Metabolism of natural antioxidants:

Evaluation of the pathways involved in the *in vivo* biotransformation of tyrosol into hydroxytyrosol

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Als meus pares, per tot el que han fet per mi

"Sir Isaac Newton is said to have avowed that he felt like a child picking up shells beside the great and unexplored ocean of truth"

Mary Shelley

Agraïments

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Abbreviations

AAPH: 2,2'-Azobis(2-methylpropionamidine) dihydrochloride

ACT: Acetyltransferase

ADH: Alcohol dehydrogenase

ALDH: Aldehyde dehydrogenase

COMT: Catechol-*O*-methyltransferase

CYP: Cytochrome P450

DMEM: Dulbecco's modified Eagle's medium

DMSO: Dimethylsulfoxide

DPPH assay: 2,2-di(4-*tert*-octylphenyl)-1-picrylhydrazyl assay

EFSA: European Food and Safety Authority

EMEM: Eagle's minimum essential medium (EMEM)

FBS: Fetal bovine serum

FITC: Fluorescein isothiocyanate

FRAP: Ferric reducing antioxidant power

g: Gram

GC-MS: Gas chromatography coupled to mass spectrometry

GlcA: Glucuronic acid

GGT: γ-Glutamyltransferase

HBSS: Hank's balanced salt solution

HDL: High-density lipoprotein

4-HPAA: 4-hydroxyphenylacetic acid

HVA: Homovanillic acid

HVAlc: Homovanillyl alcohol

IOC: International Olive Council

LC-MS/MS: Liquid chromatography coupled to tandem mass spectrometry

LDL: Low-density lipoprotein

MAO: Monoamine oxidase

MeOH: Methanol

min: Minute

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NAT: *N*-acetyl transferase

ORAC: Oxygen radical absorbance capacity

PBS: Phosphate-buffered saline

qNMR: Quantitative nuclear magnetic resonance

ROS: Reactive oxygen species

rpm: Revolutions per minute

s: Second

SD: Standard deviation

SEM: Standard error of the mean

SULT: Sulfotransferases

TEAC: Trolox equivalent antioxidant capacity

TPTZ: 2,4,6-tripyridyl-*S*-triazine

TRAP: Total radical-trapping antioxidant parameter

Tyr: Tyrosol

UGT: Uridine 5'-diphosphoglucuronosyl transferase

vs: Versus

WW: White wine

Abstract

Hydroxytyrosol [2-(3,4-dihydroxyphenyl)ethanol], a potent bioactive molecule mainly present in virgin olive oil and to a lower extent in wine, is also a by-product of dopamine metabolism. In a previous clinical trial designed to assess the effects of moderate wine intake it was found that hydroxytyrosol urinary recoveries were higher than the corresponding to the dose administered, suggesting an endogenous formation. The aim of the present work was to assess new mechanisms responsible for hydroxytyrosol generation by using an array of methodologies and studies ranging from *in vitro* assays to *in vivo* experiments in animal models and humans. The mechanisms identified as being involved in the generation of hydroxytyrosol were (1) CYP2A6/CYP2D6-catalyzed tyrosol-to-hydroxytyrosol biotransformation, (2) ethanol-induced increase in tyrosol bioavailability, and (3) alteration of dopamine oxidative metabolism due to ethanol. Considering these observations, it is postulated that hydroxytyrosol may contribute significantly to the health effects derived from moderate wine intake.

Resum

L'hidroxitirosol [2-(3,4-dihidroxiphenil)etanol], una potent molècula bioactiva present de forma rellevant a l'oli d'oliva verge i minoritàriament al vi, és també un producte del metabolisme de la dopamina. En un assaig clínic previ dissenyat per avaluar els efectes del consum moderat de vi es trobà que les recuperacions urinàries d'hidroxitirosol eren superiors a la dosi administrada, suggerint-ne una gènesi endògena. L'objectiu del present treball fou estudiar nous mecanismes responsables de la generació d'hidroxitirosol mitjançant diverses tècniques i estudis, des d'assajos *in vitro* a experiments *in vivo* en animals i humans. Els mecanismes involucrats a la generació d'hidroxitirosol són (1) la biotransformació de tirosol a hidroxitirosol catalitzada per CYP2A6/CYP2D6, (2) l'augment a la biodisponibilitat del tirosol induïda per l'etanol, i (3) l'alteració del metabolisme oxidatiu de la dopamina per l'etanol. Globalment, aquestes observacions donen a l'hidroxitirosol un paper clau en la comprensió dels efectes beneficiosos associats al consum moderat de vi.

Preface

This PhD thesis dissertation is divided into several sections as follows: Firstly, the ***Introduction*** provides a comprehensive context in order to understand the background research related to the Mediterranean Diet and the beneficial health effects associated with virgin olive oil and moderate wine consumption. A particular emphasis is placed on dietary antioxidant hydroxytyrosol, its pharmacokinetics, and the clinical trials evaluating hydroxytyrosol disposition previously performed by our research group. Secondly, on the basis of some unanswered questions regarding the potential endogenous generation of hydroxytyrosol, the ***Objectives and Hypotheses*** section states the aim of the present work and the postulated hypotheses. After briefly explaining the experimental strategy designed to achieve the goals of this thesis, the ***Methods and Results*** section details the experimental approach and the results that were obtained. ***Methods and Results*** section is made up of 7 chapters. In each one the description of the methods employed and the results obtained is preceded by a brief *Introduction* and is followed by a short *Discussion* (around 1 page each). The former establishes the context of the experimental design, its objective, and the approach used; the latter aims to help the reader interpret the results. Finally, the ***General Discussion*** provides a critical description of the implications of the findings, states the interpretation and assessment of the results, and suggests future research directions.

In the table below the scientific publications corresponding to each thesis section are summarized. In brief, the contents of the present thesis have contributed to two already published scientific articles, one manuscript that has been recently accepted, one manuscript that has been submitted and is under revision, and two future oral presentations in international congresses.

Scientific publications derived from this doctoral thesis.

Thesis Section	Scientific Publications	Status
Introduction	**Rodríguez-Morató* J**, Xicota* L, Fitó M, Farré M, Dierssen M, and De la Torre R (2015) Potential role of olive oil phenolic compounds in the prevention of neurodegenerative diseases. *Molecules* **20:**4655-4680.	Published
Introduction	**Rodríguez-Morató J**, Boronat A, Kotronoulas A, Pujadas M, Pastor A, Olesti E, Pérez-Mañá C, Khymenets O, Fitó M, Farré M, De la Torre R (2016) Metabolic disposition and biological significance of simple phenols of dietary origin: hydroxytyrosol and tyrosol. *Drug Metabolism Reviews*	Accepted
Chapter 1	Oral presentation: **Rodríguez-Morató J**, De la Torre R, Blumberg JB, Oliver Chen D-Y (2016) Hydroxytyrosol is more a more potent antioxidant than tyrosol. *Experimental Biology Conference*, San Diego (USA).	Accepted
Chapter 2 Chapter 3	Pérez-Mañá* C, Farré* M, **Rodríguez-Morató* J**, Papaseit E, Pujadas M, Fitó M, Robledo P, Covas M-I, Cheynier V, Meudec E, Escudier J-L, and De la Torre R (2015) Moderate consumption of wine, through both its phenolic compounds and alcohol content, promotes hydroxytyrosol endogenous generation in humans. A randomized controlled trial. *Molecular Nutrition & Food Research* **59:**1213-1216.	Published
Chapter 4 Chapter 5 Chapter 6	**Rodríguez-Morató J**, Robledo P, Tanner JA, Boronat A, Pérez-Mañá C, Chen O, Tyndale RF, De la Torre R (2016) CYP2D6 and CYP2A6 biotransform dietary tyrosol into hydroxytyrosol. *Food Chemistry*	Submitted
Final Chapter	Oral presentation: **Rodríguez-Morató J**, Robledo P, Pérez-Mañá C, Farré M, Fitó M, Covas MI, De la Torre R (2016). Contribution of tyrosol and ethanol to the endogenous formation of hydroxytyrosol in humans (2016) ISANH Polyphenols World Congress, Porto (Portugal).	Accepted

Throughout the chapters the contents of the previous table are developed in detail, and the full-text of the four articles included in four appendices can be found at the end of the work. Moreover, during the time period of this PhD thesis, the author was offered the possibility of participating in other research topics. These contributions, although not strictly related to the present thesis, have enriched the research experience and culminated in the publication of the following scientific articles:

1. **Rodríguez-Morató* J**, Farré* M, Pérez-Mañá C, Papaseit E, Martínez-Riera R, de la Torre R, and Pizarro N (2015) Pharmacokinetic comparison of soy isoflavone extracts in human plasma. *Journal of Agricultural and Food Chemistry* **63**:6946-6953.
2. Pastor* A, **Rodríguez-Morató* J**, Olesti E, Pujadas-Bastardes M, Pérez-Mañá C, Khymenets O, Fitó M, Covas MI, Solà R, Motilva MJ, Farré M, de la Torre R (2016). Analysis of free hydroxytyrosol in human plasma following the administration of olive oil. *Journal of Chromatography A* **1437**: 183-190
3. Xicota* L, **Rodríguez-Morató* J**, Dierssen M, De la Torre R. Potential Role of (-)-epigallocatechin-3-gallate (EGCG) in the Secondary Prevention of Alzheimer Disease (2016). *Current Drug Targets*

The contents of the previous three publications have not, however, been included in the core thesis as they are beyond the scope of this work.

