GRN 876

Nova Mentis
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Dublin 4
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June 11, 2019

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 10.8.

If you have any questions or require additional information, please do not hesitate to contact me, 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 000876	DATE OF RECEIPT 6/17/2019
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 Kevin O Connor		Position or Title Chief Scientific officer	
	Organization *(if applicable)* Nova Mentis ltd			
	Mailing Address *(number and street)* Nova UCD Belfield innovation Park			
City Dublin		State or Province	Zip Code/Postal Code D04 V1W8	Country Ireland
Telephone Number +353 85 7227580		Fax Number	E-Mail Address 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

☒ Paper

☒ Electronic files on physical media

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 92

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 _____

☐ 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)* _____

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; 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.
(name of notifier)

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.

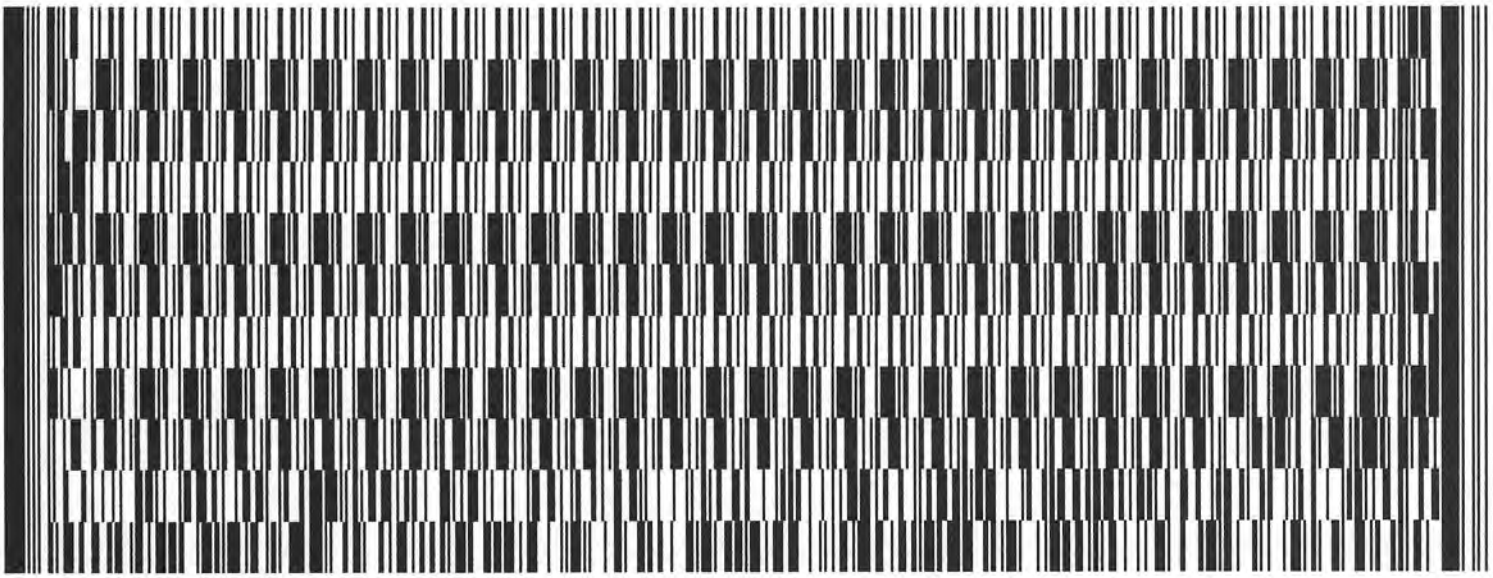
3. Signature of Responsible Official, Agent, or Attorney	Printed Name and Title	Date (mm/dd/yyyy)
[redacted]	Kevin O Connor CSO	06/11/2019

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)
	US FDA GRAS HT Nova Mentis cover letter signed.pdf	Submission
	NovaMentis Grass Dossier new 2019 June 11.pdf	Submission
	US FDA GRAS form 3667 2019 June 11 signed.pdf	Submission

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

June 11, 2019

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 April 2019. 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 (GRN 600) received no question letter from FDA 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. Also an olive preparation containing 40% hydroxytyrosol (GRN726) received no question letter from FDA 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

June 11, 2019

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

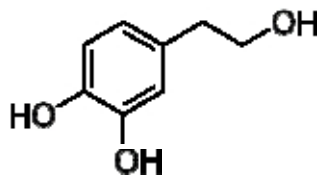


Figure 1: Chemical structure of Hydroxytyrosol

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 an enzyme, as a processing aid. The production step (Figure 2) involves cultivation of biocatalyst *E. coli* BL21(DE3) to a desired cell density, cell harvest to remove fermentation media components, washing of cells to remove any media components, breakage of cells by homogenization to produce a broken cell preparation (biocatalyst), conversion of tyrosol to hydroxytyrosol using the biocatalyst as a processing aid. Downstream processing of hydroxytyrosol from the biotransformation liquid was carried out by 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.

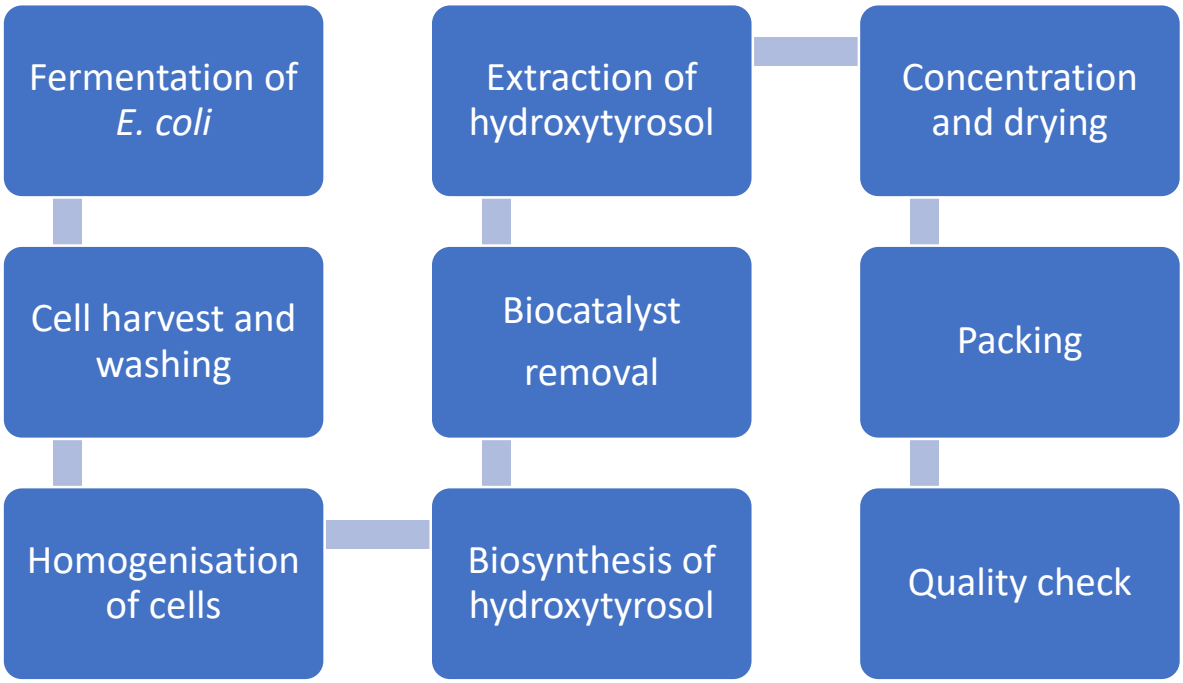


Figure 2: Flow diagram of the Nova Mentis hydroxytyrosol production process

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.

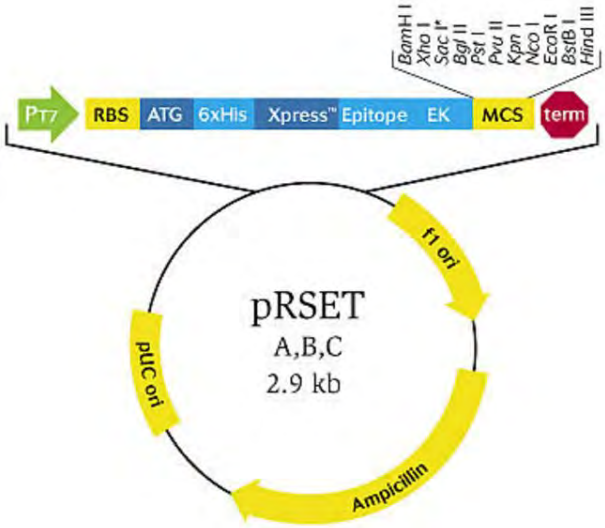


Figure 3: The plasmid pRSETB

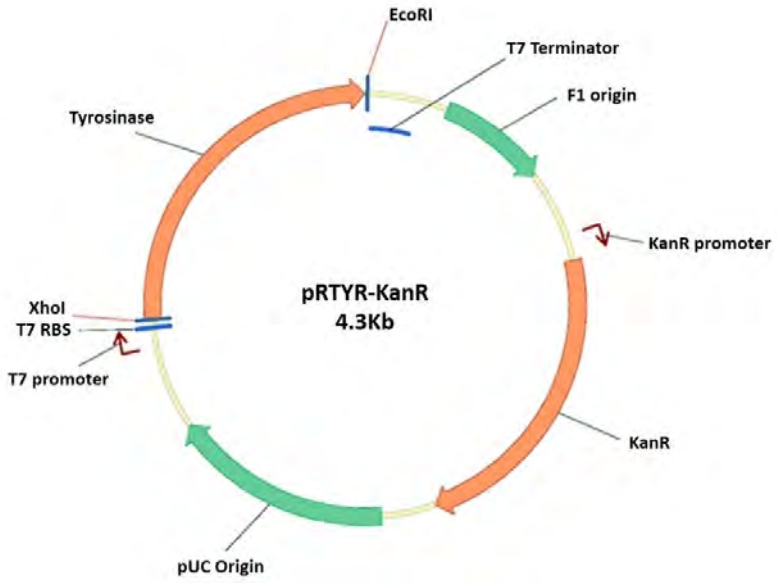


Figure 4: pRTYR-KanR construct (Ampicillin gene replaced with Kanamycin gene)

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.

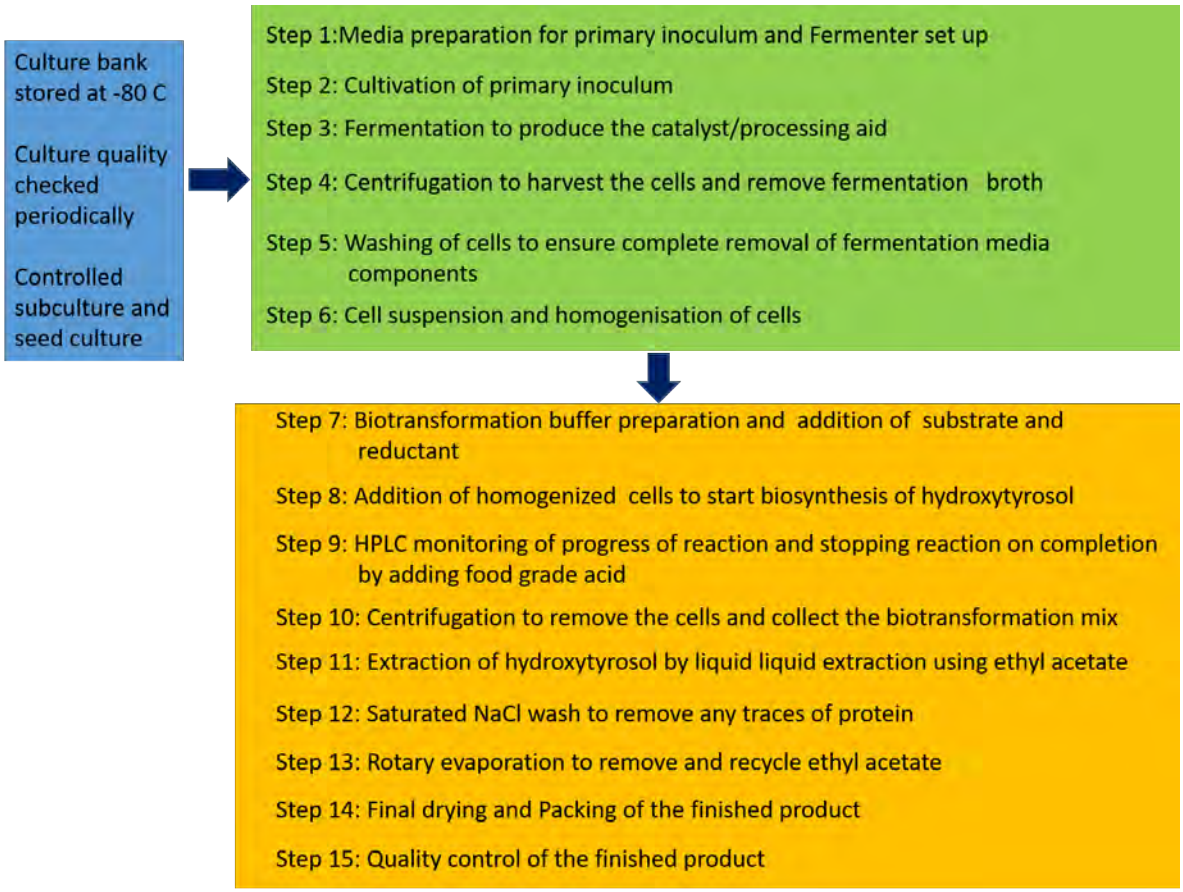


Figure 5. A stepwise flow chart of the Nova Mentis hydroxytyrosol manufacturing process

The temperature is maintained at 37 ± 0.1ºC 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± 0.1ºC 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, ascorbic acid and homogenised 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 using 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 dried to remove traces of water and solvent. The finished product is packed in food grade containers and stored at 4-8 °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% 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	< 500 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	< 500 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.

