Nova Mentis
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December 18, 2018

837

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

Sub: GRAS notice for pure hydroxytyrosol

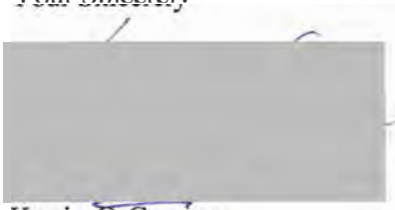
Dear Sir/Madame,

In accordance with 21 CFR §170 Subpart E consisting of §170.203 through 170.285, Nova Mentis Ltd is submitting a GRAS notification for high purity (>99% pure) hydroxytyrosol produced by biotechnological means for its intended use as a food ingredient on the basis of Scientific procedure. Therefore, the use of the hydroxytyrosol as described in this GRAS Notification is exempt from the requirement of premarket approval as set forth in the Federal Food, Drug, and Cosmetic Act.

Information supporting the GRAS status of the high-purity hydroxytyrosol and the completed FDA from 3667 are enclosed for your review. The electronic files are submitted in a USB drive and a CD ROM. I hereby certify that the enclosed electronic files were scanned for viruses prior to submission and are thus certified as being virus-free using Sophos Ltd Antivirus software version 9.7.7 threat detection engine 3.74.0.

If you have any questions or require additional information please do not hesitate to contact the undersigned at your convenience.

Your Sincerely



Kevin O Connor

CSO Nova Mentis

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

GENERALLY RECOGNIZED AS SAFE (GRAS) NOTICE (Subpart E of Part 170)

Form Approved: OMB No. 0910-0342; Expiration Date: 09/30/2019
(See last page for OMB Statement)

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
000837	1/28/2019
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

Transmit completed form and attachments electronically via the Electronic Submission Gateway *(see Instructions)*; OR Transmit completed form and attachments in paper format or on physical media to: Office of Food Additive Safety *(HFS-200)*, Center for Food Safety and Applied Nutrition, Food and Drug Administration,5001 Campus Drive, College Park, MD 20740-3835.

SECTION A – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

1. Type of Submission *(Check one)*

☒ New ☐ Amendment to GRN No._____ ☐ Supplement to GRN No. _____

2. ☒ All electronic files included in this submission have been checked and found to be virus free. *(Check box to verify)*

3 Most recent presubmission meeting *(if any)* with FDA on the subject substance *(yyyy/mm/dd)*: 2018-11-20

4 For Amendments or Supplements: Is your amendment or supplement submitted in response to a communication from FDA? *(Check one)* ☐ Yes ☐ No If yes, enter the date of communication *(yyyy/mm/dd)*: _____

SECTION B – INFORMATION ABOUT THE NOTIFIER

1a. Notifier

Name of Contact Person	Position or Title
Kevin O Connor	Chief Scientific officer

Organization *(if applicable)*: Nova Mentis ltd

Mailing Address *(number and street)*: Nova UCD Belfield innovation Park

City	State or Province	Zip Code/Postal Code	Country
Dublin		D04 V1W8	Ireland

Telephone Number	Fax Number	E-Mail Address
+353 85 7227580		koconnor@novamentis.eu

1b. Agent or Attorney *(if applicable)*

Name of Contact Person	Position or Title

Organization *(if applicable)*:

Mailing Address *(number and street)*:

City	State or Province	Zip Code/Postal Code	Country

Telephone Number	Fax Number	E-Mail Address

SECTION C – GENERAL ADMINISTRATIVE INFORMATION

1. Name of notified substance, using an appropriately descriptive term

Hydroxytyrosol

2. Submission Format: *(Check appropriate box(es))*

☐ Electronic Submission Gateway
☒ Electronic files on physical media
☒ Paper

If applicable give number and type of physical media
1 USB key

3. For paper submissions only:

Number of volumes 1

Total number of pages 88

4. Does this submission incorporate any information in CFSAN's files? *(Check one)*

☒ Yes *(Proceed to Item 5)* ☐ No *(Proceed to Item 6)*

5. The submission incorporates information from a previous submission to FDA as indicated below *(Check all that apply)*

☐ a) GRAS Notice No. GRN 726
☐ b) GRAS Affirmation Petition No. GRP
☐ c) Food Additive Petition No. FAP
☐ d) Food Master File No. FMF
☐ e) Other or Additional *(describe or enter information as above)* Information from GRN 600 and GRN 726

6. Statutory basis for conclusions of GRAS status *(Check one)*

☒ Scientific procedures *(21 CFR 170.30(a) and (b))* ☐ Experience based on common use in food *(21 CFR 170.30(a) and (c))*

7. Does the submission (including information that you are incorporating) contain information that you view as trade secret or as confidential commercial or financial information? (see 21 CFR 170.225(c)(8))

☐ Yes *(Proceed to Item 8*
☒ No *(Proceed to Section D)*

8. Have you designated information in your submission that you view as trade secret or as confidential commercial or financial information *(Check all that apply)*

☐ Yes, information is designated at the place where it occurs in the submission
☐ No

9. Have you attached a redacted copy of some or all of the submission? *(Check one)*

☐ Yes, a redacted copy of the complete submission
☐ Yes, a redacted copy of part(s) of the submission
☐ No

SECTION D – INTENDED USE

1. Describe the intended conditions of use of the notified substance, including the foods in which the substance will be used, the levels of use in such foods, and the purposes for which the substance will be used, including, when appropriate, a description of a subpopulation expected to consume the notified substance.

Nova Mentis intends to use hydroxytyrosol as an antioxidant [21 CFR 170.3(o)(3)] in 11 broad food categories: bakery products; beverages; dairy products and substitutes; desserts; fats and oils; fruit juices and nectars; dry seasoning mixes for meat, poultry and fish; chewing gum; sauces, dips, gravies and condiments; snacks; and vegetable juices to deliver 5 to 10 mg of hydroxytyrosol per serving.

2. Does the intended use of the notified substance include any use in product(s) subject to regulation by the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture?

(Check one)

☐ Yes ☒ No

3. If your submission contains trade secrets, do you authorize FDA to provide this information to the Food Safety and Inspection Service of the U.S. Department of Agriculture?

(Check one)

☐ Yes ☐ No , you ask us to exclude trade secrets from the information FDA will send to FSIS.

SECTION E – PARTS 2 -7 OF YOUR GRAS NOTICE

(check list to help ensure your submission is complete – PART 1 is addressed in other sections of this form)

☒ PART 2 of a GRAS notice: Identity, method of manufacture, specifications, and physical or technical effect (170.230).

☒ PART 3 of a GRAS notice: Dietary exposure (170.235).

☒ PART 4 of a GRAS notice: Self-limiting levels of use (170.240).

☒ PART 5 of a GRAS notice: Experience based on common use in foods before 1958 (170.245).

☒ PART 6 of a GRAS notice: Narrative (170.250).

☒ PART 7 of a GRAS notice: List of supporting data and information in your GRAS notice (170.255)

Other Information

Did you include any other information that you want FDA to consider in evaluating your GRAS notice?

☐ Yes ☒ No

Did you include this other information in the list of attachments?

☐ Yes ☐ No

SECTION F – SIGNATURE AND CERTIFICATION STATEMENTS

1. The undersigned is informing FDA that Kevin O Connor

(name of notifier)

has concluded that the intended use*(s)* of Hydroxytyrosol

(name of notified substance)

described on this form, as discussed in the attached notice, is (are) not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on your conclusion that the substance is generally recognized as safe recognized as safe under the conditions of its intended use in accordance with § 170.30.

2. Kevin O Connor (Nova Mentis) agrees to make the data and information that are the basis for the conclusion of GRAS status available to FDA if FDA asks to see them;

(name of notifier)

agrees to allow FDA to review and copy these data and information during customary business hours at the following location if FDA asks to do so; agrees to send these data and information to FDA if FDA asks to do so.

Nova UCD Belfield innovation park, UCD, Dublin D04 V1W8 Ireland

(address of notifier or other location)

The notifying party certifies that this GRAS notice is a complete, representative, and balanced submission that includes unfavorable, as well as favorable information, pertinent to the evaluation of the safety and GRAS status of the use of the substance.The notifying party certifies that the information provided herein is accurate and complete to the best or his/her knowledge. Any knowing and willful misinterpretation is subject to criminal penalty pursuant to 18 U.S.C. 1001.

3. Signature of Responsible Official, Agent or Attorney	Printed Name and Title	Date (mm/dd/yyyy)
[redacted]	Kevin O Connor CSO	12/13/2018

SECTION G – LIST OF ATTACHMENTS

List your attached files or documents containing your submission, forms, amendments or supplements, and other pertinent information. Clearly identify the attachment with appropriate descriptive file names (or titles for paper documents), preferably as suggested in the guidance associated with this form. Number your attachments consecutively. When submitting paper documents, enter the inclusive page numbers of each portion of the document below.

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
1	Insert / Clear — Nova Mentis GRAS Dossier 181213.pdf	Submission
2	Insert / Clear — Nova Mentis GRAS Dossier cover letter signed Kevin.pdf	Submission
3	Insert / Clear — US FDA GRAS form 3667 20181217.pdf	Submission
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Add Continuation Page

OMB Statement: Public reporting burden for this collection of information is estimated to average 170 hours per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to: Department of Health and Human Services,Food and Drug Administration, Office of Chief Information Officer, PRAStaff@fda.hhs.gov. (Please do NOT return the form to this address.). An agency may not conduct or sponsor, and a person is not required to respond to, a collection of information unless it displays a currently valid OMB control number.

Summary of Information Supporting the Generally Recognized As Safe (GRAS) Status of Hydroxytyrosol (>99 % pure) for Use as an Ingredient in Selected Foods

Prepared by Nova Mentis

Nova UCD, Belfield innovation park, Dublin 4 D04 V1W8
Tel: +353857227580

December 18, 2018

Summary of Information Supporting the Generally Recognized As Safe (GRAS) Status of Hydroxytyrosol (>99 % pure) for Use as an Ingredient in Selected Foods

Table of Contents **Page number**

1. STATEMENT AND CERTIFICATION

1.1. Compliance with 21 C.F.R. § 170.30

Nova Mentis, Ireland has determined that hydroxytyrosol (> 99% pure) manufactured using recombinant *Escherichia coli* BL21 (DE3) #145 strain as a processing aid 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. This determination is in compliance with proposed Sec. 170.30 of Part 21 of the Code of Federal Regulations (21 CFR § 170.30), as published in the Federal Register, Vol. 62, No. 74, FR 18937, April17, 1997

1.2. Name and Address of Notifier

Kevin O Connor, Ph.D.
Director
Nova Mentis
Nova UCD, Belfield, Dublin 4
Tel: +353 85 7227580
Email: koconnor@novamentis.eu

1.3. Chemical names and common or usual name of the notified substance

Hydroxytyrosol (abbreviation: HT) is known by the chemical names 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). The common name of the substance of this notification is hydroxytyrosol.

1.4. Intended conditions of use and technical effect

Nova Mentis intends to use hydroxytyrosol as an antioxidant in 11 broad food categories: bakery products; beverages; dairy products and substitutes; desserts; fats and oils; fruit juices and nectars; dry seasoning mixes for meat, poultry and fish; chewing gum; sauces, dips, gravies and condiments; snacks; and vegetable juices to deliver 5 to 10 mg of hydroxytyrosol per serving of food.

1.5. Basis for GRAS Determination

In accordance with 21 CFR170.30, the intended use of hydroxytyrosol has been determined to be Generally Recognized As Safe (GRAS) based on scientific procedures. In the present GRAS dossier, Nova Mentis provides detailed information about the identity and specifications for hydroxytyrosol, batch analyses, the manufacturing process, the intended foods and use levels.

A comprehensive search of the scientific literature for safety and toxicity information on hydroxytyrosol was conducted through September 2018. 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 support the safe 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 and that up to 100 mg per day was safe for adults and 200 mg was safe for older adults. Since January 1, 2018 hydroxytyrosol is approved for use in oils and spreadable fats in the European Union. Chemically produced pure (>99%) hydroxytyrosol was GRAS notified to FDA (GRN 600) for use as an antioxidant in beverages, fats and oils, fresh and processed fruits and vegetables, fresh and processed fruit and vegetable juices, and gravies and sauces at a level of 5 milligrams (mg) per serving. FDA had no questions at the time of submission. Also an olive preparation containing 40% hydroxytyrosol was GRAS notified to FDA (GRN726) for use in bakery products; beverages; dairy products and substitutes; desserts; fats and oils; fruit juices and nectars; dry seasoning mixes for meat, poultry and fish; chewing gum; sauces, dips, gravies and condiments; snacks; and vegetable juices to deliver 5 to10 mg of hydroxytyrosol per serving of food

1.6. Availability of Information

The data and information that forms the basis for this GRAS determination will be provided by Nova Mentis to Food and Drug Administration upon request

1.7. Signature

NOVA MENTIS hereby makes and submits this notice of a GRAS
Exemption Claim for hydroxytyrosol under the intended conditions of use

Prof. Kevin O'Connor
CSO
Nova Mentis

December 18, 2018

2. IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS, AND PHYSICAL OR TECHNICAL EFFECT

2.1. Information about the Identity of the notified substance:

Hydroxytyrosol is a standardized off-white powder obtained by biological synthesis according to a well-established process protocol (described in method of manufacture). The product is chemically pure and contains >99% hydroxytyrosol. The characteristics of the hydroxytyrosol product are described in Table 1 and the chemical structure is shown in Figure 1. Hydroxytyrosol is a phenylethanoid, a type of phenolic phytochemical believed to be one of the most powerful natural antioxidants. Hydroxytyrosol is naturally present in olives and olive oil along with other polyphenols.

Table 1. Characteristics of hydroxytyrosol

Parameter	Description
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	Off-white powder
Solubility	Soluble in water in all proportions; Highly soluble in polar organic solvents
Color	Off-White
Odor	Mild
Taste	Mild bitterness
Molecular weight	154.16
Chemical formula	$C_8H_{10}O_3$
Melting point	54ºC
Storage	Store in the dark at 4-8ºC; protect from oxidizing atmospheres
Stability	At least three years under recommended storage conditions

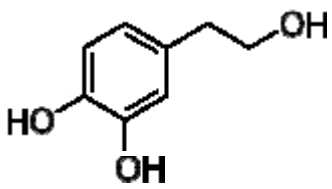

Figure 1: Chemical structure of Hydroxytyrosol

2.2. Manufacturing process of hydroxytyrosol

Nova Mentis hydroxytyrosol is a biosynthesized, purified, fermentation product manufactured using a non-pathogenic commensal strain of *Escherichia coli* designated as *E. coli* BL21 (DE3) #145, expressing a tyrosinase enzyme, as a processing aid. The production step (Figure 2) involves cultivation of biocatalyst *E. coli* BL21(DE3) #145 to a desired cell density, cell harvest to remove fermentation media components, washing of cells to remove any trace contamination of media components, breakage of cells by homogenization to produce a broken cell preparation (biocatalyst), conversion of tyrosol substrate to hydroxytyrosol using the biocatalyst as a processing aid, followed by centrifugation to remove the biocatalyst after the reaction is completed. Downstream processing of hydroxytyrosol from the biotransformation liquid was carried out by simple liquid-liquid extraction followed by rotary evaporation. Only food grade chemicals or high-grade pure chemicals, solvents and processing aids are used in the manufacture of Nova Mentis hydroxytyrosol.

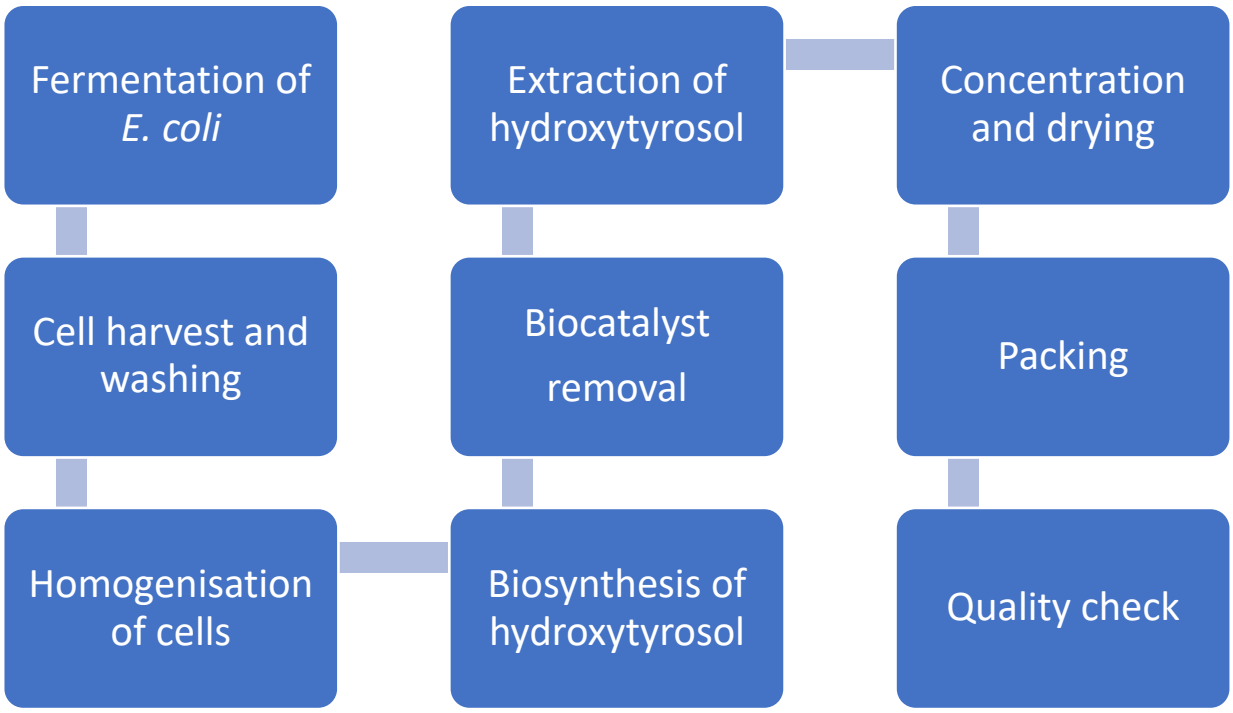

Figure 2: Flow diagram of the Nova Mentis hydroxytyrosol production process

2.2.1. Construction of the production strain

The oxidoreductase gene *(tyrosinase: E.C.1.14.18.1)* (GenBank NP518458) was amplified by polymerase chain reaction from the genomic DNA of *Ralstonia solanacearum* and the gene was cloned into a T7 promoter expression vector, pRSET B (Invitrogen, Figure 3) and the resultant plasmid was designated pRTYR-AmpR. Expression of the oxidoreductase gene from pRSET B is under the control of the lactose repressor gene (*lacI*), and therefore expression of the gene (and synthesis of the enzyme) occurs following induction with isopropyl thio-β-galactoside (IPTG), a lactose analog. The ampicillin resistance gene on the pRSETB plasmid was replaced with the kanamycin resistance gene using Gibson Assembly® Cloning Kit (New England Biolab) and the

resultant plasmid designated as pRTYR-KanR (Figure 4) was then transformed into *E. coli* BL21(DE3) and the resultant production strain was designated as *E. coli* BL21(DE3)#145. *E. coli* BL21 (DE3) #145 was grown on nutrient medium containing the antibiotic kanamycin for maintenance of the plasmid construct. The genomic stability of the organism is maintained using master and working cultures, and the fermentation procedure is subject to strict quality control measures.