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INTRODUCTION

1. The traditional Mediterranean Diet: more than just a diet

Over the last decades, several investigators have described that adherence to a healthy dietary pattern is associated with increased longevity and low incidence of chronic degenerative diseases. Consequently, several types of diet have been widely studied in order to find an optimal feeding pattern able to contribute to a favorable health status and to attain a better quality of life. Among these different diets, the Mediterranean Diet (MD) is the one that has attracted the greatest interest between researchers due to its beneficial health effects (Sofi et al., 2013).

The origin of the term MD dates back to the 1960's, when Ancel Keys was explaining the results of an epidemiological study that demonstrated that Italian and Greek populations had lower mortality rates, and a reduced incidence of cancer and cardiovascular diseases, compared to other populations (Keys et al., 1986). The study (known as the *Seven Countries Study*) included almost 13,000 people from America, Europe, and Asia, and set a precedent in the clinical and epidemiological research fields. As an ascertainment of this fact, the number of scientific articles on MD published since the beginning of this century has increased exponentially (Serra-Majem et al., 2006).

From a dietetic point of view, the traditional MD is characterized by a high intake of **olive oil** (as a source of unsaturated fatty acids); a high intake of plant foods (cereals, vegetables, legumes, fruits, nuts, seeds and olives); a moderate intake of fish and dairy products; a low intake of meat, poultry and saturated fatty acids; and a moderate but regular intake of **ethanol**, mainly in the form of **wine** and generally during meals (Trichopoulou et al., 2003; Trichopoulou, 2004).

The MD gained popularity since 1995, when the world famous pyramid was used to graphically represent the foods and their frequency of recommended intake. In 2010, the MD was acknowledged as an Intangible Cultural Heritage of Humanity by UNESCO.

One year later, a new graphic representation taking into account lifestyle and cultural factors was adopted (**Figure 1**) (Bach-Faig et al., 2011).

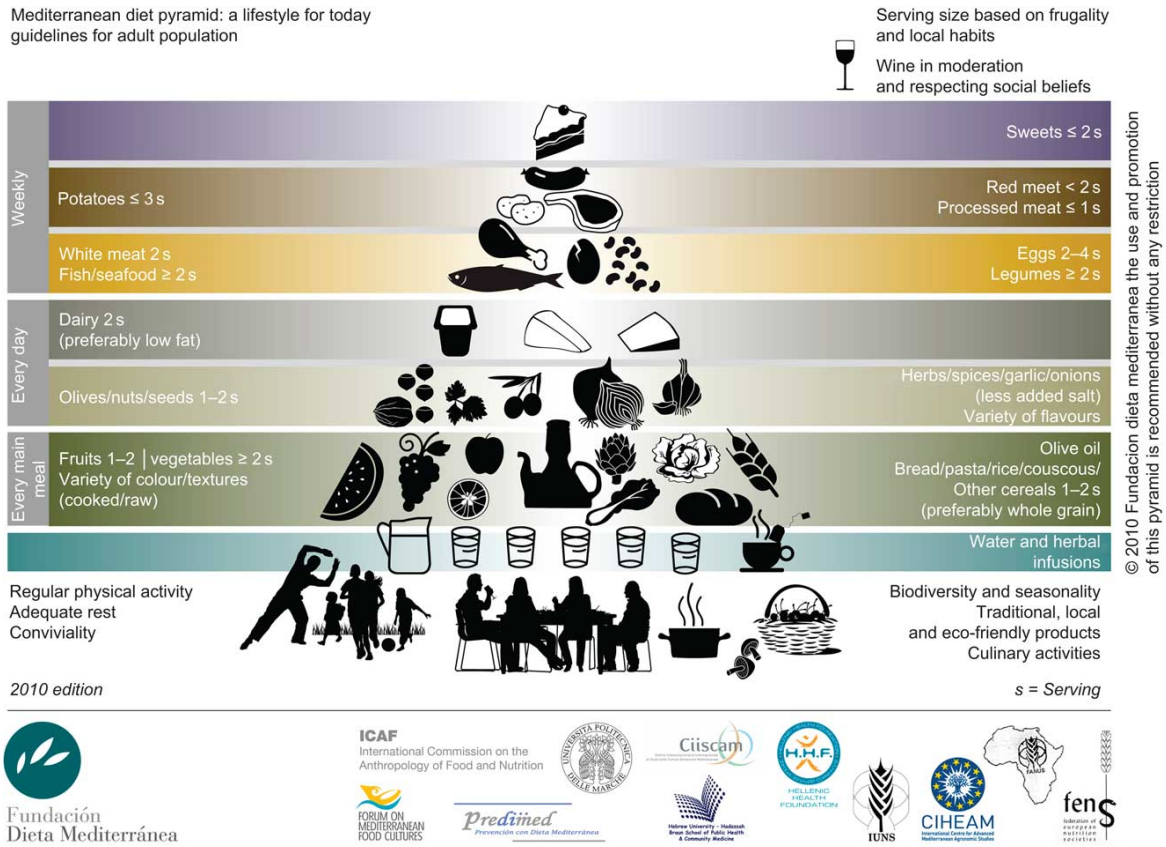


***Figure 1**. Mediterranean diet pyramid updated in 2010 by the Mediterranean Diet Foundation Expert Group.*

Different meta-analyses of prospective cohort studies have evidenced that adherence to the MD confers a significant protection for overall mortality, cardiovascular disease mortality, and incidence of cancer and degenerative diseases (Sofi et al., 2008; Sofi et al., 2010). In this sense, a high adherence to the MD diminishes the risk of suffering from cardiovascular and cerebrovascular diseases, diabetes mellitus, metabolic syndrome, certain cancers, and also neurodegenerative diseases (Sofi et al., 2013).

Nowadays, and because of the abovementioned studies, the MD is known (and even recognized by the World Health Organization) as an ideal dietary pattern that can

reduce the occurrence of the most important chronic diseases helping to preserve the health status (Sofi et al., 2013; Martínez-González et al., 2015).

Amongst the components of the MD, the present work focuses on **wine** (and particularly red wine) and **olive oil** (and particularly virgin olive oil). As discussed in further detail in *Subheadings 2* (wine) and *3* (olive oil), both foods have in common several facts: they are characteristic constituents of the MD, (ii) their consumption has proven to exert multiple beneficial health effects, and (iii) they contain phenolic compounds with potent antioxidant activities, like tyrosol or hydroxytyrosol.

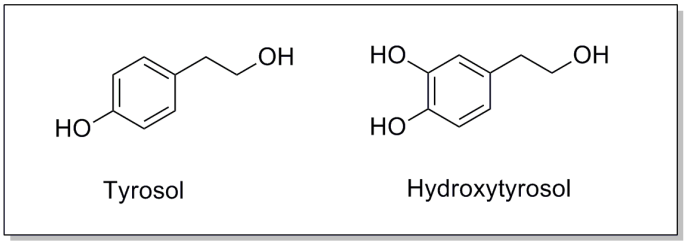


Figure 2. *Chemical structures of tyrosol and hydroxytyrosol*

2. Wine ≠ grape juice + ethanol

There is no doubt that the abuse of ethanol is unquestionably harmful. However, there is strong evidence suggesting that light-to-moderate consumption of ethanol protects against cardiovascular diseases, diabetes and depression (Nichols et al., 2012; Gea et al., 2013). The relationship between ethanol and total mortality follows a J-shaped curve: low levels of alcohol intake (1-2 drinks per day for women and 2-4 drinks per day for men) are inversely associated with total mortality, whereas higher doses of ethanol are associated with increased mortality (Di Castelnuovo et al., 2006). Similar results have been found for coronary heart disease (Reynolds et al., 2003). The mechanisms considered to be responsible for these cardioprotective effects of ethanol are the ability to: (1) increase high-density lipoprotein (HDL) cholesterol, (2) act as

antiplatelet agent, (3) improve insulin sensitivity, and (4) exert an anti-inflammatory activity by lowering circulating concentrations of fibrinogen (Mukamal, 2012; Fernández-Solà, 2015).

In the case of wine, epidemiological studies have consistently identified its moderate consumption with increased longevity and reduced cardiovascular mortality, compared to heavy drinkers or even abstainers (German and Walzem, 2000). However, it is not clear if these effects are due to ethanol, to other wine components or to a combination of both. Wine is a beverage made of the fermented juice of the must, or juice, pressed from grapes. During the fermentation process, which is mediated by yeasts, multiple chemical changes take place. These reactions are responsible for the generation of ethanol but they also alter the chemical composition of the phytochemicals that are present in the grape fruit and give rise to a great variety (more than 200) of phenolic compounds and polyphenols. As a result, wine cannot simply be considered as grape juice with ethanol added (German and Walzem, 2000). Indeed, both phenolic compounds and polyphenols have antioxidant properties which could confer wine with beneficial properties for health.

There are important differences in the level of phenolic compounds in wine depending on the grape source, the variety and the processing. During wine preparation, skins and stems are left in contact with the must for prolonged periods of time to produce red wines, whereas stems, skins and must are rapidly separated after the crush to make white wine. As a natural consequence, red wine has higher phenolic compounds and polyphenols than white wine (Vinson and Hontz, 1995). As an example, Paganga *et al.* compared the antioxidant activity equivalents between different foods and beverages and found that the antioxidant activity of 1 glass of red wine (150 mL) was equivalent to 12 glasses of white wine (Paganga et al., 1999). The total phenolic

acids and polyphenols in white wine range from 190 to 290 mg/L, whereas in red wine they range from 900-2500 mg/L (German and Walzem, 2000).

