 mg/kg in the final product.
BASIS FOR GRAS DETERMINATION:	Scientific Procedures

The data and information that are the basis of Phenofarm's GRAS determination are available for FDA's review and copying upon request at reasonable time at this office:

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Respectfully submitted

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Stefano Germani,  
Phenofarm CEO

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**PhenoFarm S.r.l.**

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## GLOSSARY AND ABBREVIATIONS

OPE :	Olive Pulp Extract
FDA:	Food and Drug Administration
OMW:	Olive mill waters/vegetation water
MF :	Microfiltration
HPLC:	High pressure liquid chromatography
HPLC- DAD	High pressure liquid chromatography, diode array detection
ROS	Reactive oxygen species
LDL:	low density lipoprotein
oxLDL:	oxidized low density lipoprotein
CHD:	coronary heart disease
DPPH:	is a common abbreviation for an organic chemical <b>compound 2,2-diphenyl-1-picrylhydrazyl</b> . It is a dark-colored crystalline powder composed of stable free-radical molecules. DPPH is a monitor of chemical reactions involving radicals, most notably it is a common antioxidant assay.
BHT:	<b>Butylated hydroxytoluene (BHT)</b> , also known as <b>butylhydroxytoluene</b> , is a lipophilic (fat-soluble) organic compound, chemically a derivative of phenol, that is useful for its antioxidant properties.
EFSA:	European Food Safety Authority
GLP:	Good Laboratory Practice
NOAEL:	No Observable Adverse Effect Level
Cmax:	Maximum Concentration
ALT:	Alanine Aminotransferase
AST:	Aspartate Aminotransferase
SDH:	Sorbytol Deidrogenase
Folin-C:	Folin-Ciocalteu, colorimetric method for total polyphenols quantization.
GAE:	Gallic Acid Equivalent, the measurement units for Folin-C method
EGCG:	Epi-Gallo-Cathechin Gallate (green tea polyphenolic molecule)

## **NOTIFIER**

Phenofarm s.r.l.  
Via Domenico Chelini 5  
00197, Roma  
Italy

## **SUBSTANCE**

Aqueous olive pulp extract (OPE) named Phenolea®Complex rich in polyphenols compounds sourced from traced olive pulp by physical process and extracted without solvents except water. Phenolea®Complex is a food grade ingredient with antioxidant properties.

### ***Declaration of intent***

Phenofarm company wishes to market OPE, denominated Phenolea®Complex as antioxidant in foods as: baked products, meat, dressing/seasonings, vegetables, canned product, etc (see below, table 5) as natural extract with protective efficacy against food oxidation phenomena. It is commonly recognized that polyphenols are effective as antioxidant added to food in order to retard deterioration derived from oxidation processes. Polyphenols antioxidant activity may also provide a nutritional benefit, in scavenging reactive oxygen species (ROS). Further discussion on biological activities of olive polyphenols is reported in the addendum section of this notice.

The purpose of this notification is to summarize available scientific data used to support a self-determination by the Phenofarm as to the GRAS status of OPE Phenolea®Complex as antioxidant substance added to food products.

A comprehensive search of the scientific literature for safety and toxicological information on OPE, constituent and related polyphenols was conducted together with an history of safe consumption.

For intended use in food, the maximum dosage of OPE Phenolea®Complex is 3000 ppm (w/w) in the finished food products.

On the basis of scientific data collected and reviewed, Phenolea®Complex could be considered GRAS and exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act (the act).

### ***Regulatory Basis For GRAS Determination***

As per volume 62 of the Federal Register, page 18938 of April 17 1997 the Phenofarm wishes to notify the Food and Drug Administration (FDA) that it has determined that the use of its OPE Phenolea®Complex, is a Generally Recognized as Safe (GRAS) substance for use in food preparation. Under the ; 21 CFR §170.30(b) and §170.30 (c); therefore:

The determination that the Phenolea®Complex natural extract is GRAS was conducted mainly through evaluation and review of publicly available scientific documents. The available scientific documents and information summarized in this report reflect a thorough review of the relevant literature dealing with olive nutrients, including the toxicity assessment on other similar OPE's already available in the market and accepted as GRAS. Relevance of the published studies was established through a comparison of polyphenolic species and chemical profiles of the respective products. All of these data has been supplemented by the use of scientifically relevant statistical reference sources, compendia, books, and reviews. All studies were conducted in accordance with generally accepted scientific procedures. In addition, for a better safety assessment, of OPE Phenolea®Complex as food ingredient, a brief history of safe consumption of comparable foods was achieved by screening of concerning scientific literature.

The GRAS determination is the direct result of a consensus among scientific community that there is a reasonable certainty that OPE Phenolea®Complex and its constituents (including olive polyphenols), will not be harmful under the intended conditions of use.



### **Summary Basis for GRAS Status**

A summary basis for the evaluation of GRAS status for the OPE Phenolea® Complex has been based on the following items:

- the food origin of the basic raw material, namely the aqueous olive pulp of the olive fruit.
- extraction process (physical extraction and concentration with water as only solvent)
- chemical composition
- condition of use
- history of safety usage of a substantially equivalent foods;
- safety assessment: based on publicly available scientific studies .

Exhaustive discussion of each of these points can be found below in this document.

Among the major constituents of olive pulp (carbohydrates, protein, fibers, salts, fats) a 4.5% abt. is represented by polyphenolic compounds. (determined by FolinC.-GAE).

Nutritional composition of Phenolea® Complex was achieved and is reported in table 2.

The chemical composition of OPE extracts is well characterized and its composition reflects the natural one present in the olive fruit. Phenolea® Complex specifications comply with limits and guidance established by FDA as to the presence of heavy metals and pesticide residues. The functionality of the constituent polyphenols in OPE as an antioxidant is well recognized in extra virgin olive oil, with strong supporting evidence demonstrating that antioxidant activity is extended to biological systems. (as recently confirmed by the EFSA opinion: <http://www.efsa.europa.eu/en/efsajournal/.../2033.pdf>)

There is supporting evidence for the safety olive polyphenols extracts in humans from clinical investigations of possible beneficial health effects. (Covas, Nyyssonen et al. 2006).

### **PHENOLEA® COMPLEX: (information about the identity of the substance)**

OPE Phenolea® Complex is a natural hydrophilic extract obtained directly from aqueous olive pulp and the OMW (olive mill water). OMW is a natural by-product of olive crushing during olive oil production. Olives (*Olea europaea* L., cultivars: *Leccino* and *Carboncella*) are sourced from traced cultivations surrounding the production site.

as follows are described some topics related to OPE Phenolea® Complex origin, manufacturing, characterization and usage:

- raw material;
- manufacture process;
- Phenolea complex chemical and nutritional characterization;
- dosage for intended use;

### **Raw material olive pulp and OMW.**

The olive pulp and OMW are collected during olive oil production, liquid olive pulp is collected and quickly processed as described below.

Biochemical composition and safety assessment of the initial matrix are both performed routinely: toxins, heavy metals, pesticides and microbial contaminants are monitored constantly. Phenolea® Complex contains all polyphenolic families belonging in the olive pulp. The original pool is preserved during the different stages of production so the final product can be considered as a concentrated liquid olive pulp. In table 1 are reported the HPLC polyphenolic profile of olive pulp/OMW sourced from *Carboncella* and *Leccino* cultivars.