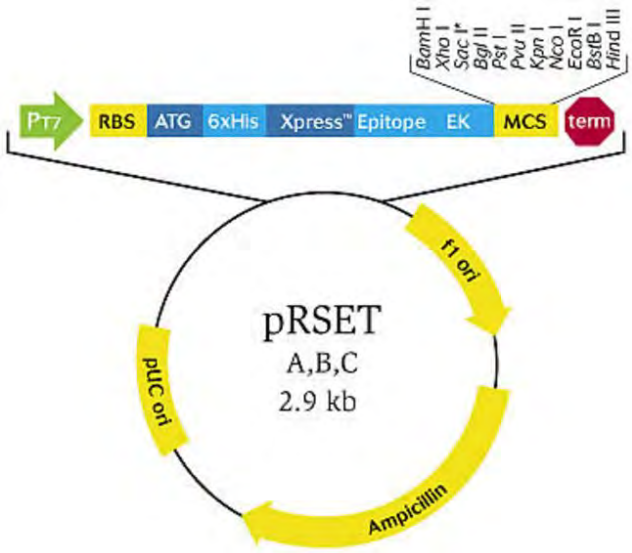

Figure 3: The plasmid pRSETB

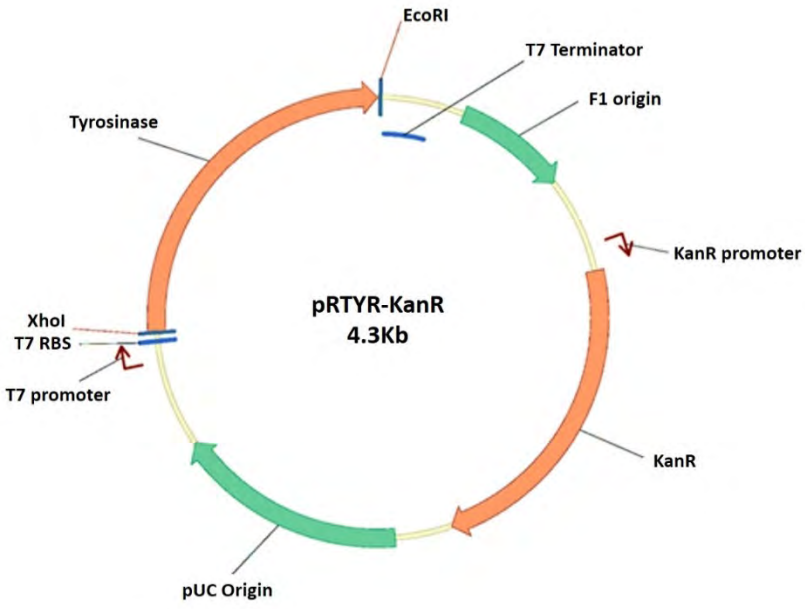

Figure 4: pRTYR-KanR construct (Ampicillin gene replaced with Kanamycin gene)

2.2.2. Characteristics and safety of the host microorganism

E. coli BL21 (DE3) #145 was used as processing aid for the efficient synthesis of Hydroxytyrosol. The parent strain *E. coli* strain BL21 (DE3) is a common laboratory strain used for expression of biotechnology products. The strain was obtained from Invitrogen and has an

established and verifiable identity. The complete gene sequence of *E. coli* BL21(DE3) was published by Jeong et al. (2009), and comprehensive bioinformatics analyses of the organism are described by Studier et al. (2009).
E. coli was first identified in the stool of breastfed infants (Escherich, 1885) and the *E. coli* B strain was isolated from apparently normal commensals of the human gut by d'Herelle in 1918 (Daegelen et al., 2009). This bacterium is better characterised at the genetic level than any other microorganism. Laboratory strains of *E. coli* were among the first organisms to have complete genome sequences published and they are widely used models of bacterial physiology, genetics, biochemistry, molecular biology and now systems biology (Blattner et al., 1997; Hayashi et al., 2006). The first biopharmaceutical product produced in genetically modified cells was manufactured by the fermentation of an engineered *E. coli* B strain (recombinant insulin, marketed as Humulin, approved in 1982). *E. coli* BL21 (DE3) does not carry the well-recognized pathogenic components required by *E. coli* strains that cause most enteric infections. *E. coli* BL21 (DE3) is therefore considered to be non-pathogenic and unlikely to survive in host tissues or to cause disease (Chart et al., 2000). The genome sequence of *E. coli* BL21 (DE3) revealed the absence of genes encoding invasion factors, adhesion molecules and enterotoxins associated with virulence (Jeong et al., 2009). The strain is widely used in the biopharmaceutical industry for the manufacture of recombinant pharmaceutical proteins and used in the manufacture of at least two ingredient intended for use as a food ingredient that is "generally recognized as safe" (GRAS) (FDA GRN 485 and GRN571).

2.2.3. Taxonomic information and genotype on the parental organism

Domain: Bacteria
Kingdom: Bacteria
Phylum: Proteobacteria
Class: Gamma-Proteobacteria
Order: Enterobacteriales
Family: Enterobacteriaceae
Genus: *Escherichia*
Species: *Escherichia coli*
Strain: *Escherichia coli* BL21 (DE3)
Commercial name: The *E. coli* BL21 (DE3) strain was purchased from Invitrogen. *E. coli* is a facultative anaerobic enterobacterium that can grow in the presence and absence of oxygen. The genotype of *E. coli* BL21 (DE3) is F– *omp*T *hsd*SB *(rB –mB–) gal dcm* (DE3). The *E. coli* BL21 (DE3) strain was developed for T7 RNA polymerase-based gene expression by introducing a lambda prophage containing a T7 RNA polymerase under the control of the *lac*UVA promoter. The integration of the lambda prophage generated a Gal– phenotype. The T7 RNA polymerase enables the high-level inducible expression of genes driven by the T7 promoter. The expression of the T7 RNA polymerase in *E. coli* BL21 (DE3) is usually achieved by the addition of 0.1–1 mM IPTG.

2.2.4. Source and habitat of the parental microorganism

E. coli is a ubiquitous inhabitant of the mammalian colon. The ancestor of the *E. coli* B strain used in our *E. coli* fermentation process was derived from apparently normal commensals of the

human gut by d'Herelle at the Institut Pasteur, Paris, 1918 (Daegelen et al., 2009). The *E. coli B* strain has been used in the laboratory for almost 100 years, and was developed for T7 RNA polymerase-based gene expression by DE3 prophage integration, UV treatment and selection. The resulting *E. coli* BL 21 (DE3) strain is commercially available and has become established as a host for recombinant protein expression worldwide.

2.2.5. Raw Materials/Processing-aids for growth of *E. coli* BL21 (DE3) #145

The *E. coli* was grown to high cell density using a fed batch fermentation system, and the fermentation medium contains de-ionised water, yeast extract, tryptone, dipotassium phosphate (K_2HPO_4), dihydrogen potassium phosphate KH_2PO_4) citric acid, minerals, pH regulators, kanamycin as the antibiotic, IPTG as inducer, and glucose as a carbon sources (Table 2). Kanamycin, IPTG, minerals, glucose and magnesium sulphate are added to the media post-sterilization. Kanamycin is used during fermentation to maintain stable growth of the recombinant strain during fermentation, and IPTG is used to induce expression of the tyrosinase gene. The suitable use of IPTG and antibiotics during fermentation of enzyme preparations has been reviewed previously (GRN 126, GRN 485). The removal of residual fermentation chemicals in the processing aid is achieved by centrifugation and washing. The trace mineral solution was used as a growth supplement for bacterial growth. The trace mineral solution contains cobalt chloride ($CoCl_2 \cdot 6H_2O$) as an ingredient. The complete removal of culture broth by centrifugation and washing steps ensures that all the additives in the media are removed and not carried over to the next step of production process as can be verified from the batch quality data on Cobalt and Kanamycin of the final product. Furthermore no inducer, antibiotics or minerals were used during the biosynthesis step of the final product hydroxytyrosol. All raw materials and processing-aids are food grade or high grade pure chemicals in accordance with FDA regulation and where applicable comply with limits established in the Food Chemicals Codex (FCC).

Table 2. Media Components for the growth of Bacteria

Components	Stock solution	Final concentration
Yeast extract		5g/L
Tryptone		10g/L
K_2HPO_4		5g/L
KH_2PO_4		3g/L
$(NH_4)_2SO_4$		1.6g/L
Citric acid		1.7g/L
Glucose feed	700 g glucose and 12.5 g $MgSO_4 \cdot 7H_2O$ per liter	20g/L
Trace mineral solution #		3mL/Litre
Inducer	1 Molar solution of IPTG	1 mM
Antibiotic	Kanamycin 50 mg/mL	50 microgram/mL

#*The Trace mineral solution:* Dissolve the following trace elements in 1L 1NHCl and store at room temperature: 20.0 g FeSO4·7H2O, 6.0 g CaCl2·2H2O, 4.4 g ZnSO4·7H2O, 1.0 g MnSO4·4H2O, 0.6g H3BO3, 0.4 g CoCl2·6H2O, 0.3 g Na2MoO4·2H2O, 0.04 g NiCl2·6H2O, 2.0 g CuSO4·5H2O

2.2.6. Manufacturing process steps

Hydroxytyrosol is manufactured according to a well-established process protocol at Nova Mentis, Ireland.

2.2.6.1. Fermentation to grow *E. coli*

The manufacturing flow scheme describing the fermentation process to produce the processing aid (biocatalyst *E. coli* BL21 (DE3) #145) and manufacture of hydroxytyrosol and its downstream processing are represented in Figure 2 and 5. As mentioned above, the production strain is grown in an aqueous solution containing appropriate sources of carbon, nitrogen, mineral salts and other miscellaneous organic and inorganic compounds together with 50 µg/mL of the antibiotic, kanamycin. A series of control measures are in place to prevent contamination by foreign microorganisms and to ensure that the fermentation conditions remain optimal. These measures include storage of the culture bank at -80°C, the control and limiting of sub-culturing, the preparation of seed cultures under aseptic conditions, the sterilization of fermenters and interconnecting lines prior to use, the monitoring and control of pH, temperature, and dissolved oxygen, and the use of food grade or high-grade pure chemicals.

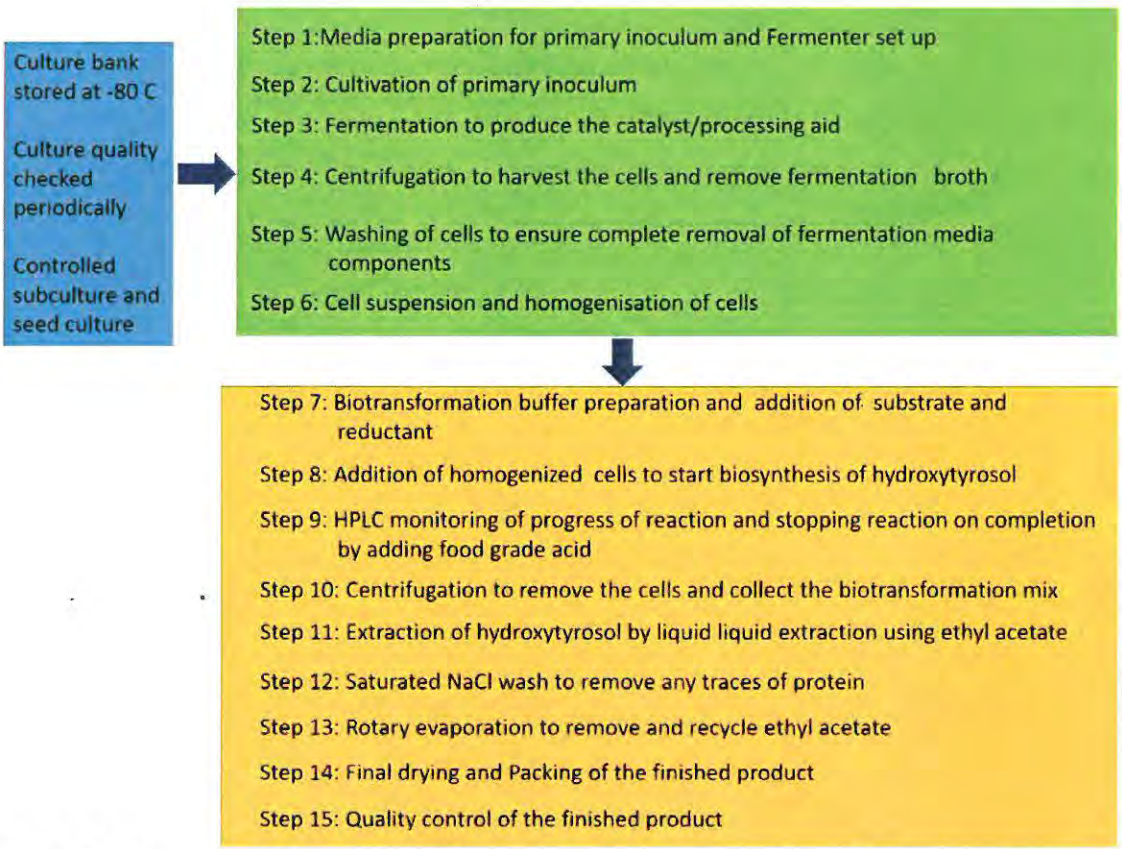

Figure 5. A stepwise flow chart of the Nova Mentis hydroxytyrosol manufacturing process

The temperature is maintained at 37 ± 0.1°C automatically at the beginning of the cultivation. The pH is automatically controlled at 7 +/- 0.1 by the addition of acid and base; foaming is controlled by manual addition of antifoam (polypropylene glycol P2000) when required. The fed batch fermentation is started with addition of 10% primary inoculum. The OD_{600} is monitored every hour and once the OD reaches 10-14 the temperature is reduced to 30± 0.1°C and expression of the tyrosinase gene is induced with IPTG. Pulse feeds of glucose are added manually when needed, based on the increase of DO value. Fermentation is stopped when a certain cell density is reached (80-90 g/L wet weight basis). The cells are harvested by centrifugation and any waste culture or broth from the fermenter is autoclaved and disposed appropriately. The harvested cells are suspended in phosphate buffer for washing and centrifuged to remove the phosphate buffer containing any media components like antibiotics, inducer, sugar and minerals. The washing steps ensured the complete removal of media components as can be seen from the batch analysis results. The washed and harvested cells are then subjected to homogenization. The homogenized cells are stored under refrigeration condition until used as the processing aid (biocatalyst).

2.2.6.2. Biosynthesis of hydroxytyrosol

As mentioned above, the homogenized cells of *E. coli* BL21 (DE3) # 145 are used as a processing aid (biocatalyst) for the conversion of tyrosol into hydroxytyrosol in an aqueous buffer (50mM potassium phosphate buffer) at 30°C and at a pH of 7.0. No antibiotics, inhibitors, or inducers

are used in the biosynthetic production process of hydroxytyrosol. Food grade ascorbic acid is used as a reducing agent. Tyrosol and ascorbic acid are dissolved in phosphate buffer and added into the bioreactor. Homogenized cells are added to the fermenter to start the biosynthesis. Antifoam is added manually when needed to prevent any foaming. After the biotransformation is completed food grade acid is added and the biotransformation mix is centrifuged to remove any homogenized cells (clarification). The resultant clarified reaction medium containing hydroxytyrosol is retained for downstream processing.

2.2.6.3. Downstream processing of hydroxytyrosol

Hydroxytyrosol from the clarified acidified reaction medium is extracted by 2 sequential batch extractions using 2 volume of ethyl acetate and separating the phases. The organic phase is washed with saturated NaCl solution (20% v/v) and later evaporated by rotary evaporation and finally freeze dried to remove traces of water and solvent. The finished product is packed in food grade containers and stored at 4-8 ^{o}C. The production procedure assures a consistent and high-quality product. Ethyl acetate, a class 3 solvent is one of the extraction solvents used in compliance with good manufacturing practice for all uses according to "2009/32/EC of the European parliament and the council" on the approximation of the laws of the Member States on extraction solvents used in the production of foodstuffs and food ingredients. The suitable use of ethyl acetate for food application and hydroxytyrosol extraction has been reviewed in GRN 600.

2.3. Specification and Identity of the Nova Mentis hydroxytyrosol

2.3.1 Identity of the Nova Mentis hydroxytyrosol

Absolute and comparative methods were used to confirm the identity of the Nova Mentis hydroxytyrosol, which is identical in chemical composition and structure to the chemically synthesised hydroxytyrosol. Chemically synthesised hydroxytyrosol for use in food as an antioxidant is claimed to be GRAS (GRN 600). To confirm that the Nova Mentis hydroxytyrosol product was comparable in structure to chemically synthesised HT, a proton and carbon NMR spectroscopy was conducted demonstrating that the chemical composition of HT (Appendix 1). Furthermore an HPLC and LC-MS (Appendix 2) analysis were carried out to determine the identity and % hydroxytyrosol content in the finished product. The chromatogram revealed comparable retention times for the Nova Mentis HT product in comparison to the reference standard of hydroxytyrosol (> 98% pure) from Tokyo Chemical Incorporated (TCI).

2.3.2. Specifications of Nova Mentis hydroxytyrosol

The methods employed by Nova Mentis to classify its hydroxytyrosol product, determine and quantify other constituents including contaminants of the product are described in this section and associated appendices. To ensure that a consistent food-grade product is produced, Nova Mentis has established specifications for our hydroxytyrosol. The chemical, physical and microbiological specifications of the product are presented in Table 3. Three batches of product were analyzed with regard to the chemical and microbiological parameters listed in the specifications. The product is highly pure and contains >99% hydroxytyrosol with less than 0.6% of starting material tyrosol detected by HPLC (Diode array detector (DAD)). Tyrosol is a

natural antioxidant present in wine, olives, and olive oil and has multiple beneficial health effect. It has been consumed as part of Mediterranean diet for centuries and has no toxic effect on human beings (Babich and Visioli, 2003). Hence the presence of tyrosol at low concentration (<0.6%) does not raise any safety concern.

A broad range of microbiological analyses were conducted to demonstrate that the Nova Mentis hydroxytyrosol product meet the specification (Table 3). Analysis of 3 independent batches of hydroxytyrosol showed absence of microorganisms including aerobic plate count, yeast and mold, Enterobacteriacea, *E. coli*, coliforms and Salmonella (Table 4).

In order to prove the absence of recombinant DNA in the finished hydroxytyrosol samples a quantitative real-time polymerase chain reaction (qPCR) test method was carried out. This method detects the antibiotic gene (kanamycin gene) and tyrosinase gene used in the construction of the recombinant *E. coli* BL21 (DE3) #145 production strain. These two genes are therefore served as appropriate marker of the existence of DNA from the production strain. A standard curve was plotted using different concentration of the gene product and limit of detection was established (7.5×10^{-5} ng/uL for kanamycin marker and 7.5×10^{-4} ng/uL for tyrosinase marker). For this assay the DNA was isolated from hydroxytyrosol samples using Macherey-Nagel™ NucleoSpin™ Food Column. The assay was validated and eliminated any possible interference by hydroxytyrosol by spiking known quantity of plasmid DNA (pRTYR-KanR) into hydroxytyrosol sample followed by extraction of DNA and detection by qPCR (Appendix 3).

Heavy metals, trace solvents and antibiotic residue (kanamycin) analysis were conducted on three independently manufactured batches of hydroxytyrosol by Eurofins Food Testing Ireland Limited. The corresponding certificates of analysis for 3 batches of hydroxytyrosol are in Appendix 4. Analytical data from three manufacturing lots are presented in Table 4. All tested samples met the established specifications demonstrating that the Nova Mentis product complies with appropriate specifications for food-grade materials and that a consistent product can and is produced.