According to human clinical trials and large cohort studies, sustained wine consumption provides antioxidant benefits in healthy volunteers. However, to date, there is no evidence that sustained wine consumption affords antioxidant benefits other than to counteract possible pro-oxidant effects of ethanol (Covas et al., 2010). On the other hand, there are some promising results concerning the antioxidant protective effect of red wine, by counteracting the postprandial oxidative stress that takes place after a meal (Covas et al., 2010).

Despite the general consensus regarding the beneficial effects of light-to-moderate wine consumption, the active constituent(s) responsible for these activities and the corresponding mechanism(s) of action remain unknown. Taking into account that wine is a complex mixture containing ethanol (which has a great variety of effects itself) and a high number of phenolic compounds (each of them with different bioavailability characteristics and mechanisms of action), it is not easy to elucidate which compounds (and their corresponding actions) are behind these beneficial health effects.

Amongst red wine polyphenols, resveratrol has being attracting researchers' attention during the last 20 years due to its multiple *in vitro* biological activities which identified this molecule as the potential active principle responsible for the "French Paradox" (i.e. the low incidence of coronary heart disease in the presence of a high dietary intake of cholesterol and saturated fat in France) (Liu et al., 2007). Despite large amounts of resources invested in research and despite promising *in vitro* results, these effects do not seem to take place *in vivo* as suggested by several human studies (Visioli, 2014). As an example, in a recent report on an 11-years-long prospective cohort study (InCHIANTI) no relationship was found between resveratrol metabolites urinary

concentration and inflammatory markers, cardiovascular disease, cancer or all-cause mortality (Semba et al., 2014). The low bioavailability of this compound has been identified as one of the factors for discrepancy between the *in vitro* results and those obtained *in vivo*. In the light of these results, the mechanisms that could explain the beneficial effects associated to moderate wine consumption remain to be elucidated.

Recently, a randomized clinical trial performed in Israel evaluated the cardiometabolic effects of initiating white wine or red wine consumption in alcohol-abstaining adults with type 2 diabetes. Patients (n=224) were assigned to 150 mL of mineral water, white wine or red wine for two years. Both wines were nearly equal in ethanol and caloric content, but the levels of total phenols in red wine were 7 times higher. Two interesting results were observed: (1) only slow ethanol metabolizers significantly benefited from the effect of both wines on glycemic control, suggesting that ethanol plays an important role in glucose metabolism; and (2) red wine (but not white wine) resulted in a beneficial effect on lipid profile, as shown by an increase in HDL cholesterol and apolipoprotein(a)$_1$ and a decrease in HDL/cholesterol ratio (Gepner et al., 2015). Taken together, these results suggest that the beneficial health effects of wine could derive from a potential synergy of moderate ethanol intake with specific non-alcoholic wine constituents.

3. Olive oil: liquid gold with cardio and neuroprotective properties

Between 1992 and 2004, the *European Prospective Investigation into Cancer and Nutrition (EPIC) Study* recruited more than half a million participants of 10 different European countries. This study aimed to evaluate the relationship between nutritional, lifestyle, metabolic, and genetic factors and the risk of cancer and other chronic diseases. The Spanish cohort of EPIC study included more than 41,000 healthy volunteers and had a follow-up of 10.4 years. The results provided suggestive evidence that a greater

intake of olive oil was associated with a reduction in primary incidence of coronary heart disease events (Buckland et al., 2012b) and a decreased risk of overall mortality (Buckland et al., 2012a).

Between 2003 and 2011, a nutritional intervention study was performed in Spain whose aim was to assess the long-term effects of the MD in the primary prevention of cardiovascular diseases. The study, called PREDIMED as an acronym for *PREvención con DIeta MEDiterránea*, was a randomized clinical trial of dietary intervention and included more than 7,200 individuals of both genders at high cardiovascular risk. The main objective of PREDIMED was to evaluate whether the MD supplemented with extra-virgin olive oil or mixed nuts prevented cardiovascular diseases, by comparison with a low-fat diet.

The results from this study demonstrated that extra-virgin olive oil consumption was associated with a reduction in the overall risk of cardiovascular disease (by 30%) and mortality in individuals at high cardiovascular risk (Guasch-Ferré et al., 2014). Additionally, the MD supplemented with extra-virgin olive oil improved the lipid profile, decreased blood pressure, and reduced the risk of major cardiovascular events (Estruch, 2010; Estruch et al., 2013).

Recent results from a subgroup from PREDIMED have shown that the beneficial health effects of the MD are not limited to the cardiovascular protection as a MD enriched with extra-virgin olive oil was also associated with improved cognitive function (executive function and global cognition) (Valls-Pedret et al., 2015).

4. Olive oil phenolic compounds: good things come in small packages

4.1. Background

Initially, the virgin olive oil beneficial effects were attributed in general to the high content of monounsaturated fatty acids, and specifically to oleic acid (Bulotta et al., 2014). However, other foods that are rich in oleic acid (like rapeseeds, soybeans, sunflower, pork or poultry) are not associated to the healthy properties attributed to olive oil (such as the prevention of LDL oxidation) (Aguilera et al., 2004; Covas et al., 2006c; Bulotta et al., 2014).

A decade ago, a study aiming to assess the impact of olive oil consumption on oxidative damage in European populations concluded that olive oil is not just a source of monounsaturated fatty acids, and stressed the important role of olive oil phenolic compounds. In that study (known as EUROLIVE), two hundred healthy young men from 5 different countries received three similar olive oils differing only from the phenolic content. A refined olive oil had the lowest phenolic content (2.7 mg/kg), a mixture of refined and virgin olive oil had a medium (164 mg/kg), and a virgin olive oil had the highest amount of phenolic compounds (366 mg/kg). Participants received daily 25 mL of one of the olive oils for three weeks followed by the other olive oils, with a washout period of two weeks between treatments. The results evidenced that the phenolic content of olive oil was able to provide health benefits, as there was an increase in HDL-cholesterol and a decrease in the lipid oxidative damage, and both were linear with the phenolic content of the olive oil consumed (Covas et al., 2006b). The relevance of these results lies on the fact that (i) the doses of olive oil used in the study were based on the daily dietary MD pattern consumption, and (ii) that they evidenced that the phenolic compounds have a protective role *in vivo*. Recent results from a subsample of the PREDIMED have shown that the supplementation of the MD

with extra-virgin olive oil decreases *in vivo* oxidized LDL and lipoprotein(a) plasma concentrations (a traditional predictor of cardiovascular risk) corroborating EUROLIVE main findings (Fitó et al., 2014).

At this point, it is worth mentioning that the health effects of phenolic compounds (and polyphenols in general) can be hindered by their low bioavailability in the human body, as it happened with the already mentioned example of resveratrol. Generally, polyphenols have a poor absorption and distribution, and a high metabolism and excretion. This fact makes difficult the translation of promising *in vitro* activities to realistic *in vivo* effects (Fantini et al., 2015). In this sense, studies (like EUROLIVE or PREDIMED) reporting *in vivo* activity of phenolic compounds are of utmost importance as, despite the extensive *in vitro* reports evaluating its activity and metabolism, little is known about what happens to these compounds once they are in the high complexity of the human body (Santos et al., 2014). On the other hand, these evidences are secondary variables and it is difficult to point at a single phenol as the main responsible for the observed effects.

4.2. Olive oil chemical composition

The composition of virgin olive oil consists of two different fractions: the **major components** (which constitute the 98-99% of the weight) and the **minor components** (which represent around 1-2%).

The **major components** (also known as the saponifiable fraction) are mainly triacylglycerols and small amounts of free fatty acids. Amongst the fatty acids that constitute the triacylglycerols, the monounsaturated oleic acid is the main representative (55-83%), followed by polyunsaturated (4-20%) and saturated fatty acids (8-14%). The major fatty acids present in olive include oleic (55-83%), linoleic (3.5-21%), palmitic

(7.5-20%), stearic (0.5-5%), palmitoleic (0.3-3.5%) and α-linolenic acid (<1%) (Beltrán et al., 2004; Covas et al., 2015). Fatty acid composition differs depending on the zone of production, the latitude, the climate, the olive cultivar, and the fruit ripening state (Quintero-Flórez et al., 2015).