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 6. Information on the intended food uses and use levels was used to estimate consumer intakes, which are discussed further below

Table 6 Summary of all proposed foods and use levels

Food category	HT[a] use levels (mg/ serving	RACC [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. Available Data and Methods

3.3.1 Consumption Data

The daily intake of HT is estimated using "maximum" intended use levels and mean consumption estimates of designated food categories based on food consumption records collected in the What We Eat in America (WWEIA) component of the National Health and Nutrition Examination Surveys (NHANES) conducted in 2007-2008 and 2009-2010 (2007-2010).

For the existing dietary exposure to hydroxytyrosol from olives and olive oil, the 2-day average intake of hydroxytyrosol was estimated by multiplying the reported intake of foods from the 24-hr recall with the hydroxytyrosol concentration derived from the literature and the cumulative sum over the two 24-hr recalls was divided by two. Estimates were also derived on a body weight basis based on each participant's reported body weight.

The cumulative estimated daily intake (CEDI) for hydroxytyrosol was calculated by summing at the individual level the EDI from existing dietary sources with the EDI from proposed uses of Nova Mentis HT.

3.3.2 Existing dietary exposure

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 7 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 7 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).

3.3.4 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.

3.3.5. 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 90th 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 90th percentile *per user* CEDI for the U.S. population 2 years and older was 52.4 mg/day (0.9 mg/kg-bw/day).

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).

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).

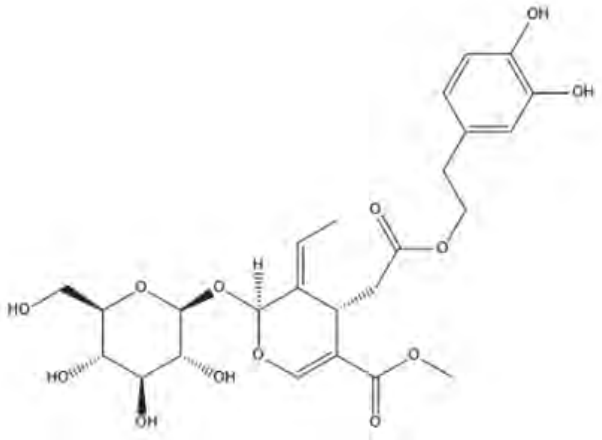


Figure 6: Molecular structure of Oleuropein (Adapted from Sahin and Bilgin, 2018).

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. Sub chronic toxicity studies

There are three published major sub chronic toxicity studies have been carried out with hydroxytyrosol or olive extract containing hydroxytyrosol (Auñon-Calles et al., 2013a; Christian et. al*,* 2004; Heilman et al*.,* 2015). These studies and their results are explained below.

6.1.1.1. Study 1 by Auñon-Calles et al. (2013a): 90 day study with pure hydroxytyrosol

Method

This study (Auñon-Calles et al., 2013) was performed in accordance with internationally accepted guidelines and OECD Guideline 408, and GLP. Hydroxytyrosol was administered orally daily by gavage to 10 rats/sex/group for a 13-week period at dose levels of 0, 5, 50, and 500 mg/kg/day to rats (Wistar Hannover RccHan™: WIST, from Harlan Laboratories, B.V.). Five additional animals per sex in groups 1 and 4 were used for a four-week recovery period. 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).

Results

During the course of the study, no mortality was noted in any group. Salivation was recorded in all animals treated at the high dose and sporadically in some animals from groups treated at the intermediate- and low-doses. This phenomenon was attributed to the bitter taste of hydroxytyrosol and/or the physical characteristics of the formulation (slightly oily and dense). Because the effects on body weight and body-weight gain in males and females treated at the high dose level were modest and a recovery was observed after four treatment-free weeks, these outcomes were not considered by the authors as toxicologically relevant. However, there was a lower weight gain in males at the high dosage of 500 mg/kg bw/day.

Some haematological and biochemical changes were recorded and included higher mean cell volume (MCV) and mean cell haemoglobin (MCH) in females treated at the high- and intermediate-doses; higher reticulocytes with high fluorescence (HFR) and white blood cells (WBC) values in females treated at the high dose; lower creatinine and higher albumin values in males treated at the high dose; and higher calcium values in males treated at intermediate- and high-doses. Higher relative kidneys weights were observed in males and females from the 500 mg/kg group. Differences were statistically significant in females as related to brain weight and males and females as related to body weight. Some other statistically significant differences in organ weights relative to body weight were observed in animals from the 500 mg/kg 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. Microscopy observations did not reveal any morphological alteration in any of the organs or tissues examined. There were no differences between controls and test-item treated animals.

Conclusion

Based on the results obtained, daily oral administration of hydroxytyrosol to rats for a period of 13 weeks did not induce effects that can be considered of toxicological relevance. However, considering the statistically significant higher relative kidney weights in males and females from the 500 mg/kg bw/d group it can be concluded that hydroxytyrosol at dose level up to 50 mg/kg bw/day is unlikely to cause any adverse effects.

6.1.1.2. Study 2 by Christian et al. (2004): 90-day rat study with olive pulp extract (HIDROX)

Method

In this 90-day study, 20 rats/sex/group (excluding satellite animals) of the Sprague Dawley (CD1) strain were administered HIDROX® (hydrolysed aqueous olive pulp extract; OPE) by oral gavage at 0, 1000, 1500 and 2000 mg/kg bw/day; corresponding to dosages in terms of hydroxytyrosol to 0, 24, 36 and 48 mg/kg bw/day, respectively (Christian *et al.,* 2004). The study was performed following international guidelines including OECD 408 requirements and in accord with GLP. Study included additional satellite animals for toxicokinetic sampling, MNT genotoxicity element (after 4 weeks treatment) and a single dose acute phase at 5000 mg/kg bw in terms of olive pulp extract. The study included a micronucleus (MN) evaluation and an acute phase element. Blood samples (from 6/sex/group) were collected on day 90, prior to dosing and at 0.5, 1, 2, 4 and 8 h post-dose for hydroxytyrosol measurement.

Results

Daily oral dosages of 1000, 1500 and 2000 mg/kg bw/day for 90 days produced small decreases in body weight gains at 2000 mg/kg bw/day in the male rats and in all groups of female rats. Feed consumption was comparable to controls. There were no adverse effects upon clinical, hematologic, biochemical, organ weight or gross necropsy parameters. 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/day; this change was attributed to local irritation by repeated intubation of large volumes of viscous, granular dosing suspension. In the acute phase element at 5000 mg/kg, there were no deaths or clinical signs of toxicity.

Plasma data for hydroxytyrosol indicated that hydroxytyrosol was rapidly absorbed. Mean concentrations were measurable through 1 to 4hr at 1000 and 1500 mg/kg/day and through 8 hours at 2000 mg/kg/day. AUC last and Cmax were similar for males and females at the corresponding dosages.

Conclusions

Based on the study results the authors observed no significant adverse effect and they established a NOAEL at the high dose of 2000 mg HIDROX/kg/day, or 48 mg/kg/day in terms of hydroxytyrosol

6.1.1.3. Study 3 by Heilman et al. (2015): 90-Day rat study with olive extract containing 35% HT

Method

In this study a an olive extract containing 35% hydroxytyrosol (H35) was administered orally (gavage) to male and female Wistar rats for 13 weeks, followed by a 4-week treatment-free period, at doses of 0, 345, 691 and 1381 mg /kg bw/day, which were equivalent to doses of 0, 125, 250 and 500 mg/kg/day in terms of hydroxytyrosol. The study was performed following OECD guideline 408 and GLP. The study included additional elements in addition to the standard OECD guideline 408 endpoints. These included neurobehavioral observations, seminology, estrous cycling and a MNT genotoxicity element. Also, blood samples were collected 30 minutes after dosing one day in weeks 4, 8, and 13 for hydroxytyrosol analysis.

Results

With respect to end-points in the main OECD 408 phase, no mortality or morbidity was observed during the study period. Animals from the high dose group showed signs of mild to moderate salivation intermittently from weeks 1 to 13. Similarly, in the intermediate dose group salivation was observed during weeks 2 to 13 in 3 to 5 animals. The observation of salivation occurred beginning at approximately 15 minutes post-dosing and persisted for approximately 40 to 50 minutes. This effect was considered to be related to the test article but not an adverse treatment-related effect.

A statistically significant lower body weight was observed during weeks 6 to 10 in males of the high dose group compared with controls ($P<0.05$). The body weight deficit in the high dose group males was approximately 9% at 13 weeks compared to control males. No significant changes were observed in body weight and percent body weight change for male or female rats in the low and intermediate dose groups, except for a statistically significant decrease in body weight gain ($P<0.01$) observed during the first week of treatment for the low dose group males compared to controls. This difference quickly recovered and by the second week, animals were gaining weight at a rate not statistically different from control animals. In females, a statistically significant decrease in body weight ($P<0.05$) was observed during week 2 in the high dose recovery group when compared with the control recovery group, while in all other weeks of the treatment period, reductions in body weight in both the high dose and high dose recovery groups were not statistically significant.
The reduction in body weight at 13 weeks of study in the high dose males was considered treatment-related, and it corresponded with a reduction in mean body weight gain (17%) which also showed statistical significance. No significant changes in mean food consumption were observed for treated males or females when compared with controls during the treatment phase.

Statistically significant increases in MCV, MCH, neutrophil count and platelet count were observed in high dose male animals compared to corresponding controls as well as a statistically significant

reduction in lymphocyte count. Increases in MCV and MCH values were observed in female rats of the intermediate dose group, and these changes were also statistically significant. However, there was no apparent dose-response and the changes did not reach significance in the low- and high- dose groups. A statistically significant increase in WBC count was observed in female rats of the low- and high-dose groups, however significance was not achieved in the intermediate dose group. Platelet counts were significantly increased in females at the high dose compared to controls and significant decreases in HCT, MCV, MCH and platelet counts were observed, along with an increase in MCHC in male rats of the high dose recovery group when compared with the control recovery group. All haematological variations observed following treatment with H35 at any dose level and during recovery were inconsistent without dose- response apart from minor differences in males of the high dose group. The observed variations were not considered toxicologically significant.

Compared to controls, increased serum albumin levels were observed with statistical significance in all dose groups tested and significant increases in total serum protein levels were observed in males at the low- and high-dose groups, but not in the intermediate-dose group. Serum phosphorus was significantly increased in males at the intermediate- and high-doses compared to controls, and serum chloride and sodium were decreased significantly in females of the low- and high-dose groups. In high-dose females, serum alkaline phosphatase was significantly increased compared to controls, and in males of the high-dose recovery group, serum triglyceride levels were significantly increased compared to the level in the recovery high- dose males. Alterations in clinical chemistry parameters were determined to be unrelated to treatment due to their lack of dose dependence, spontaneous nature, and their concordance with historical control ranges. Urine volume was significantly increased and urine pH significantly decreased for males in the high-dose group compared to controls, and urine pH was significantly decreased in males of the intermediate-dose group compared to controls. Urine pH was also significantly decreased in females at the high dose, and increased in females of the high-dose recovery group. All noted observations in urinalysis parameters were without dose-response, spontaneous in nature, and within historical control range, and were therefore considered not to be of toxicological relevance.

Macroscopic external examination of animals of both sexes and across dose groups did not reveal any treatment-related abnormalities of pathological significance. Spontaneous observations that were not determined to be treatment-related included instances of enlargement of the spleen, hydronephrosis/distended pelvis in kidney, small-sized testes and epididymis, and distension of uterus and oviduct. Microscopic examination of tissues collected revealed various minimal lesions, not related to treatment and within historical control ranges, present in the liver, kidneys, spleen, thymus, pituitary, eye, Harderian gland, heart, vagina, testes, epididymis, thyroid, adrenals and oviducts. Although some instances of lesions were observed with greater frequency in the high dose group, for example, in the spleen, extramedullary haematopoiesis (EMH) and hemosiderosis occurred in 3/10 females at the high dose, while EMH only occurred in control females at an incidence of 1/10 animals, none of the observations were considered treatment-related as all were within

normal historical control ranges. Similarly, adrenal lipidosis occurred at a higher incidence (4/10) in high dose group males than controls (0/10), however, lipidosis is to some extent a normal finding which is increased with stress, and lesions were minimal and without corresponding changes in other parameters, thus the finding was considered spontaneous and not treatment-related. Significant decreases in absolute weights of the thymus were observed in females of the intermediate-dose group compared with controls and a significant increase in absolute kidney weight was observed in females of the high dose group compared to controls. For males in the high dose group, significant increases in relative weights of the liver, heart, spleen and kidneys were observed, and a significant increase in relative weight of the kidneys was observed in male rats of the intermediate dose group compared to control animals. Similar to the findings in high dose males, a significant increase in relative weights of the liver, heart and kidneys was observed in female rats of the high dose group compared with controls. A significant decrease in the relative weight of the thymus was observed in female rats of the intermediate dose group compared with control females. The significant increase in relative weights of liver, thymus, kidneys and spleen of the high dose group which appeared to occur with a dose-response, could be considered treatment-related effects. However, in the absence of any corresponding or related clinical, gross or microscopic lesion, this could not be explained pathologically, and these effects could be considered non- adverse.