Table 3. Specifications of Nova Mentis hydroxytyrosol

Parameter	**Specification**	**Assay Method**
Appearance	Off-white powder	Visual
Identity	Corresponds	NMR and HPLC
Odor	Mild	Organoleptic
Taste	Slightly bitter	Organoleptic
Solubility (water)	Miscible in water	In house
Appearance in solution	Clear slightly yellow	Visual
Moisture	<0.5%	Halogen moisture analyser
pH (IM water solution)	3.0-4.5	pH meter
Chemical assay		
Hydroxytyrosol	> 99.0%	In house LC-MS and HPLC-DAD
Tyrosol	<0.6%	In house HPLC- DAD
Antibiotic residue	<0.1ppm	LC-MS/MS §64 LFGB L 06.00-62
Total protein	<200 mg/Kg	Modified Bradford assay
Heavy metals		
Lead	<0.05 ppm	ICP-MS
Cadmium	<0.05 ppm	ICP-MS
Arsenic	<0.05ppm	ICP-MS
Mercury	<0.05 ppm	ICP-MS
Cobalt	<0.05ppm	ICP-OES
Residual solvents		
Ethyl acetate	< 100 ppm	Head space GC/MS
Microbiology		
Total aerobic plate count	<10 cfu/g	AOAC 990.12
Total yeast and mold count	< 10 cfu/g	AOAC 997.02
Enterobacteriacea	negative in 10 g	AOAC 2003.01
Coliforms	negative in 10 g	AOAC 991.14
Escherichia coli	negative in 10 g	AOAC 991.14
Salmonella species	negative in 25 g	ISO 6579-1:2017
GMO detection	negative	qPCR

Table 4. Confirmatory analyses for 3 independent batches of Nova Mentis hydroxytyrosol

Parameter	**Specification**	**Batch numbers**		
Appearance	Off-white powder	Complies	Complies	Complies
Identity	Corresponds	Complies	Complies	Complies
Odor	Mild	Complies	Complies	Complies
Taste	mildly bitter	Complies	Complies	Complies
Solubility (water)	Miscible in water	Complies	Complies	Complies
Appearance in solution	Clear slightly yellow	Complies	Complies	Complies
Moisture	<0.5%	0.28%	0. 39 %	0. 46 %
pH (IM water solution)	3.0-4.5	3.94	3.98	3.94
Chemical assay				
Hydroxytyrosol	> 99.0%	99.16%	99.26%	99.18%
Tyrosol	<0.6%	0.53%	0.32%	0.33%
Antibiotic residue	<0.1 ppm	<0.1 ppm	<0.1 ppm	<0.1 ppm
Total protein	<200 mg/Kg	37 mg/Kg	50 mg/Kg	9 mg/Kg
Heavy metals				
Lead	<0.05 ppm	<0.005 ppm	<0.005ppm	<0.005ppm
Cadmium	<0.05 ppm	<0.001 ppm	<0.001 ppm	<0.001 ppm
Arsenic	<0.05ppm	0.004 ppm	<0.002 ppm	<0.002 ppm
Mercury	<0.05 ppm	<0.001 ppm	0.002 ppm	<0.001 ppm
Cobalt	<0.05ppm	0.010 ppm	0.00075ppm	<0.0005 ppm
Residual solvents				
Ethyl acetate	< 100 ppm	58 ppm	44 ppm	86 ppm
Microbiology				
Total aerobic plate count	<10 cfu/g	Complies	Complies	Complies
Total yeast and mold count	< 10 cfu/g	Complies	Complies	Complies
Enterobacteriacea	negative in 10 g	Complies	Complies	Complies
Coliforms	negative in 10 g	Complies	Complies	Complies
Escherichia coli	negative in 10 g	Complies	Complies	Complies
Salmonella species	negative in 25 g	Complies	Complies	Complies
GMO detection	Negative	Negative	Negative	Negative

2.4. Additional information on safety

2.4.1. Allergenicity information on the gene product

A tyrosinase (polyphenol oxidase) gene from *R. solanacearum* was cloned into the *E. coli* strain used as a processing aid in the manufacture of Nova Mentis hydroxytyrosol. Tyrosinase is a ubiquitous enzyme present in human, animals, microorganism plants fruits and vegetables. The widespread exposure of all humans at all ages of life, to the tyrosinase enzyme from different food sources is therefore expected, and allergenicity concerns are not expected. Polyphenol oxidase are used as food processing enzymes during the manufacture of coffee and tea. The Allergen Online database version 18B (Updated March 23, 2018) was used to conduct a Preliminary screen of tyrosinase from *R. solanacearum* for relevant matches against to known putative allergens. This database is maintained by the Food Allergy Research and Resource Program of the University of Nebraska. A FASTA3 overall search of Allergen Online was conducted using default settings (E cutoff = 1 and maximum alignments of 20). No matches to any of the major allergens were identified.

An 80 amino acid sliding window (segments 1-80, 2-81, 3-82, etc.) also was used to scan the amino acid sequence of tyrosinase against the allergen database using FASTA to search for matches of 35% identity or more. This 35% identity for 80 amino acid segments is a suggested guideline proposed by Codex for evaluating proteins in genetically modified crops (Codex, 2003; Goodman et al., 2008). As shown in Table: 5 below the results of the FASTA3 alignments of all possible 80 amino acid segments of tyrosinase against all putative allergen sequences in the database were all less than the 35% threshold over 80 amino acids. Based on the information presented herein it was therefore concluded that the tyrosinase catalyst used for the manufacture of hydroxytyrosol do not raise allergenic risks.

Table 5: 80mer Sliding Window Search Results

Database	Allergen Online Database v18B (23 March 2018)
Input Query	>query MVVRRTVLKAIAGTSVATVFAGKLTGLSAVAADAAPLRVRRNLHGMKMD DPDLSAYREFVGIMKGKDQTQALSWLGFANQHGTLNGGYKYCPHGDWYF LPWHRGFVLMYERAVAALTGYKTFAMPYWNWTEDRLLPEAFTAKTYNG KTNPLYVPNRNELTGPYALTDAIVGQKEVMDKIYAETNFEVFGTSRSVDRS VRPPLVQNSLDPKWVPMGGGNQGILERTPHNTVHNNIGAFMPTAASPRDP VFMMHHGNIDRVWATWNALGRKNSTDPLWLGMKFPNNYIDPQGRYYTQ GVSDLLSTEALGYRYDVMPRADNKVVNNARAEHLLALFKTGDSVKLADHI RLRSVLKGEHPVATAVEPLNSAVQFEAGTVTGALGADVGTGSTTEVVALIK NIRIPYNVISIRVFVNLPNANLDVPETDPHFVTSLSFLTHAAGHDHHALPSTM VNLTDTLKALNIRDDNFSINLVAVPQPGVAVESSGGVTPESIEVAVI
Length	496
Number of 80 mers	417
Number of Sequences with hits	0

No Matches of Greater than 35% Identity Found
***Allergen Online* Database v18B (23 March 2018)**

2.5. Physical or technical effects

Nova Mentis intends to use hydroxytyrosol as an antioxidant in 11 broad food categories: bakery products; beverages; dairy products and substitutes; desserts; fats and oils; fruit juices and nectars; dry seasoning mixes for meat, poultry and fish; chewing gum; sauces, dips, gravies and condiments; snacks; and vegetable juices to deliver 5 to 10 mg of hydroxytyrosol per serving of food. No specific physical or technical effects are proposed for Nova Mentis hydroxytyrosol at this time.

3. INTENDED FOOD USES AND PROJECTED DIETARY EXPOSURE

3.1. History of exposure and use

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 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.

The estimated daily intakes of hydroxytyrosol from existing dietary sources (i.e. olives and olive oil) in units of mg/day and mg/kg-bw/day are provided in Table 6 for the U.S. population ages 2 years and older and four subpopulations. The highest 90th percentile per user reported intake of

hydroxytyrosol from existing dietary sources was 1.2 mg/day (0.01 mg/kg-bw/day) among adults ages 19 years and older. The existing EDI at 90th percentile per user for U.S. population 2 years and older was 1.0 mg/day (0.01 mg/kg-bw/day). Approximately 50% of the U.S. population ages 2+ years reported eating a food containing hydroxytyrosol.

Table 6 U.S. Population ages 2+ years average daily hydroxytyrosol intake from olives and olive oil (NHANES[a] 2007-2010)

			2 Day Average (mg/day)				**2 Day Average (mg/kg-bw/day)**			
			Per Capita		**Per User**		**Per Capita**		**Per User**	
Subpopulation	**N[b]**	**%User**	**Mean**	**90th**	**Mean**	**90th**	**Mean**	**90th**	**Mean**	**90th**
Children 2-5 y	649	47.2%	0.1	0.05	0.2	0.1	0.005	0.003	0.01	0.006
Children 6-12 y	1010	44.0%	0.1	0.1	0.2	0.4	0.003	0.002	0.008	0.009
Teens 13-18 y	685	40.6%	0.1	0.1	0.3	0.5	0.002	0.002	0.005	0.009
Adults 19+ y	5540	54.1%	0.3	0.4	0.6	1.2	0.004	0.005	0.007	0.01
U.S. population 2+ y	7884	51.5%	0.3	0.3	0.5	1.0	0.004	0.004	0.007	0.01

a NHANES = National Health and Nutrition Examination Survey, [b] Unweighted number of users; % user, per capita and per user estimates derived using the statistical weights (National Center for Health Statistics (NCHS).

3.2 Intended use levels and food categories

Hydroxytyrosol (HT) is naturally occurring polyphenol found in olives and processed olive products such as olive oil. Nova Mentis intends to use HT as an antioxidant [21 CFR 170.3(o)(3)] in 11 broad food categories: bakery products; beverages; dairy products and substitutes; desserts; fats and oils; fruit juices and nectars; dry seasoning mixes for meat, poultry and fish; chewing gum; sauces, dips, gravies and condiments; snacks; and vegetable juices to deliver 5 to 10 mg of HT per serving of food. Based on the FDA reference amounts customarily consumed per eating occasion (RACC) outlined in 21 Code of Federal Regulations (CFR) 101.121, the use of Nova Mentis HT imparts 5 to 10 mg of hydroxytyrosol per serving for each of the 11 food categories, as summarized in Table 7. Information on the intended food uses and use levels was used to estimate consumer intakes, which are discussed further below.

Table 7. Intended use levels and possible daily intake of hydroxytyrosol based on USDA data[1]

Food category	HT[a] use levels (mg/ serving	RAC C[b] (g)	Use level HT (PPM)
Bakery products			
Crackers that are usually used as snacks	5	30	167
Croutons	5	7	714
Grain based bars with or without filling or coating (e.g breakfast bars, granola bars, rice cereal bars)	10	40	210
Protein based meal replacement and energy bars	10	40	250
Beverages			
Sports drinks, energy drinks, milk-based meal replacement, flavored waters, fruit flavored drinks	5	240	8
Dairy Products and Substitutes			
Yogurt	10	225	44
Desserts			
Frozen Yogurt	10	120	83
Fats and Oils			
Butter, margarine, oil and shortening	5	15	333
Dressing for salads	5	30	167
Mayonnaise, sandwich spreads, mayonnaise-type	5	15	333
Fruit and Fruit Juices			
Fruit juices and fruit nectars	5	240	21
Miscellaneous			
Meat, poultry, and fish coating mixes, dry; seasoning mixes, dry (e.g., chilli seasoning mixes, pasta salad seasoning mixes)[b]	5	4.5	1111
Chewing gum	10	3	3333
Sauces, Dips, Gravies, Condiments			
Major main entree sauces (e.g., spaghetti sauce)	5	125	40
Minor main entree sauces (e.g., pizza sauce, pesto sauce), other sauces used as toppings (e.g. gravy, white sauce, cheese sauce), cocktail sauce	5	60	83
Major condiments: catsup only dairy-based dips, salsa)	5	15	333
Barbecue sauce, hollandaise sauce, tartar sauce, other sauces for dipping (e.g., mustard sauce, sweet and sour sauce), all dips (e.g., bean dips,	5	30	167
Snacks			
All varieties, chips, pretzels, popcorns, extruded snacks, fruit-based snacks (e.g., fruit chips), grain-based snack mixes	5	30	167
Vegetable Juices			
Vegetable juice	5	240	21

aNova Mentis hydroxytyrosol (HT) contains >99% Hydroxytyrosol **b**U.S. FDA reference amounts customarily consumed (RACC) refers to Reference Amounts Customarily Consumed per eating occasion – 21 CFR §101.12 (CFR, 2014). When a range of values is reported for a particular food-use, particular foods within that food-use may differ with respect to their RACC. **b**The estimated RACC for dry seasoning mixes was estimated to be 4.5 g dry spice rub (i.e., 2 teaspoons per serving) based upon publicly available food recipes for mixed dishes containing dry seasonings and rubs from McCormick Spices (http://www.mccormick.com/Grill-Mates/Recipes). This is the lowest value, which would provide a worst-case scenario for estimating exposure to a food additive in dry seasonings and rubs.

3.3. Estimated daily intake from the intended uses

3.3.1. Use of USDA Data

The daily intake of HT 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 (90th 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 90th percentile consumption of hydroxytyrosol, the corresponding mean total intake value from all food categories was multiplied by two on the grounds that the 90th 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 for which Nova Mentis HT is proposed to be added, the possible mean and maximum daily intake of hydroxytyrosol from each of the categories is summarized in Table 7. The estimated daily intake of HT exclusively from proposed uses of Nova Mentis HT is shown in Table 8 and the Cumulative estimated daily intake (CEDI) of hydroxytyrosol from existing dietary exposure plus proposed uses from Nova Mentis HT are shown in Table 9.

Table 8 Estimated daily intake of hydroxytyrosol exclusively from proposed uses of Nova Mentis HT [a, b] (NHANES 2007-2010)

			2 Day Average (mg/day)[a, b]				**2 Day Average (mg/kg-bw/day)[a,b,c]**			
			Per Capita Per User				**Per Capita Per User**			
Population	**N [c]**	**%User**	**Mean**	**90th**	**Mean**	**90th**	**Mean**	**90th**	**Mean**	**90th**
Children 2-5 y	1374	99.8%	19.6	32.9	19.6	32.9	1.2	2.0	1.2	2.0
Children 6-12 y	2127	99.9%	24.3	39.1	24.3	39.1	0.7	1.3	0.7	1.3
Teens 13-18 y	1563	100%	30.5	54.7	30.5	54.7	0.5	0.9	0.5	0.9
Adults 19+ y	9950	99.8%	30.5	53.6	30.5	53.6	0.4	0.7	0.4	0.7
U.S. Population 2+ Years	15014	99.9%	29.3	51.9	29.3	51.9	0.5	0.9	0.5	0.9

[a] Based upon use of 5-10 mg of Nova Mentis hydroxytyrosol per serving of food. [b] Nova Mentis' HT is proposed for use in 11 broad food categories including bakery products; beverages; dairy products and substitutes; desserts; fats and oils; fruit juices and nectars; dry seasoning mixes for meat, poultry and fish; chewing gum; sauces, dips, gravies and condiments; snacks; and vegetable juices.

[c] Unweighted number of users; % user, per capita and per user estimates derived using statistical weights (NCHS).

Table 9 Cumulative estimated daily intake (CEDI) of hydroxytyrosol from existing Dietary exposure plus proposed uses from Nova Mentis HT (NHANES 2007-2010)

Population	N[a]	% User	2 Day Average (mg/day)[b, c]				2 Day Average (mg/kg-bw/day)[b,c]			
			Per Capita Per User				Per Capita Per User			
			Mean	90th	Mean	90th	Mean	90th	Mean	90th
Children 2-5 y	1374	99.8%	19.6	33.0	19.7	33.0	1.2	2.0	1.2	2.0
Children 6-12 y	2127	99.9%	24.4	39.9	24.4	39.9	0.7	1.3	0.7	1.3
Teens 13-18 y	1563	100%	30.6	55.1	30.6	55.1	0.5	0.9	0.5	0.9
Adults 19+ y	9950	99.8%	30.8	53.9	30.8	53.9	0.4	0.7	0.4	0.7
U.S. Population 2+ Years	15014	99.9%	29.5	52.4	29.5	52.4	0.5	0.9	0.5	0.9

[a] Unweighted number of users; % user, per capita and per user estimates derived using the statistical weights (NCHS).

[b] Cumulative EDI of hydroxytyrosol based upon existing uses of hydroxytyrosol in olive and olive oil and proposed uses of Nova Metnis' hydroxytyrosol in 11 broad food categories including bakery products; beverages; dairy products and substitutes; desserts; fats and oils; fruit juices and nectars; dry seasoning mixes for meat, poultry and fish; chewing gum; sauces, dips, gravies and condiments; snacks; and vegetable juices at a use rate of 5-10 mg of hydroxytyrosol per serving of food.

[c] Unweighted number of users; % user, per capita and per user estimates derived using the statistical weights (NCHS).

3.3.2. Exposure based on Daily Servings

The estimated daily intake of Nova Mentis HT from its proposed uses in 11 broad categories of food in units of mg/day and mg/kg-bw/day are provided in Table 8 for the U.S. population ages 2 years and older and in four sub populations. The highest 90th percentile *per user* EDI of Nova Mentis HT was 54.7 mg/day among teenagers ages 13 to 18 years (0.9 mg/kg-bw/day). The 90 th percentile *per user* EDI of Nova Mentis HT for U.S. population 2 years and older was 51.9 mg/day (0.9 mg/kg-bw/day). Nearly everyone 2 years and older in the U.S. population reported eating a food with proposed uses of Nova Mentis HT.

3.3.3. Cumulative estimated intake of hydroxytyrosol

The cumulative estimated daily intake (EDI) of hydroxytyrosol from existing dietary sources and Nova Mentis' proposed uses of HT (to deliver 5 to 10 mg/serving of HT in 11 food categories) in units of mg/day and mg/kg-bw/day are provided in Table 9 for the U.S. population ages 2 years and older and in four sub populations. The highest 90^{th} percentile *per user* cumulative estimated dietary intake (CEDI) of hydroxytyrosol was 55.1 mg/day among teenagers ages 13 to 18 years (0.9 mg/kg-bw/day). The 90^{th} percentile *per user* CEDI for the U.S. population 2 years and older was 52.4 mg/day (0.9 mg/kg-bw/day).

4. SELF-LIMITING LEVELS OF USE

In keeping with CFR 170.240 Part 4 of a GRAS notice, in circumstances where the amount of the notified substance that can be added to food is limited because food containing levels of the notified substance above a particular level would become unpalatable or technologically impractical must be described, including data and information on such self-limiting levels of use.

Nova Mentis is unaware of any specific physical or technically impractical effects for nova Mentis HT at this time. The intended uses and use levels for Nova Mentis HT are intended exclusively as commercial products in the United States of America.

5. EXPERIENCE BASED ON COMMON USE IN FOOD BEFORE 1958

5.1 Scientific Procedures

The statutory basis for Nova Mentis' conclusion of GRAS status is not through experience based on common use in food use by a significant number of consumers prior to January 1, 1958. A self-affirmation of GRAS status by Nova Mentis was instead based upon scientific procedures including the application of scientific data (including data from human, animal, analytical, or other scientific studies), information, and methods, as well as the application of scientific principles, appropriate to establish the safety of a substance under the conditions of its intended use.