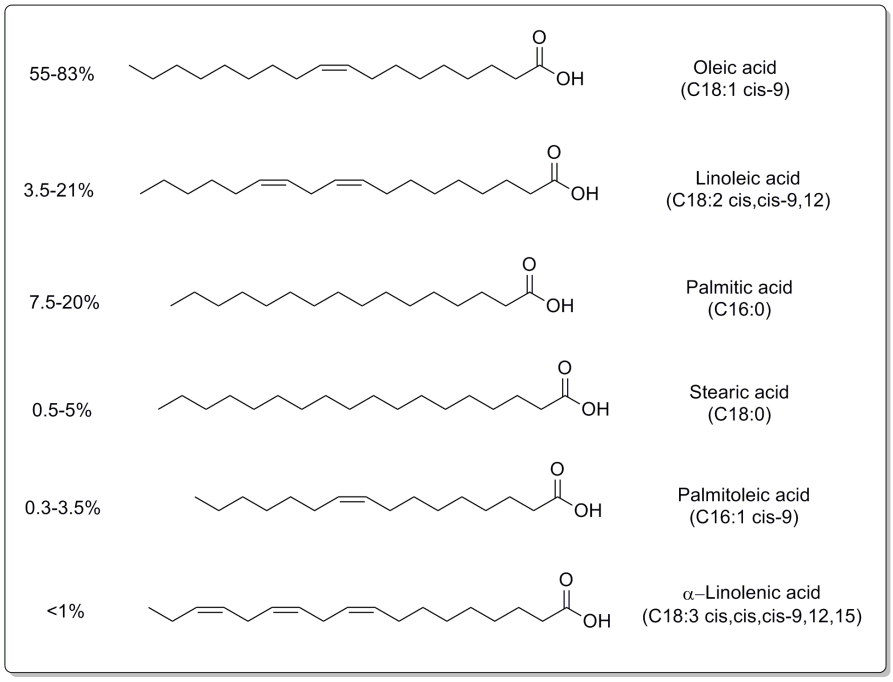


***Figure 3**. Chemical structures of the major fatty acids in olive oil, ordered from greater to smaller presence in virgin olive oil. Generally, these fatty acids are esterified with glycerol forming triacylglycerols.*

On the other hand, the unsaponifiable fraction contains **minor components** and does not have fatty compounds. There are more than 230 components found in virgin olive oil and, despite being in a low proportion, the *phenolic compounds* are the most studied and best-known components due to its beneficial health effects (Servili and Montedoro, 2002; Covas et al., 2015). As some of these compounds (such as tyrosol) do not possess two aromatic hydroxyl groups, the term *polyphenols* seems less appropriate than *phenolic compounds* (Visioli et al., 2002). Interestingly, these compounds are also responsible for the organoleptic characteristics of extra-virgin olive oil. Phenolic

compounds are exclusive components of the virgin olive oils (they are not present in other vegetable oils) although they are also present in wine and plant foods.

A wide range (50-1,000 mg/kg) of phenolic compounds in olive oil has been described. Tyrosol, hydroxytyrosol, and its secoiridoid derivatives constitute around 90% of total phenolic content of virgin olive oil and have antioxidant and anti-inflammatory activities. However, the proportion of these compounds differs from its origin in the olive tree and its final stage in virgin olive oil. In the olive tree, the secoiridoids glycosides (i.e. ligstroside and oleuropein) are the main compounds. During the process of crushing and malaxation, endogenous β-glucosidases catalyze the hydrolysis that results in ligstroside and oleuropein aglycones, that are the most abundant phenolic compounds present in virgin olive oil (Bendini et al., 2007). The dialdehydic forms of decarboxymethyl elenoic acid linked to tyrosol and hydroxytyrosol are known as oleocanthal and oleacein, respectively (See **Figure 4**) (Karkoula et al., 2012). According to a recent study in which secoiridoids from 340 extra virgin olive oils were quantified using qNMR, the amount of phenolic compounds was as follows: oleocanthal > oleacein > ligstroside and oleuropein aglycones, all of them being tyrosol or hydroxytyrosol esters (Karkoula et al., 2014). The free forms of hydroxytyrosol and tyrosol are present only in very low amounts in extra-virgin olive oils, although they can increase with storage as a consequence of the hydrolysis of the secoiridoid aglycones (Brenes et al., 2001; Romani et al., 2007). Other olive oil secoiridoids that also present a hydroxytyrosol or tyrosol moiety in their structure are demethyloleuropein and nüzhenide, respectively (Tripoli et al., 2005).

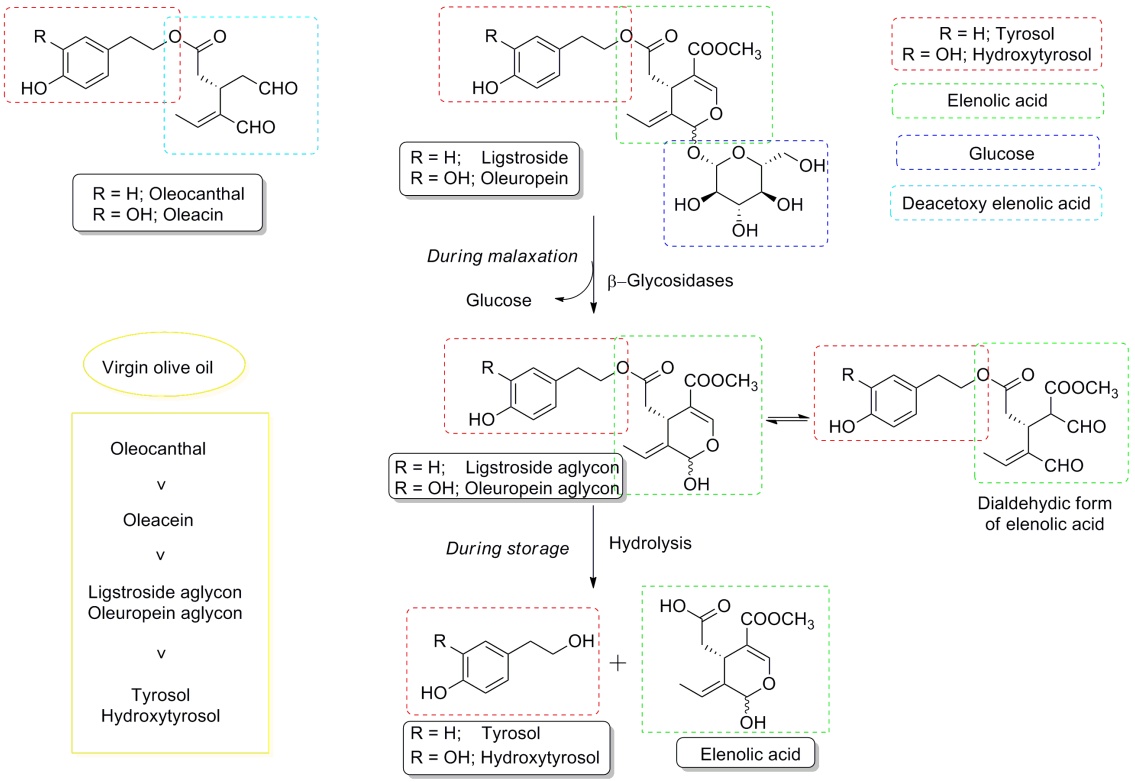


Figure 4. *Chemical structures of the major phenolic compounds present in virgin olive oil. The secoiridoid derivatives are constituted by a tyrosol or hydroxytyrosol moiety (red) esterified with elenolic acid (green) or deacetoxy elenolic acid (blue).*

4.3. EFSA Scientific opinion on olive oil phenolic compounds and its consequences

In November 2011, the European Food and Safety Authority (EFSA) released a claim regarding the benefits of daily ingestion of olive oil rich in phenolic compounds. According to EFSA, there is a cause-effect relationship between the intake of olive oil phenolic compounds (standardized by the content of tyrosol, hydroxytyrosol, and their derivatives) and the protection from LDL oxidation. In order to obtain these beneficial effects, EFSA established a minimum daily intake requirement of 5 mg of hydroxytyrosol and their derivatives (EFSA, 2011).

Despite the previously mentioned health claim, at present there is a lack of a standardized analytical method to quantify the individual phenolic compounds on olive oil that belong to the group of tyrosol, hydroxytyrosol, and their derivatives. Different analytical methods have been published (Mulinacci et al., 2006; Romero and Brenes, 2012; Purcaro et al., 2014) and the International Olive Council (IOC) is currently evaluating them to propose an official one to standardize the quantification of olive oil phenols (Mastralexi et al., 2014; Romero and Brenes, 2014). A total quantification of these compounds is challenging due to the great diversity of compounds involved, the lack of commercially available standards, the high number of possible isomers for each one, and the low stability of some of them. As an example, 11 different isomers of oleuropein aglycon and 10 of ligstroside aglycon have been found in olive oils (Fu et al., 2009a; Fu et al., 2009b). Moreover, the artificial formation of some isomers during chromatographic separation due to the mobile phase used has been recently identified as an additional problem when trying to characterize the composition of olive oil secoiridoids (Karkoula et al., 2014) and, consequently, total hydroxytyrosol equivalents.

At this point, it is worth mentioning that extra-virgin olive oil is the one that has a higher amount of phenolic compounds, compared to refined oil or seed oils (Owen et al., 2000). Virgin olive oils are defined as those *"obtained from the fruit of the olive tree solely by mechanical or other physical means under conditions that do not lead to an alteration in the oil, which have not undergone any treatment other than washing, decantation, centrifugation or filtration"*. There are not considered virgin olive oils those obtained *"using solvents or using adjuvants having a chemical or biochemical action, or by re-esterification process and any mixture with oils of other kinds"* (Covas et al., 2015).

During the refining process, up to an 80% of the phenolic compounds are lost. Therefore, virgin olive oils are richer in phenolic compounds than other oils. As an example, a study that quantified the phenolic content of Spanish olive oils found that virgin olive oils were the richest in phenolic compounds (330-500 mg/kg), followed by olive and olive-pomace oils (which contained only about 30% and 15% of that found in the virgin ones). The amount of phenolic compounds in other edible vegetable oils (i.e. soya, sunflower, peanut) is even lower (García et al., 2003). It is also known that lower quality olive oils (such as refined olive oil) have lower antioxidant and anti-inflammatory capacities due to their lack of polyphenols (Estruch and Salas-Salvadó, 2013).