Neurobehavioral observations conducted weekly in the home cage, during handling and in the open field did not reveal any test item-related abnormality in treated animals. Neurobehavioral observations made during removal and handling of animals did not reveal any abnormalities related to treatment. Normal gait and mobility were observed during open field observations in all treated groups and controls, and there were no alterations observed in rearing, or urination and defecation counts for treated males and females compared to controls. In the high dose recovery group males, a significant increase in rearing count was observed during week 8 and week 11, however, this was not considered treatment-related and it did not correspond to any other findings of toxicological significance. Likewise, no alterations were observed in mean grip strength values in groups treated with H35 compared with controls, except for a significant decrease in forelimb grip strength was observed in male rats of the high dose recovery group when compared with the control recovery group. This finding was not considered treatment-related in absence of further supportive findings.

Ophthalmological examinations conducted as part of the neurological testing set for the study did not reveal any abnormalities in any treatment groups compared to controls. In male rats, there no apparent treatment related effects on sperm motility, percent abnormal sperm and no significant changes in Homogenisation Resistant Spermatid count from testicular and cauda epididymis samples of treated male rats compared with respective controls. Estrus cycle length and pattern of all treated female rats were comparable with female rats of the control group. Recovered plasma hydroxytyrosol concentrations (unconjugated or “free” HT) ranged from approximately 1543 to 2635 ng/mL in the low-dose group, 2623 to 5096 ng/mL in the mid- dose group and 5535 to 7229 ng/mL in the high-dose group. Plasma concentration of hydroxytyrosol did not differ significantly

within same dose levels at different occasions of blood collection (weeks 4, 8, and 13). Total plasma hydroxytyrosol (after enzymatic de-conjugation) was not measured.

Conclusion

Daily oral administration of H35 to male and female Wistar rats for a period of 90 days did not induce any effect on body organs that could be regarded as toxicologically relevant. No reduction in food consumption was observed to explain the slightly lower weight gain in the high dose male rats (500 mg/kg bw/day). Based on the reduction in body weight gain in the high dose males, it was concluded that the NOAEL of H35 is 250 mg hydroxytyrosol/kg bw/day (equivalent to 691 mg H35/kg bw/day). The high dose, equivalent to 500 mg hydroxytyrosol/kg bw/day, can also be considered to be the lowest observed adverse effect level (LOAEL).

6.1.2. Acute toxicity studies with hydroxytyrosol

6.1.2.1. Study 1 by Christian et al. (2014): Acute oral study in the mouse and rat with HIDROX

Method

CD-1 mice (Charles River Laboratories) and Crl:CD1(SD)IGS BR VAF/Plus1 rats (Charles River Laboratories, Portage, Michigan) were used in the studies. After one week of acclimation, all animals were randomly assigned to groups of five males and five females by a computer-generated weight-ordered distribution such that individual body weights did not exceed ± 20% of the mean weight for each sex. On Study Day (DS) 1, the mice were seven weeks old; males weighed 27–29 g, and females weighed 21–24 g. The rats were 70 days old; males weighed 310–375 g, and females weighed 175–244 g. On Day 1 a single dosage of 2000 mg/kg (limit dosage) of hydrolysed aqueous olive pulp extract (OPE) was administered to each fasted mouse via gavage; animals were then allowed to recover for 14 days. Single dosages of 0 (aqueous 0.5% methylcellulose), 1000, 1500 or 2000 mg/kg OPE (corresponding to 0, 24, 36 and 48 mg of hydroxytyrosol) were administered to each of the rats in the four dosage groups. An additional group of 5 male and 5 female rats was gavaged with a single higher limit dosage of 5000 mg/kg OPE and then observed for six days, after which 5000 mg/kg was given for 29 consecutive days. Oral dosage volumes were 10 mL/kg. The following parameters were recorded during the studies: clinical signs (hourly for four hours during the first day; twice daily thereafter); daily body weights and feed consumption in rats; weekly body weights in mice (no feed consumption in mice); daily water consumption in rats only; observations of gross lesions at necropsy on day 15, following euthanasia by carbon dioxide asphyxiation.

Results

A single limit dose of 2000 mg/kg of OPE, administered by gavage and followed by a 14-day recovery period, produced no mortality or morbidity. No abnormal clinical signs or gross morphologic changes were noted in either male or female mice. There was a mean body weight gain of 5.4 grams in male mice and 4.2 grams in female mice during the 15 days on study. Due to the lack of toxicity at the limit dose, lower doses of OPE were not investigated in this study. In rats, a single dosage of 0 (vehicle), 1000, 1500 or 2000 mg/kg OPE produced no mortality, morbidity, abnormal clinical signs or gross changes at necropsy. Body weight gains for the entire study period were generally comparable for the male rats in the four dosage groups. For female rats, weight gains were reduced at 1500 and 2000 mg/kg although absolute (g/day) or relative (g/kg/day) feed consumption was generally comparable among the four dosage groups. Non-dosage-dependent reductions in absolute and relative water consumption occurred in male rats administered the test article, while values for female rats were comparable to or increased over control group values for the same periods No mortality was produced in the five rats/sex administered single doses of 5000 mg/kg and then observed for another six days. No clinical signs of toxicity were revealed, and both males and females continued to gain weight, although at a reduced rate when compared to control rats from an equivalent weight period in the 90-day study. Absolute (g/day) or relative (g/kg/day) feed consumption was similar to that for the controls. Administration of 5000 mg/kg/day dosages of OPE for 29 consecutive days was well tolerated by the rats although it resulted in decreased body weight gains. It is noteworthy to mention hematology and serum chemistry determinations and histopathologic examination of tissues were not performed for this group of animals.

Conclusions

According to study results the authors conclude the acute oral NOAEL was considered to be 2000 mg/kg of OPE (equal to 48 mg/kg of hydroxytyrosol) in the mouse studies and 1000 mg/kg in the oral rat study (24 mg/kg of hydroxytyrosol). Based on the lack of significant adverse OPE-related effects at 2000 mg/kg/day, a NOAEL of 2000 mg/kg/day was established for the repeat-dosage studies in rats.

6.1.2.2. Study 2 by D'Angelo et al. (2001): Acute oral toxicity study in the rat with pure hydroxytyrosol

Method

The study was carried out using young adult Sprague-Dawley rats by RBM-Laboratories & Clinics Group (Colleretto Giacosa, Italy). Six male and six female rats, about 3-months old and weighing

210 to 262 g, were used for the experiment. They were acclimatized at least 5 days before starting the test and fasted about 16 h before the experiment. A single dose of 2 g/kg BW of pure hydroxytyrosol was administered by gavage. The hydroxytyrosol was chemically synthesized.

Three hours after treatment, diet was made available “ad libitum”. During the study period, rats were housed under controlled environmental conditions. The rats were observed and weighed daily, after administration of hydroxytyrosol until day 14. At the end of the test, rats were sacrificed, and gross pathological changes in main organs were evaluated. Toxicity was determined from the death/survival ratio of treated animals

Results

During the study period, no death occurred in the treated animals; the only clinical sign observed in males and females was piloerection, which started 2 h after gavage and disappeared within 48 h from treatment. Body weight did not vary after substance administration, and the autoptic analysis failed to show appreciable macroscopic alterations of internal organs.

Conclusions

The acute oral LD50 value for hydroxytyrosol is greater than 2000 mg/kg/BW.

6.1.2.3. Study 3 by Martínez et al.(2018) acute and repeat dose oral safety study in the rat with phosphatidyl-hydroxytyrosol

Method

In the acute (limit test) study, 24 rats (Wistar 12 males, 12 females) were distributed into two groups of 6 males and 6 females each. After an overnight fast each rat received distillate water orally (control group or Group 1), or a single oral dose of 2000 mg PHT/kg bw; PHT dissolved in distilled water (treated group or Group 2). Doses of the test and control articles were administered by gavage at a volume of 15 mL/kg bw based on the individual animal body weights obtained on the day dosing. Animals were checked for clinical signs and mortality twice a day (a.m.and p.m). At the end of a 14 days observation period, the rats were weighed, killed by decapitation, then exsanguinated, and necropsie. The repeated dose (28 days) (limit test) study was conducted in 48 rats (24 males, 24 females) divided in four groups of 6 males and 6 females each (control group or Group 3; treated group or Group 4;satellite control group or Group 5; and satellite treated group or Group 6). Rats received a daily dose of either distilled water (Groups 3 and 5) or 2000 mg/kg bw of PHT dissolved in distilled water (Groups 4 and 6) orally once a day over 28 days. All animals were observed twice daily for general appearance, behaviour, sings of morbidity and mortality (once before treatment and once daily thereafter). Rats were observed for their general condition and the condition of the skin

and fur, eyes, nose, oral cavity, abdomen and external genitalia, evaluated for respiration rat and palpated for masses. Behavioural parameters checked were abnormal movements (tremor, convulsion, and muscular contractions), reactions to handling and behaviour in open field (excitability, responsiveness to touch and to sharp noise), changes in ordinary behaviour (changes in grooming, head shaking, and gyration), abnormal behaviour (autophagy, backward motion) and aggression. Body weight, body weight gain and food and water consumption were measured daily and at the end of the observation periods the rats were examined by necropsy, and the weights of the organs recorded

Results

Phosphatidyl-hydroxytyrosol administered in a single oral gavage dose of 2000 mg/kg of body weight (bw) resulted in no adverse events or mortality. There were no statistical differences in bodyweights among groups. Similarly, no statistically significant differences in body weight gain, food and water consumption were noted. Body weight, daily body weight gain, food and water consumption thus were unaffected by the treatment. The haematological and clinical chemistry parameters assessed 14 days after administration of the PHT product as a single oral dose of 2000 mg/kg bw were not significantly different compared with those of controls. No treatment-related changes were noted. In addition, phosphatidyl-hydroxytyrosol administered as a daily dose of 2000 mg/kg bw for 28 days by gavage resulted in no adverse events or mortality. Data analysis of body weight gain, food consumption, clinical observations, blood biochemical, haematology, organ weight ratios and histopathological findings did not show significant differences between control and treated groups.

Conclusions

It is concluded that phosphatidyl-hydroxytyrosol orally administered to rats was safe and 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.

6.1.3. Reproductive Toxicity

In a reproductive toxicity study in rats, Christian et al. (2004) evaluated the potential adverse effects of olive pulp extract containing 24 mg hydroxytyrosol/g of the extract. Sprague Dawley rats (8/sex/group) were administered once daily with the extract at a dose level of 0, 500, 1000, 1500 and 2000 mg/kg bw/day for 14 days before cohabitation. The equivalent dose of hydroxytyrosol for each group was 0, 12, 24, 36 and 48 mg/kg bw/day, respectively. The treatment was continued until the day before necropsy (males were euthanized after being administered a total of 49 daily doses of the extract; females were euthanized after completion of the 22-day post-partum period). All 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 epididymis 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.

6.1.4. 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.

6.1.5. Genotoxicity studies

6.1.5.1. Ames Assay (*in vitro* studies for mutation)

Ames assays have been carried out with olive extract containing 35% HT (H35) (Kirkland et al. 2015) HIDROX olive extract with 2.4% HT (Christian et al. 2004) and with pure hydroxytyrosol (Aunon-Calles et al. 2013 b). These studies and their results are explained below.

6.1.5.1.1. Ames test with H35 (Kirkland et al., 2015)

This study was performed following OECD guidelines and done under GLP. This study used Salmonella typhimurium strains TA98, TA100, TA1535, TA1537, and TA102

The study included concentration analysis of the formulation in terms of hydroxytyrosol.

Based on the results of the cytotoxicity test, test concentrations of 156.25, 312.5, 625, 1250, 2500 and 5000 µg/plate of hydroxytyrosol both in the absence and presence of metabolic activation (5% v/v S9 mix) were selected for Trial I. Trial I did not show any positive mutagenic responses when compared with the negative control at any of the tested concentrations in any of the 5 strains.

Trial II was conducted to confirm the negative results of Trial I with concentrations separated by

2.5 fold i.e., 51.2, 128, 320, 800, 2000 and 5000 µg/plate of active ingredient hydroxytyrosol both in the absence and presence of metabolic activation (S9 concentration was increased to 10% v/v). No mutagenic responses were observed in Trial II confirming the results of Trial I. The efficiency of the test system was demonstrated by clear increases in numbers of revertant colonies observed with the positive controls both in the absence and presence of metabolic activation in both trials.

The formulation concentrations tested were confirmed analytically. The active ingredient hydroxytyrosol was found to be within an acceptable range of ± 10 % in sterile reverse osmosis water at the tested concentrations of 1562 and 50003 µg/mL (stock solutions from which 0.1 mL was added to each plate) during the main study (Trial I). Average recoveries were 99% and 100% at these test concentrations, respectively.

Therefore, the doses complied with the presence of test item for claimed concentration (± 10%) of active ingredient. The results of this study with H35 were clearly negative, without any indication of gene mutation potential.

6.1.5.1.2. Ames test with HIDROX (Christian et al., 2004)

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).

6.1.5.1.3. Ames test with pure hydroxytyrosol (Auñon-Calles et al., 2013b)

This test was performed in accordance with international guidelines and GLP. *Salmonella typhimurium* strains TA 100, TA98, TA1535, and TA1537 and *Escherichia coli* strain WP2(pKM101) were exposed to pure HT at 5 concentrations (5 µL/plate down to 0.06 µL/plate) with and without S9 under the direct incorporation (main study) and the pre-incubation (confirmatory study) procedures.

None of the concentrations assayed for HT were stated by the authors to show an increase in the revertant counts relative to control (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. Therefore, there was no indication of mutagenic potential.

6.1.5.2. *In vitro* Micronucleus and Chromosomal Aberration

In an *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.