5.2. Natural occurrence of hydroxytyrosol

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 are found both as simple (hydroxytyrosol and tyrosol) and complex compounds (hydroxytyrosol or tyrosol esterified to elenolic acid, in the form of oleuropein (Figure 6) and ligstroside, respectively). 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 (2001) 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).

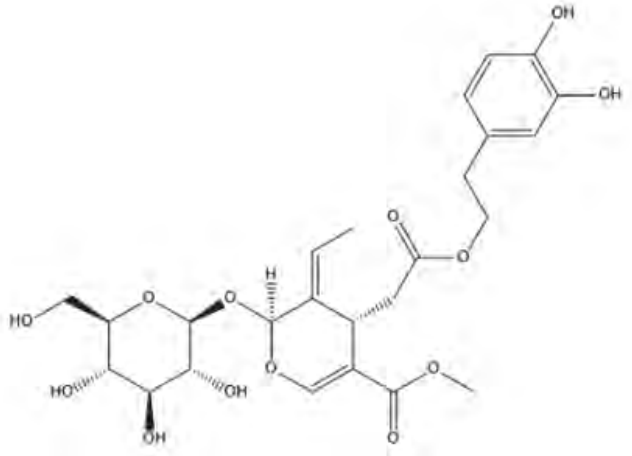

Figure 6: Molecular structure of Oleuropein (Adapted from Sahin and Bilgin, 2018).

5.3. Benefits of hydroxytyrosol

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 (HT) 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 HT, 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 HT.

HT is currently being actively marketed as a potential supplement or preservative in the nutraceutical, cosmeceutical, and food industries (Visioli and Bernardini, 2011). HT has been proposed as a cardioprotective (Visioli, 2012), anti-inflammatory (Lopez et al., 2017), and chemopreventive (Bernini et al., 2013) agent. Given the potential health benefits of phenolic compounds, particularly HT, Nova Mentis intends to market HT for use as a food ingredient in selected foods as described in this dossier.

6. NARRATIVE ON SAFETY

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.

6.1. Toxicology

6.1.1. Specific Studies with Hydroxytyrosol

The acute toxicity of HT has been studied in cells and animal models (Auñon-Calles et al., 2013a; Christian et al., 2004; Soni, et al., 2006). Studies in rodents carried out in 2013 by Aunon-Calles showed no toxic effects at an administration of 500mg/kg/day and no genotoxic or carcinogenic effects in *in vitro* human models up to a media concentration of 10 mM of HT. Martinez and co-workers reported that phosphatidyl-hydroxytyrosol orally administered to rats was safe. Furthermore they conclude that no treatment-related toxicity was detected even at the high doses investigated in both acute (2000 mg/kg bw) and repeated dose (28-day) oral (2000 mg/kg bw) toxicity studies (Martinez et al., 2018). Several human studies have been carried out to test the clinical efficacy of HT and its effects on different parts of human physiology. Two of the most recent of these, by Heilman et al. (2015), and Khymenets et al. (2016), respectively have used concentrations of 25 mg of HT per day and they reported no adverse or toxic effects.

6.1.1.1. Subchronic Study

In a dose-response study, Auñon-Calles et al. (2013a) investigated the potential toxicity of hydroxytyrosol in rats. 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 guide lines and recommendations by the European Commission Directives and OECD. The study was performed in compliance with 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. 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). Urine analysis 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 epididymis 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).

6.1.1.2. 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 µ L/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. 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 suggests 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% CO2 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). Furthermore, in the *in vivo* micronucleus assay in mice following administration of olive pulp extract containing hydroxytyrosol, Christian et al. (2004) did not observe 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.

6.1.2. Other Studies

6.1.2.1. 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 LD50 of olive pulp extract was greater than 2000 mg/kg (72 mg hydroxytyrosol/kg bw). In a recent 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).

6.1.2.2. 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 F1 generation pups were weaned on day 21 post-partum. Two male and two female from the F1 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 F0 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 Fo 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.

6.1.3. 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.

6.1.4. Genotoxicity studies

6.1.4.1. 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 uvrA 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).

6.1.4.2. *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.

6.1.4.3. *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. 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*.

6.2. 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 10. 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 10 Summary of human studies of olive phenolics*

Reference	Test component/	Study type	Duration	Subjects	Primary Outcome
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
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 isoprostanes) and increased final products of nitric oxide

*Adapted from Raederstorff 2009 and other studies

Table 10 (continued) Summary of human studies of olive phenolics*

Reference	Test component /	Study type	Duration on Intake	Subjects	Primary Outcome
Fito et al., 2005	Olive oil low phenolic content (1 mg) Olive oil high phenolic content (8 mg)	Randomized, cross-over, controlled	3 weeks	40 males with stable coronary heart disease	Consumption of virgin olive oil rich in polyphenolics decreased *in vivo* oxidized LDL and lipid peroxide plasma levels and increased glutathione peroxidase also observed with the high phenolic content product. activity as compared to refined olive oil consumption. A decrease in systolic blood pressure was observed with the high phenolic content product.
Leger et al., 2005	Olive phenolic concentrate (first day 5 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	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

*Adapted from Raederstorff 2009 and other studies

Table 10 (continued) Summary of human studies of olive phenolics*

Reference	Test component / daily dose	Study type	Duration on Intake	Subjects	Primary Outcome
Gimeno et al., 2007	Olive oil low phenolic content (0 mg) Olive oil medium phenolic content (9 mg). Olive oil high 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 LD L 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 mg) Olive oil high phenolic content (9 mg)	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-deoxy guanosine, 8-oxo-guanosine)
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. 2002	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.

*Adapted from Raederstorff 2009 and other studies

Table 10 (continued) Summary of human studies of olive phenolics*

Reference	Test component/ daily dose	Study type	Duration on Intake	Subjects	Primary Outcome
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 virgin olive oil group as compared to the sunflower oil group
Gimeno et al., 2007	Olive oil low phenolic content (0 mg) Olive oil medium phenolic content (9 mg). Olive oil high 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 mg) Olive oil high phenolic content (9 mg)	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-deoxy guanosine, 8-oxo-guanosine)

*Adapted from Raederstorff 2009 and other studies

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/m2) 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.

6.3. Evaluation by EFSA

Hydroxytyrosol is on the European Union list of novel foods in accordance with Regulation (EU) 2015/2283 of the European Parliament and of the Council on novel foods. Specifically, hydroxtyrosol is approved in the European Union for use in fish oils, vegetable oils and spreadable fats (Table 11). 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.

Table 11. Authorized uses of hydroxytyrosol as a novel food in the European Union

Specified food category	**Maximum levels**	**Additional specific labelling requirements**
Fish and vegetable oils, (except olive oils and olive pomace oils as defined in Part VIII of Annex VII of Regulation (EU) No 1308/2013 (6)), placed as such on the market	0,215 g/kg	The designation of the novel food on the labelling of the food products containing shall be 'hydroxytyrosol'. The labelling of the food products containing hydroxytyrosol shall bear the following statements: a) This food product should not be consumed by children under the age of three years, pregnant women, and lactating women; b) This food product should not be used for cooking, baking or frying
Spreadable fats as defined in Part VII of Annex VII of Regulation (EU) No 1308/2013, placed as such on the market	0.175 g/kg	

6.4. Absorption, distribution, metabolism and excretion

The bioavailability of polyphenolic compounds, including hydroxytyrosol, from olive oil has been extensively reviewed and summarized in an 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 C_{max} 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, catechol methyltransferase, and sulfotransferases. In 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-methylhydroxytyrosol 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 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. Recently, the first direct method to measure free HT in human plasma has been reported (Pastor et al., 2016). In that study, the authors report Cmax of HT of 2.8 $X10^{-6}$ mol/L, following ingestion of EVOO. According to the authors (Pastor et al., 2016), the low amounts of free HT present in plasma after dietary doses (0.3% of the dose administered) cannot explain a direct in vivo antioxidant activity of HT, but could be the result of secondary mechanisms (e.g. transcriptomic effects or the activity of metabolites)

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.

6.5. 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 (Olsen et al., 2000). 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 evidence of hydroxytyrosol toxicity when administered to rats, even in high doses. 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. There are two studies which used pure hydroxytyrosol in human to study absorption and metabolism. Khymenets et al. (2016) measured HT urinary concentrations and reported HT-S-3' as the major metabolites. Of note, Gonzalez-Santiago et al. (2010) described the association of HT to LDL after intake of the pure molecule. This might be important in light of the purported activities of HT in reducing ox-LDL concentrations, as per the EFSA health claim. 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.

6.6. 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 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 10. 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.

6.7 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. Nova Mentis intends to use hydroxytyrosol (> 99% pure) as an food ingredient (antioxidant) in selected food products. The product is an off-white powder with a mild odor and mildly bitter taste. The proposed food categories for the use of hydroxytyrosol at levels up to 5 -10 mg per serving are bakery products; beverages; dairy products and substitutes; desserts; fats and oils; fruit juices and nectars; dry seasoning mixes for meat, poultry and fish; chewing gum; sauces, dips, gravies and condiments; snacks; and vegetable juices. 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. Nova Mentis HT is proposed for use in 11 broad categories of food in units of mg/serving (Table 7), mg/day and mg/kg-bw/day (Table 8 and 9) for the U.S. population ages 2 years and older and in four sub populations. The highest 90th percentile *per user* EDI of Nova Mentis HT was 54.7 mg/day among teenagers ages 13 to 18 years (0.9 mg/kg-bw/day). The 90th percentile *per user* EDI of Nova Mentis HT for U.S.

population 2 years and older was 51.9 mg/day (0.9 mg/kg-bw/day). Nearly everyone 2 years and older in the U.S. population reported eating a food with proposed uses of Nova Mentis HT. 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 LD50 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 (90th percentile) intake of hydroxytyrosol from the proposed food uses is estimated as 0.85 mg/kg bw/day. The sub chroni 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 54.7 mg/day (Table 8) is unlikely to cause any adverse effects. Chemically produced pure (>99%) hydroxytyrosol was GRAS notified to FDA (GRN 600) for use as an antioxidant in beverages, fats and oils, fresh and processed fruits and vegetables, fresh and processed fruit and vegetable juices, and gravies and sauces at a level of 5 milligrams (mg) per serving. FDA had no questions at the time of submission. Also an olive preparation containing 40% hydroxytyrosol was GRAS notified to FDA (GRN726) for use in bakery products; beverages; dairy products and substitutes; desserts; fats and oils; fruit juices and nectars; dry seasoning mixes for meat, poultry and fish; chewing gum; sauces, dips, gravies and condiments; snacks; and vegetable juices to deliver 5 to10 mg of hydroxytyrosol per serving of food. 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 and that up to 100 mg per day was safe for adults and 200 mg was safe for older adults. Since January 1, 2018 hydroxytyrosol is approved for use in oils and spreadable fats in the European Union.

In summary, on the basis of scientific procedures , history of exposure and use, consumption of hydroxytyrosol as a food ingredient (antioxidant) at use levels of up to 5-10 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 170.30 (3) in bakery products; beverages; dairy products and substitutes; desserts; fats and oils; fruit juices and nectars; dry seasoning mixes for meat, poultry and fish; chewing gum; sauces, dips, gravies and condiments; snacks; and vegetable juices when not otherwise precluded by a Standard of Identity, and is produced as described in this document.

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Appendix 1: NMR Analysis

NMR analysis for the Identity of 3 independent batches of hydroxytyrosol produced by Nova Mentis

Biotechnologically produced hydroxytyrosol by Nova Mentis was subject to proton and carbon NMR analysis. For spectra recording hydroxytyrosol was dissolved in hexadeuterodimethyl sulfoxide (DMSO-d6).

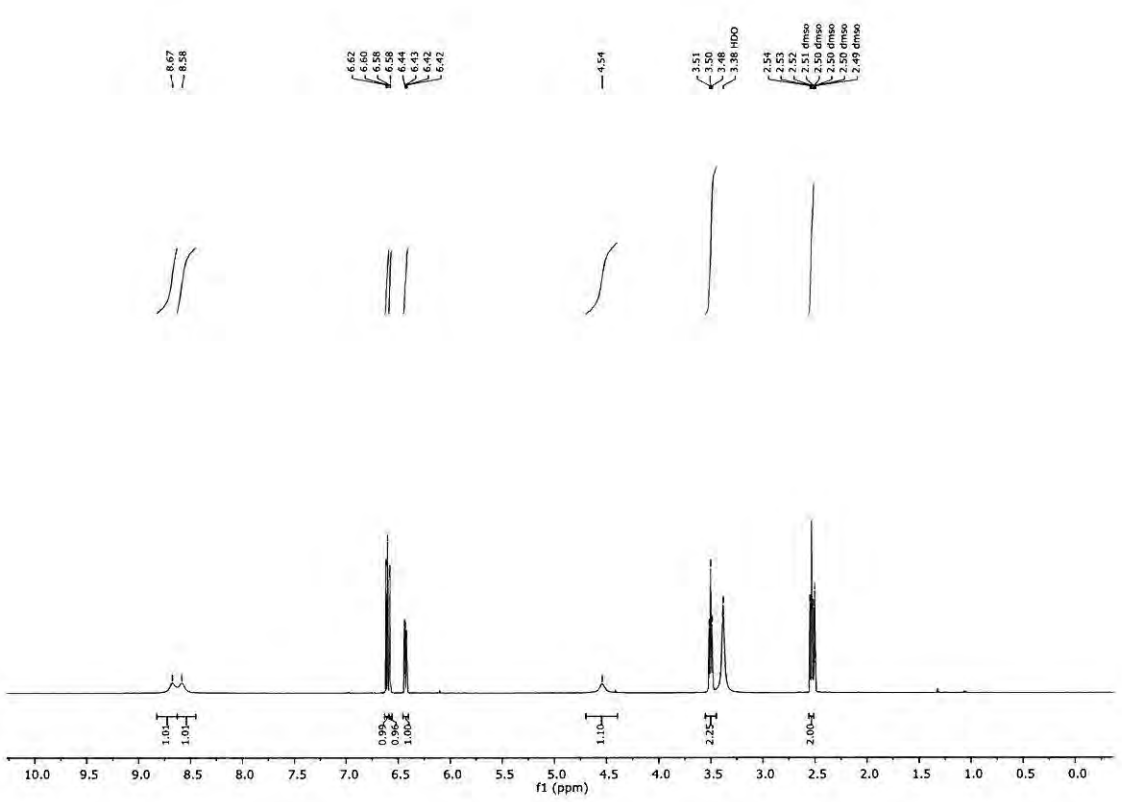

Figure 1: ^{1}H-NMR of batch [redacted]