Taking into account (i) the EFSA scientific opinion, (ii) the currently available data on randomized controlled human studies, and (iii) the differences on phenolic content between different commercial oils, the best olive oils concerning nutritional purposes are extra-virgin olive oils rich in phenolic compounds (Covas et al., 2015). Once the IOC has approved a standardized analytical methodology to quantify olive oil phenolic compounds, the European Union legislation could potentially regulate how this information can be used in terms of oil marketing and labelling. Another possibility that arises as a consequence of EFSA scientific opinion is the utilization of hydroxytyrosol or tyrosol as nutraceuticals.

5. Hydroxytyrosol: beyond the direct antioxidant capacity

Hydroxytyrosol [2-(3,4-dihydroxyphenyl)ethanol] presents a great variety of health promoting properties including antioxidant, anti-inflammatory, cardioprotective, antitumor, antimicrobial, antidiabetic, and neuroprotective activities (Fernández-Mar et al., 2012). The strong antioxidant capacity of this compound has attracted scientists

attention, being specially relevant in the field of prevention of cardiovascular diseases (Covas et al., 2006c). Recently, an increasing number of research articles suggest that olive oil phenolic compounds in general, and hydroxytyrosol in particular, could be used in the secondary prevention of neurodegenerative diseases. In this context, we have recently summarized the state-of-the-art knowledge on this field and published a review in the journal *Molecules* (Rodríguez-Morató et al., 2015). The original article can be found in *Appendix I*.

As for the mechanism of action, hydroxytyrosol antioxidant activity has a direct action by its ability to scavenge reactive oxygen species (ROS) generated during oxidative stress. However, its antioxidant action is not limited to this intrinsic antioxidant capacity as it also has the ability to activate different cellular signaling pathways that increase the organism's defense against an oxidative stress (i.e. Nrf2) (Martín et al., 2010). Additionally, as described for other polyphenols, the interaction of hydroxytyrosol with miRNA could be evaluated as a potential molecular target for eliciting its biological effects (Bladé et al., 2013; Milenkovic et al., 2013). In a similar way, effects secondary to hydroxytyrosol (and its metabolites) interaction with biological systems (nutrigenomics) must be considered (Konstantinidou et al., 2013).

5.1. Sources of hydroxytyrosol

There are two known sources of hydroxytyrosol: an exogenous one (which follows the ingestion of natural products that contain hydroxytyrosol or its precursors) and an endogenous one (derived from dopamine oxidative metabolism) (Goldstein et al., 1961).

5.1.1. Exogenous sources of hydroxytyrosol

Hydroxytyrosol is the principle phenolic compound found in olives and olive products. A double hydrolysis of oleuropein is necessary to release hydroxytyrosol. As previously mentioned, the first hydrolysis happens during olive maturation and storage, when the enzymatic removal of glucose from oleuropein and ligstroside takes place and the corresponding aglycones are released (Vissers et al., 2002). Later on, a second hydrolysis in the gastrointestinal tract, breaks the ester bound and gives rise to free hydroxytyrosol and elenolic acid (Granados-Principal et al., 2010) (**Figure 4**).

Although virgin olive oil is the main dietary source of hydroxytyrosol, in 1998 the presence of this compound (although in lower amounts) was described for the first time in wine. That study also revealed that red wines present higher amounts of hydroxytyrosol, compared to white wines (3.89 versus 1.80 mg/L) (Di Tommaso et al., 1998). The presence of tyrosol has also been described in wine, where it is more abundant than hydroxytyrosol. As an example, the quantification of both phenols in 30 red wines showed levels of hydroxytyrosol ranging from non-detectable to 5.0 mg/L, and levels of tyrosol ranging from 20.5 to 44.5 mg/L (Piñeiro et al., 2011). As the reader will see later, this fact is of special relevance to understand some of the results of the present thesis.

In the case of wine, tyrosol and hydroxytyrosol are formed as catabolites of the amino acid tyrosine. During the fermentation process, yeasts like *Saccharomyces cerevisiae* carry out alcoholic fermentation and convert glucose (and other sugars like fructose and sucrose) in carbon dioxide and ethanol under anaerobic conditions (**Figure 5A**). In a similar way, tyrosine is metabolized by yeasts following the Ehrlich pathway (involving transamination, decarboxylation and reduction reactions) that generate

aromatic alcohols known as *fusel* alcohols (from the German word *fusel*, meaning *bad liquor*) (**Figure 5B**) (Hazelwood et al., 2008).

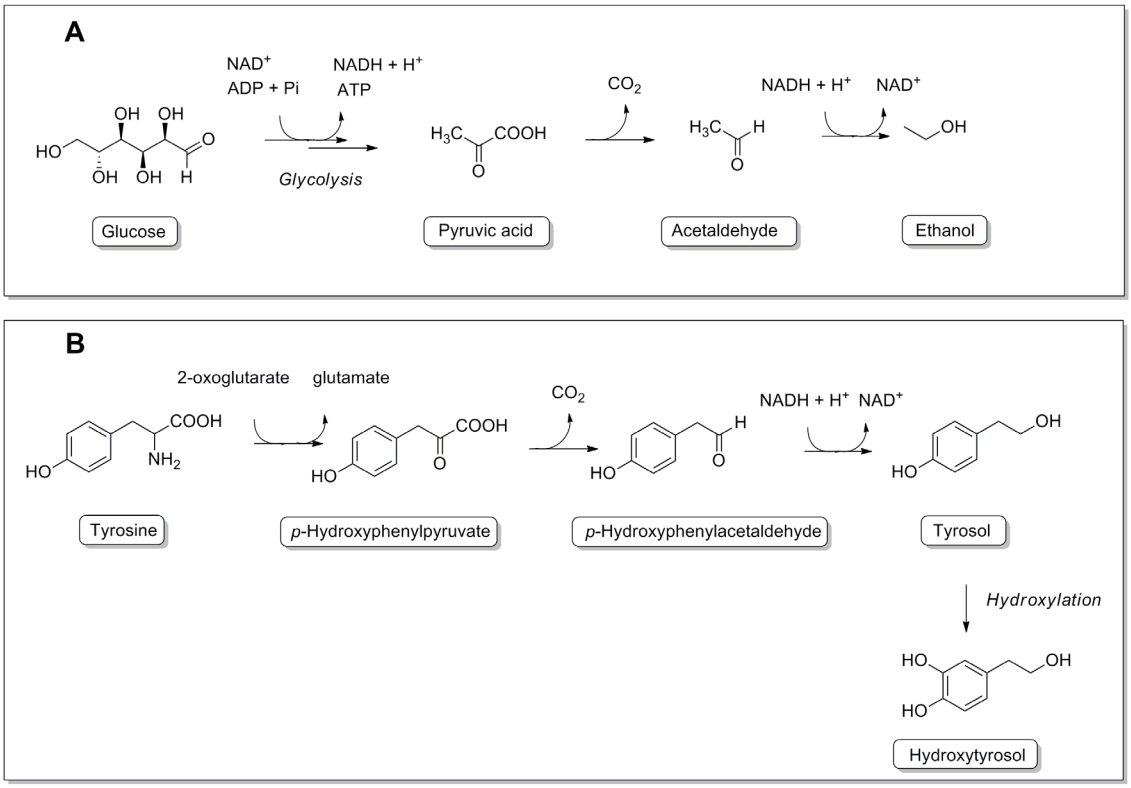


***Figure 5**. Schematic representation of ethanol fermentation (**A**) and the formation of tyrosol and hydroxytyrosol by yeasts according to Ehrlich pathway (**B**). A parallel representation of both pathways is depicted to highlight that the reactions involved are the same ones.*

5.1.2. Endogenous sources of hydroxytyrosol

Hydroxytyrosol is also formed in the brain as a metabolite of dopamine (Goldstein et al., 1961). Indeed, this *ortho*-diphenolic compound can be produced endogenously as a by-product of dopamine oxidative metabolism. The by-product formed is known as DOPET (3,4-**D**ihydr**o**xy**p**henyl**et**hanol) (De la Torre, 2008). In a similar way to what happens to other biogenic amines (like serotonin or noradrenalin) monoamine oxidase (MAO) catalyzes the oxidative-deamination of dopamine giving rise to the aldehyde metabolite DOPAL (3,4-dihydroxyphenylacetaldehyde), which can then be subsequently oxidized by aldehyde dehydrogenase to the corresponding

carboxylic acid DOPAC (3,4-dihydroxyphenylacetic acid). DOPAC is the major metabolite of dopamine in biological matrices, but a small portion of DOPAL is reduced to DOPET by aldehyde or aldose reductase (Lamensdorf et al., 2000; Marchitti et al., 2007). After ethanol intake, DOPET becomes more relevant quantitatively due to the interaction of ethanol with the dopamine oxidative metabolism (Tank and Weiner, 1979) (**Figure 6**).