OMW ( <i>Carboncella</i> cultivar)	mg/L
oleoside gluc deriv (390)	682.9
oleoside gluc (390)	2238.9
elenolic acid AE (242)	471.6
OH-Tyr glic (170)	55.3
OH-Tyr der.	71.9
OH-Tyr (hydroxytyrosol)	228.6
OH-Tyr gluc (316)	268.6
Tyr	74.3
Tyr gluc (300)	0.0
Tyr der.	48.3
vanillic acid	0.0
demethyl oleurop	0.0
secoiridoid derivative	0.0
DACOLAG	768.2
oleocanthal	0.0
caffeic derivative	34.7
caffeic acid	0.0
p-cumaric acid	0.0
bOH verbascoside (isomers)	63.7
verbascoside	68.7
isoverbascoside	10.6
secologanoside caffeeoil ester	62.0
secologanoside p-cumaroil ester	78.3
total	5226.7

Table 1: OMW phenolic composition by HPLC-DAD analysis

### **Manufacture Process Of Phenolea® Complex**

The production of Phenolea® Complex is performed without any kind of organic solvent, so the biochemical composition of the final extract reflects the original composition of olive fruit but in a more concentrated formulation.

The production process comprises the following steps:

- 1) collection of olive pulp and OMW after the olive milling process;
- 2) pre-treatment;
- 3) tangential filtration: ceramic microfiltration (MF);
- 4) vacuum evaporation of the permeate phase obtained in step 3.

Pre-treatment stage is carried out on liquid olive pulp with the aim of reducing and separating solid suspensions present in the matrix (cellulosic fibres, oil globules, pulp residues), improving the filterability of the raw extract; Olive pulp/OMW is treated immediately, within 24 hours from the olive crushing, in order to avoid the oxidation phenomena of the biophenols

#### **Pre-Treatment**

Pre-treatment of liquid olive pulp comprises:

- a) pH adjustment (with different food grade acids can be used: citric acid, phosphoric acid, sulphuric acid)
- b) enzymatic hydrolysis (with food grade enzymes)
- c) solid removal

after these steps the liquid extract is sent by pumping to the subsequent filtering section the liquid mass which is collected on the bottom of the pre-treatment tank.

#### **Filtration process**

The liquid mass deriving from pre-treatment step is subjected to Microfiltration, the permeate is constituted by a solution, typically red-coloured due to the presence of antocyanic pigments having molecular weight comprised between 400 and 500 Da, and it comprises the entire pool of polyphenols and also all organic and inorganic substances (sugar, proteins, salts).

#### **Vacuum evaporation:**

After microfiltration the permeate is placed in an evaporator.

The vacuum allows the evaporation occurring at very low temperature so any kind of thermal damage to final extract is avoided.

### **Phenolea® Complex composition and technical specifications.**

The final product is obtained without requiring technological supports such as maltodextrins, gum arabic or others usually used in phyto-extracts but with the sole use of mechanical means and at low temperature.

Appearance and organoleptic properties: Phenolea® Complex in its final formulation is a semi-solid paste (mollis extract) with a residual humidity of 20%, it present a dark red colour due to the presence of natural pigments in olives (anthocyanines), the bitter taste derives from the polyphenolic compounds while flavour is related to volatile molecules. The mollis extract is 100% water soluble, and it is suitable for application in which water or water solution are employed.

Nutritional composition of the Phenolea®Complex reflects the profile of olive pulp In table 2 are reported nutritional values including microbiological, heavy metals, and pesticides content.

<b>PHENOLEA®COMPLEX NUTRITIONAL COMPOSITION</b>	
<b>proteins</b>	2.50 g/100 g
<b>fats</b>	0.10 g/100 g
<b>dietary fibres</b>	2.0 g/100 g
<b>ashes</b>	6.00 g/100 g
<b>carbohydrates</b>	61.00 g/100 g
<b>sugars</b>	12.00 g/100 g
<b>sodium</b>	360 mg/kg
<b>heavy metals:</b>	<0.1 mg/kg
<b>pesticides:</b>	Absent
<b>mould</b>	<10 UFC/1 g
<b>yeast</b>	<10 UFC/1 g
<b>total polyphenols</b>	50 mg/g

**Table 2: nutritional composition of OPE Phenolea®Complex, Total polyphenols are measured by Folin C. analysis. Values are Expressed in Gallic Acid Equivalents (GAE).**

Polyphenolic profile: The final product can be considered as a concentrated liquid olive pulp. In Table 3 is reported the typical HPLC analysis on Phenolea® Complex for polyphenolic determination.

<b>PHENOLEA® COMPLEX POLYPHENOLS CONTENT BY HPLC</b>		
<b>Total polyphenols</b>	mg/kg	45261
<b>Total aromatic alcohols</b>	mg/kg	21328
Hydroxytyrosol	mg/kg	20131
Tyrosol	mg/kg	1197
<b>Oleuropein derivatives</b>	mg/kg	23005
<b>Ligstroside derivatives</b>	mg/kg	1710
<b>Verbascoside</b>	mg/kg	1089
<b>Oleocanthal</b>	mg/kg	1020
<b>Total Lignans</b>	mg/kg	36
(Pinoresinol and Acetoxypinoresinol)		
<b>Total Phenolic Acids</b>	mg/kg	4784
(Protocatechuic Acid, Vanillic Acid, Caffeic Acid, p-Coumaric Acid, Ferulic Acid)		
<b>Total Flavonoids</b>	mg/kg	222
Luteolin	mg/kg	222
Apigenin	mg/kg	n.d.
<b>Total Secoiridoid Acids</b>	mg/kg	7695
Decarboxymethyl Elenolic Acid	mg/kg	2408
Elenolic Acid	mg/kg	5287

**Table 3: typical HPLC Polyphenolic profile of OPE Phenolea® Complex.**

Phenolea® Complex comply with international standards as to toxins, heavy metals and pesticides in table 4 is reported an example of technical sheet and MSDS sheet.

#### TOXICOLOGICAL, CHEMICAL AND PESTICIDE COMPLIANCE OF PHENOLEA® COMPLEX WITH INTERNATIONAL STANDARDS

<b>MICROBIOLOGICAL QUALITY</b>	(Eu. Ph 6.7: 5.1.8 Cat. B) or according to current Pharmacopea Reg. CE 629/2008, Reg. 1881/2006	Yeasts and moulds $\leq 5 \times 10^2$ UFC/g
		Total plate count: $\leq 5 \times 10^5$ UFC/g
		Escherichia coli: absent in 1g
		Enterobacteria: $\leq 10^2$ UFC/g
		Salmonellae SPP: Absent in 25g
<b>HEAVY METALS</b>		Pb: < 3 ppm Cd: < 1 ppm Hg: < 0,01 ppm As: < 1 ppm
<b>PESTICIDES</b>	Reg 396/2005 and following DM 27 August 2004 and following	Conform
<b>AFLATOXINS</b>	Reg. CE 1881/2006 and following updates Reg. CE 401/2006 and following updates	Absent
<b>OCHRATOXIN</b>	Reg. CE 105/2010 Reg. CE 401/2006 and following updates	Absent
<b>PAHs (POLYCYCLIC AROMATIC HYDROCARBONS)</b>	Reg. CE 1881/2006 Codex Erbarum: vegetal ingredients Benzopirene: 10ppb	< 1 µg/kg
<b>EXTRACTION SOLVENTS</b>		No chemical solvents
<b>RESIDUAL MOISTURE</b>		<30%
<b>EXTRANEIOUS MATERIAL</b>	Metallic and non metallic	Absent
<b>PESTS CONTAMINATION</b>		Absent

Table 4: analysis of levels for main risk classes (microbia, pesticides, heavy metals, chemicals)

### ***Phenolea® Complex intended use in food***

The combination of polyphenolic molecules belonging to *Leccino* and *Carboncella* varieties confers distinctive characteristics to Phenolea® Complex. Said characteristics make it particularly suitable for food applications.