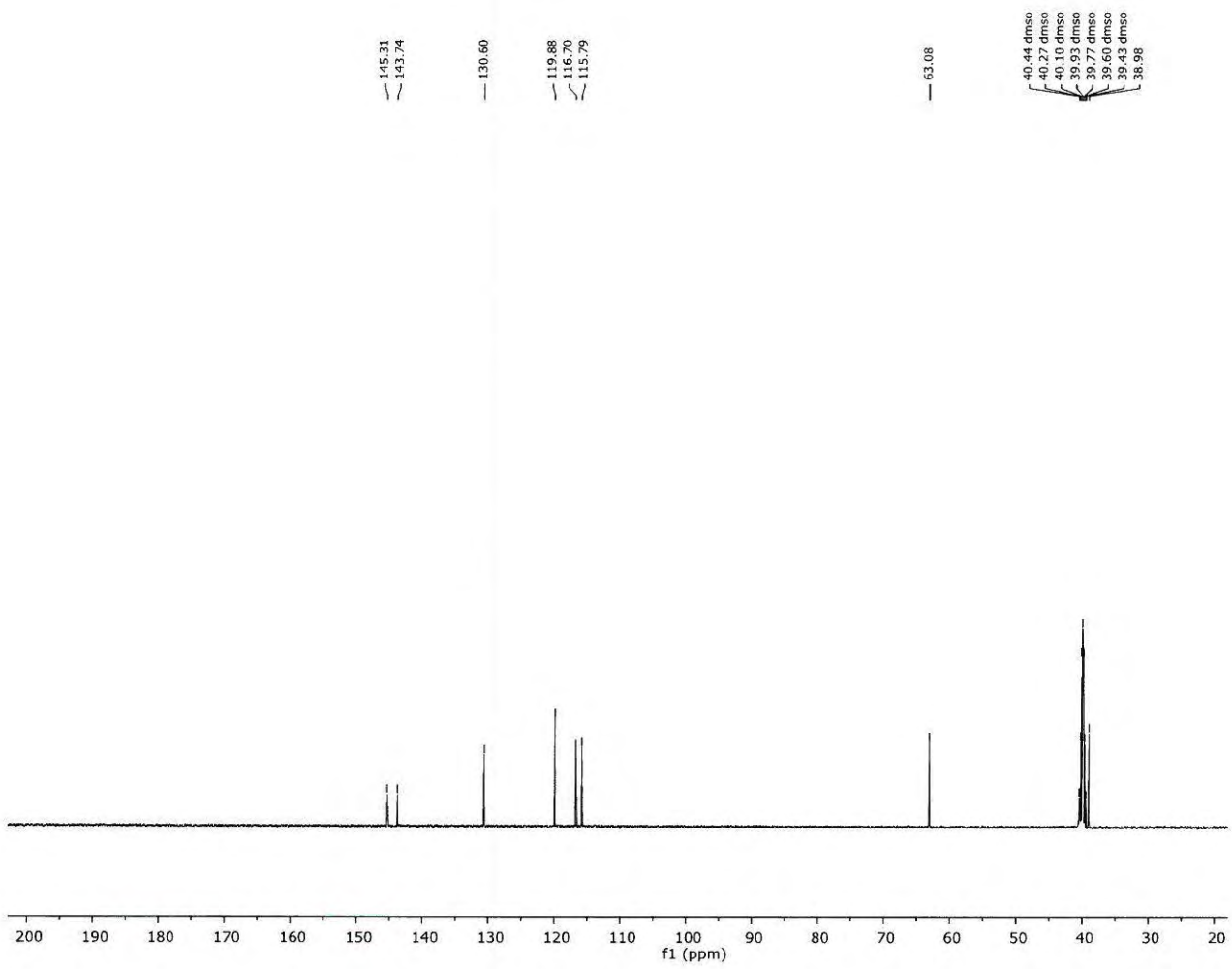

Figure 2: ^{13}C-NMR of batch

Figure 3: ^{1}H-NMR of batch

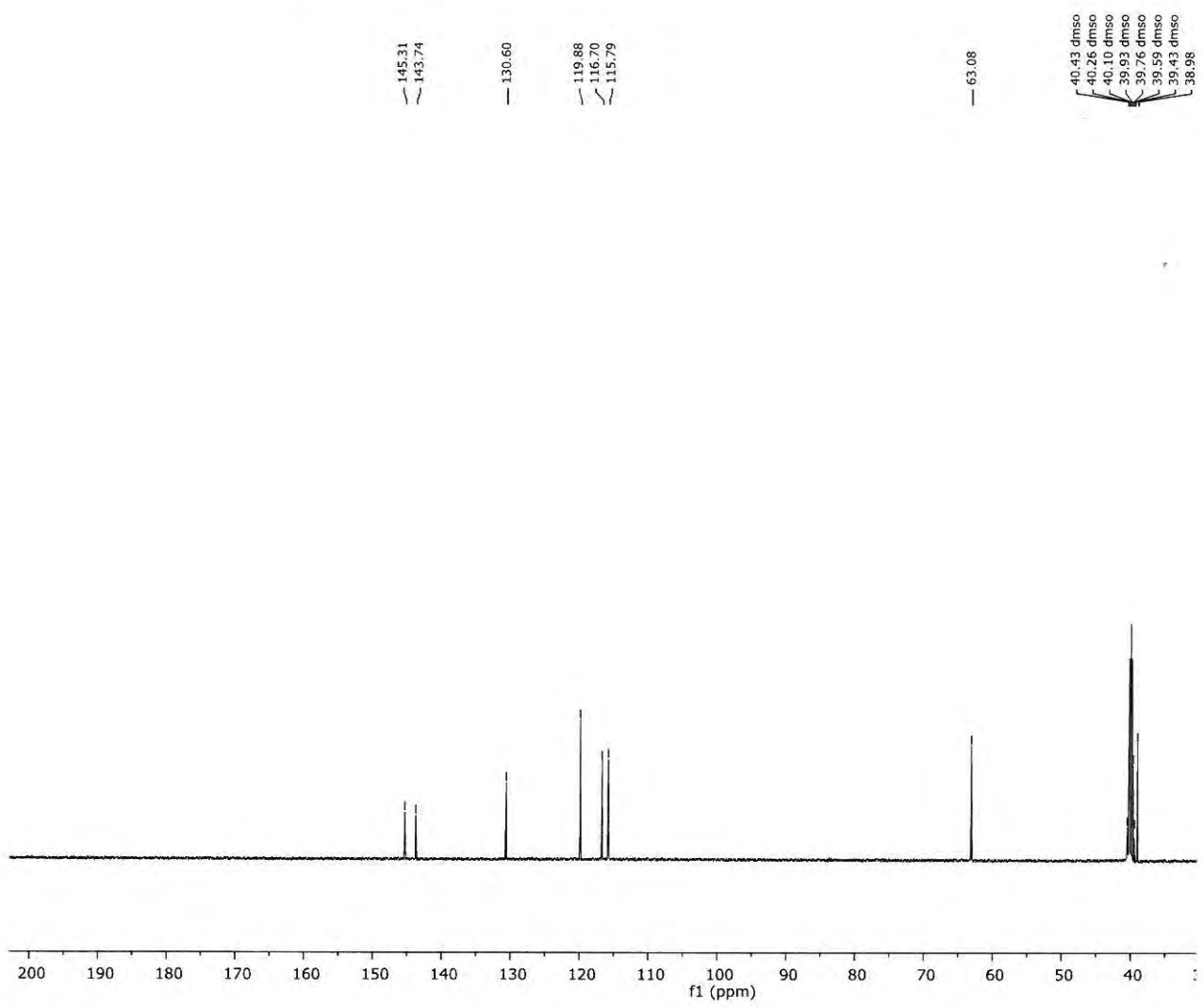

Figure 4: ^{13}C-NMR of batch

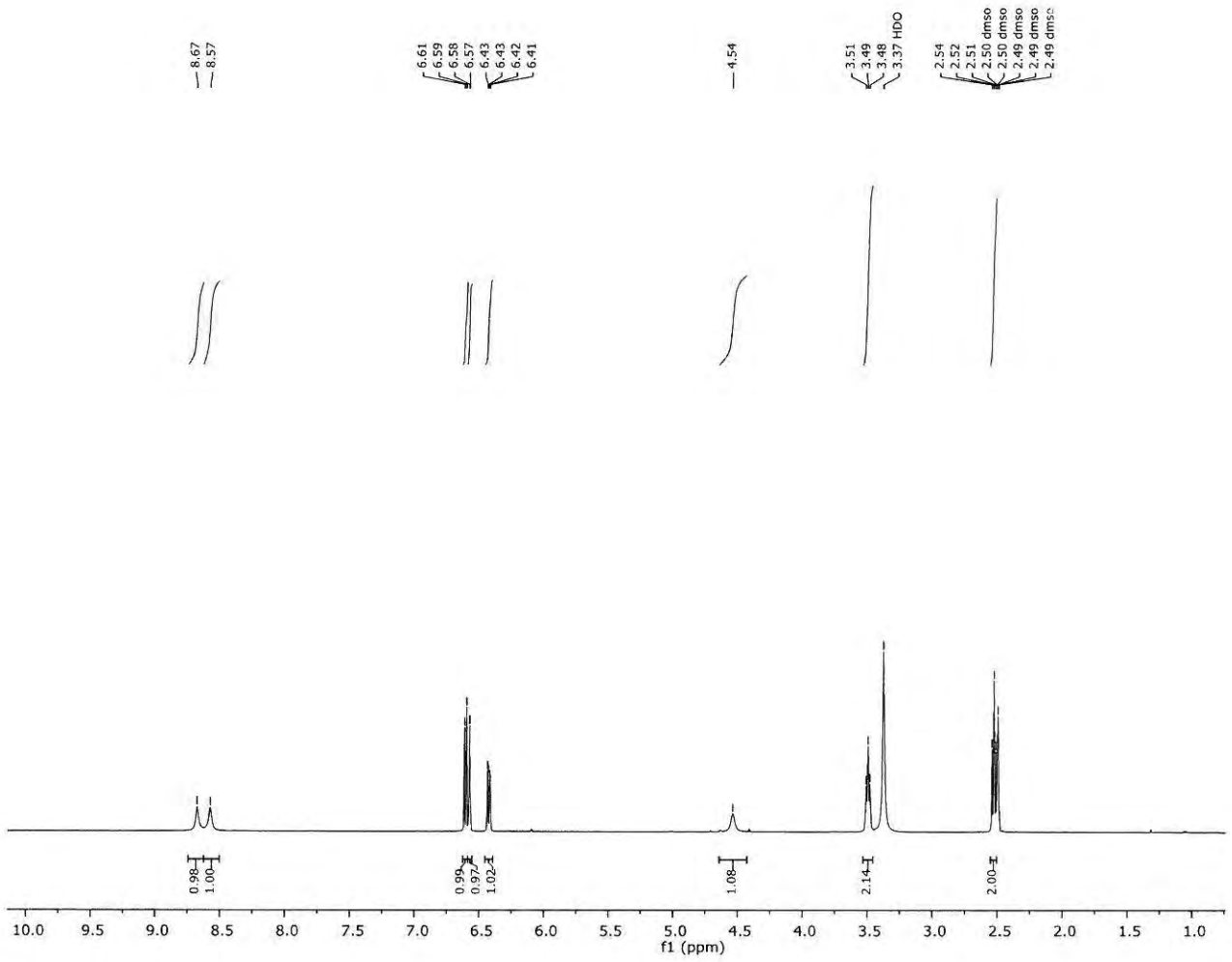

Figure 5: ^{1}H-NMR of batch

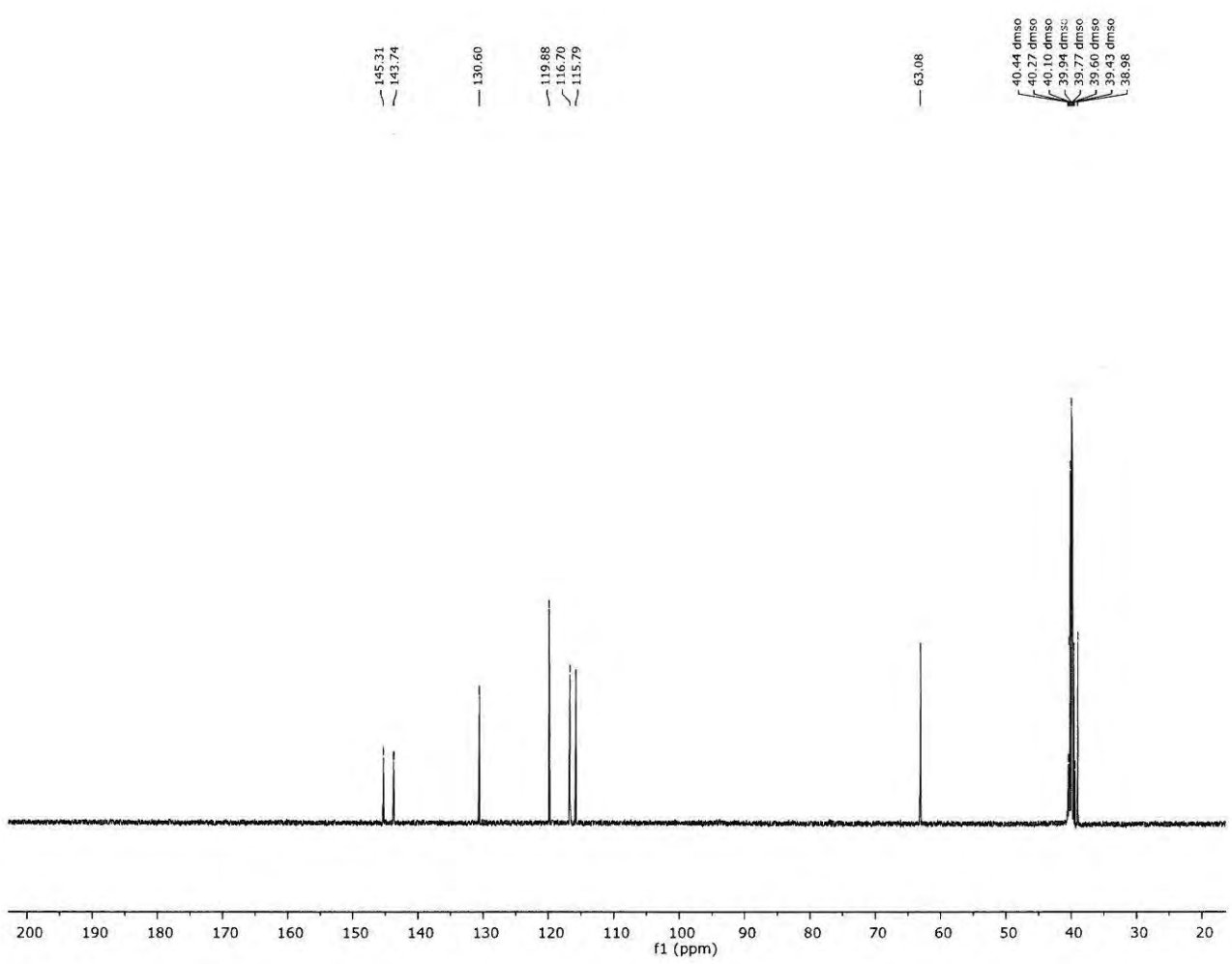

Figure 6: ^{13}C-NMR of batch

Appendix 2: LC-MS Analysis

LC-MS analysis report for identification and purity of 3 independent batches of Nova Mentis hydroxytyrosol.

The sample arrived as a white dried powder was reconstituted in methanol 80% and, after vortexing, it was diluted until a concentration of 250mg/ml was achieved. The chromatographic separation was acquired by using an Agilent C-18 column, Eclipse plus RRHD C18 (2.1 x 50 mm, 1.8 mm) and commonly mobile phase composition was used: ultrapure water with 0.1% formic acid (A) and methanol (B).

Chromatographic runs were conducted with an Agilent 1290 Infinity UHPLC system equipped with a binary pump and a thermostatic column compartment. The column was maintained at 30˚ C and using a ramp gradient with a flow rate of 200 µl/min, mobile phases were pumped into the UHPLC system with a following elution program: 0-8 min, 15-25% B; 1 min, 25% B; 1 min, 25-15% B. Subsequently, column was re-equilibrated to the initial conditions for 5 min before the next injection (total run time 15 min). The injection volume of each samples was 16 µl and all samples were analysed sequentially in a single batch experiment.

Mass spectral analysis was performed by an Agilent 6550A iFunnel QTOF-MS instrument, where the full scan mode acquisition and the MS/MS experiment in negative ion polarity were obtained. The QTOF instrument was equipped with an ESI source in negative-ion mode operated by capillary voltage +3.0 kV, nozzle 1.5 kV, fragmentor 275 V, nebulizer 30 psi, dry gas flow 11.0 L/min at 250˚ C and mass range 50-1200 m/z.

MS/MS data were acquired in a data-dependent strategy selecting the targeted mass at 153.055 m/z from the survey scan (50 - 1200 m/z) for CID fragmentation. Mass range of CID spectra was depended on the precursor ion (50 - 300 m/z), in which 3 microscan at isolation width of 4 amu with 5 V as fixed collision energy were set.

Formula Confirmation Report

Sample Name	BI_005	**Position**	P1-A3	**Instrument Name**	LC_QTOF
User Name		**Inj Vol**	10	**IRM Calibration Status**	Success
Data Filename	NM20180704.d	**ACQ Method**	HTyrosol_4_neg_MSMS.	**Comment**	-neg
Acquired Time	08/11/2018 18:27:18				

Compound Label	RT	Mass	Abund	Formula	Tot Mass	Purity Value	Purity Result
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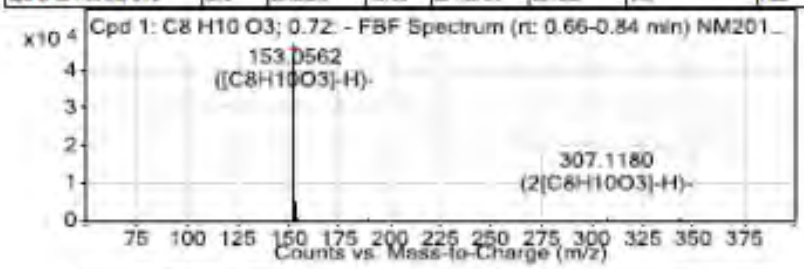
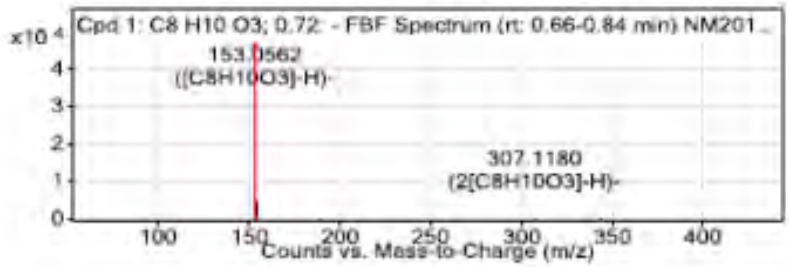
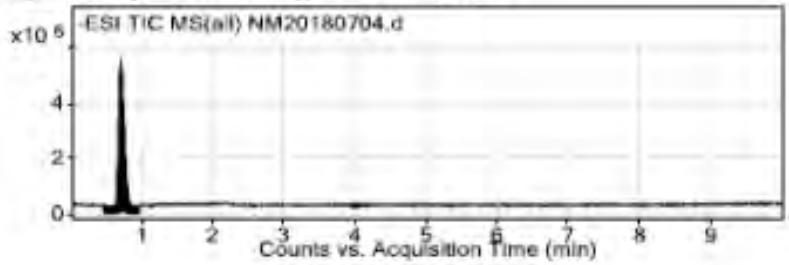
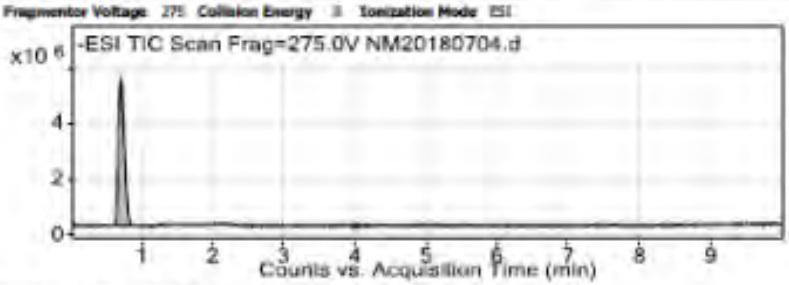




Fragmentor Voltage 0 Collision Energy 0 Ionization Mode ESI



Fragmentor Voltage 275 Collision Energy 0 Ionization Mode ESI



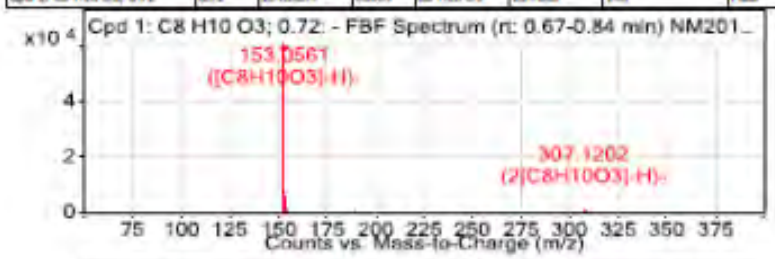
User Chromatogram Peak List

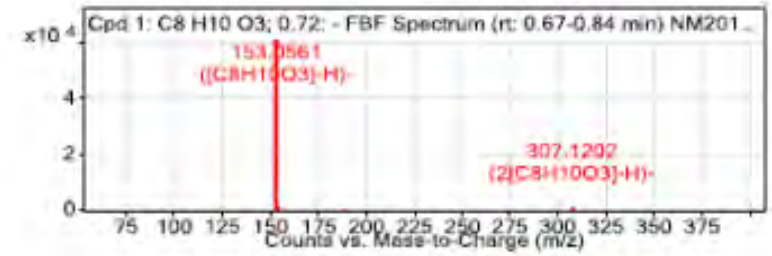
Compound Name	RT	Height	Height %	Area	Area %	Area Sum %	Basic Peak m/z	Width
Cpd 1: 0.72	0.72	5336468	100	30986707	100	100	1234.0627	0.3

Formula Confirmation Report

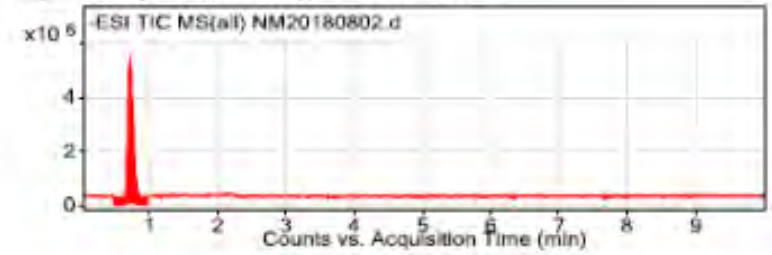
Sample Name	E2_009	Position	F1-A4	Instrument Name	LC_QTOF
User Name		Inj Vol	10	IRM Calibration Status	Success
Data Filename	NM20180802.d	ACQ Method	HTyrosol_4_neg_MSMS.	Comment	-ve
Acquired Time	08/11/2018 18:43:06				

Compound Label	RT	Mass	Abund	Formula	Tot Mass	Purity Value	Purity Result
Cpd 1: C8 H10 O3: 0.72	0.72	154.0634	60669	C8 H10 O3	154.063	100	Pass





Fragmentor Voltage 0 Collision Energy 0 Ionization Mode ESI



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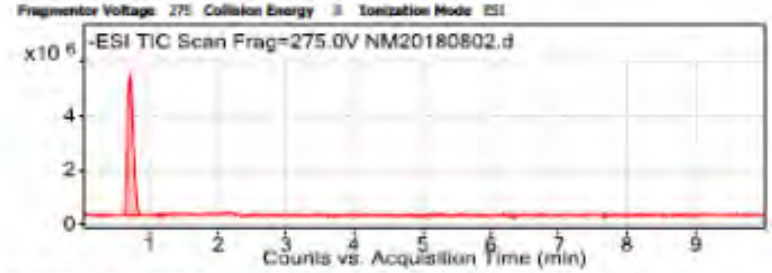