In a similar study Aunon Calles et al. (2013b) first assessed pure HT, dissolved in deionised water, for its potential to induce chromosomal aberrations in human lymphocytes in vitro in the absence and presence of metabolic activation by S9 mix. The highest treatment concentration in this study, 1542.0 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 observed. No relevant influence on osmolarity or pH value was observed. In the absence of S9 mix one statistically significant increase in the number of aberrant cells, excluding gaps (9.0%) was observed after treatment with 503.5 µg/mL In the presence of S9mix after treatment with 287.7 and 503.5µg/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. Either EMS (770.0 µg/mL) or CPA (15.0 µg/mL) were used as positive controls and showed distinct increases in cells with structural chromosome aberrations.

6.1.5.3 *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 micro nucleated polymorphic erythrocytes were not significantly increased in any of the extract treated groups. Similarly, the ratio of polychromatic erythrocytes to norm chromatic 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.

Reference	Test component/ daily	Study type	Duration	Subjects	Safety 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 adverse effect or positive effect reported
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	No adverse effect reported
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	No adverse effect reported
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	No adverse effect reported.
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	No adverse effect reported
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	No adverse effects reported
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	No adverse effect reported.

Table 10 Summary of human studies of olive phenolics*

*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	Safety Outcome
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	No adverse effect reported
Covas et al., 2006b	Olive oil low phenolic content (0 mg) Olive oil medium phenolic content (6 mg) Olive	Randomized, cross-over, controlled,	Single dose	12 healthy male volunteers	No adverse effect reported
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	No adverse effect reported
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	No adverse effect reported
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	No adverse effect reported
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	No adverse effects reported
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	No adverse effect reported

*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.

In a study in human subjects (21 healthy volunteers), 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 (after one-week wash out,i.e. olive-free diet, subjects were randomly assigned to either the placebo (maltodextrin), 5 mg/d HT, or 25 mg/d HT group Administration of each treatment was carried out for one week, followed by a one-week washout after which treatments were switched).. 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. Both 5 and 25 mg HT doses were well-tolerated and no adverse effect was reported. No differences in anthropometric variables, such as weight or body mass index, were recorded and no significant variations in vital signs such as blood pressure were noted either (Crespo et al. 2015) 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.

Colica et al. (2017) carried out a randomized double-blinded, placebo-controlled crossover trial to determined the effect in healthy volunteers of two gastro resistant capsules containing 15 mg/day of HT, for a 3-week period. Evaluation of nutritional status, serum metabolites, oxidative stress biomarkers, and gene expression of 9 genes related to oxidative stress, inflammation, and CVDs was performed. This study did not report any adverse effect.

Lopez-Huertas and Fonolla (2017) studied the chronic effects (eight weeks) produced by the intake of pure HT administered in humans as a supplement in an aqueous solution. HT was administered at a daily dosage of 45 mg for 8 weeks to volunteers with mild hyperlipidemia (n=14). This study measured markers of cardiovascular disease risk, enzyme markers of several clinical conditions, hematology, antioxidant parameters, vitamins and minerals at baseline (T0), 4 weeks (T4) and 8 weeks (T8). The values obtained at T4 and T8 were compared with baseline. The HT dose administered was well tolerated, safe and did not influence markers of cardiovascular disease, blood lipids, inflammatory markers, liver or kidney functions and the electrolyte balance in healthy subjects with borderline high levels of cholesterol. Some minor changes were detected in biochemical parameters analysed in serum like a decrease in lactate dehydrogenase or an increase in creatine phosphokinase enzymes, but their values were kept within the normal range and the changes have little clinical relevance. Serum iron levels remained constant but a significant ($P < 0.05$) decrease in ferritin at T4 and T8 was found albeit witin the physiological range. Serum folate and red blood cell folate levels were also reduced at T4 and T8. Finally, vitamin C increased by two-fold at T4 and T8 compared with levels at baseline.

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, hydroxytyrosol 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
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
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 phenol sulfotransferase.

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. ribed 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 6), 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 (48 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 (D'Angelo et al., 2001) indicates that the oral LD50 of hydroxytyrosol is greater than 2000 mg/kg/bw. In a recent 90-day dose-response study (Heilman et al, 2015) safety of olive extract H35 containing 35% hydroxytyrosol revealed statistically significant reductions in body weight

gain (9%) and decreased absolute body weight (17%) in male rats. 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 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.. 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. 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 (GRN726) received no question letter from FDA 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.

7. LIST OF SUPPORTING DATA AND REFERENCES

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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). 1H NMR and 13C NMR spectra of three batches of hydroxytyrosol were recorded on Varian Inova spectrometer (500 MHz). The chemical shifts for 1H NMR and 13C NMR are as follows:
1H NMR (500 MHz, DMSO-d6): δ 8.67 (s, 1H), 8.58 (s, 1H), 6.61 (d, J = 7.9 Hz, 1H), 6.58 (d, J = 2.1 Hz, 1H), 6.43 (dd, J = 8.0, 2.1 Hz, 1H), 4.54 (s, 1H), 3.50 (t, J = 7.3 Hz, 2H), 2.53 (t, J = 7.3 Hz, 2H).

13C NMR (126 MHz, DMSO-d6): δ 145.3, 143.7, 130.6, 119.9, 116.7, 115.8, 63.1, 39.0.

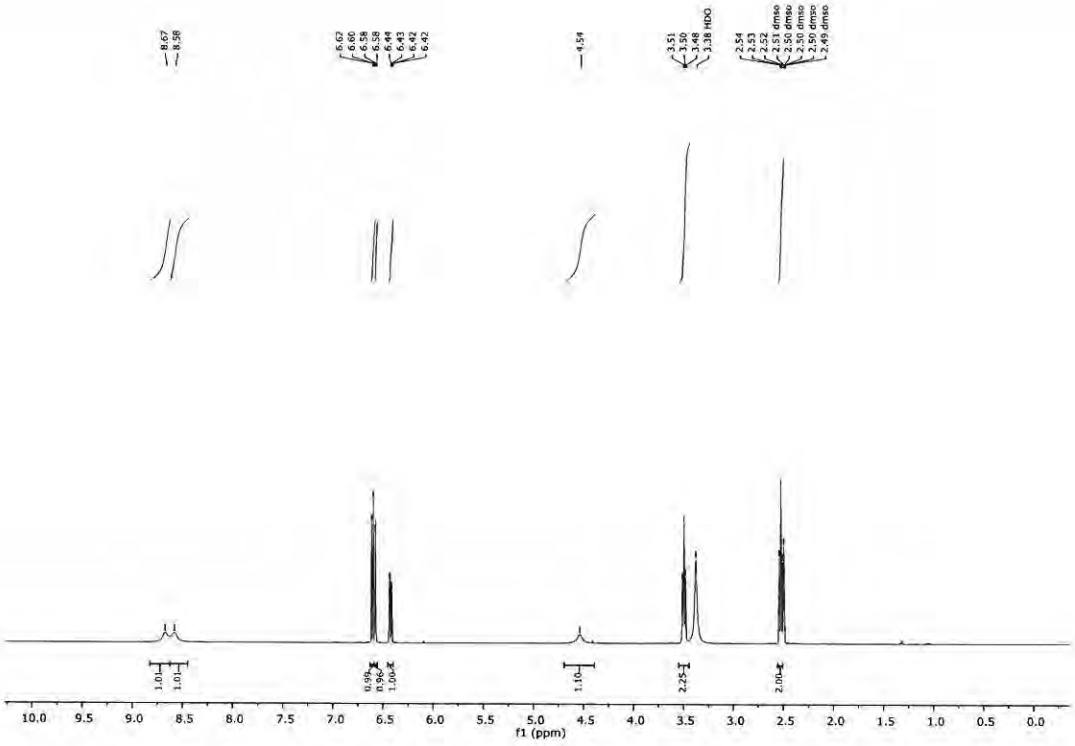

Figure 1: ^{1}H-NMR of batch (b) (4)

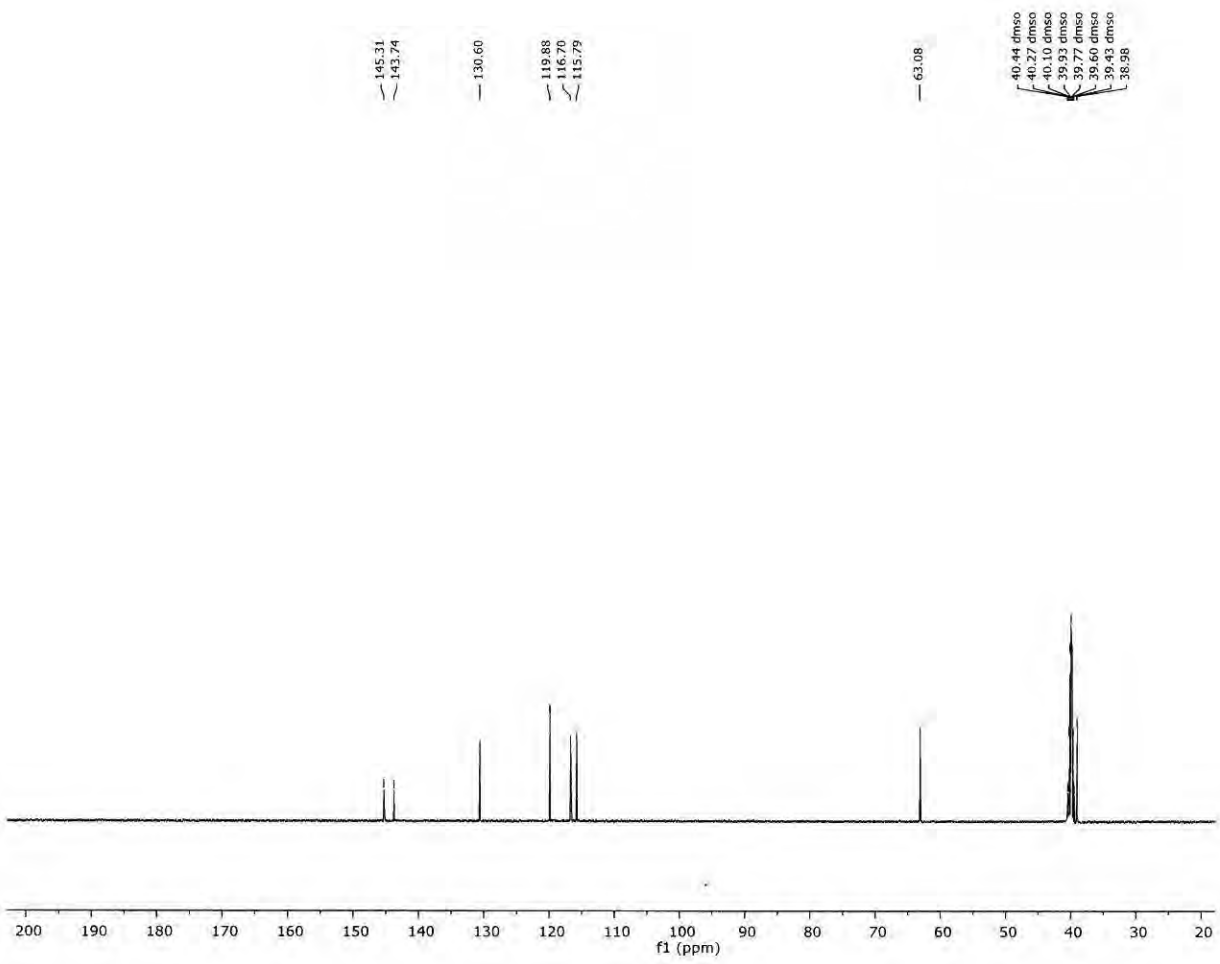

Figure 2: ^{13}C-NMR of batch (b) (4)

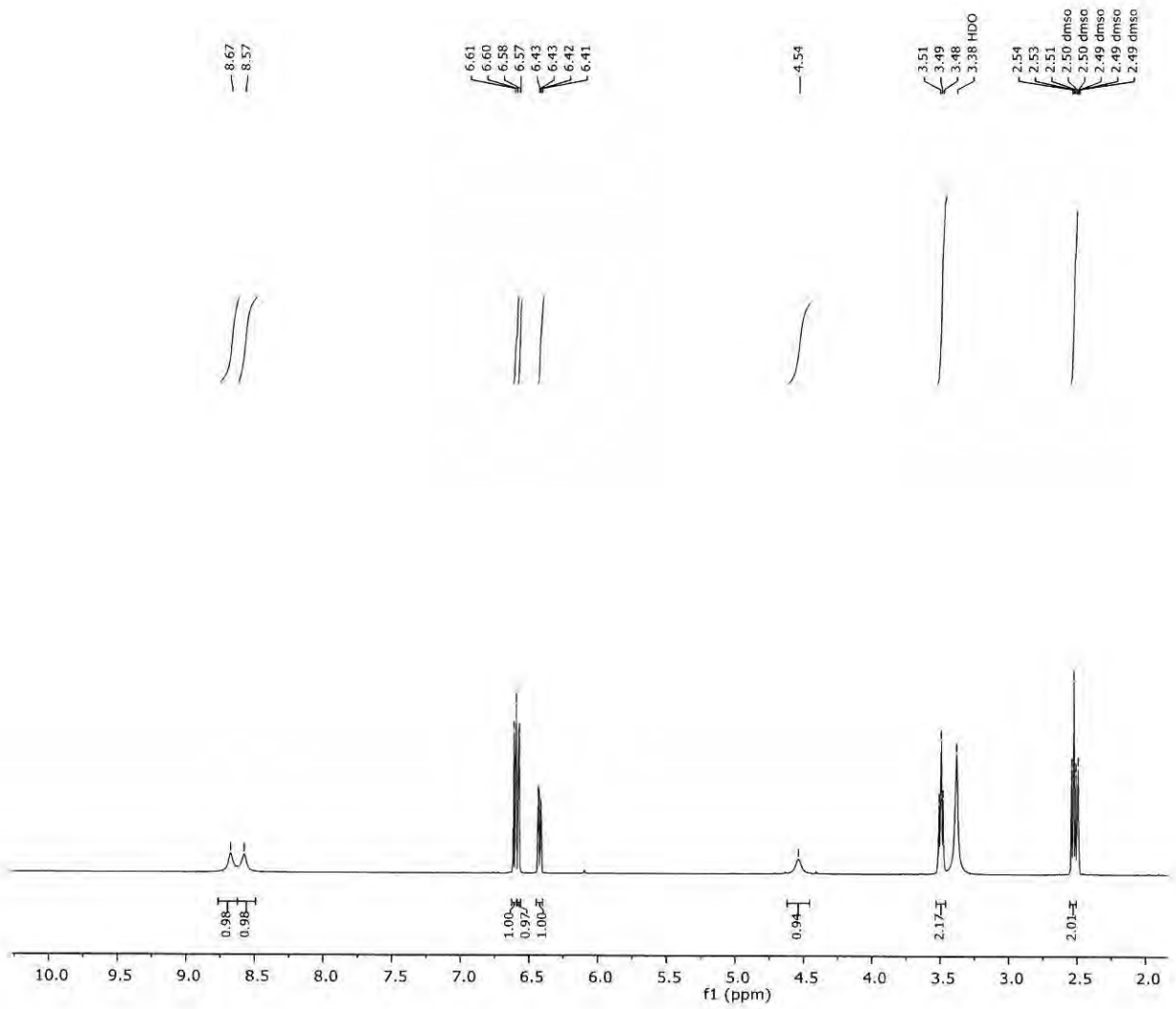

Figure 3: ^{1}H-NMR of batch (b) (4)