Phenolea® Complex, is mainly applied in the food production industry, as antioxidant for preventing rancidity, possibly as antimicrobial preservative, in fresh and/or frozen meat, sausage products, baked products, sauces and seasonings and generally in food products containing a fat part subjected to oxidation (see table 5).

<b>FOOD ANTIOXIDANT</b>	
<b>FOOD SPECIALTIES</b>	<b>Phenolea® Complex</b>
	<b>Mollis extract</b>
BAKED GOODS	X
BEVERAGES	X
CEREALS	X
SAUCES AND DRESSINGS	X
MEAT AND POULTRY	X
SAUSAGES	X
SEASONINGS	X
SNACKS	X
FUNCTIONAL FOOD	
SUPPLEMENTS	

**Table 5: Food specialties where Phenolea® Complex can be employed as antioxidant**

Intended use: The maximum dosage of Phenolea® Complex for food application is 3000 mg/kg in final preparation.



As an example, in table 6 is reported the nutrient contribution of Phenolea®Complex in some food specialties (salami, cookies and white bread) in comparison with the nutrient intake of an edible portion of olives 20/40 g. edible portions were retrieved from USDA publication "Nutritive value of foods" (Gebhardt and G.T. 2002).

	<b>Table olive 20/40 g (edible portion min/max )<sup>1</sup></b>	<b>Salami + Phenolea®Complex (edible portion 20g)</b>	<b>cookie+ Phenolea®Complex (edible port. 5g, 1 piece)</b>	<b>White bread + Phenolea®Complex (edible portion, 1 slice 25 g)</b>
	mg	mg	mg	mg
<b>Proteins</b>	tr	1.55	0.45	2.28
<b>Carbohydrates</b>	700/1400	37	10.86	54.33
<b>Fats</b>	2000/4000	0.066	0.03	0.09
<b>Fibers</b>	1000/2000	1.2	0.36	1.77
<b>Ashes</b>		3.65	1.08	5.37
<b>Sodium</b>	192/384	0.024	0,001	0.03
<b>Polyphenols</b>	200/400*	3.0	0,75	3.75
<b>Hydroxytyrosol</b>	10/20	1.5	0.375	1.87

**Table 6. comparison of nutrient intake in salami, cookies and white bread related with the addition of Phenolea®Complex at the maximum dosage of 3000 mg/kg. (edible portions are obtained from USDA publication "Nutritive value of foods" (Gebhardt and G.T. 2002))**

The addition of Phenolea®Complex in the referred foods (salami, cookies, white bread) at the maximum dosage (3000 mg/kg) provide a nutrient intake well below the standard portion of olives normally consumed 20/40g.

Self limiting levels individuated for Phenolea®Complex are related to toxicological data reported in the paper of Christian et al 2004 (Christian, Sharper et al. 2004) (see below under section "preclinical and toxicological") where acute studies of toxicity were conducted with a maximum dosage of 5000 mg/kg.



## OLIVE POLYPHENOLS GENERAL FACTS:

The following section provides:

1. a basic discussion of the general chemistry of polyphenolic compounds, including their nomenclature and categorization;
2. a background discussion regarding the long history of safe olive polyphenols human consumption is provided with evaluation of polyphenols intake from table olives.

These concepts together with the safety assessment based on review of publicly available scientific data (see below), provide a framework to facilitate evaluation of safety of OPE Phenolea®Complex extract as antioxidant ingredient for use in food specialties. A further discussion was provided in addendum on the topic of biological activities of olive polyphenols.

### **Polyphenolic Compounds**

The terms “*phenols*”, “*polyphenols*” or “*biophenols*” refer to any chemical species bearing one or more aromatic ring substituted with one or more hydroxyl groups. This includes priority phenols that are important synthetic chemicals.

In the olive literature, the term “*biophenol*” has gained widespread usage, this term is sufficient to distinguish between industrial phenols and those of plant origin.

The term covers those phenolic compounds that have been of interest in the chemistry of olive oil as well as those of more recent pharmaceutical interest. It also covers compounds that have been traditionally known as polyphenols, such as the flavonoids whose presence in olive may be important. Within this communication the term “polyphenols” or “phenolic compounds” identify the Biophenols of the olive pulp.

Biophenolic compounds or “*biophenols*” constitute an extremely complex and widely distributed group of plant substances. Polyphenols are the products of plant metabolism and arise from two main synthetic pathways, the Shikimate and the acetate pathways. Natural polyphenols can range from simple molecules, such as phenolic acids, to highly polymerized compounds.

### **Polyphenol chemical classes and occurrence in olives and olive oil.**

Olive fruit is known to contain simple, as well as complex phenolic substances. The phenolic content and the specific composition of these phenols in whole olives depend on the altitude where the olive trees are grown, the harvesting time and the processing conditions. Similarly, the levels of phenolics in olive oil depend upon several factors (cultivar, climate, ripeness of olives, preparation and storage of the oil). These phenolics are responsible for the stability of the oil from oxidation and for the organoleptic properties (Galli and Visioli 2001)

There are more than 100 different phenolic molecules reported in olive products (fruit, oil, leaves, and by-products).

The phenolic composition of olives differs among different products (fruit, oil, leaves and by-product) and different dirrues (pulp and stone). Further, physiological, seasonal, geographic, environmental, varietal, agronomic, and pathological, factors have shown a significant impact on the phenolic composition of olives.

Moreover the olive oil extraction condition affect both the type and amount of polyphenols in both olive oil and olive mill waters (OMW).

Major polyphenolic compounds detected in olive products include hydroxytyrosol, tyrosol and their secoiridoid derivatives oleuropein, oleuropein aglycone and elenolic acid dialdehydes, verbascoside, lignans (acetoxypinoresinol) and flavonoids (rutin and glycosides of luteolin and apigenin). Whereas the main phenolic species in olive fruits, oil and OMW showed a variation amongst different cultivars and different countries, oleuropein is considered universally the most abundant biophenol in olive leave constituting up to 9% of the dry matter weight. In virgin and extra virgin olive oil hydroxytyrosol and tyrosol in addition to lignans are the most abundant components (Obied, Prenzler et al. 2012).

In the intact olive, oleuropein and ligstroside are present in the glycosidic, relatively polar form. 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 during production (crushing), while a substantial proportion remains in the water phase (OMW).