User Chromatogram Peak List

Compound Name	RT	Height	Height %	Area	Area %	Area Sum %	Base Peak m/z	Width
Cpd 1: 0.72	0.72	5245730	100	30730611	100	100	1034.0029	0.31

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Formula Confirmation Report

Sample Name	ES_013	**Position**	P1-A5	**Instrument Name**	LC_QTOF
User Name		**Inj Vol**	10	**IRM Calibration Status**	Success
Data Filename	NM20180815.d	**ACQ Method**	HTyrosol_4_neg_MSMS.	**Comment**	-Neg
Acquired Time	08/11/2018 18:58:54				

Compound Label	RT	Mass	Abund	Formula	Tgt Mass	Purity Value	Purity Result
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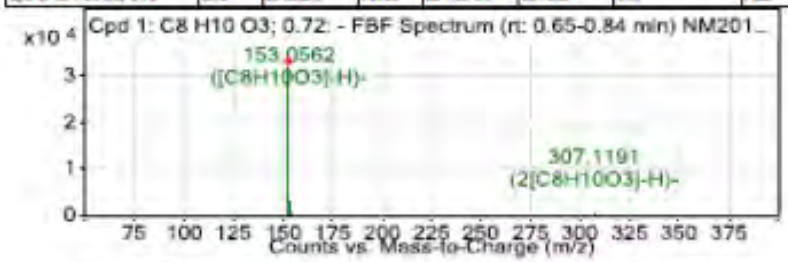
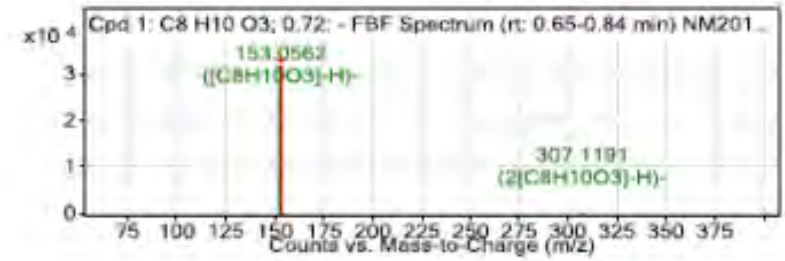
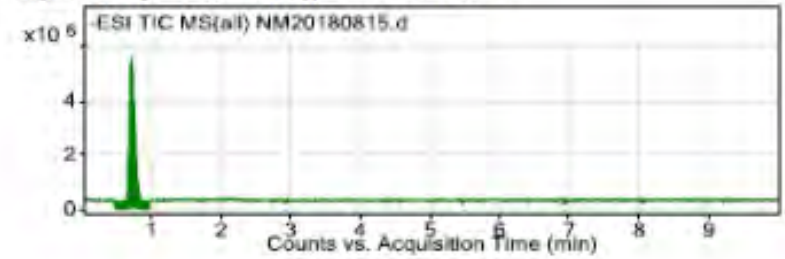




Fragmentor Voltage 0 Collision Energy 0 Ionization Mode ESI



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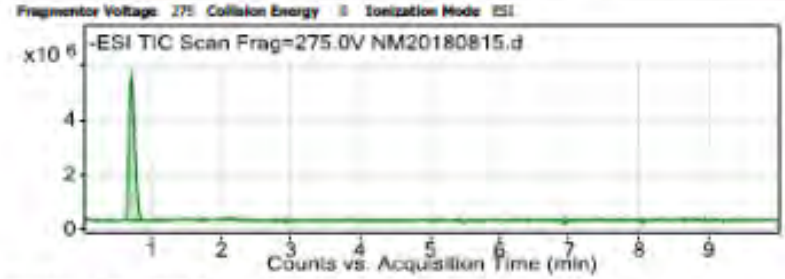

User Chromatogram Peak List

Compound Name	RT	Height	Height %	Area	Area %	Area Sum %	Base Peak m/z	Width
Cpd 1: 0.72	0.72	5318797	100	31169754	100	100	1034.0015	0.31

Appendix 3: GMO Detection

Development and validation of a detection system for genes from genetically modified *E.coli* by quantitative *real-time* PCR for the purposes of determining DNA contamination of hydroxytyrosol

1. Introduction

The production system used to produce Hydroxytyrosol (HT) employs a recombinant *E. coli* which contains a high copy number plasmid which contains the kanamycin resistance gene ($Kanamycin^R$) and the tyrosinase gene. As these genes are both in multiple copies in each cell during the process they were chosen as targets to determine DNA contamination in the finished product using Quantitative Real-Time Polymerase Chain Reaction (qPCR).

1.1. The Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) is a method of detecting specific DNA sequences in solution via the amplification of target sequences using a DNA polymerase enzyme which catalyses DNA synthesis.
PCR reactions contain the following:

- A target DNA sequence to be amplified, known as the template.
- A DNA polymerase enzyme suitable to work under reaction conditions
- DNA nucleotides (dATP, dGTP, dCTP, dTTP), the raw material for DNA synthesis
- Two Primers, short DNA fragments which define the starting and end points of DNA synthesis

DNA synthesis during PCR reaction takes place in a piece of equipment known as a Thermocycler. Thermocyclers aid the synthesis of new DNA by altering the reaction temperature in the following steps:

1. *Denaturation*
   The double stranded DNA of the template is separated into two separate single stranded fragments. This is carried out at a high temperature (90-95ºC) to melt the bonds between DNA strands.
2. *Primer Annealing*
   The temperature cools which allows primer strands to bind to the single stranded DNA strands. The temperature depends on the sequence and length of the primers but is usually between 50ºC and 60ºC.
3. *Extension*
   After primers have annealed the DNA polymerase acts on the template DNA adding free DNA nucleotides to the 3’ end of the primer. The primers define the starting point for DNA polymerase action. This reaction takes place at a temperature suitable for the specific enzyme being used, usually in the range of 68ºC to 72ºC.

These steps should be repeated 35 – 45 times to allow for the logarithmic amplification of the selected sequence of the DNA template.

In a standard PCR, after the reaction is completed a dye can be added to the reaction mixture which associates with double stranded DNA (dsDNA) and fluoresces under UV radiation. This allows the DNA to be visible in an agarose gel.

1.2. Quantitative real-time PCR

Quantitative real-time PCR (qPCR) allows for the detection of specific concentrations of nucleotides in any given sample. In this reaction the fluorescent dye which associates with dsDNA is added directly into the reaction prior to the PCR step. At the end of each cycle the fluorescence of each sample is measured which allows for a quantitative calculation of the new DNA. By creating a reference standard of DNA concentrations, the levels of particular sequences of a gene in any given sample can be determined.

2. Materials

For the detection of the kanamycin^R and the tyrosinase genes, qPCR systems were developed and validated. The following is a list of the materials used in these reactions:

- Promega GoTaq qPCR Master Mix
- Ultrapure H_2O
- Primers pairs
- Template DNA: Plasmid Isolated from GMO host/DNA isolated from samples and spiked samples
- Accustart II PCR tough mix
- Thermo Scientific GeneJET gel extraction kit
- Macherey-Nagel NucleoSpin Food column kit

2.1. Gene Sequences

The following DNA sequences correspond to the Kanamycin^R and Tyrosinase genes which were targeted by qPCR:

1. **Kanamycin^R gene**
2. **Tyrosinase gene**

2.2. Primer Design

Two sets of primers were designed for the genes to be detected, one specific to the 5’ region and one to the 3’ region of each gene. Primers were designed using the primer design tool Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) with the following parameters:

- 0.2µM primer concentration in the reaction volume
- 60ºC melting temperature
- GC-clamp ≥ 1
- PCR product size 100 – 200 base pairs (bp)
- Template melting point 80 – 90ºC
- Guanine - Cytosine content ~50%

The following primer pairs were made to fulfil the above criteria:

Kanamycin P1:
Forward Primer – CTCACCTTGCTCCTGCCGAGA
Reverse Primer – CGCCTTGAGCCTGGCGAACAG

Kanamycin P2:
Forward Primer – ACACGTAGAAAGCCAGTCCG
Reverse Primer – GCTATCGCCATGTAAGCCCA

Tyrosinase P1:
Forward Primer – AAGCTGGCCGATCATATCCG
Reverse Primer – CTTGATCAGGGCCACGACTT

Tyrosinase P2:
Forward Primer – CGGCATCATGAAAGGCAAGG
Reverse Primer – GAAGGTCTTGAAGCCGGTGA

3. Validation of detection systems

3.1. Testing Primers & generating Template DNA

Template DNA was generated by plasmid isolation of a sample of the GM-organism using a Thermo Scientific GeneJET plasmid min-prep kit.
All DNA concentrations measured were determined by use of a BioDrop µLite. Using the following PCR conditions all four primer sets were tested using the plasmid isolate as the DNA template in a 25µL reaction:

PCR Reaction makeup:

- 12.5µL 2X Accustart PCR tough Mix
- 0.5µL of 10µM Forward Primer
- 0.5µL of 10µM Reverse Primer
- 10.5µL Ultrapure H_2O
- 1µL Template DNA

Total volume: 25µL

PCR Reaction conditions:

1. 95ºC for 30 seconds
2. 95ºC for 30seconds
3. 60ºC for 20 seconds
4. 72ºC for 30 seconds
5. 4ºC forever

Repeat steps 2 – 4 35 times. Remove samples from thermocycler when step 5 has been reached.

The PCR products from these reactions were tested for their DNA concentration and run through an agarose gel to determine the size and specificity of the DNA fragments obtained.

3.2. qPCR reaction parameters

The following parameters were used to carry out the reaction in an Applied Biosystems QuantStudio 7 Real-Time PCR system:

qPCR Reaction Makeup:

- 12.5µL qPCR Master mix
- 0.25µL Rox qPCR dye
- 0.5µL Forward Primer (2.5µM stock)
- 0.5µL Reverse Primer (2.5µM stock)
- 1µL Template DNA

Total Volume: 25µL

qPCR Reaction Conditions:

- 95ºC for 30 seconds
- 95ºC for 3 seconds
- 60ºC for 20 seconds
- 95ºC for 15 seconds
- 60ºC for 1 minute
- 95ºC for 15 seconds

Repeat steps 2 and 3 x40

3.3. qPCR fluorescence curves and melting point specificity analysis

To determine the concentrations of kanamycin^R and tyrosinase DNA in unknown samples a standard curve of known concentrations was created. For this both the full length kanamycin^R and tyrosinase genes were amplified by PCR as described above and purified from an agarose gel. DNA concentrations of both kanamycin^R and tyrosinase DNA were normalised to a concentration of 18.75µg/ml.
Initially a single sample of each gene were put through qPCR analysis to determine the base fluorescence curves and melting point analysis for DNA specificity.

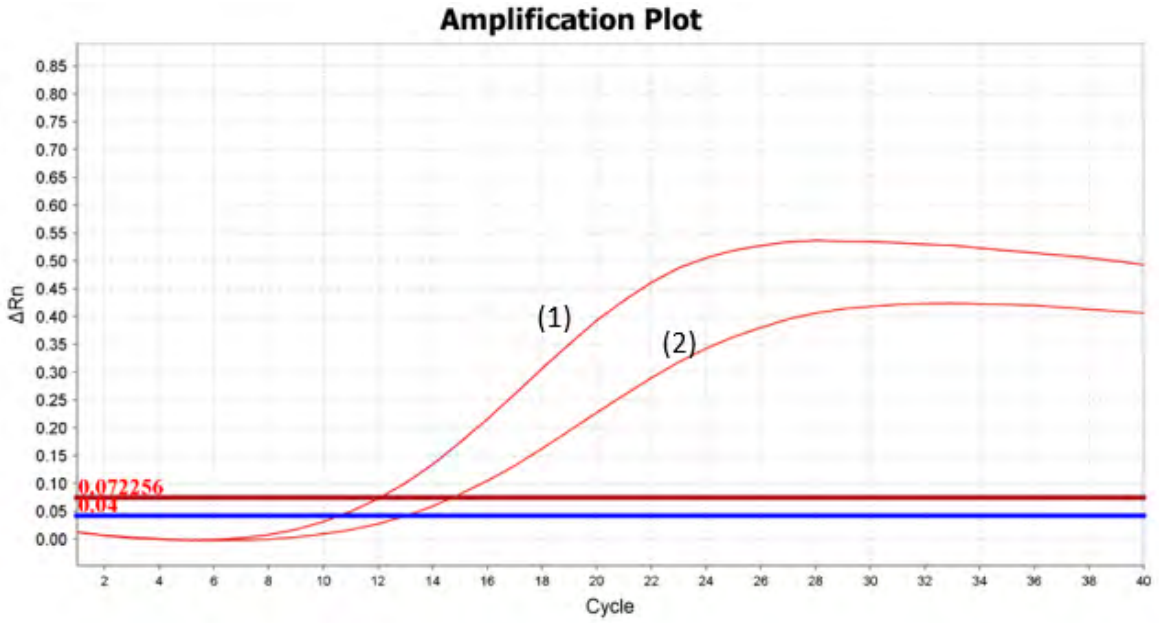

Figure 1. Amplification fluorescence curves during qPCR for Kanamycin^{R} fragment P2 (Kan-P2) (1) and Tyrosinase P1 (Tyr-P1) (2) gene fragments. On the X-axis is the number of PCR cycles, on the Y-axis the fluorescence coefficient ΔRn. DNA concentration in the pcr reaction was 0.75μg/ml.

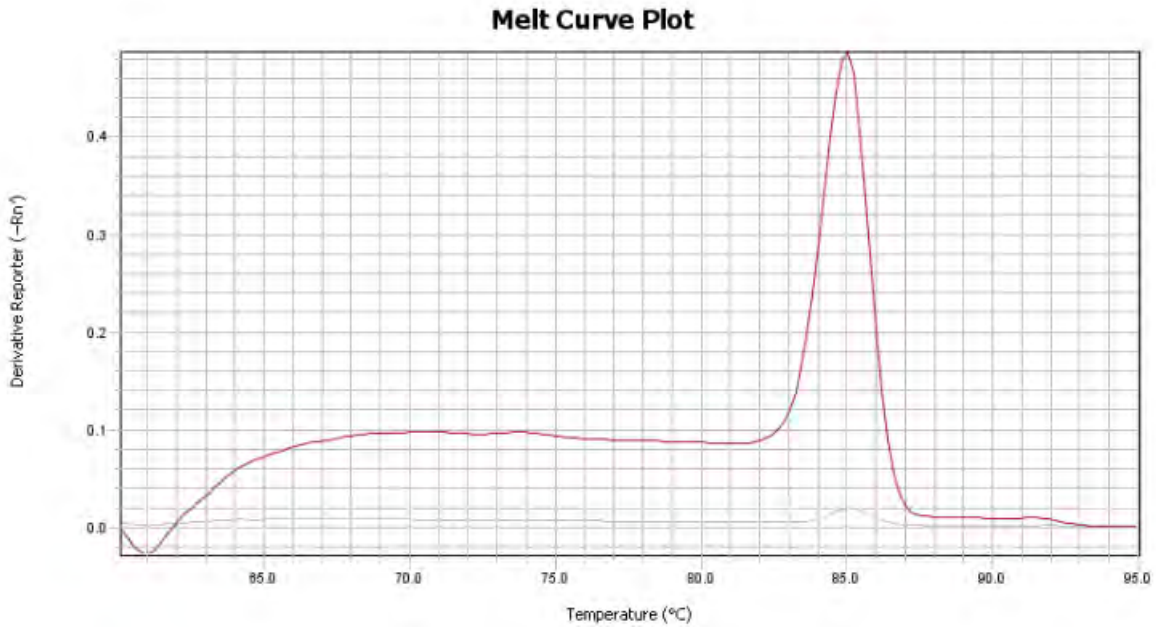

Figure 2. Kan-P2 Melting point analysis (~85ºC). X-axis corresponds to temperature in ºC, Y-axis corresponds to the relative fluorescence derivative (-Rn).

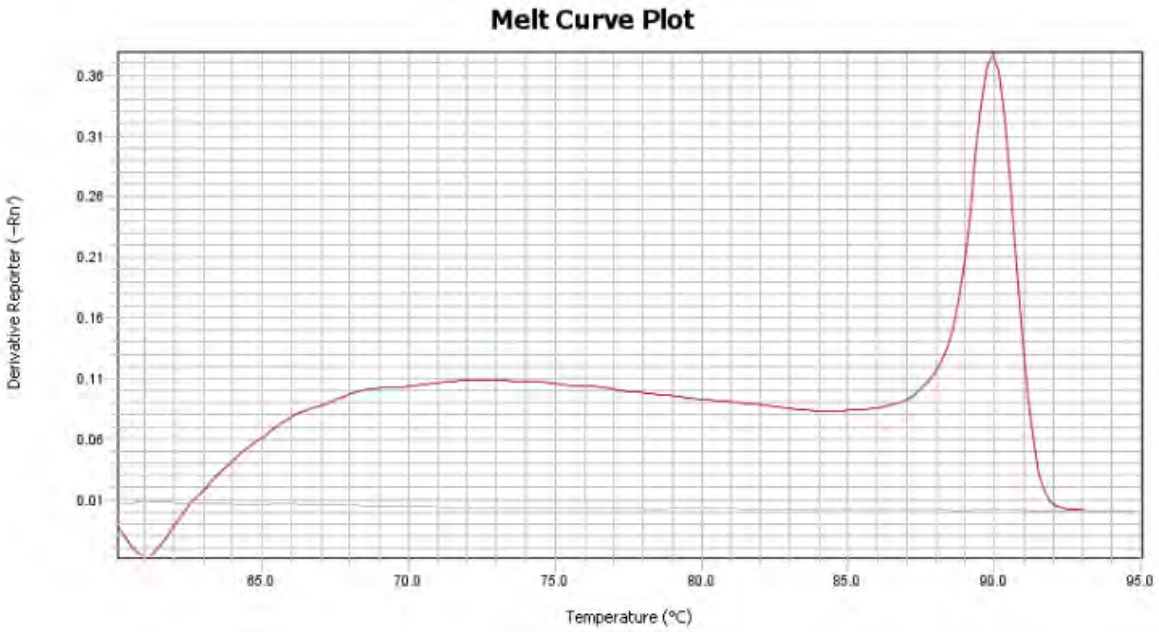

Figure 3. Tyr-P1 Melting point analysis (~90ºC). X-axis corresponds to temperature in ºC, Y-axis corresponds to the relative fluorescence derivative (-Rn).