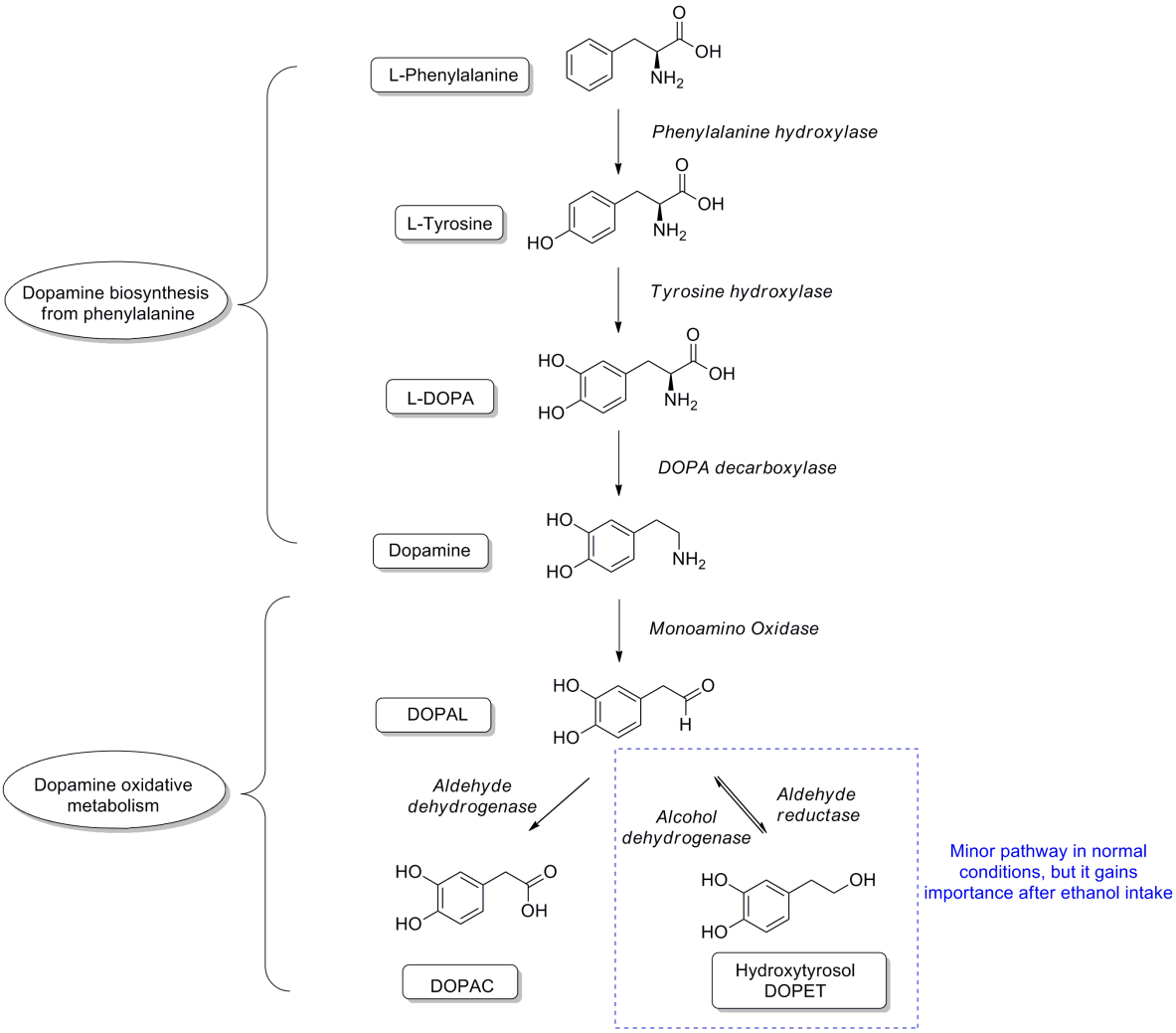


Figure 6. *Schematic representation of the biosynthesis of dopamine from phenylalanine* ***(up)*** *and dopamine oxidative metabolism* ***(down)***.

This same shift in the ratio alcohol/acid metabolites is observed in a greater extent in serotonin metabolism, where the levels of the corresponding alcohol (5-hydroxytryptophol) increase dramatically after ethanol intake at the expense of the

levels of the corresponding carboxylic acid (5-hydroxyindoleacetic acid). As a consequence, the ratio of these two metabolites in urine is a robust ethanol biomarker that lasts about 5-15 hours longer than conventional ethanol tests (Beck and Helander, 2003). The postulated mechanisms than explain this shift in the ratio are (i) the competitive inhibition of aldehyde dehydrogenase by acetaldehyde, and (ii) the alteration of the NADH/NAD ratio that results from ethanol metabolism (Beck and Helander, 2003).

5.2. Pharmacokinetics of hydroxytyrosol

The pharmacokinetic properties of a chemical compound are a key prerequisite that needs to be taken into consideration when evaluating *in vivo* biological effects. These include the processes of absorption, distribution, metabolism and excretion (ADME), which define its bioavailability. In this regard, the bioavailability and metabolism of hydroxytyrosol have been widely studied in both animal and human models (Miró-Casas et al., 2003a; Miró-Casas et al., 2003b; De la Torre-Carbot et al., 2007; González-Santiago et al., 2010). We have recently reviewed in detail the state of the art of the scientific evidence regarding the metabolic disposition of tyrosol and hydroxytyrosol. The full text version of the manuscript recently accepted for publication by *Drug Metabolism Reviews* can be found in *Appendix II*. The most relevant aspects of the processes of absorption, distribution, metabolism and excretion of tyrosol and hydroxytyrosol are summarized below.

5.2.1. Absorption

The currently available information regarding hydroxytyrosol absorption comes from experiments in animals (mainly rats) and humans. We can differentiate two different approaches: (i) a nutritional approach where hydroxytyrosol is presented in its dietary source (mainly virgin olive oil or polyphenol-enriched olive oils), and (ii) a

pharmacological approach where pure hydroxytyrosol (chemically synthesized or purified) is administered.

Following the administration of olive oil, hydroxytyrosol as well as tyrosol have been found in urine, evidencing that these phenolic compounds are absorbed in a dose-dependent manner in humans (Visioli et al., 2000). After the administration of dietary doses of olive oil (25-50 mL/day), hydroxytyrosol and tyrosol have been detected and quantified in urine (Miró-Casas et al., 2001; Weinbrenner et al., 2004), plasma (Covas et al., 2006a) as well as in low density lipoproteins (De la Torre-Carbot et al., 2006; De la Torre-Carbot et al., 2007).

The vehicle of administration is another important factor, as it determines the absorption to a considerable extent. In humans, important differences in bioavailability have been described after the administration of the same amount of hydroxytyrosol in different matrices. A higher urinary excretion of this phenolic compound was found when it was administered as a natural component of extra-virgin olive oil (44% of recovery), compared to hydroxytyrosol-supplemented refined olive oil (23%) or yogurt (6%) (Visioli et al., 2003). In line with these results, when an aqueous solution of pure hydroxytyrosol was administered in high amounts (2.5 mg/kg) to healthy volunteers, the bioavailability was below 10% of the dose administered (González-Santiago et al., 2010).

De Bock *et al.* administered concomitantly pure hydroxytyrosol and oleuropein to humans and evidenced that these phenolic compounds are rapidly absorbed, as their metabolites are detected in plasma after ingestion (23-93 min), with oleuropein concentrations peaking earlier than its conjugates (De Bock et al., 2013). In a similar study in which important amounts of pure hydroxytyrosol (around 170 mg) were

administered to healthy volunteers, plasma T_{max} was 13 minutes (González-Santiago et al., 2010).

5.2.2. Distribution

Once absorbed, hydroxytyrosol is widely distributed throughout the organism. The first study in this field was carried out by D'Angelo *et al.*, who administered an intravenous dose of 1.5 mg/kg of [^{14}C]-labeled hydroxytyrosol to rats. The pharmacokinetic analysis indicated an extensive and fast uptake of this antioxidant by different organs including the skeletal muscle, kidneys, liver, lungs, heart and brain (D'Angelo et al., 2001). The ability of hydroxytyrosol to cross the blood-brain barrier, despite having a low brain uptake, demonstrated *in vivo* that it fulfills this essential requirement to be used as a neuroprotective agent.

A recent study in rats showed that, after the oral administration of increasing doses (1, 10 and 100 mg/kg) of this compound (given in an olive oil matrix), hydroxytyrosol was accumulated in a dose-dependent manner not only in urine and plasma but also in the liver, kidney and brain (López de las Hazas et al., 2015).

In the two previously mentioned studies in rats, the kidneys and the liver were the organs that presented higher uptake of hydroxytyrosol and its metabolites.

5.2.3. Metabolism

Despite its good absorption and wide distribution, hydroxytyrosol bioavailability is poor due to an extensive pre-systemic metabolism. Before entering the portal bloodstream, it undergoes phase I/II metabolism in the enterocytes and after having reached the liver through portal circulation it is subject of additional phase II metabolism. After the intake of olive oil, around 98% of hydroxytyrosol and tyrosol in urine and plasma appear to be conjugated (Miró-Casas et al., 2003a). The remaining

amount of unaltered hydroxytyrosol in the bloodstream is low, to such an extent that the free form has been deemed undetectable in plasma (De la Torre, 2008).

To date, more than 10 hydroxytyrosol metabolites have been described (**Figure 7**). These include sulfates, glucuronides, *O*-methylated forms, *N*-acetylcysteine derivatives (Kotronoulas et al., 2013) as well as oxidized forms of the aliphatic alcohol (D'Angelo et al., 2001).