## Polyphenols in vegetation water (olive mill waters OMW)

The polyphenolic fraction of olive oil comprises only 2% of the total phenolic content of the olive fruits, with the remaining 98% being in olive mill water (OMW) (Rodis, Karathanos et al. 2002).

It is noteworthy that during the olive milling process, for olive oil production, the olive paste is continuously hosed with lukewarm water during the milling, a process that is called malaxation (Aktas, Imre et al. 2001). The OMW is produced in extremely large quantities (~800,000 tons/year in Italy) and, despite the fact that it contains a considerable amount of phenols (more than 1% w/v), is currently disposed of. More than a decade ago, Visioli *et al.* demonstrated that OMW extracts have powerful (in the ppm range) *in vitro* antioxidant activity (Aktas, Imre et al. 2001; Longhi, Vodopivec et al. 2001); In animal experiments (Visioli, Galli et al. 2000; Visioli, Caruso et al. 2001) and a couple of human studies (Visioli and Galli 2003; Leger, Carbonneau et al. 2005) confirmed that waste waters are a source of bioactive phenols with a wide array of biological activities and dietary supplements derived from olive mill waste water are already available in the market. The latest of such studies showed that OMWW increases glutathione levels in healthy volunteers (Visioli, Wolfram et al. 2009) (see below in addendum “*Biological activities of olive polyphenols*”).

### Principal olive polyphenolic components. (detailed information about the identity of the notified substance)

**Oleuropein.** Oleuropein is a phenolic secoiridoid glycoside found in the bark, leaves and fruit of the olive tree, as well as in some other genera of the *Oleaceae*. The most abundant phenolic substance in the drupe is oleuropein, a bitter glycoside that constitutes up to 14% of the fruit's dry weight. Oleuropein (CAS No.: 32619-42-4) has the chemical formula C<sub>25</sub>H<sub>32</sub>O<sub>13</sub> and a molecular weight of 541 Da. (Fig.1)

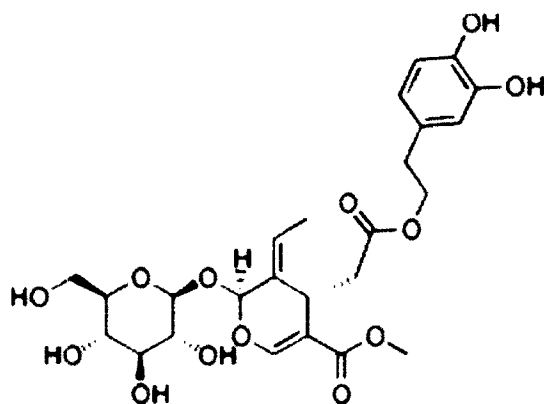


Figure 1: chemical structure of oleuropein

**Tyrosol.** Tyrosol (CAS No.: 501-94-0), a minor component of OPE, has a faint sweet fruity-floral odor and a sweet but very weak taste. Tyrosol has the chemical formula C<sub>8</sub>H<sub>10</sub>O<sub>2</sub> (Fig. 2) and a molecular weight of 138 Da. (Soni, Burdock et al. 2006)

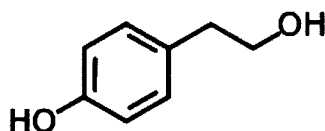
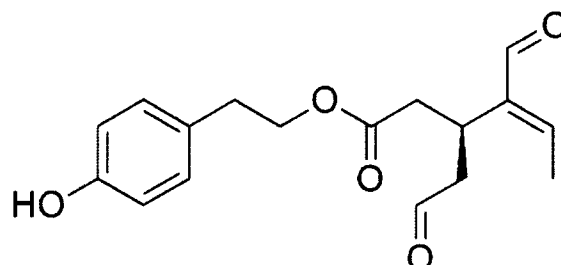


Figure 2: chemical structure of tyrosol

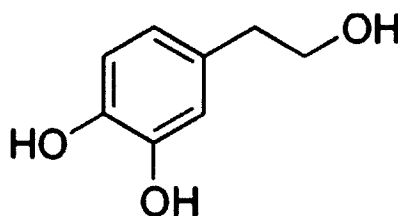
**Oleocanthal:** Oleocanthal (CAS No: 289030-99-5) (Fig. 3) is a natural organic compound isolated from extra virgin olive oil. It is responsible for the slightly peppery "bite" of extra virgin olive oil. Oleocanthal is a tyrosol

ester and its chemical structure is related to oleuropein that is also found in olive oil. Oleocanthal has been found to have anti-inflammatory and antioxidant properties. 50g of olive oil per day is thought to have the same effect as 1/10 of the adult ibuprofen dose (Beauchamp, Keast et al. 2005).



**Figure 3: chemical structure of oleocanthal**

**Hydroxytyrosol:** Hydroxytyrosol, also known as 3,4-dihydroxytyrosol or 3,4-dihydroxyphenylethanol (CAS No.: 10597-60-1); is the major component of the phenolic fraction of olive extract and olive oil (Fig. 4); hydroxytyrosol is present in olive oil either as simple phenol or esterified with elenolic acid to form oleuropein aglycone. Hydroxytyrosol, is the most investigated molecule among olive polyphenols, and it represents the biochemical target in the majority of bioavailability studies performed on humans and animal systems (see below in following sections) .



**Figure 4: chemical structure of Hydroxytyrosol**

**History of safe olive polyphenols consumption** (brief discussion and citation of available scientific data and publications)

#### Historical data

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

#### Olive and olive oil use and consumption.

Owen et al. (2003) investigated the phenolic content of two brined olive drupe types (black and green) (Table 7). The green type contained predominantly hydroxytyrosol, 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. They also reported that consumption of approximately 50 g of black olive pericarp would provide about 400 mg of phenolic substances to the daily dietary intake, while a similar quantity of extra virgin olive oil (produced with conventional methods) provides about 12 mg (Owen, Haubner et al. 2003). The percent of wet weight for

phenolics in black and green olives was reported as 0.082 and 0.118, respectively. In a recent analysis carried out on 48 olive samples, Romero et al. (2004) reported that the 'turning color olives' in brine had the highest concentration of poly- phenols (0.12%)(Romero, Brenes et al. 2004).

Component	Olive type	
	Black	Green
<i>Pericarp</i>		
Total g wet wt.	71.78	111.6
Total g dry wt.	35.89	29.3
G dry wt. per drupe	1.794	1.465
Water (% of wet wt.)	50	73.7
<i>Phenolics in pericarp</i>		
Mg per drupe	29.43	6.56
% of wet wt.	0.82	0.118
% of dry wt.	1.64	0.448
<i>Oil</i>		
Total g	5.52	18.22
% of wet wt.	7.69	16.3
% of dry wt.	15.4	62.2

Values for 20 drupes of each olive type.

**Table 7:Some basic characteristics and phenolics in black vs. green-brined olives (Owen et al., 2003)**

The current primary source of exposure to the constituents (phenolics) of OPE as Phenolea®Complex is via consumer use of olives and their products.