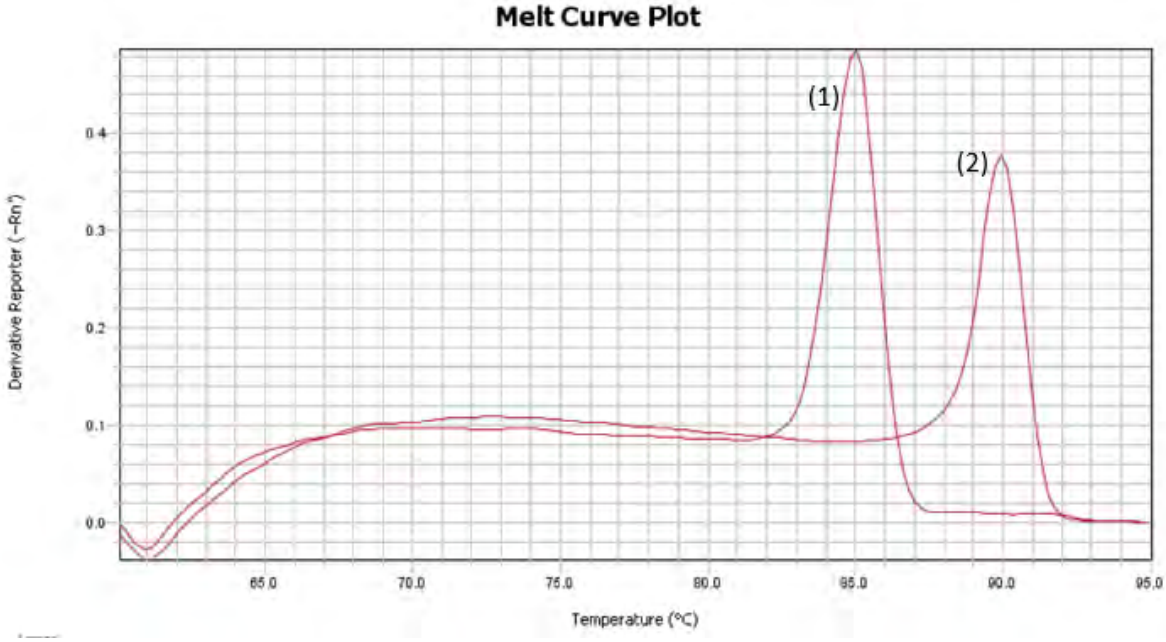

Figure 4. A comparison of the melting point analysis of the Kan-P2 fragment (1) and the Tyr-P1 fragment (2).

As can be seen from figures 1 – 4, both fragments have distinct amplification fluorescence curves and melting points, the fragments can also both be amplified under the same conditions.

3.4 qPCR Standard Curves

To determine the concentrations of kanamycin^{R} and tyrosinase DNA in unknown samples a standard curve of known concentrations was created. For this the concentration normalised (18.75µg/ml) DNA fragments of both kanamycin and tyrosinase were diluted serially in order to have DNA concentrations ranging from 0.75µg/ml to 7.5x10^{-6}µg/ml in the final reaction mixture.

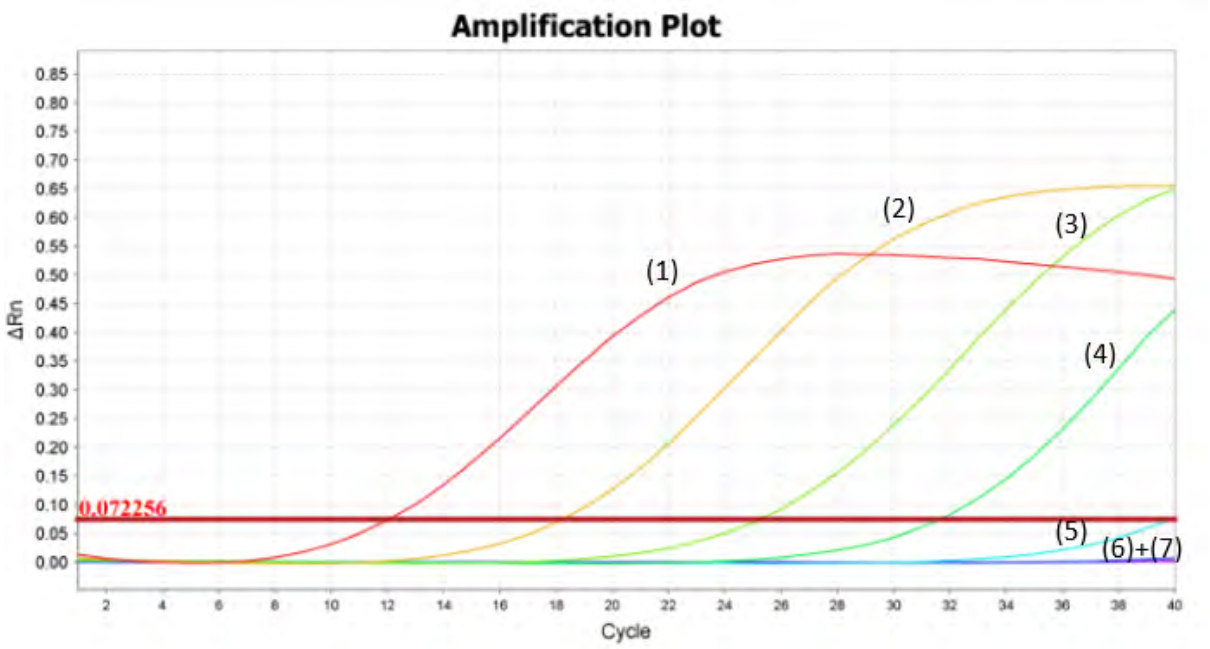

Figure 5. Fluorescence curves during qPCR amplification of the Kan-P2 fragment from the Kanamycin^{R} gene template at different template concentrations to determine the limit for detection. DNA concentrations in template samples (1) 0.75 ng/µL ,(2) 7.5X 10^{-2}ng/µL , (3) 7.5X 10^{-3}ng/µL,(4) 7.5X 10^{-4}ng/µL,(5) 7.5X 10^{-5}ng/µL ,(6) 7.5X 10^{-6}ng/µL ,(7) 7.5X 10^{-7}ng/µL .

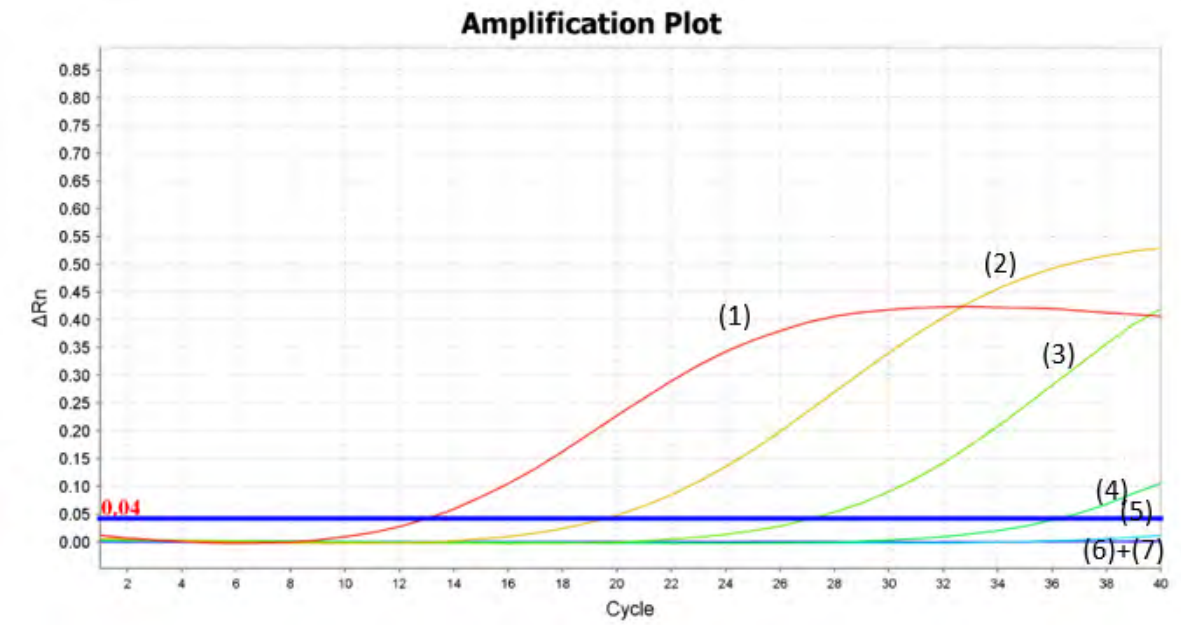

Figure 6. Fluorescence curves during qPCR amplification of the Tyr-P1 fragment from the Tyrosinase gene template at different template concentrations to determine the limit for detection. DNA concentrations in template samples (1) 0.75 ng/µL ,(2) 7.5X 10^{-2}ng/µL , (3) 7.5X 10^{-3}ng/µL,(4) 7.5X 10^{-4}ng/µL,(5) 7.5X 10^{-5}ng/µL ,(6) 7.5X 10^{-6}ng/µL ,(7) 7.5X 10^{-7}ng/µL .

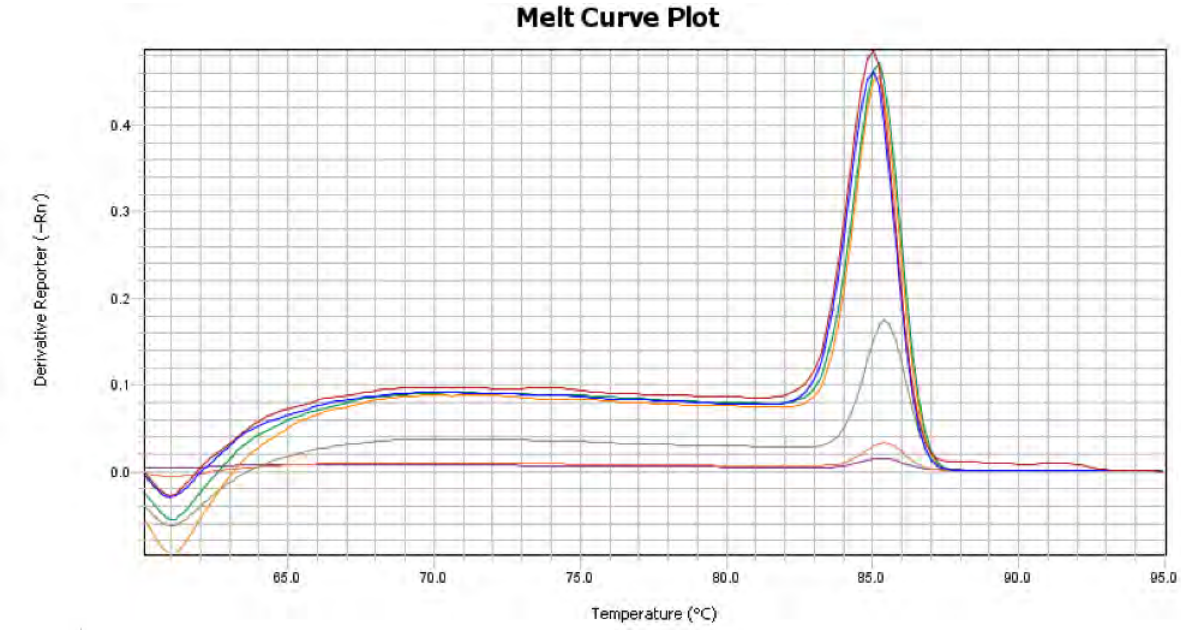

Figure 7. Melting point analysis of serially diluted DNA amplified from the kanamycin^{R} gene using primers Kan-P2.

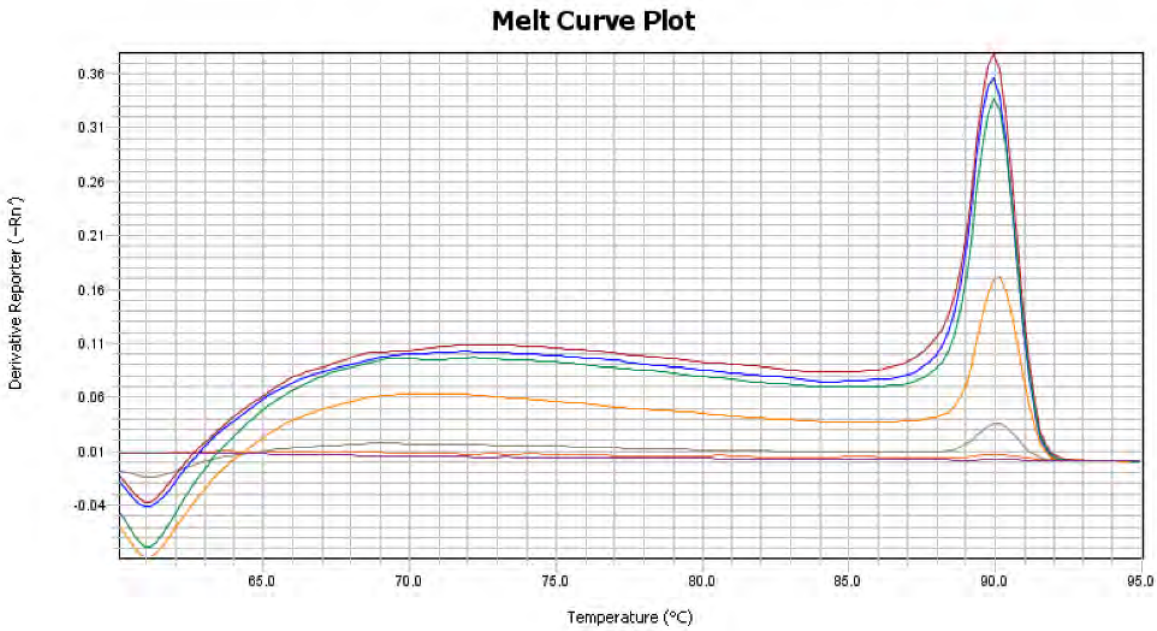

Figure 8. Melting point analysis of serially diluted DNA amplified from the Tyrosinase gene using primers Tyr-P1.

Using the fluorescence curves shown in figures 5 & 6 the limit of detection for both genes were established:

Kanamycin^{R} gene detection limit: 7.5 X 10^{-5} ng/uL
Tyrosinase gene detection limit: 7.5 X 10^{-4} ng/uL

3.5. Validation of matrix effects / Spike tests

Spike tests were carried out to determine the validity of the detection systems. To do this amplification with a DNA positive standard (tyrosinase or kanamycin^{R} gene isolate) was compared with the amplification of a real sample (hydroxytyrosol) spiked with the same or comparable DNA positive material using qPCR. For this 200mg of hydroxytyrosol (HT) was spiked with known quantity of plasmid DNA and the DNA was extracted using the Machery Nagel NucleoSpin food columns. The resulting 50µl eluate was then used as the spiked sample for PCR. The following qPCR was carried out using the same reaction make up as for the standard curve, however the spiked sample was used as template.

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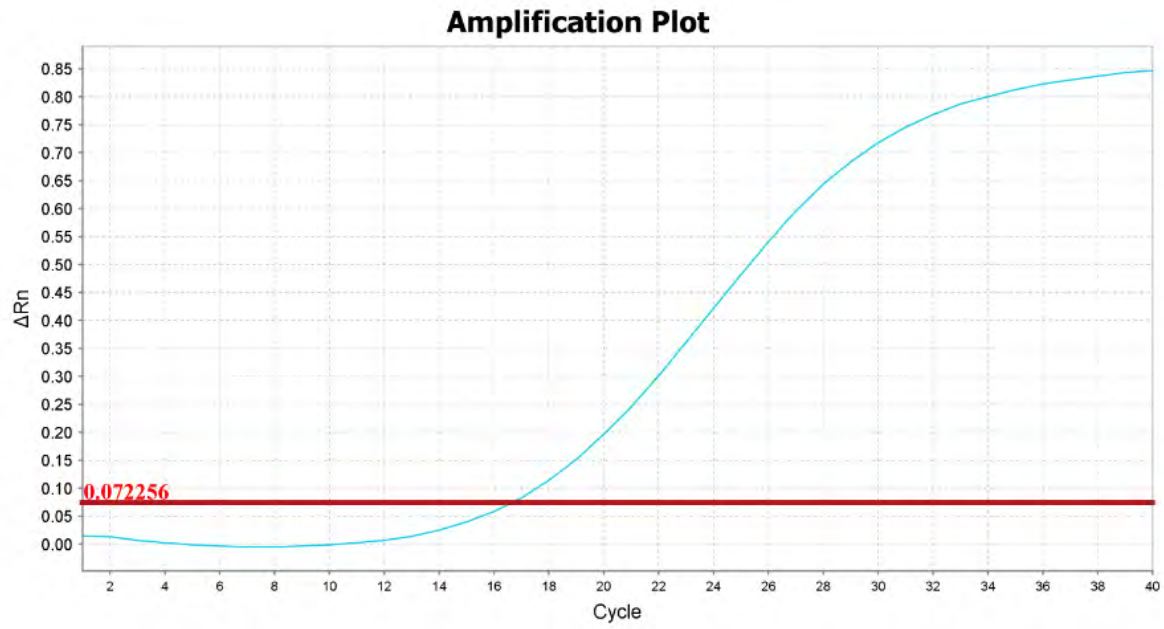

Figure 9. Fluorescence curve during qPCR amplification of Kan-P2 fragment using spiked HT sample as template.

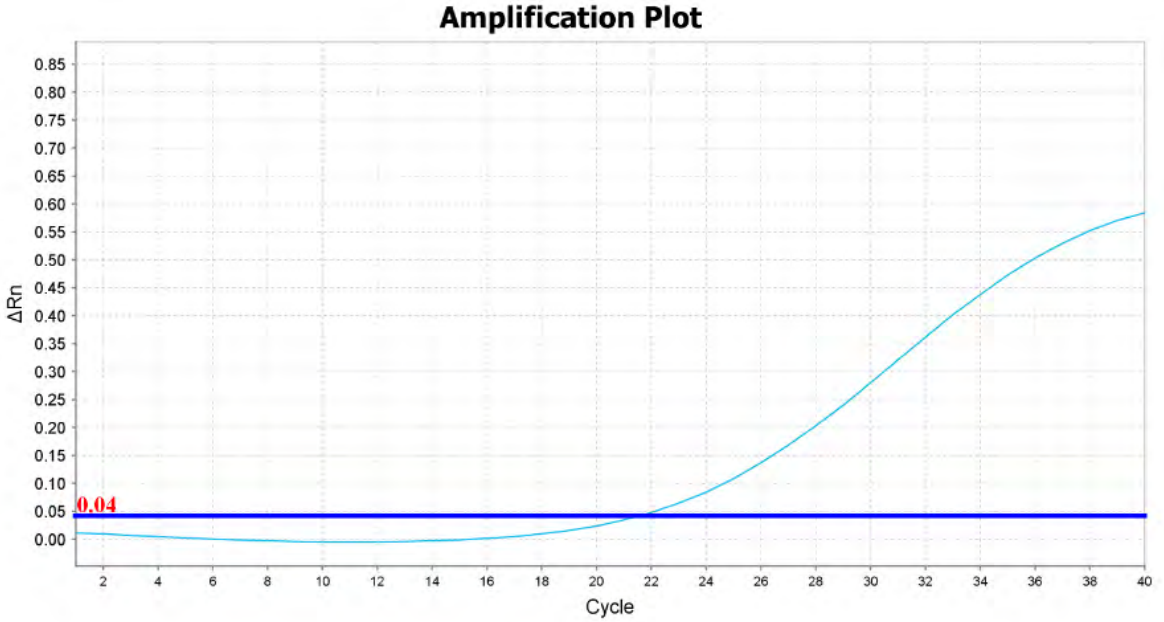

Figure 10. Fluorescence curve during qPCR amplification of Tyr-P1 fragment using spiked HT sample as template.

As can be seen from figures 9 & 10 the spiked samples passed the absorbance threshold at cycles 17 and 22 (Kan-P2 & Tyr-P1 respectively).

3.6 Testing of "Real" samples (3 independent batches of hydroxytyrosol) for the presence of DNA.

Once the validation of the qPCR system had been completed three independent batches of Hydroxytyrosol were tested for the presence of Kanamycin^R and Tyrosinase DNA in duplicate.