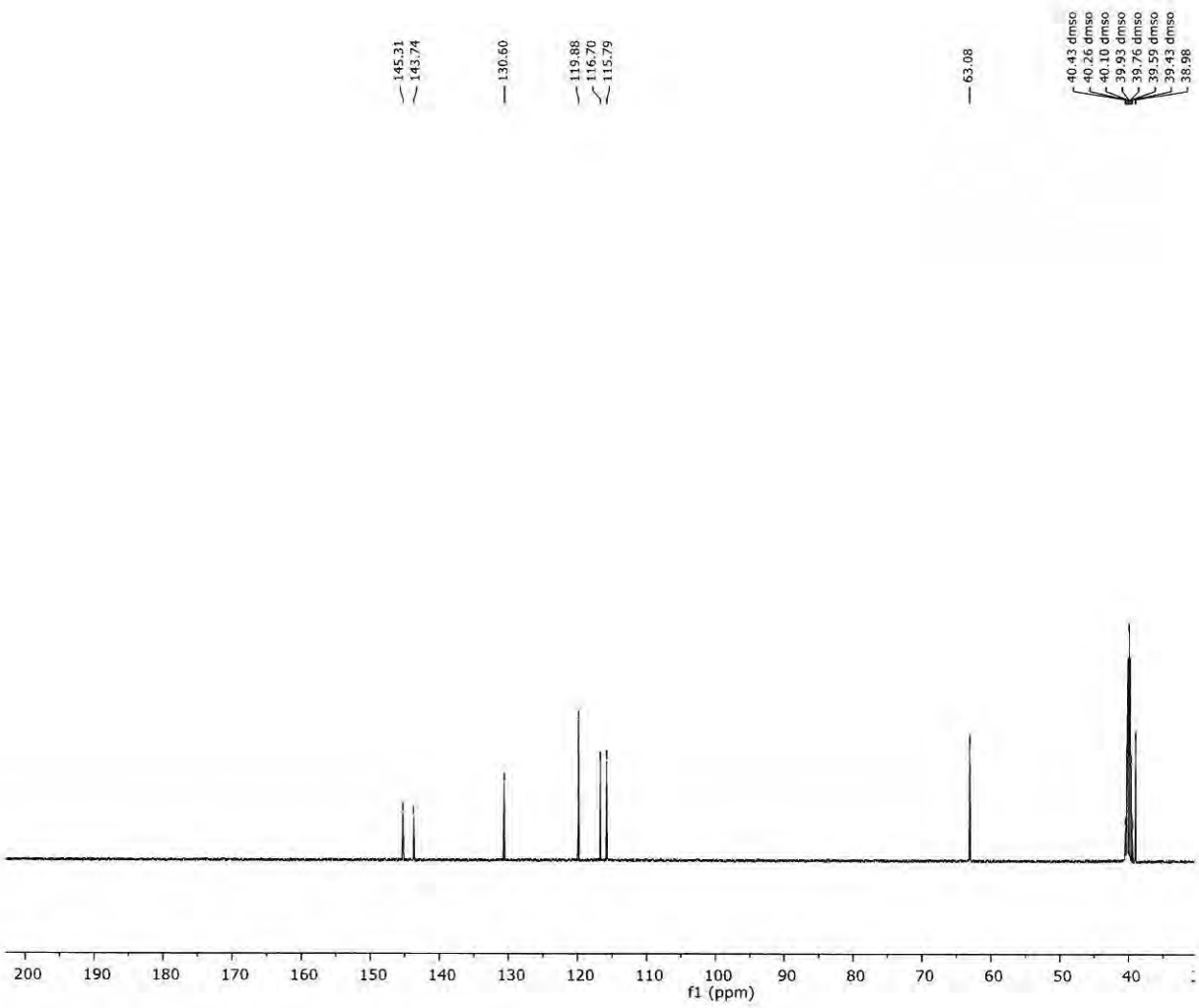

Figure 4: ^{13}C-NMR of batch (b) (4)

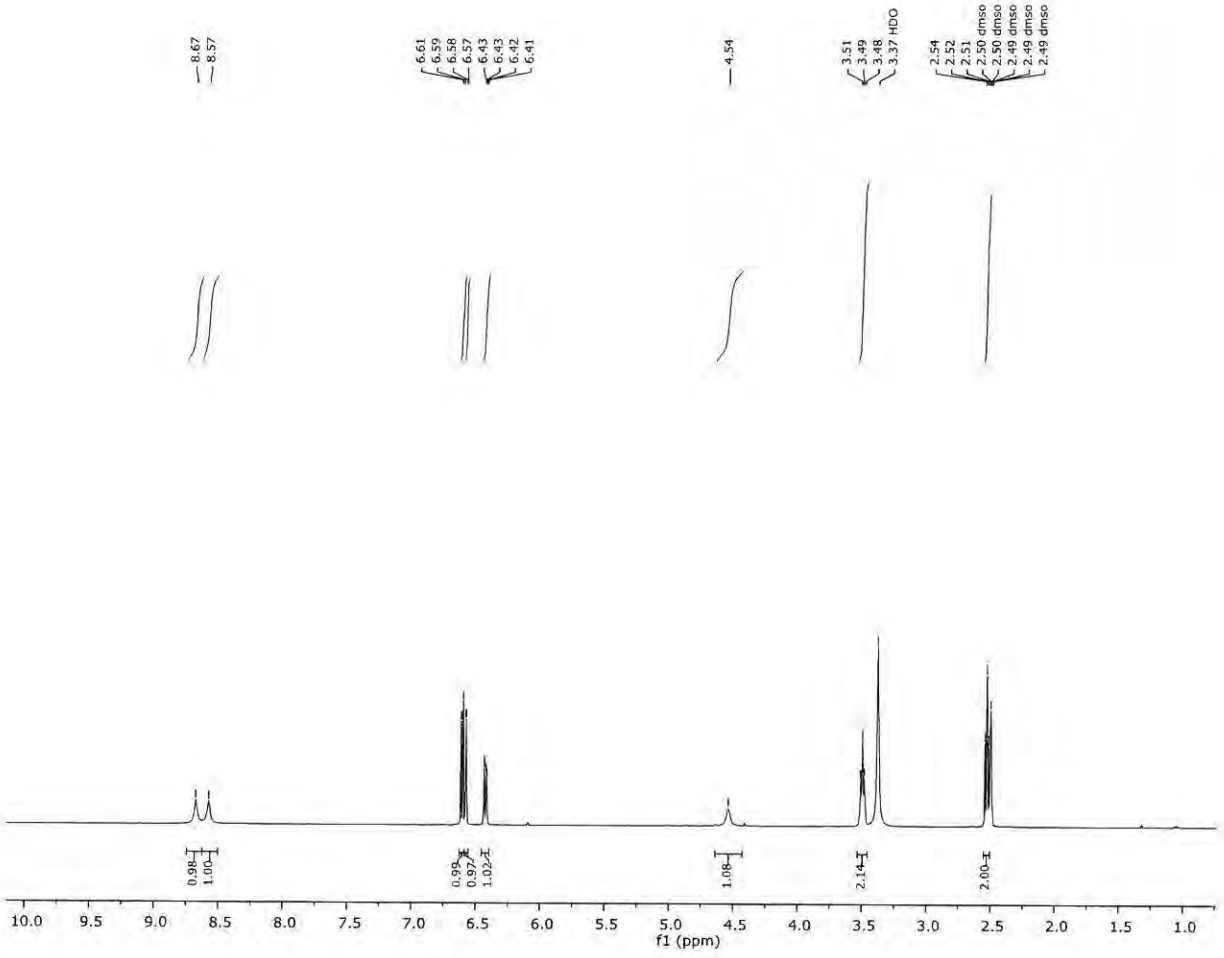

Figure 5: ^{1}H-NMR of batch (b) (4)

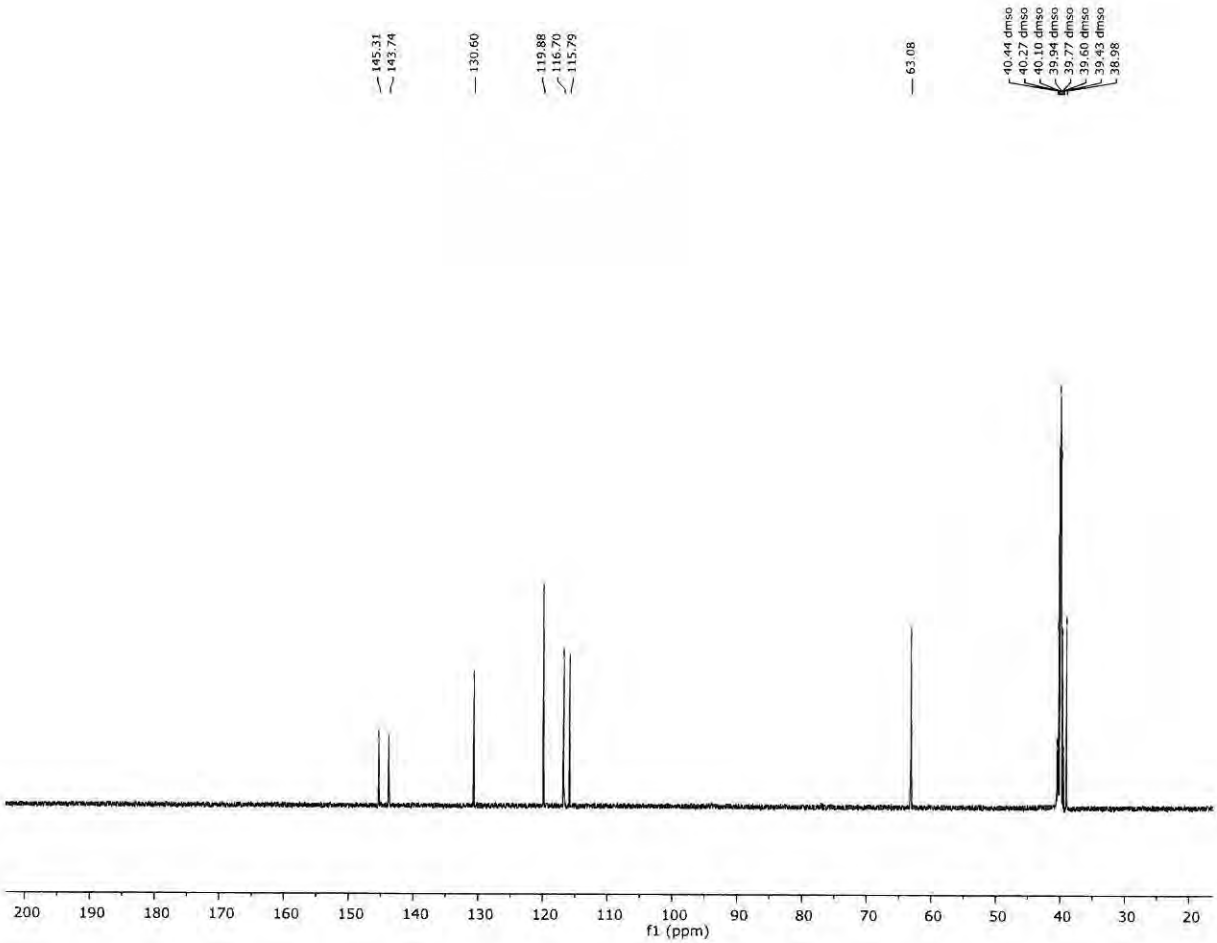

Figure 6: ^{13}C-NMR of batch (b) (4)

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	-ESI
Acquired Time	06/11/2018 18:27:18				