Blekas et al. (2002) reported hydroxytyrosol (unbound) content of table olives as 250–750 mg/kg ( $\approx$ 0.5 mg/g) in two cultivars (Blekas, Vassilakis et al. 2002). Based on this information and United States Department of Agriculture (USDA) eaters-only data, per capita consumption of hydroxytyrosol can be determined (USDA Agricultural Research Service)

#### **Polyphenols intake in table olives.**

Eaters-only data describe the amount of substance naturally present in food consumed only by those individuals that actually consume the particular food. The eaters-only per capita mean and 90th percentile consumption of olives in the US is 20.15 and 40.50 g/day, respectively.

As table olives contain approximately 0.5 mg hydroxytyrosol/g of olive, the mean (20 g) and 90th percentile (40 g) consumption of table olives may result in a daily intake of 10 or 20 mg of hydroxytyrosol.

As before mentioned Owen et al in 2003 reported that consumption of 50 g of black olive pericarp provides approximately 400 mg of phenolic substances, while a similar quantity of extra virgin olive oil provides about 12 mg. Of the phenolic compounds found in olives, approximately 10% was reported as hydroxytyrosol (Marsilio, Campestre et al. 2001). As olives contain approximately 0.8 mg hydroxytyrosol/g of olive, the mean (20 g) and 90th percentile (40 g) consumption of olives will result in a daily intake of 16 or 32 mg of hydroxytyrosol.

#### **SAFETY ASSESSMENT** (comprehensive discussion and citation of available scientific data, information methods.)

Several scientific works have been conducted in order to establish and demonstrate *in vivo* the effects of olive oil phenolics to assess their bioavailability. In fact, experimental evidence that phenolic compounds are absorbed from the diet is accumulating (Manach, Williamson et al. 2005; Williamson and Manach 2005).

An olive mill waste water nutraceutical, i.e. HIDROX®, containing 12% of polyphenols (6% hydroxytyrosol) has been granted the GRAS status (Visioli and Bernardini 2011).

#### **Absorption and elimination**

The experimental aiming to unravel the mechanisms of absorption, excretion and metabolism of oil phenolic compounds, were conducted both on animal and human model. Many of them have been focused on

evaluation of metabolic behavior of specific molecules as hydroxytyrosol, tyrosol or oleuropein some the most known and effective molecules within the pool of olive polyphenolics.

### Animal studies

Bai and his collaborators supplemented rats via oral gavages with hydroxytyrosol, this approach evidenced a rapid appearance of phenolic substances in blood; the mean peak was observed after 180 min (Bai, Yan et al. 1998).

A further study of tracking the metabolic fate of hydroxytyrosol after intravenous injection, was performed rats using a radio-labeled hydroxytyrosol ([<sup>14</sup>C]-hydroxytyrosol): less than 8% of the administered radioactivity was detected in the blood stream 5 min after injection. Only 0.1% of the administered hydroxytyrosol dose was detectable in the blood 5 h after administration. Approximately 90% of the administered radioactivity was detected in urine within 5 h, while about 5% was detected in feces and the gastrointestinal content. [<sup>14</sup>C]-hydroxytyrosol was enzymatically converted to four oxidized and/or methylated derivatives. A significant fraction of total radioactivity was associated with sulfo-conjugated forms, which also represented the major urinary excretion products (D'Angelo, Manna et al. 2001).

Tuck et al in 2001 also demonstrated the absorption of phenolic species (hydroxytyrosol and tyrosol) in rats, after intravenous and oral (in oil and water based solutions) Sampling on urines revealed different pattern of excretion when phenolics were administered in an olive oil and in an aqueous solution : for hydroxytyrosol were 99% in oil and 75 % in water, for tyrosol were 98% and 71 respectively (Tuck, Freeman et al. 2001).

Edgecomb et al in 2000 demonstrated that oleuropein is poorly absorbed from isolated perfused rat intestine. Therefore, it is possible that it or its metabolites may confer a positive health benefit after the consumption of olive oil, most likely via an antioxidant mechanism (Edgecombe, Stretch et al. 2000).

In another study, Sprague Dawley rats were administered with daily dosage of an OPE (1000, 1500 and 2000 mg/kg/day; corresponding to hydroxytyrosol at 24, 36 and 48 mg/kg/day, respectively) by oral gavage for 90 days (Christian, Sharper et al. 2004) (see below under "*preclinical toxicological studies*" section).

Blood samples were collected on Day 90, prior to dosing and at 0.5, 1, 2, 4 and 8 h post-dose. Pre-dose plasma samples contained no measurable mean concentrations of hydroxytyrosol, suggesting minimal carry-over of hydroxytyrosol from prior doses. The results of this study suggest that hydroxytyrosol was rapidly absorbed, and mean concentrations were measurable through 1–4 h at 1000 and 1500 mg/kg and through 8 h at 2000 mg/kg. (a better discussion on this paper is performed below in this notice)

### Human studies

Olive oil phenolics seem to be absorbed in the human intestinal tract probably in the post-prandial phase (Bonanome, Pagnan et al. 2000) and further insights revealed that the small intestine is involved in phenolics absorption (Vissers, Zock et al. 2002) The mechanisms for absorption at intestinal level were still investigated by several works in models of colon and stomach environments (Corona, Tzounis et al. 2006).

In the years 2000-2001, it was observed that olive oil phenolics are dose-dependently absorbed in humans and their excretion in the urine, mainly in the form of a glucuronide conjugates and, to a lesser extent, as sulfates. Basal levels of homovanillic acid are also detected during excretion. In this study are elucidated metabolic pathways of other phenolic compounds as oleuropein that can be absorbed and hydrolyzed to hydroxytyrosol. (Caruso, Visioli et al. 2001). The aforementioned formation of homovanillyl alcohol and acid was interpreted as a consequence of the cerebral production of hydroxytyrosol as a metabolite of dopamine (Lamensdorf, Eisenhofer et al. 2000); it is noteworthy that increasing amounts of phenolics administered with olive oil stimulated the rate of conjugation with glucuronide (Visioli, Galli et al. 2000).

Miro-Casas and his colleagues investigate extensively the bioavailability of olive phenolics in humans (Covas, Miro-Casas et al. 2003; Miro-Casas, Covas et al. 2003; Miro-Casas, Covas et al. 2003) they developed a method to quantify hydroxytyrosol and its metabolites in plasma the results reported that, absorption of hydroxytyrosol in nearly complete and its plasma half-life is 2.43 h.

Covas et al in 2006 performed the most important human study on the effect of olive oil polyphenols. This work was considered meaningful for the reduction of lipid oxidation in blood markers as LDL by EFSA (<http://www.efsa.europa.eu/it/efsajournal/doc/2033.pdf>). A Randomized, crossover, controlled trial was

conducted on 200 healthy male volunteers to evaluate whether the phenolic content of olive oil could have benefits on plasma lipid levels and lipid oxidative damage compared with monounsaturated acid content. 6 research centers from 5 European countries were involved in the project.

**measurements:** Glucose levels, plasma lipid levels, oxidative damage to lipid levels, and endogenous and exogenous antioxidants at baseline and before and after each intervention.

**intervention:** In a crossover study, participants were randomly assigned to 3 sequences of daily administration of 25 mL of 3 olive oils. Olive oils had 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 3 weeks preceded by 2-week washout periods.