The hydroxytyrosol sample was treated by following the protocol and using reagents supplied in the Macherey-Nagel NucleoSpin Food column to extract DNA.
1µl of these DNA extracts were then used as templates for qPCR reactions, made up as above.

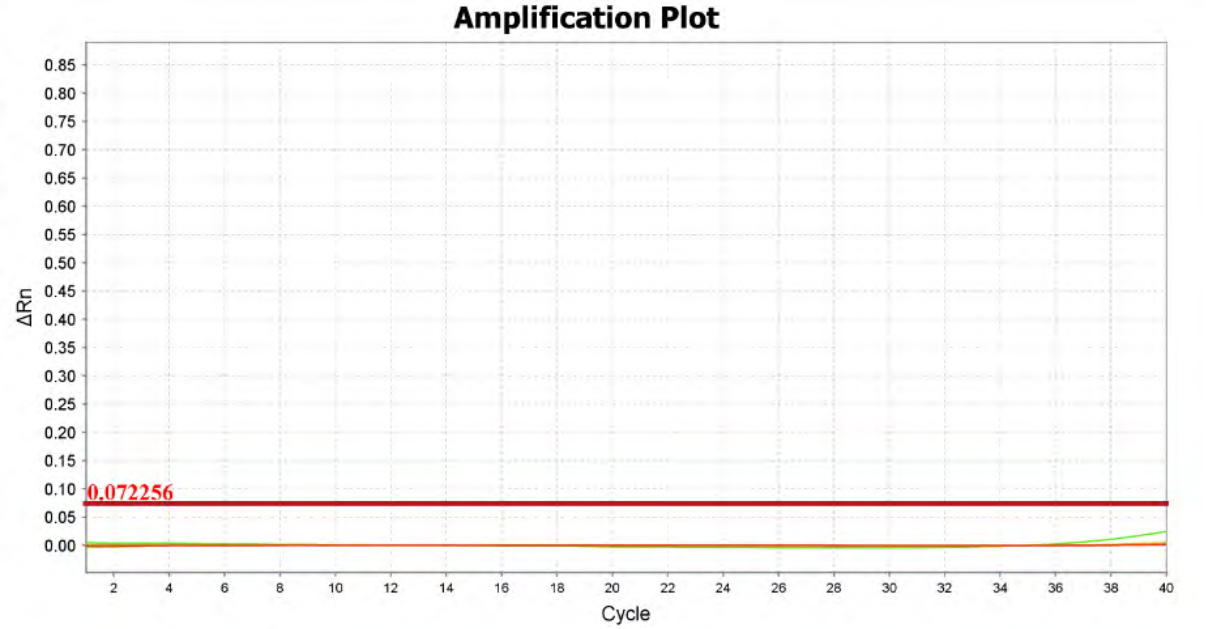

Figure 11. Fluorescence amplification during PCR of the Kan-P2 fragment using HT samples (, ,) from three independent batches in duplicate.

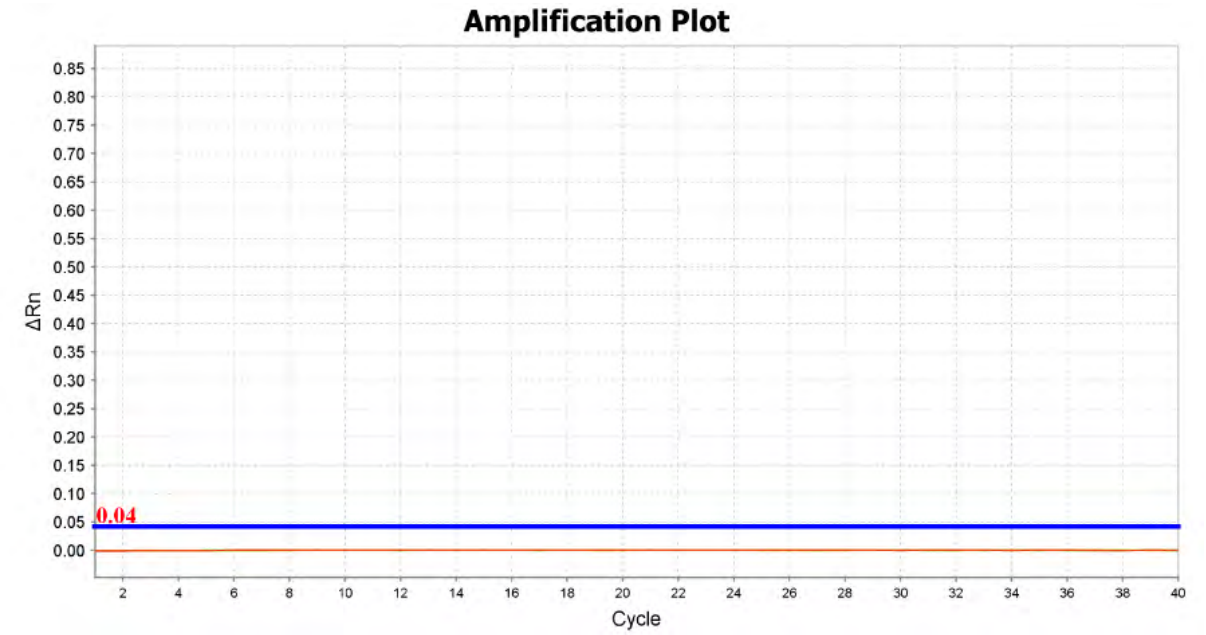

Figure 12. Fluorescence amplification during PCR of the Tyr-P1 fragment using HT samples (, ,) from three independent batches in duplicate.
As can be seen from figures 12 and 13, no kanamycin^{R} or tyrosinase DNA could be detected on the samples taken from three independent HT batches.

3.7 Summary

For both kanamycin^R and tyrosinase DNA detection real-time qPCR systems were successfully developed and validated. Melting point analysis showed specific curves for both DNA fragments amplified. The standard curve was created using known concentrations of amplified kanamycin^R and tyrosinase DNA and had a high correlation coefficient (R^2=0.998). Spiked positive samples showed that targeted DNA could accurately be detected and quantified in given samples. The true samples (3 independent batches of Novamentis hydroxytyrosol ,) tested returned negative results for the presence of both kanamycin^R and tyrosinase DNA.

Appendix 4: Certificate of Analyses

Analysis Certificate for 3 independent batches of Nova Mentis hydroxytyrosol for heavy metal, antibiotic residue, and trace solvent

Client: **Nova Mentis Ltd.**
c/o Lahiff & Company
Unit 9 - Block C
Cashel Business Park
Cashel road
Dublin 12
IRELAND

Certificate Code: **AR-18-AH-021166-02**
Page Number: **Page 1 of 2**
PO reference:
Reported On: **26/11/2018**

Certificate of Analysis

This certificate replaces all previous certificates for these samples

Laboratory Sample Number: 322-2018-00021266
Sample reception date: 24/10/2018
Your sample description: Nova Mentis Ltd. Hydroxytyrosol Batch:
Your sample reference: Batch:

Test Code	Analyte	Results	Units	Accreditation [1]
Heavy metals				
UD032	Lead	<0.005	mg/kg	EUDLWO:L0
UD033	Cadmium	<0.001	mg/kg	EUDLWO:L0
UD401	Arsenic (As)	0.004	mg/kg	EUDLWO:L0
UD579	Mercury	<0.001	mg/kg	EUDLWO:L0
Other Analysis				
JCAM5	Amikacin	<100	µg/kg	EUHAWE3:R3
	Apramycin	<100	µg/kg	EUHAWE3:R3
	Gentamycine	<100	µg/kg	EUHAWE3:R3
	Hygromycin B	<100	µg/kg	EUHAWE3:R3
	Kanamycine	<100	µg/kg	EUHAWE3:R3
	Neomycin	<100	µg/kg	EUHAWE3:R3
	Paromomycin	<100	µg/kg	EUHAWE3:R3
	Spectinomycin	<100	µg/kg	EUHAWE3:R3
	Tobramycin	<100	µg/kg	EUHAWE3:R3
UD130	Cobalt	0.0103	mg/kg	EUDLWO:L0
JCSRA	1,1,1,2-Tetrachloroethane	<0,01	mg/kg	EUHAWE3:R3
	1,1,1-Trichloroethane	<0,01	mg/kg	EUHAWE3:R3
	1,1,2-Trichloroethane	<0,01	mg/kg	EUHAWE3:R3
	1,1-Dichloroethane	<0,05	mg/kg	EUHAWE3:R3
	1,2-Dichloroethane	<0,05	mg/kg	EUHAWE3:R3
	2-Butanon (Methylethylketon)	<1	mg/kg	EUHAWE3:R3
	2-Methylpentane	<1	mg/kg	EUHAWE3:R3
	3-Methylpentane	<1	mg/kg	EUHAWE3:R3
	Benzene	<0,01	mg/kg	EUHAWE3:R3
	Bromodichloromethane	<0,05	mg/kg	EUHAWE3:R3
	Chloroform (Trichloromethane)	0.012	mg/kg	EUHAWE3:R3
	cis-Dichloroethene	<0,05	mg/kg	EUHAWE3:R3
	Dibromochloromethane	<0,05	mg/kg	EUHAWE3:R3
	Dichloromethane	<0,05	mg/kg	EUHAWE3:R3
	Ethyl Acetate	58	mg/kg	EUHAWE3:R3
	Ethylbenzene	<0,01	mg/kg	EUHAWE3:R3
	m-/-p-Xylene	<0,01	mg/kg	EUHAWE3:R3
	Methyl acetate	<1	mg/kg	EUHAWE3:R3
	Methylcyclopentane	<1	mg/kg	EUHAWE3:R3
	n-Heptane	<1	mg/kg	EUHAWE3:R3

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Page Number: **Page 2 of 2**

Test Code	Analyte	Results	Units	Accreditation [1]
Other Analysis				
JCSRA	n-Hexane	<1	mg/kg	EUHAWE3:R3
	n-Pentane	<1	mg/kg	EUHAWE3:R3
	Styrene	<0,01	mg/kg	EUHAWE3:R3
	Sum 3 chlorinated solvents	0.012	mg/kg	EUHAWE3:R3
	Technical Hexane (calculated)	Not Calculable	mg/kg	EUHAWE3:R3
	Tetrachloroethene	<0,01	mg/kg	EUHAWE3:R3
	Tetrachloromethane	<0,01	mg/kg	EUHAWE3:R3
	Toluene	0.012	mg/kg	EUHAWE3:R3
	trans-Dichloroethene	<0,05	mg/kg	EUHAWE3:R3
	Tribromomethane	<0,05	mg/kg	EUHAWE3:R3
	Trichloroethene	<0,01	mg/kg	EUHAWE3:R3
	Xylene (ortho-)	<0,01	mg/kg	EUHAWE3:R3

Report validated by: Fiona McMahon , Analytical Services Manager on 26/11/2018

[1] Accreditation key:

Unless otherwise stated, all results are expressed on a sample as receved basis.

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Certificate Code: **AR-18-AH-021165-02**
Page Number: **Page 1 of 2**
PO reference:
Reported On: **26/11/2018**

Certificate of Analysis

This certificate replaces all previous certificates for these samples

Laboratory Sample Number: 322-2018-00021265
Sample reception date: 24/10/2018
Your sample description: Nova Mentis Ltd. Hydroxytyrosol Batch:
Your sample reference:

Test Code	Analyte	Results	Units	Accreditation [1]
Heavy metals				
UD032	Lead	<0.005	mg/kg	EUDLWO:L0
UD033	Cadmium	<0.001	mg/kg	EUDLWO:L0
UD401	Arsenic (As)	<0.002	mg/kg	EUDLWO:L0
UD579	Mercury	0.002	mg/kg	EUDLWO:L0
Other Analysis				
JCAM5	Amikacin	<100	µg/kg	EUHAWE3:R3
	Apramycin	<100	µg/kg	EUHAWE3:R3
	Gentamycine	<100	µg/kg	EUHAWE3:R3
	Hygromycin B	<100	µg/kg	EUHAWE3:R3
	Kanamycine	<100	µg/kg	EUHAWE3:R3
	Neomycin	<100	µg/kg	EUHAWE3:R3
	Paromomycin	<100	µg/kg	EUHAWE3:R3
	Spectinomycin	<100	µg/kg	EUHAWE3:R3
	Tobramycin	<100	µg/kg	EUHAWE3:R3
UD130	Cobalt	0.000750	mg/kg	EUDLWO:L0
JCSRA	1,1,1,2-Tetrachloroethane	<0,01	mg/kg	EUHAWE3:R3
	1,1,1-Trichloroethane	<0,01	mg/kg	EUHAWE3:R3
	1,1,2-Trichloroethane	<0,01	mg/kg	EUHAWE3:R3
	1,1-Dichloroethane	<0,05	mg/kg	EUHAWE3:R3
	1,2-Dichloroethane	<0,05	mg/kg	EUHAWE3:R3
	2-Butanon (Methylethylketon)	<1	mg/kg	EUHAWE3:R3
	2-Methylpentane	<1	mg/kg	EUHAWE3:R3
	3-Methylpentane	<1	mg/kg	EUHAWE3:R3
	Benzene	<0,01	mg/kg	EUHAWE3:R3
	Bromodichloromethane	<0,05	mg/kg	EUHAWE3:R3
	Chloroform (Trichloromethane)	0.013	mg/kg	EUHAWE3:R3
	cis-Dichloroethene	<0,05	mg/kg	EUHAWE3:R3
	Dibromochloromethane	<0,05	mg/kg	EUHAWE3:R3
	Dichloromethane	<0,05	mg/kg	EUHAWE3:R3
	Ethyl Acetate	44	mg/kg	EUHAWE3:R3
	Ethylbenzene	<0,01	mg/kg	EUHAWE3:R3
	m-/-p-Xylene	0.017	mg/kg	EUHAWE3:R3
	Methyl acetate	<1	mg/kg	EUHAWE3:R3
	Methylcyclopentane	<1	mg/kg	EUHAWE3:R3
	n-Heptane	<1	mg/kg	EUHAWE3:R3

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Page Number: **Page 2 of 2**

Test Code	Analyte	Results	Units	Accreditation [1]
Other Analysis				
JCSRA	n-Hexane	<1	mg/kg	EUHAWE3:R3
	n-Pentane	<1	mg/kg	EUHAWE3:R3
	Styrene	<0,01	mg/kg	EUHAWE3:R3
	Sum 3 chlorinated solvents	0.013	mg/kg	EUHAWE3:R3
	Technical Hexane (calculated)	Not Calculable	mg/kg	EUHAWE3:R3
	Tetrachloroethene	<0,01	mg/kg	EUHAWE3:R3
	Tetrachloromethane	<0,01	mg/kg	EUHAWE3:R3
	Toluene	0.047	mg/kg	EUHAWE3:R3
	trans-Dichloroethene	<0,05	mg/kg	EUHAWE3:R3
	Tribromomethane	<0,05	mg/kg	EUHAWE3:R3
	Trichloroethene	<0,01	mg/kg	EUHAWE3:R3
	Xylene (ortho-)	0.018	mg/kg	EUHAWE3:R3

Report validated by: Fiona McMahon , Analytical Services Manager on 26/11/2018

[1] Accreditation key:

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Certificate Code: **AR-18-AH-021164-02**
Page Number: **Page 1 of 2**
PO reference:
Reported On: **26/11/2018**

Certificate of Analysis

This certificate replaces all previous certificates for these samples

Laboratory Sample Number: 322-2018-00021264
Sample reception date: 24/10/2018
Your sample description: Nova Mentis Ltd. Hydroxytyrosol Batch: [redacted]
Your sample reference: [redacted]

Test Code	Analyte	Results	Units	Accreditation [1]
Heavy metals				
UD032	Lead	<0.005	mg/kg	EUDLWO:L0
UD033	Cadmium	<0.001	mg/kg	EUDLWO:L0
UD401	Arsenic (As)	<0.002	mg/kg	EUDLWO:L0
UD579	Mercury	<0.001	mg/kg	EUDLWO:L0
Other Analysis				
JCAM5	Amikacin	<100	µg/kg	EUHAWE3:R3
	Apramycin	<100	µg/kg	EUHAWE3:R3
	Gentamycine	<100	µg/kg	EUHAWE3:R3
	Hygromycin B	<100	µg/kg	EUHAWE3:R3
	Kanamycine	<100	µg/kg	EUHAWE3:R3
	Neomycin	<100	µg/kg	EUHAWE3:R3
	Paromomycin	<100	µg/kg	EUHAWE3:R3
	Spectinomycin	<100	µg/kg	EUHAWE3:R3
	Tobramycin	<100	µg/kg	EUHAWE3:R3
UD130	Cobalt	<0.0005	mg/kg	EUDLWO:L0
JCSRA	1,1,1,2-Tetrachloroethane	<0,01	mg/kg	EUHAWE3:R3
	1,1,1-Trichloroethane	<0,01	mg/kg	EUHAWE3:R3
	1,1,2-Trichloroethane	<0,01	mg/kg	EUHAWE3:R3
	1,1-Dichloroethane	<0,05	mg/kg	EUHAWE3:R3
	1,2-Dichloroethane	<0,05	mg/kg	EUHAWE3:R3
	2-Butanon (Methylethylketon)	<1	mg/kg	EUHAWE3:R3
	2-Methylpentane	<1	mg/kg	EUHAWE3:R3
	3-Methylpentane	<1	mg/kg	EUHAWE3:R3
	Benzene	<0,01	mg/kg	EUHAWE3:R3
	Bromodichloromethane	<0,05	mg/kg	EUHAWE3:R3
	Chloroform (Trichloromethane)	0.029	mg/kg	EUHAWE3:R3
	cis-Dichloroethene	<0,05	mg/kg	EUHAWE3:R3
	Dibromochloromethane	<0,05	mg/kg	EUHAWE3:R3
	Dichloromethane	<0,05	mg/kg	EUHAWE3:R3
	Ethyl Acetate	86	mg/kg	EUHAWE3:R3
	Ethylbenzene	<0,01	mg/kg	EUHAWE3:R3
	m-/-p-Xylene	0.027	mg/kg	EUHAWE3:R3
	Methyl acetate	<1	mg/kg	EUHAWE3:R3
	Methylcyclopentane	<1	mg/kg	EUHAWE3:R3
	n-Heptane	<1	mg/kg	EUHAWE3:R3

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Page Number: **Page 2 of 2**

Test Code	Analyte	Results	Units	Accreditation [1]
Other Analysis				
JCSRA	n-Hexane	<1	mg/kg	EUHAWE3:R3
	n-Pentane	<1	mg/kg	EUHAWE3:R3
	Styrene	<0,01	mg/kg	EUHAWE3:R3
	Sum 3 chlorinated solvents	0.029	mg/kg	EUHAWE3:R3
	Technical Hexane (calculated)	Not Calculable	mg/kg	EUHAWE3:R3
	Tetrachloroethene	<0,01	mg/kg	EUHAWE3:R3
	Tetrachloromethane	<0,01	mg/kg	EUHAWE3:R3
	Toluene	0.18	mg/kg	EUHAWE3:R3
	trans-Dichloroethene	<0,05	mg/kg	EUHAWE3:R3
	Tribromomethane	<0,05	mg/kg	EUHAWE3:R3
	Trichloroethene	<0,01	mg/kg	EUHAWE3:R3
	Xylene (ortho-)	0.021	mg/kg	EUHAWE3:R3

Report validated by: Fiona McMahon , Analytical Services Manager on 26/11/2018

[1] Accreditation key:

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