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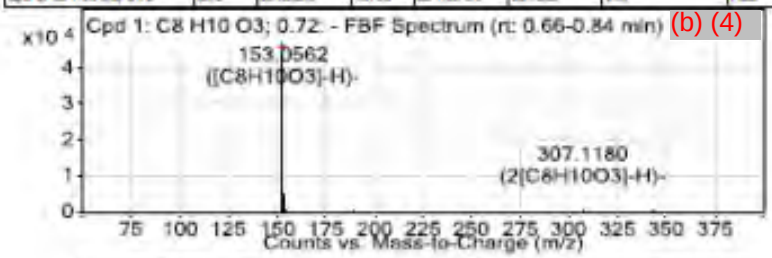
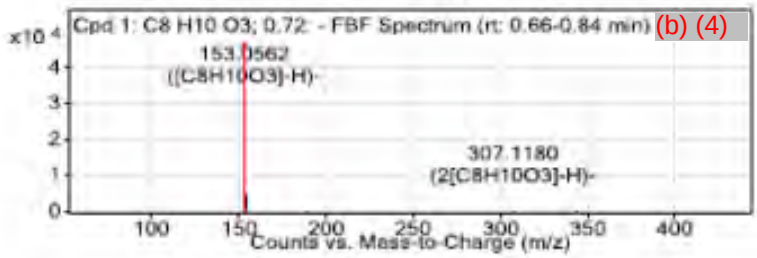
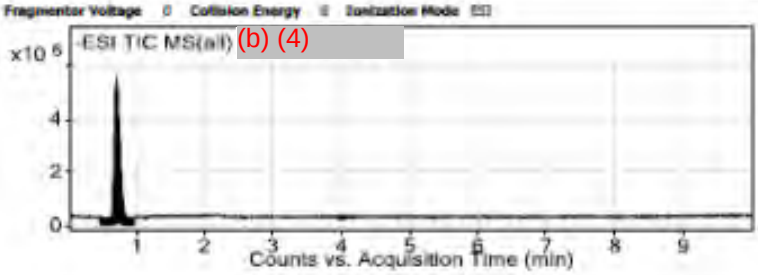
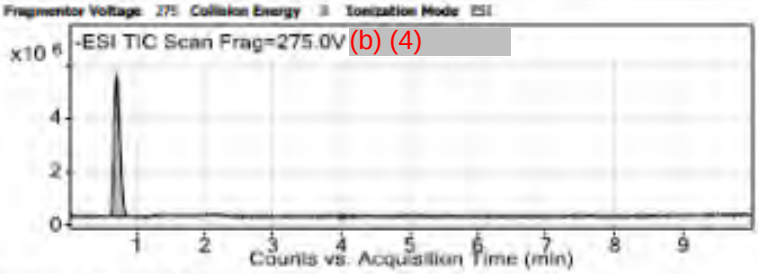




Fragmentor Voltage 0 Collision Energy 0 Ionization Mode ESI



Fragmentor Voltage 275 Collision Energy 0 Ionization Mode ESI



User Chromatogram Peak List

Compound Name	RT	Height	Height %	Area	Area %	Area Sum %	Base Peak m/z	Width
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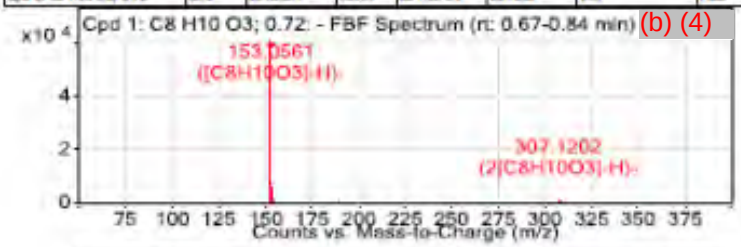
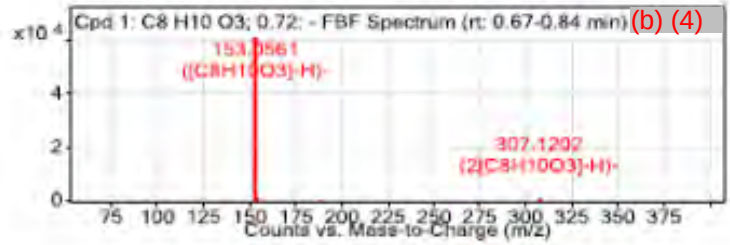
Formula Confirmation Report

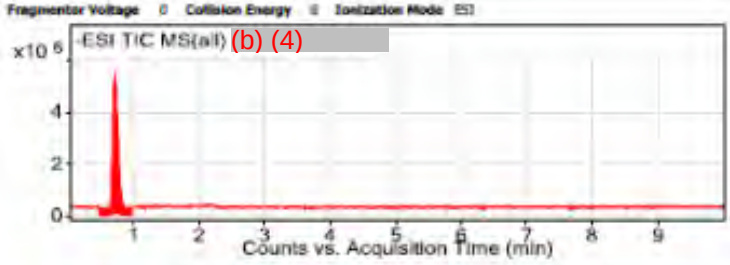
Sample Name	EI_009	**Position**	P1-A4	**Instrument Name**	LC_QTOF
User Name		**Inj Vol**	10	**IRM Calibration Status**	Success
Data Filename	NM20180802.d	**ACQ Method**	HTyrosol_4_neg_MSMS.	**Comment**	-ve
Acquired Time	08/11/2018 18:43:06				

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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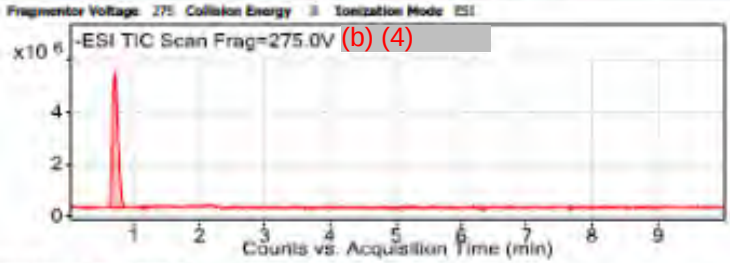


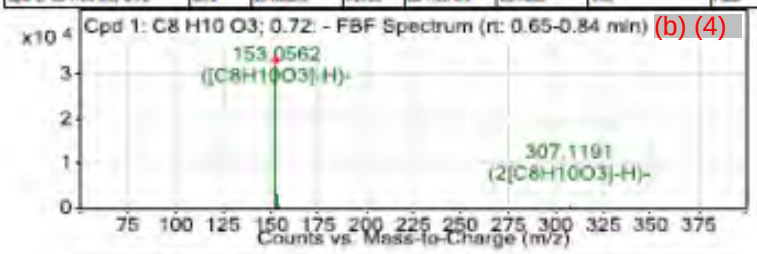
User Chromatogram Peak List

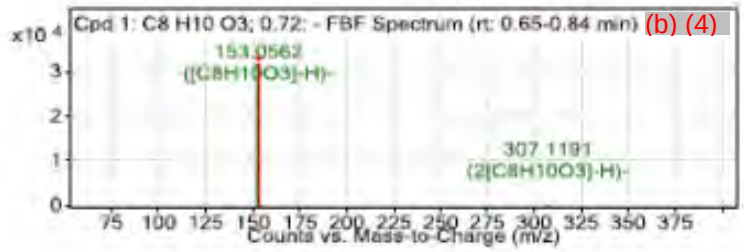
Compound Name	RT	Height	Height %	Area	Area %	Area Sum %	Base Peak m/z	Width
Cpd 1: 0.72	0.72	524573	100	30730611	100	100	1034.0029	0.31

Formula Confirmation Report

Sample Name	B3_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	Tot Mass	Purity Value	Purity Result
Cpd 1: C8 H10 O3; 0.72	0.72	154.0635	71913	C8 H10 O3	154.063	100	Pass





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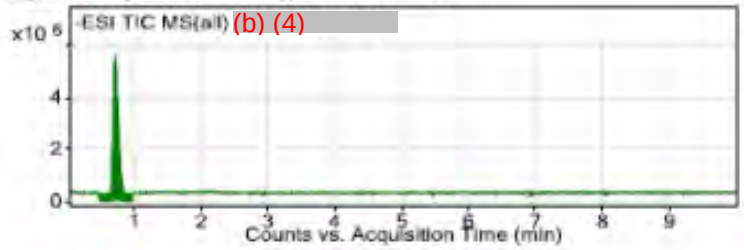

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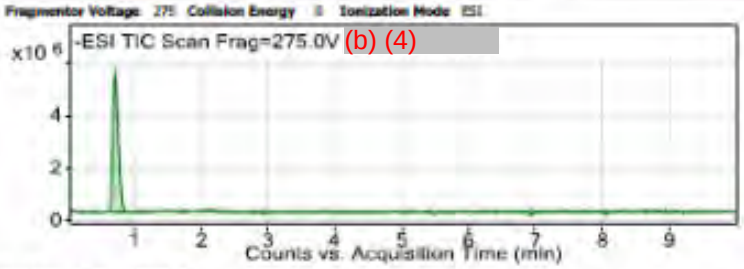

User Chromatogram Peak List

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	1334.0015	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.

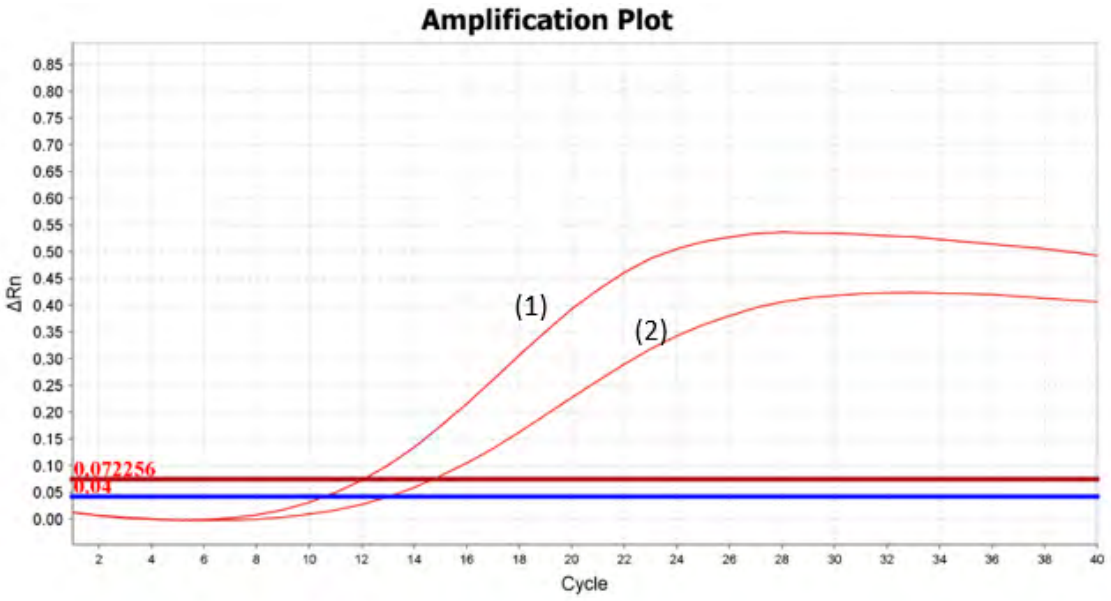

Figure 1. Amplification fluorescence curves during qPCR for Kanamycin^R fragment P2 (Kan-P2) (1) and tyrosinase P1 (Tyr-P1) (2) gene fragments. On the X-axis is the number of PCR cycles, on the Y-axis the fluorescence coefficient ΔRn. DNA concentration in the pcr reaction was 0.75µg/ml.

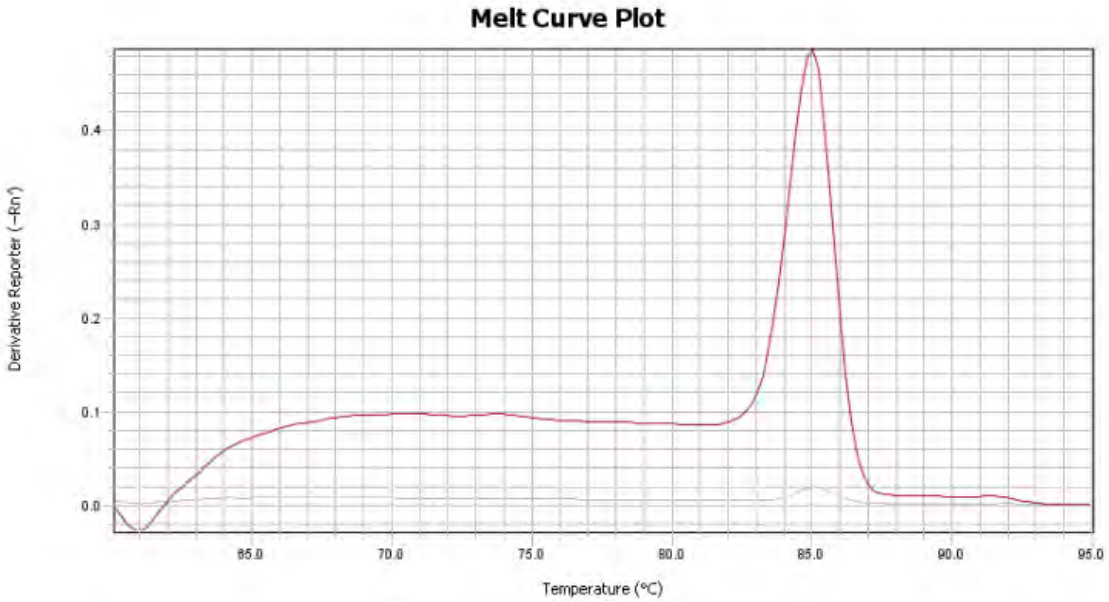

Figure 2. Kan-P2 Melting point analysis (~85ºC). X-axis corresponds to temperature in ºC, Y-axis corresponds to the relative fluorescence derivative (-Rn).