**results:** A linear increase in high-density lipoprotein (HDL) cholesterol levels was observed for low-, medium-, and high-polyphenol olive oil: mean change, 0.025 mmol/L (95% CI, 0.003 to 0.05 mmol/L), 0.032 mmol/L (CI, 0.005 to 0.05 mmol/L), and 0.045 mmol/L (CI, 0.02 to 0.06 mmol/L), respectively. Total cholesterol-HDL cholesterol ratio decreased linearly with the phenolic content of the olive oil. Triglyceride levels decreased by an average of 0.05 mmol/L for all olive oils. Oxidative stress markers decreased linearly with increasing phenolic content. Mean changes for oxidized low-density lipoprotein levels were 1.21 U/L (CI, -0.8 to 3.6 U/L), -1.48 U/L (-3.6 to 0.6 U/L), and -3.21 U/L (-5.1 to -0.8 U/L) for the low-, medium-, and high-polyphenol olive oil, respectively.

**conclusions:** Olive oil is more than a monounsaturated fat. Its phenolic content can also provide benefits for plasma lipid levels and oxidative damage. (Covas, Nyyssonen et al. 2006)

### **Preclinical, toxicological safety**

(extracted and adapted from: Christian, M.S., et al., *The toxicity profile of hydrolyzed aqueous olive pulp extract*. *Drug Chem Toxicol*, 2004. 27(4): p. 309-30.)

An exhaustive Evaluation of Toxicological effects of an OPE (olive pulp extract) was performed by Christian et al in 2004 (Christian, Sharper et al. 2004). The work of Christian et al. is focused on toxicity of OPE in several *in vivo* studies performed on mice and rat models.

Here below is resumed the discussion of the paper. Ther results of toxicology studies conducted with OPE to establish the toxicology profile for this natural product are summarized.

All studies were conducted in strict compliance with Good Laboratory Practices (GLPs), as defined by the FDA (Administration 1987). Toxicological procedures in the acute studies reflect those described in the Redbook 2000 (Administration 2000). All animal husbandry practices and procedures were in compliance with the "Guide for the Care and Use of Laboratory Animals" (Institute of Laboratory Animal Resources, 1996).

The tests performed concerned:

- acute toxicity studies;
- oral 90 -Day toxicity study;
- reproduction;
- developmental toxicity;
- mutagenicity.

The results of the above mentioned tests, summarized here below, indicate that maximal concentrations of OPE (500 mg/mL of 0.5% w/v methylcellulose) at maximally feasible oral single or repeat dosages in rats (10

mL/kg body weight) are essentially devoid of toxicity. This 5000 mg/kg/day dosage is equivalent to a dose of 350 grams in a 70 kg human on a mg/kg basis.

### Acute studies

In the acute studies in rodents, a limit dosage of 2000 mg/kg produced no toxicity in mice, thus establishing the oral no-observable-adverse-effect level (NOAEL) in mice at 2000 mg/kg. In rats, an acute oral NOAEL of 1000 mg/kg was established, based on partial reductions in weight gains in both sexes at 5000 mg/kg, and reduced weight gains in female rats at 1500 and 2000 mg/kg. As described below, these findings were not replicated in repeat dosage studies, indicating that 2000 mg/kg/day is a more appropriate NOAEL.

### 90 day toxicity studies

In the repeat-dosage studies in rats, daily oral OPE dosages of 1000, 1500 and 2000 mg/kg/day produced small decreases in body weight gains at 2000 mg/kg/day in the male rats and in all groups of female rats. The decreased body weight gains were not statistically significant or dosage-related, and, therefore, were not considered to be a toxic phenomenon. Absorption was rapid in all cases, and peak levels (C<sub>max</sub>) were most frequent at 0.5 hours post-treatment. No remarkable differences were seen between sexes or in female rats when pregnant or lactating. Minimal levels (below the level of quantification) of hydroxytyrosol were found in fetal plasma, indicating that placental passage occurred. There were no detected levels of hydroxytyrosol in maternal milk or plasma from the nursing pups after 8 days of treatment (Christian, Sharper et al. 2004).

Other effects associated with OPE were dosage-related increases in red blood cells in females statistically and corpuscular hemoglobin concentration. These findings were interpreted as a slight but beneficial erythropoietic stimulation of the bone marrow.

In similar fashion, significant decreases in serum liver "leakage" enzymes (ALT: alanine aminotransferase, AST: aspartate aminotransferase and SDH: sorbitol dehydrogenase) that were observed in most of the OPE-treated groups of male and female rats were considered to be a beneficial, and not a toxic, manifestation. The reason for decreased activity could be associated with the biliary excretion of large dosages of OPE, due to the slightly decreased serum cholesterol levels in both male and female rats. Other biomarkers of liver function (serum bilirubin, alkaline phosphatase, protein levels and histopathology) were unaffected by OPE. Administration of 5000 mg/kg/day dosages of OPE for 29 consecutive days produced only decreased body weight gains, although hematology and serum chemistry determinations and histopathologic examination of tissues were not performed. Based on the lack of significant adverse OPE-related effects at 2000 mg/kg/day, a NOAEL of 2000 mg/kg/day was established for the repeat-dosage studies in rats.

### Reproduction studies

Dosages of OPE ranging from 500 to 2000 mg/kg/day did not adversely affect any of the mating and fertility parameters investigated in an oral rat dosage-range reproduction study. Delivery and litter parameters were not significantly affected by OPE. Adverse effects were also absent in a rat developmental toxicity study in which pregnant dams were treated with 1000, 1500 or 2000 mg/kg/day dosages of OPE on days 6 through 20 of gestation.

### In vivo Mutagenicity studies

In order to assess OPE mutagenicity potential *in vivo* a micronucleus assay was conducted on rats. Mutagenicity potential *in vivo* was evaluated not only after the usual single dosage regimen but also after repeated (28 consecutive days) dosages of 1000, 1500 or 2000 mg/kg/day. In addition, rats in a supplemental study were also evaluated after being treated with a 5000 mg/kg/day dosage of OPE for 29 consecutive days. The resulting evaluations of the single-dosage and the 5000 mg/kg/day dosage supplemental studies were both negative, indicating that OPE is not a mutagen under *in vivo* conditions. In summary, the studies described indicate that single or repeated dosages of OPE up to 2000 mg/kg/day have no adverse clinical, hematological, biochemical, reproductive, developmental or mutagenic effects except slight to moderate Toxicity of Aqueous Olive Pulp Extract

Minor effects as reductions of weight gain, when compared to controls. Focal areas of gastric hyperplasia, noted at 2000 mg/kg/day, and increased salivation after intubation were considered to be caused by the viscosity and granular consistency of the formulated suspension. Single and repeated OPE dosages of 5000 mg/kg/day were also devoid of mutagenic potential or adverse clinical signs, other than minimally decreased body weight gains.