The enzymes implicated in hydroxytyrosol **phase I** metabolism are non-microsomal alcohol and aldehyde dehydrogenases, both located in the cytosol. The corresponding metabolites are DOPAL and DOPAC which, as already explained, are non-specific as they are not only metabolites of hydroxytyrosol but also metabolites of dopamine.

The enzymes involved in hydroxytyrosol **phase II** reactions are sulfotransferases (SULT), uridine 5'-diphosphoglucuronosyl transferases (UGTs), catechol-*O*-methyltransferases (COMT), and acetyltransferases. A metabolite resulting from methylation and glucuronidation has also been identified, although this represents a minor metabolic pathway (Miró-Casas et al., 2003a). Recently, hydroxytyrosol 1-acetate-4'-*O*-sulfate has also been reported as a biological metabolite of hydroxytyrosol (Rubió et al., 2012).

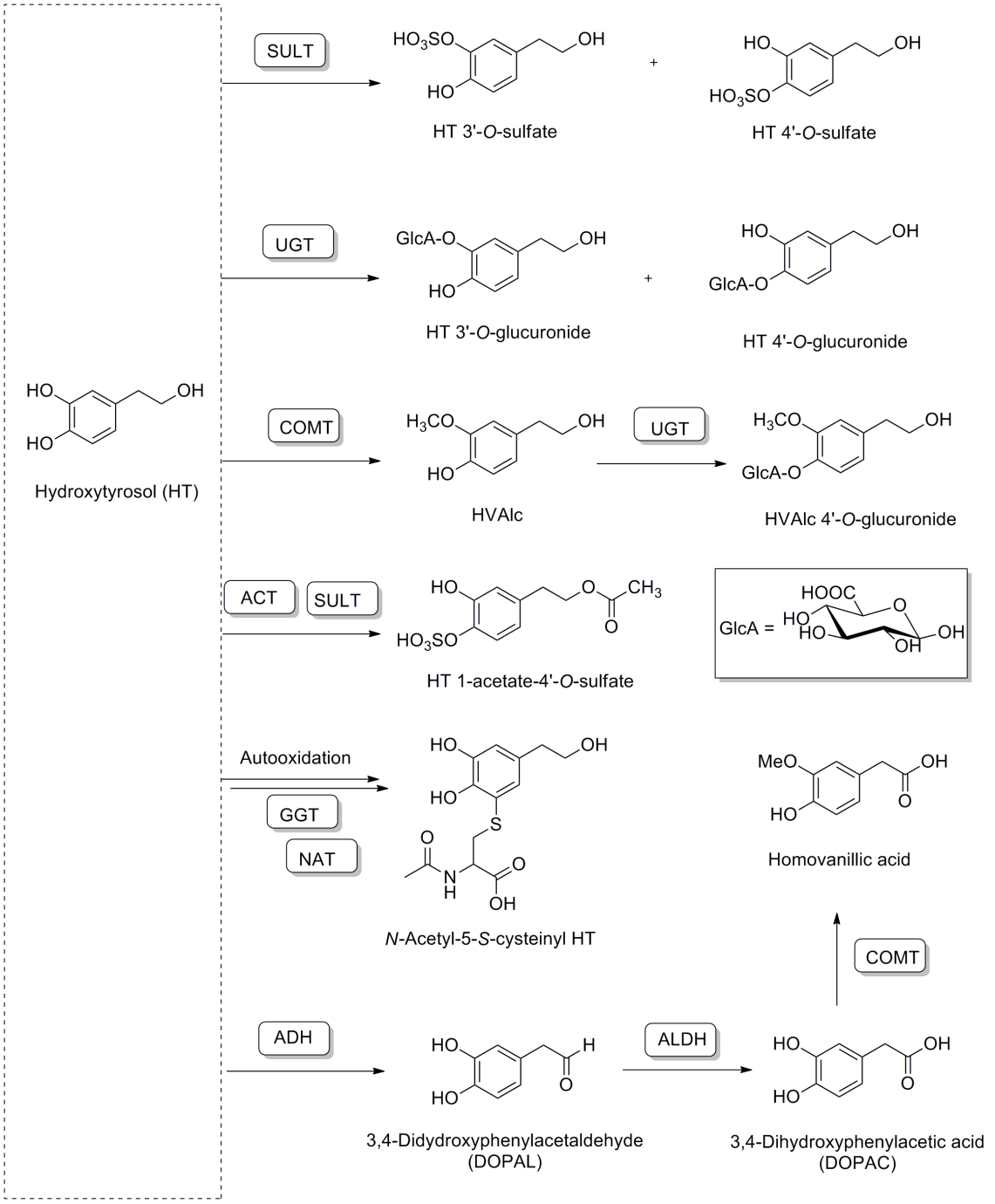


Figure 7. *Biotransformation pathways of hydroxytyrosol (HT). Abbreviations: ACT: O-Acetyl transferase; ADH: Alcohol dehydrogenase; ALDH: Aldehyde dehydrogenase; COMT: Catechol-O-methyl transferase; GGT: γ-Glutamyl transferase; GlcA: Glucuronic acid; HVAlc: Homovanillyl alcohol; NAT: N-acetyl transferase; UGT: UDP-glucuronosyl transferase; SULT: Sulfotransferase.*

Finally, homovanillic acid (4-hydroxy-3-methoxyphenylacetic acid) has also been identified as a metabolite of hydroxytyrosol (Tuck et al., 2002) although it is non-specific as it is:

(i) A metabolite of dopamine (formed by *O*-methylation of DOPAC via COMT)

(ii) A metabolite of dietary flavanols (e.g. quercetin, rutin) (Combet et al., 2011)

(iii) A phenolic compound found in olive oil (Bayram et al., 2013)

Despite the well-known activities of hydroxytyrosol and tyrosol, little is known about the activity of their corresponding metabolites. The main reason for this fact has been the lack of commercially available reference standards.

The first attempts to evaluate the activity of metabolites obtained the conjugates from the urine of rats that had been administered with labelled hydroxytyrosol. This study found radical scavenging activities of hydroxytyrosol-3-*O*-glucuronide higher than that of the parent compound (Tuck et al., 2002). A few years later, pure standards of this and other glucuronides were biosynthesized using porcine liver microsomes (Khymenets et al., 2006) and, when the radical scavenging activity was evaluated using the same test (DPPH), the glucuronides were devoid of activity (Khymenets et al., 2010).

During last years, the development of convenient chemical synthesis to obtain hydroxytyrosol glucuronides (Lucas et al., 2009), as well as the possibility of obtaining them by custom synthesis, has facilitated the assessment of the activity of these compounds. Consequently, the number of recent papers supporting the activity of hydroxytyrosol metabolites has increased, although the results vary depending on the model employed.

A protective effect comparable to that of hydroxytyrosol was reported for its glucuronides in a model of H_2O_2-induced oxidative damage in renal tubular epithelial cells (Deiana et al., 2011) and on erythrocyte oxidative-induced hemolysis (Paiva-

Martins et al., 2013). Giordano *et al.* have provided further evidence of the activity of hydroxytyrosol glucuronides assessing their ability to reduce endoplasmic reticulum stress in HepG2 cells (Giordano et al., 2015).

The chemical synthesis and activity of the sulfates has been recently reported (Gomes et al., 2015). Interestingly, both tyrosol and hydroxytyrosol sulfates showed an efficiency in protecting Caco-2 cells comparable to that of the parent compounds (Atzeri et al., 2016).

These same cells have been use to explore the activity of hydroxytyrosol using a different strategy: by biosynthesizing phase II metabolites using intestinal cells and testing the whole mixture of metabolites in human aortic endothelial cells. The results showed that hydroxytyrosol metabolites significantly reduced the TNF-α induced secretion of cell adhesion molecules (Catalán et al., 2015).

Taken together, these studies suggest that, once metabolized, hydroxytyrosol conjugates still exert biological activity. The differences in activity between the metabolites and the parent compound may lay on differences in the mechanisms of action.

5.2.4. *Excretion*

Both animal and human studies revealed that hydroxytyrosol metabolites are excreted in urine, indicating that renal excretion is their preferential route of excretion (Caruso et al., 2001; D'Angelo et al., 2001). In humans, the main metabolites found in plasma and urine after the administration of pure oleuropein and hydroxytyrosol are sulfate and glucuronide conjugates (96-99%). Similarly to absorption, excretion takes place rapidly and the metabolites are detected in plasma up to 6 hours after ingestion (De Bock et al., 2013)

Following the administration of pure hydroxytyrosol to rats, different excretion patterns have been observed between gender, the females presenting higher recoveries of hydroxytyrosol than males (Domínguez-Perles et al., 2015).

Studies in rats by a different research group have shown that there are changes in the metabolic disposition of hydroxytyrosol in a dose-dependent manner. Glucuronidation, the main metabolic pathway at low doses, shifts to sulfation at higher doses (Kotronoulas et al., 2013).

An unsolved issue related with excretion is the fact that some studies in rats (Domínguez-Perles et al., 2015) and also in humans (De la Torre et al., 2006) have revealed urinary recoveries higher than 100% of the dose of hydroxytyrosol administered. As detailed in the following section, the aim of the present work is to study this phenomenon and to explain the mechanisms responsible for it.