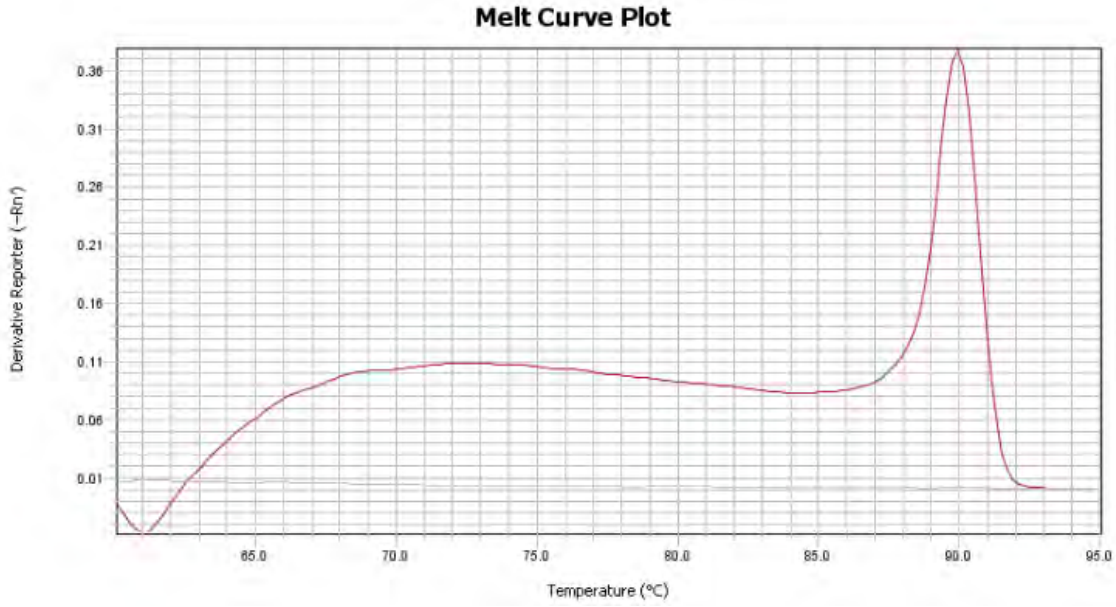

Figure 3. Tyr-P1 Melting point analysis (~90ºC). X-axis corresponds to temperature in ºC, Y-axis corresponds to the relative fluorescence derivative (-Rn).

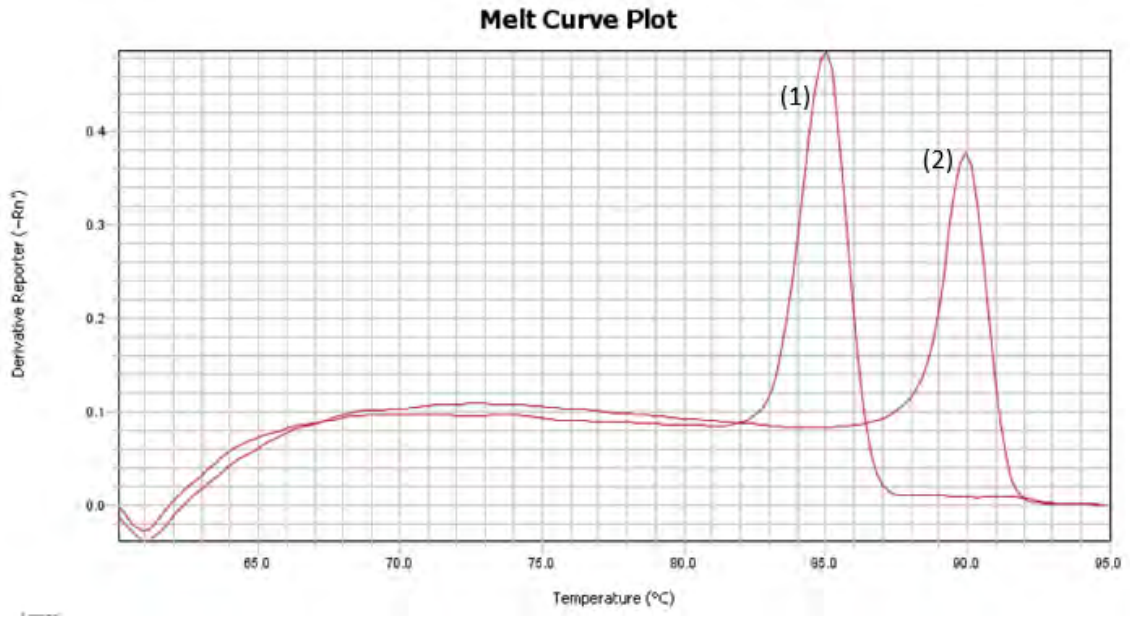

Figure 4. A comparison of the melting point analysis of the Kan-P2 fragment (1) and the Tyr-P1 fragment (2).

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.

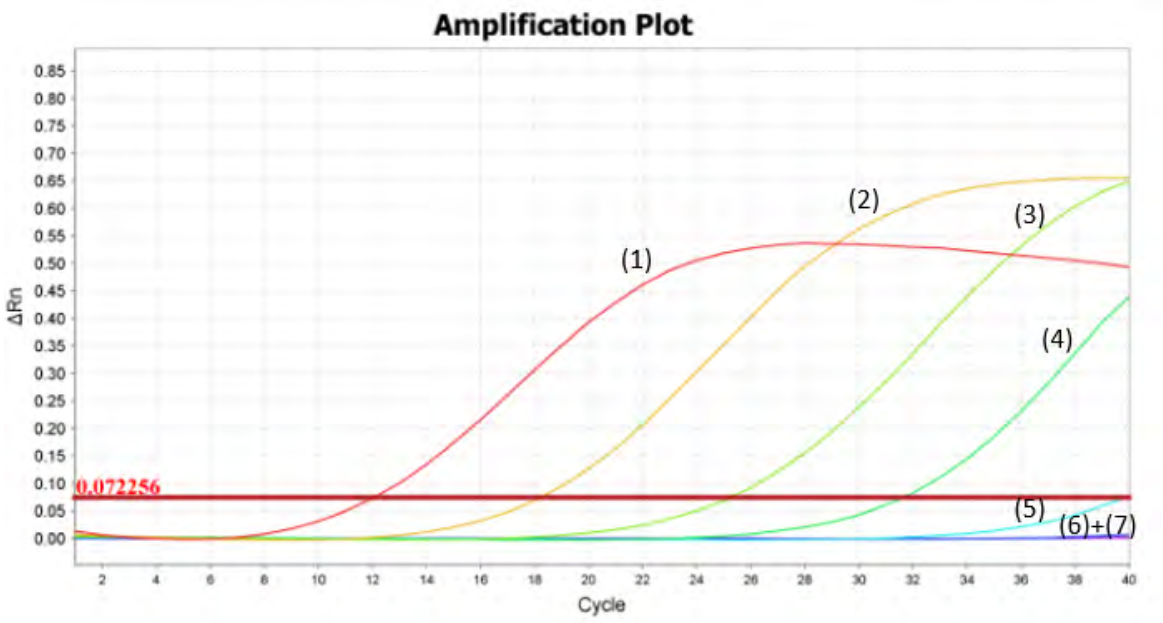

Figure 5. Fluorescence curves during qPCR amplification of the Kan-P2 fragment from the Kanamycin^{R} gene template at different template concentrations to determine the limit for detection. DNA concentrations in template samples (1) 0.75 ng/µL ,(2) 7.5X 10^{-2}ng/µL , (3) 7.5X 10^{-3}ng/µL,(4) 7.5X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

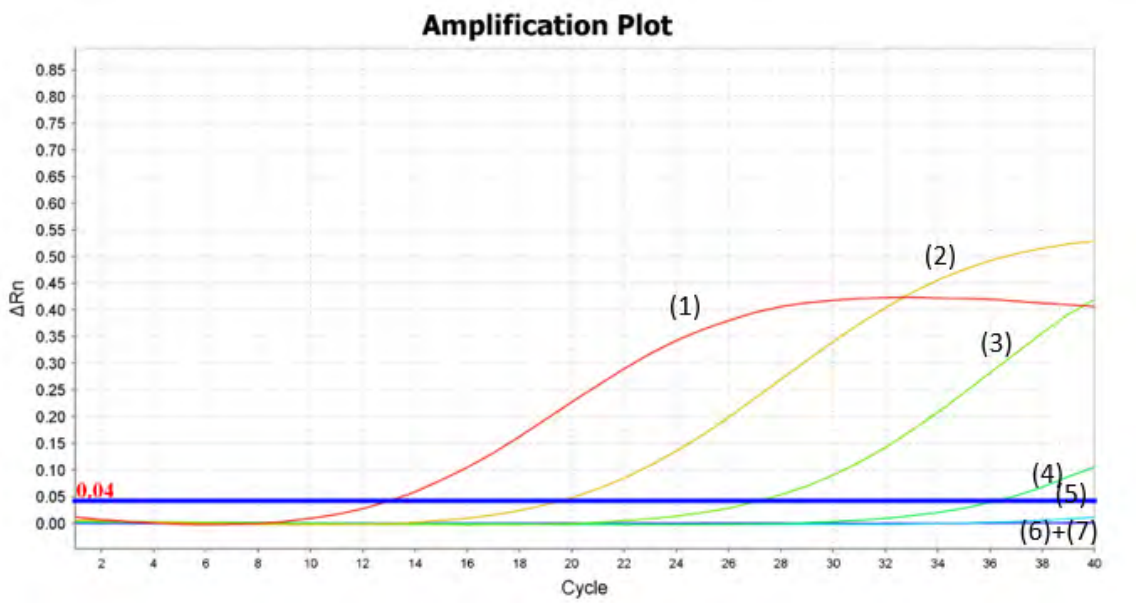

Figure 6. Fluorescence curves during qPCR amplification of the Tyr-P1 fragment from the Tyrosinase gene template at different template concentrations to determine the limit for detection. DNA concentrations in template samples (1) 0.75 ng/µL ,(2) 7.5X 10^{-2}ng/µL , (3) 7.5X 10^{-3}ng/µL,(4) 7.5X 10^{-4}ng/µL,(5)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

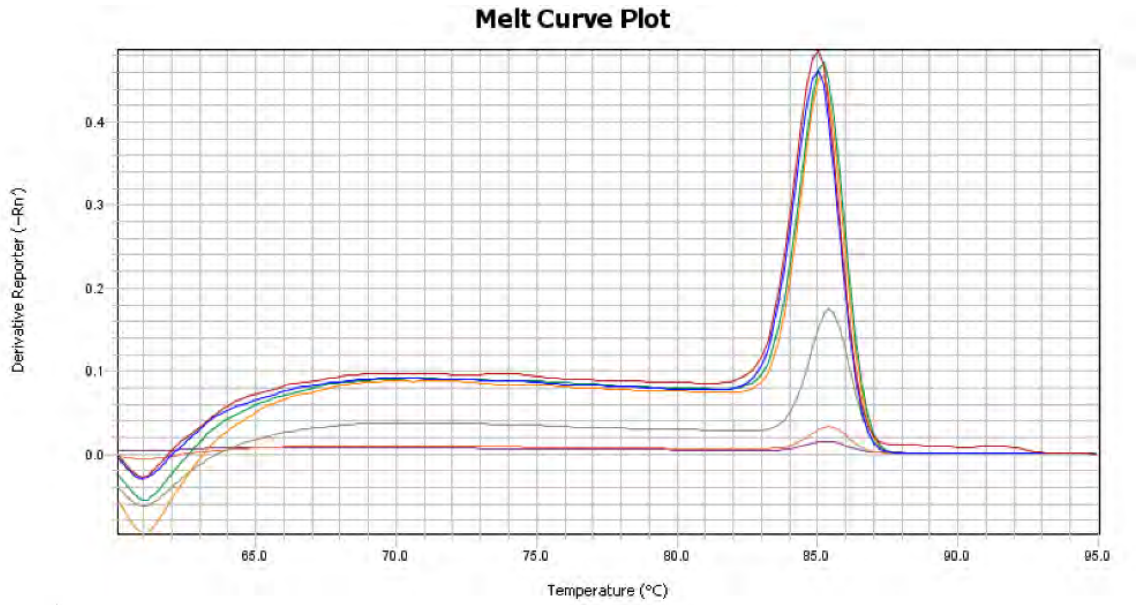

Figure 7. Melting point analysis of serially diluted DNA amplified from the kanamycin^{R} gene using primers Kan-P2.