**Polyphenols Pro-oxidative activities** (*discussion of any reports of investigations or other information that may appear to be inconsistent with the GRAS determination*)

Some authors proposed for certain polyphenolic species also pro-oxidant behaviors, *in vivo*. The ability of polyphenolic compounds to react with metal ions could make them pro-oxidant. These pro-oxidant activities are mentioned mainly for green tea polyphenols and very poor data concern with olive polyphenols. It has in fact been widely observed that caffeic acid, a simple polyphenol with an ortho-diphenolic structure, can have pro-oxidant activity on LDL oxidation induced by Cu<sup>2+</sup> (Yamanaka, Oda et al. 1997). However, this pro-oxidant activity has been found only in the propagation phase of oxidation, and not in the initiation phase, in which caffeic acid inhibits lipoprotein oxidation, as has been found in previous studies (Laranjinha, Almeida et al. 1994; Nardini, D'Aquino et al. 1995). Catechins, another class of polyphenols, oxidise readily in beverages [(Akagawa, Shigemitsu et al. 2003; Aoshima and Ayabe 2007; Lambert, Sang et al. 2010), such as green tea. They can also oxidize in cell culture media and even in the oral cavity; Often, these pro-oxidant effects involve interactions of polyphenols with transition metal ions (Halliwell 2008). However, in practice pro-oxidant effects can also be beneficial, since, by imposing a mild degree of oxidative stress, the levels of antioxidant defenses and xenobiotic-metabolising enzymes might be raised, leading to overall cytoprotection (Fahey and Kensler 2007).

No data are available on whether polyphenols are pro-oxidant *in vivo* in the human stomach, intestines, and colon, where they can be present at significant levels (Halliwell, Zhao et al. 2000; Kanner and Lapidot 2001; Manach and Donovan 2004; Jenner, Rafter et al. 2005). As for effects after absorption into the body, multiple well-designed human studies have been done using reliable biomarkers of oxidative damage in plasma (F<sub>2</sub>-isoprostanes) and urine (F<sub>2</sub>-isoprostanes, isoprostane metabolites, 8-hydroxy-2'-deoxyguanosine [8OHdG]), essentially testing for systemic antioxidant or pro-oxidant activity. The results have been reviewed in detail elsewhere (Halliwell, Rafter et al. 2005) and are quite variable, but overall no evidence for systemic pro-oxidant effects of polyphenols has emerged. A few studies report that administration of high doses of epigallocatechin gallate to animals leads to the formation of cysteine conjugates detectable in the urine, indicative of some degree of oxidation *in vivo* (Lambert, Sang et al. 2007). However, these effects may not be important at lower doses and may not be relevant to humans (Lambert, Sang et al. 2007).

**CONCLUSION:**

**Phenolea® Complex is an OPE (olive pulp extract) obtained from liquid olive pulp sourced from traced cultivation in this communication a thorough review of scientific data about safety of OPE Phenolea® Complex and its components as phenolic substances was performed.**

**The scientific literature reported that olive polyphenols in foods specialties are involved in:**

- **protection against oxidation phenomena on some blood markers as LDL (this activity is confirmed also by the EFSA),**
- **free radical scavenging;**

**Although for some polyphenolic species (green tea catechins, olive caffeic acid) and in particular conditions (presence of metal ions), pro-oxidant behaviors (production of ROS and H<sub>2</sub>O<sub>2</sub>) have been reported, the discussed scientific and technical data showed that, exist a consensus among experts qualified and food authorities on the matter that polyphenols are safe and devoid of any effect from toxicological point of view. The following "addendum" section summarize scientific data on biological activities and beneficial effects of olive polyphenols.**



Moreover brief history of safe consumption of foods containing polyphenols was provided, indeed some investigators reported that a normal consumption of olive and olive oil contributes with an average intake of about 400 and 12 mg of polyphenols respectively (Owen, Haubner et al. 2003). A comparison of polyphenols intake through foods containing maximum dosage of Phenolea®Complex (3000 ppm (w/v)) was performed revealing that the intake of polyphenols in 40g of black olives is substantially equivalent to a food containing Phenolea®Complex.

Toxicological Studies performed on rats and mice (Christian, Sharper et al. 2004) didn't show any relevant effect of toxicity in any of parameter considered for the evaluation, also for an OPE dosage of 5000 mg/kg is equivalent to a dose of 350 grams in a 70 kg human.

On the basis of aforementioned observations we can attribute to OPE Phenolea®Complex the status of GRAS for its intended use in foods.

## ADDENDUM

### BIOLOGICAL ACTIVITIES OF OLIVE POLYPHENOLS

#### Antioxidant Activity in humans.

The antioxidant properties of the olive polyphenols have been extensively studied and their effects are described in several reviews (Covas 2007) (Visioli, Poli et al. 2002) (Manna, Della Ragione et al. 1999) (Fito, de la Torre et al. 2007) (Visioli, 2002 #544). Olive polyphenols are potent radical scavengers and they inhibit the oxidation of lipids and of low-density lipoprotein (LDL) particle; their antioxidative properties have been demonstrated both *in vivo* and *in vitro*. Hydroxytyrosol, an ortho-diphenol, is considered to be a potent antioxidant due to its two adjacent hydroxyl groups. Hydroxytyrosol inhibits copper induced LDL oxidation (Visioli, Bellomo et al. 1995) while a mono-phenol such as tyrosol, has little antioxidant activity and does not protect LDL from chemically induced oxidation. Therefore, the olive oil polyphenols with a catechol moiety, such hydroxytyrosol or its derivatives are considered the major antioxidant in olive products, several investigations and studies support the antioxidant properties of hydroxytyrosol and its derivatives (Fito, Covas et al. 2000), (Schaffer, Podstawa et al. 2007) (Weinbrenner, Fito et al. 2004; Corona, Tzounis et al. 2006). Rietjens showed that hydroxytyrosol efficiently protects vascular tissue against oxidative stress (Rietjens, Bast et al. 2007). Hydroxytyrosol was also shown to reduce oxidative damage in intestinal epithelial cells (Manna, D'Angelo et al. 2002) hepatocytes (Goya, Mateos et al. 2007), and human erythrocytes (Manna, Galletti et al. 1999).

Hydroxytyrosol is an efficient radical scavenger (superoxide anion, hydroxyl radical, peroxynitrite) and has metal chelating capacities; it efficiently protects against LDL oxidation *in vitro* at relatively low concentrations. The data show that hydroxytyrosol is a potent inhibitor of lipid peroxidation which is considered to be one of the main mechanisms of tissue damage by free radicals. Finally, the antioxidant properties of olive polyphenols were also demonstrated *in vivo* in animal models (Visioli, Galli et al. 2000), (Deiana, Rosa et al. 2007). Thus, olive polyphenols and particularly hydroxytyrosol clearly can reduce oxidative damage *in vitro* and *in vivo* and protect cells from oxidative damage.

#### Markers of oxidation in Humans

Oxidized low density lipoprotein (oxLDL) are modified LDL particles exhibiting proatherogenic, proinflammatory, and highly immunogenic activities.