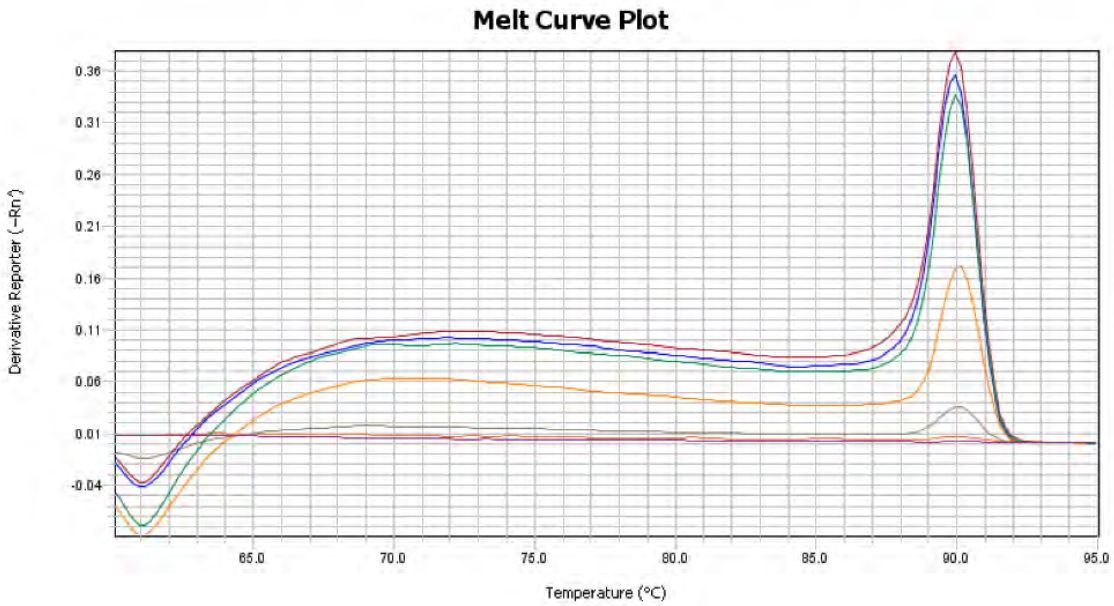

Figure 8. Melting point analysis of serially diluted DNA amplified from the tyrosinase gene using primers Tyr-P1

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.

.

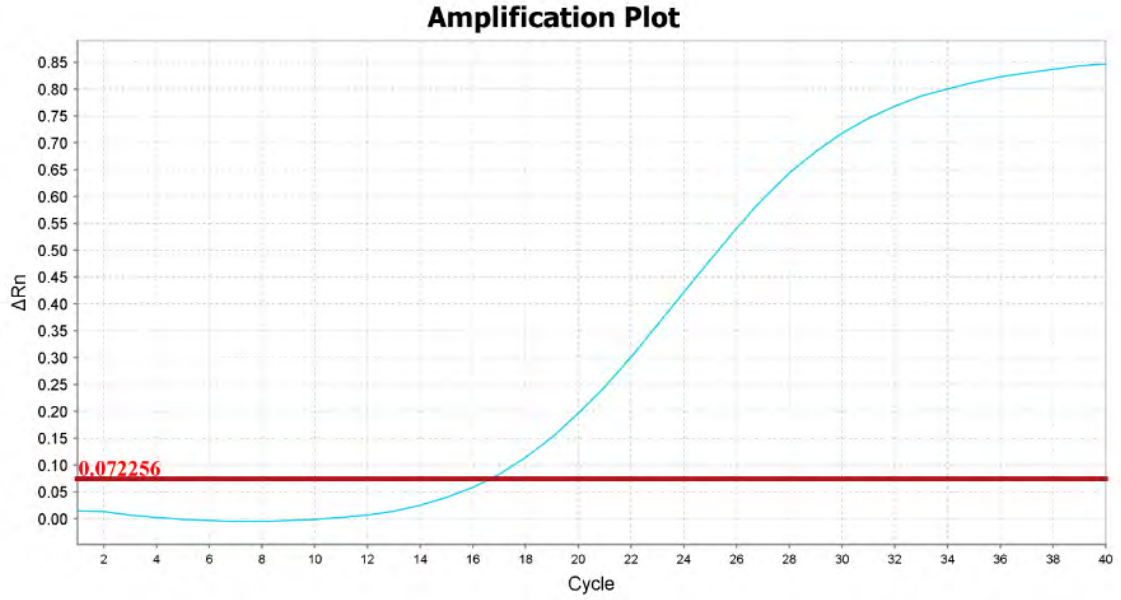

Figure 9. Fluorescence curve during qPCR amplification of Kan-P2 fragment using spiked HT sample as template.

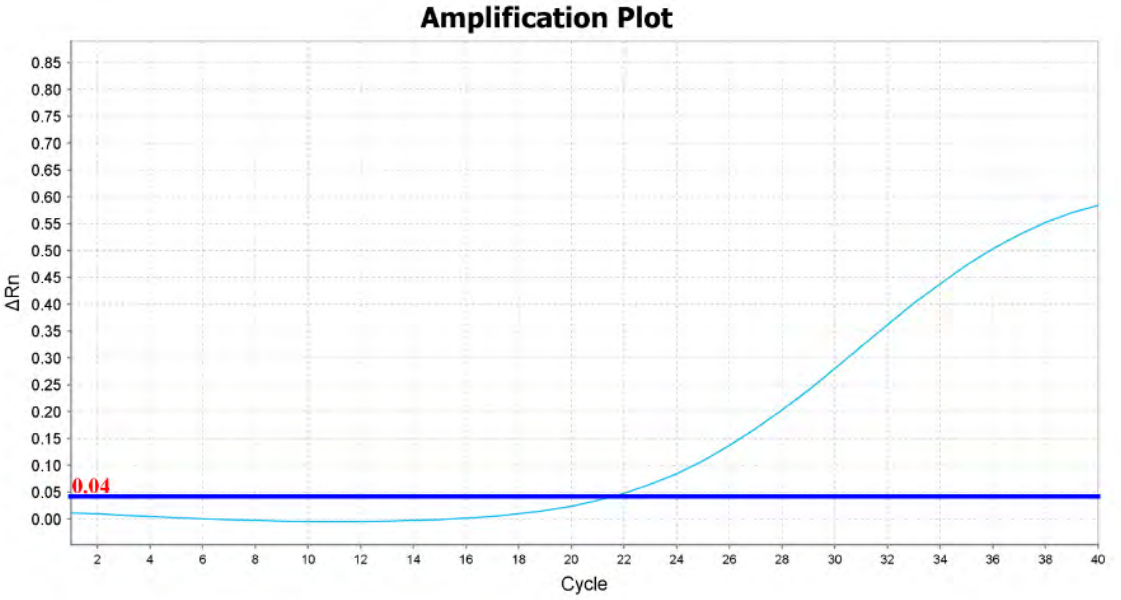

Figure 10. Fluorescence curve during qPCR amplification of Tyr-P1 fragment using spiked HT sample as template.

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.

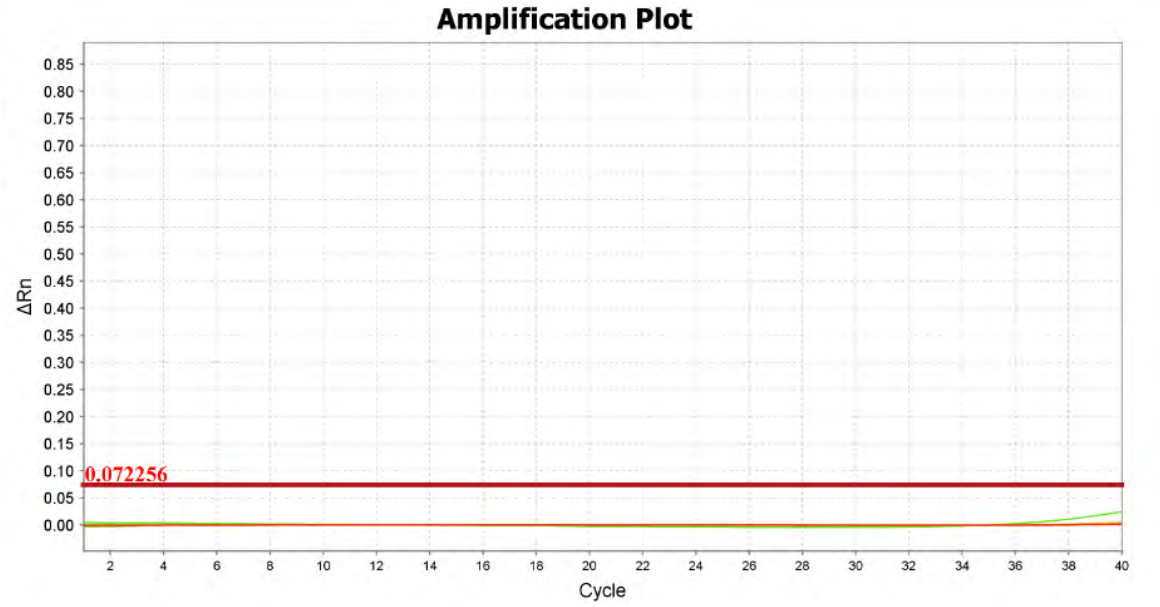

Figure 11. Fluorescence amplification during PCR of the Kan-P2 fragment using HT samples ( ) from three independent batches in duplicate.

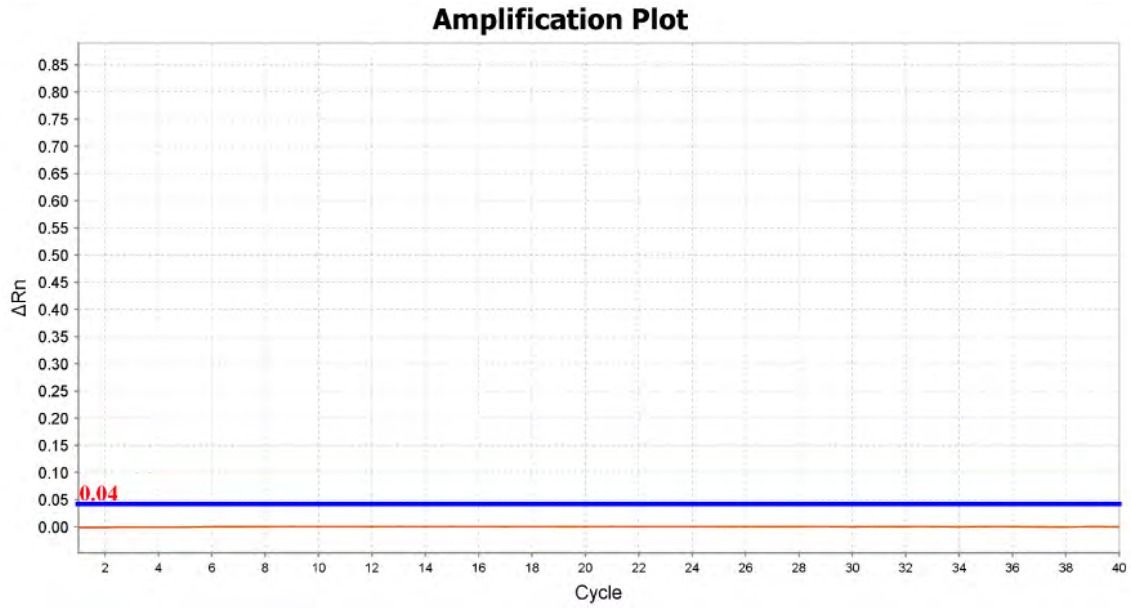

Figure 12. Fluorescence amplification during PCR of the Tyr-P1 fragment using HT samples ( ) from three independent batches in duplicate.
As can be seen from figures 12 and 13, no kanamycin^{R} or tyrosinase DNA could be detected on the samples taken from three independent HT batches.

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

eurofins

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
Your sample description: Nova Mentis Ltd. Hydroxytyrosol Batch:
Sample reception date: 24/10/2018
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

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.

Eurofins Food Testing Ireland Limited
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North City Business Park
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T: +353 1 431 1306

www.eurofins.ie

Registered Office: Clogherane
Dungarvan
Co Waterford
Registered Number: 469953

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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-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**
Your sample description: **Nova Mentis Ltd. Hydroxytyrosol Batch:**
Sample reception date: **24/10/2018**
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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Registered Office: Clogherane
Dungarvan
Co Waterford
Registered Number: 469953

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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Co Waterford
Registered Number: 469953

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Client: **Nova Mentis Ltd.**
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Cashel Business Park
Cashel road
Dublin 12
IRELAND

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**
Your sample description: **Nova Mentis Ltd. Hydroxytyrosol Batch:**
Sample reception date: **24/10/2018**
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	<0.001	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

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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Dublin 11

T: +353 1 431 1306

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Registered Office: Clogherane
Dungarvan
Co Waterford
Registered Number: 469953

From: iMac
To: Anderson, Ellen
Cc: Reeta Davis
Subject: Re: GRN 876 Hydroxytyrosol
Date: Monday, October 14, 2019 5:57:47 AM

Dear Ellen

I realise there was a typo in my original e-mail.

We confirm that

Hydroxytyrosol is not intended to be used as a color additive in food.

All analytical methods used to analyse the batches of hydroxytyrosol for conformance with the stated specification have been validated for that particular purpose.

Kind regards
Kevin

> On 14 Oct 2019, at 09:16, iMac <koconnor@novamentis.eu> wrote:
>
> Dear Ellen
>
> We confirm that
>
> Hydroxytyrosol is not intended to be used as a collar additive in food.
>
> All analytical methods used to analyse the batches of hydroxytyrosol for conformance with the stated specification have been validated for that particular purpose.
>
> Kind regards
> Kevin
>
> > On 11 Oct 2019, at 20:06, Anderson, Ellen <Ellen.Anderson@fda.hhs.gov> wrote:
> >
> > Dear Dr. O'Connor,
> >
> > After reviewing your GRAS Notice No. 000876 for hydroxytyrosol, FDA requests clarification on the following:
> >
> > 1. The notice states that the appearance of hydroxytyrosol is an off-white powder and slightly yellow in solution. Please include a statement indicating that hydroxytyrosol is not intended to be

used as a color additive in food.

2. Please provide a statement that all analytical methods used to analyze the batches of hydroxytyrosol for conformance with the stated specifications have been validated for that particular purpose.

Please insert your response after each request for clarification; a reply via email is sufficient. Thank you.

Sincerely,
Ellen

Ellen Anderson
Consumer Safety Officer

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
Tel: 240-402-1309
ellen.anderson@fda.hhs.gov