They play a key role in development atherosclerosis, coronary heart disease (CHD) (70-72). High concentrations of oxLDL are predictive of future CHD also in apparently healthy subjects and are related to metabolic syndromes. Plasma oxLDL levels were measured in 6 randomized, placebo-controlled, cross-over studies ((Marrugat, Covas et al. 2004), (Gimeno, de la Torre-Carbot et al. 2007), (Covas, Nyyssonen et al. 2006), (Salvini, Sera et al. 2006), (Covas, de la Torre et al. 2006), (Fito, Cladellas et al. 2008), (Weinbrenner, Fito et al. 2004)). Several studies investigated the effects of olive polyphenols on postprandial oxidative stress, which is linked with postprandial lipemia and hyperglycemia. These studies consistently showed that plasma levels of oxLDL level was observed in the groups with a higher intake of olive polyphenols. Moreover, in 5 of the 6 studies a significant decrease was observed in oxLDL levels between the low- and the high olive polyphenols groups. The Euroolive study (Covas, Nyyssonen et al. 2006), performed in 200 healthy subjects from five European countries is the largest clinical study showing that olive polyphenols decreased biomarkers of lipid oxidative damage, such as plasma oxLDL, conjugated dienes, and hydroxyl fatty acids and provided good evidence for the antioxidant activity of olive polyphenols increased plasma hydroxytyrosol levels, showing that olive polyphenols are systemically available. The concentration of phenolic compounds in LDL was directly correlated with the phenolic concentration in the olive oils tested. Moreover, the increase in the phenolic content of LDL could account for the increase in resistance of LDL to oxidation, and the decrease of *in vivo* oxLDL, observed in the study.

The doses of olive phenolics showing an effect on plasma oxLDL levels ranged from 4 to 20 mg per day; consistent significant effects were observed with doses of about 10 mg per day of total olive phenolic compounds. Hydroxytyrosol or derivatives thereof represent about 50 % of the olive phenolic compounds. The data clearly support the protective effects of olive polyphenols against LDL oxidation.

F2- isoprostanes are produced by nonenzymatic, free. Radical-catalyzed peroxidation of arachidonic acid. They are considered as markers of lipid peroxidation and can also exert potent biological actions. They can

be quantified in human body fluids such as plasma and urine. In several studies a trend toward a decrease in plasma isoprostane levels was observed with increasing intake of olive polyphenols (Covas, Nyyssonen et al. 2006). The olive polyphenols also tended to decrease the postprandial rise in plasma isoprostane levels (Covas, de la Torre et al. 2006). However a significant decrease in plasma isoprostane levels between the groups receiving a low phenolic diet a a high phenolic diet was only observed in one study from Ruano et al (Ruano, Lopez-Miranda et al. 2005). No effect was observed by Weinbrenner et al (Weinbrenner, Fito et al. 2004) in a small study. In another open study Leger et al (Leger, Carbonneau et al. 2005) reported that an olive polyphenolic extract had no effect on urinary isoprostane levels but significantly lowered serum thromboxane levels. Overall, the studies suggest that a modest effects on plasma isoprostane levels may be observed after ingestion of olive polyphenols.

### Hydroxytyrosol *in vivo* and *in vitro* activities studies

Within the total pool of olive polyphenols hydroxytyrosol is the most abundant (50% of total polyphenols).

It is claimed that hydroxytyrosol is formed in part as a result of hydrolysis of oleuropein (the major biophenol in many olive varieties) during oil extraction by the action of esterases (Capasso, Evidente et al. 1999). Moreover, the amount of hydroxytyrosol can be enriched by acid hydrolysis of secoiridoid derivatives and verbascoside. Recently, it has been made available commercially for research purposes and has also been introduced under different trade names as an anti- oxidant nutraceutical. Hydroxytyrosol is peculiar to olives (and, hence, to olive oil) and is being exploited as a potential supplement or preservative to be employed in the nutraceutical, cosmeceutical, and food industry.

The biological activities of hydroxytyrosol can be conveniently summarized as follows:

1) **Antioxidant activity.** Hydroxytyrosol is a potent inhibitor of metal-induced oxidation of low density lipoprotein (see above). In addition, metal-independent oxidation is also significantly retarded by hydroxytyrosol. The antioxidant activities of hydroxytyrosol, which has been proven to be more effective than BHT or vitamin E, were further confirmed, by the use of stable free radicals, such as DPPH. Also, hydroxytyrosol is a scavenger of superoxide anions generated by either human polymorphonuclear cells or by the xanthine/xanthine oxidase system. Furthermore, a scavenging effect of hydroxytyrosol was demonstrated with respect to hypochlorous acid, a potent oxidant produced *in vivo* at the site of inflammation and a major component of chlorine-based bleaches that can often come into contact with food during manufacturing. Antioxidant activities have also been demonstrated versus DNA damage, hydrogen peroxide-induced insult to red blood cells. These results have been discussed above and can be found in several reviews, e.g. (Perez-Jimenez, Alvarez de Cienfuegos et al. 2005).

The signaling pathways involved in the biological activities of hydroxytyrosol are being elucidated (Corona, Deiana et al. 2009; Incani, Deiana et al. 2010) and will likely be the subject of several future investigations.

2) **Modulation of enzymes.** Hydroxytyrosol is able to modulate several enzymatic activities linked to cardiovascular disease. Among them, inhibition of platelet aggregation (Petroni, Blasevich et al. 1995) and pro- inflammatory enzymes such as 5-lipoxygenase (Kohyama, Nagata et al. 1997; Petroni, Blasevich et al. 1997; de la Puerta, Ruiz Gutierrez et al. 1999), and stimulation of the inducible form of nitric oxide synthase [(Visioli, Bellosta et al. 1998) have been demonstrated *in vitro*. In *in vitro* models, hydroxytyrosol is not able to upregulate the activity of the endothelial form of nitric oxide synthase, leaving its role in modulation of vasomotion unresolved (Schmitt, Handler et al. 2007). Notably, the vasomodulating effects of olive leaves extracts have been demonstrated in a rabbit model (Zarzuelo, Duarte et al. 1991), but the human relevance of these findings needs further investigations.

While the majority of data have been obtained *in vitro*, several experiments have been performed in laboratory animals. In addition, there are approximately 15 human experiments that compared olive oil with extra virgin olive oil (which, however, contains phenols other than hydroxytyrosol) (Covas 2007). Finally, hydroxytyrosol and related olive phenols have been tested, as supplements, in humans. The most notable result is the inhibition of thromboxane B2 production by whole blood, suggesting antithrombotic activity *in vivo* (Leger, Carbonneau et al. 2005).

Animal experiments confirm, *in vivo*, most of the evidence obtained *in vitro*. In particular, hydroxytyrosol retains its antioxidant activity once ingested (though the human metabolic pathway has been elucidated and shows extensive glucuronidation and subsequent urinary excretion) (Visioli, Caruso et al. 2001), protects from second hand smoke induced oxidative damage (Visioli, Galli et al. 2000), inhibits platelet aggregation (Priora, Summa et al. 2008), ameliorates lipid profile and decreases atherosclerosis development (Gonzalez-Santiago, Martin-Bautista et al. 2006), increases brain cell resistance to oxidation and mitochondrial membrane potential [(Schaffer, Podstawa et al. 2007). Further experiments confirmed hydroxytyrosol's anti-inflammatory (Bitler, Viale et al. 2005), and anti-thrombotic potential (Leger, Carbonneau et al. 2005), and its ability to ameliorate osteoarthritis (Bitler, Matt et al. 2007). As a caveat, such experiments have been performed with mixtures of olive phenols in which Hydroxytyrosol was the most active ingredient, but not the exclusive one. Synergy with other olive phenols cannot, at present, be excluded.

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