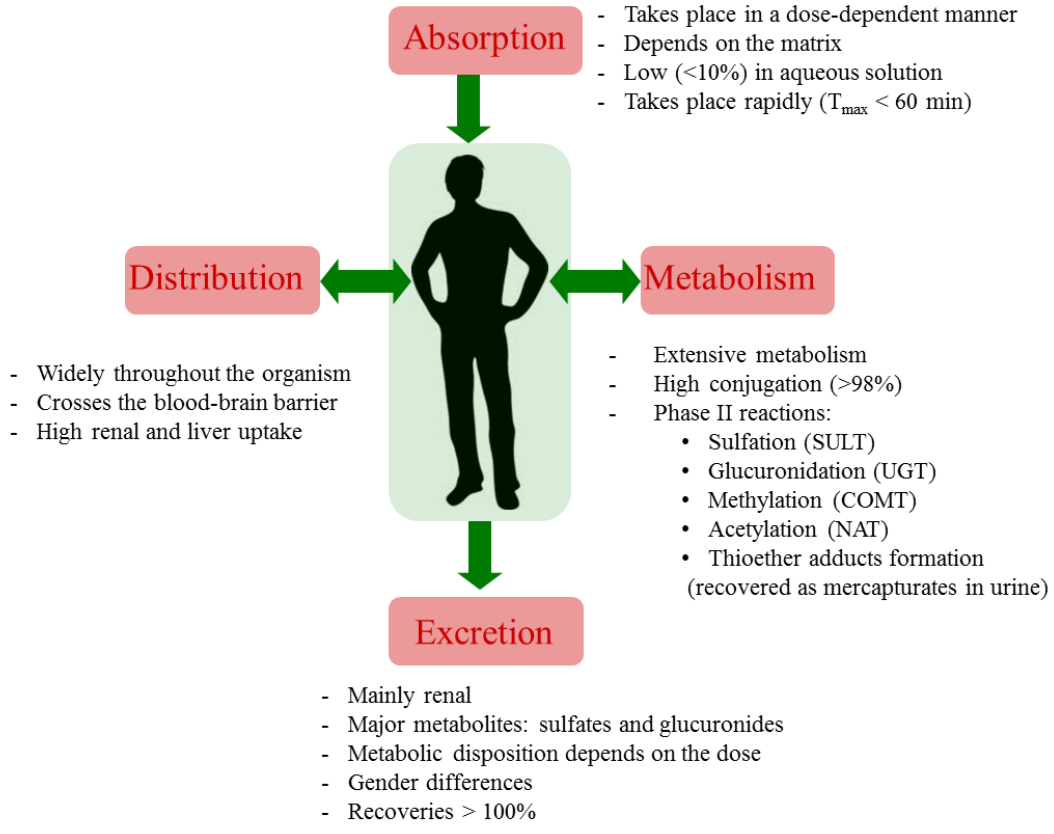


Figure 8. *Schematic summary of hydroxytyrosol disposition*

6. Background: Defying the principle of mass conservation?

6.1. Detection of abnormally high amounts of hydroxytyrosol following the intake of red wine

In the frame of a clinical study designed to assess the postprandial and short-term effects of moderate doses of red wine it was found that hydroxytyrosol was endogenously produced in significant amounts. In that study, hydroxytyrosol pharmacokinetics after red wine (250 mL/day) and virgin olive oil (25 mL/day) administration were compared. The dose of hydroxytyrosol administered with a glass of red wine was 5 times lower than one administered in 25 mL olive oil (0.35 versus 1.70 mg). In the case of olive oil, hydroxytyrosol urinary recoveries were around 30%. Unexpectedly, urinary recoveries following red wine administration were not only higher than those following olive oil intake, but also exceeded the amount of hydroxytyrosol administered (200%), suggesting an endogenous formation of this phenolic compound (De la Torre et al., 2006) (**Figure 9**).

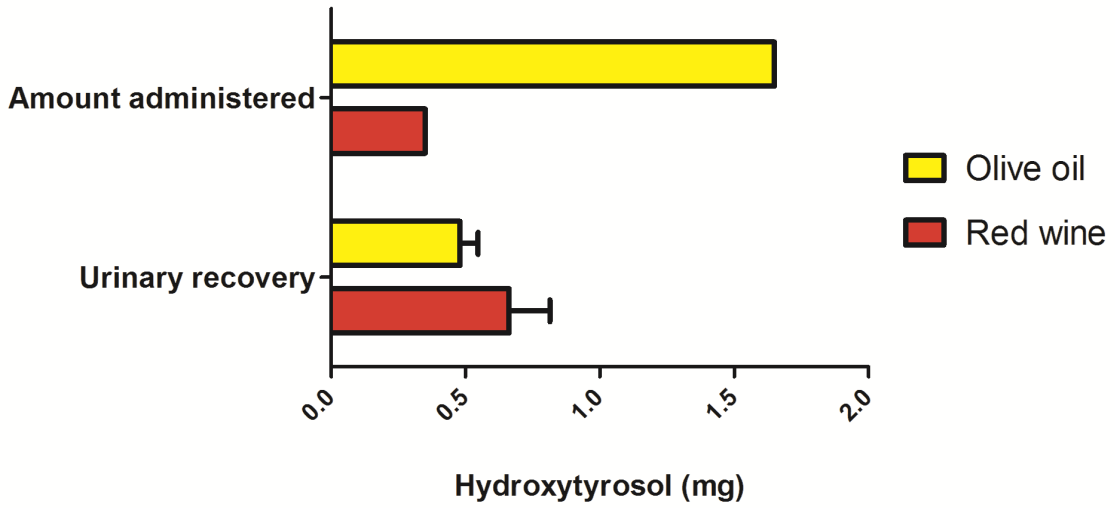


Figure 9. *Amount of hydroxytyrosol administered and corresponding urinary recoveries following the intake of olive oil (25 mL) and red wine (250 mL).*

These high levels of hydroxytyrosol following wine intake are relevant since some authors have questioned the *in vivo* concentrations after the ingestion of olive oil polyphenols are too low to explain the *in vitro* observed biological activities (Vissers et al., 2004). Another fact that adds relevance to this finding is revealed when looking at the different studies evaluating the contribution of phenolic compounds to the beneficial effects of wine consumption. So far, neither catechins (Boban et al., 2006) nor the already explained case of resveratrol (Semba et al., 2014) have shown to contribute to the beneficial effects associated to moderate wine consumption.

6.2. The role of ethanol as a minor contributor to the endogenous generation of hydroxytyrosol

As previously mentioned, during the sixties and seventies, the alteration of the metabolism of biogenic amines (serotonin (Davis et al., 1967), tyramine (Tacker et al., 1970) and dopamine (Tank and Weiner, 1979)) by ethanol was reported. Under normal conditions, these primary amines are firstly oxidized to aldehydes and then they are further oxidized to the corresponding carboxylic acids. However, in the presence of ethanol, the reduction of the aldehydes to alcohols gains importance.

In the case of dopamine, it was found that DOPET (=hydroxytyrosol) becomes more relevant quantitatively after ethanol intake at the expense of its oxidized analogue DOPAC (Tank and Weiner, 1979).

Taking into account this observation, and in order to study if the high recovery of urinary hydroxytyrosol could be caused by the ethanol present in wine, another intervention study was performed. Twenty-four male volunteers received different doses of ethanol (6-42 g) or placebo. In agreement with the findings of Tank and Weiner, urinary excretion of hydroxytyrosol (and also of tyrosol) increased with the dose of

ethanol administered, this being compatible with a shift in dopamine (and tyramine) oxidative metabolism (Pérez-Mañá et al., 2015a) (**Figure 10**).

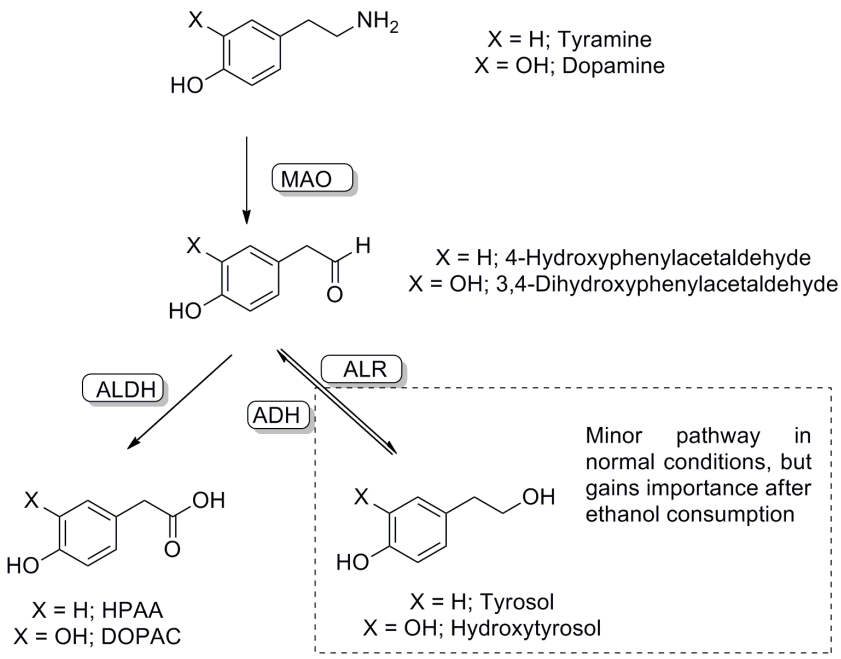


Figure 10. *Tyramine and dopamine oxidative metabolism diagram. Abbreviations: ALR: Aldehyde/aldose reductase; ADH: Alcohol dehydrogenase; ALDH: Aldehyde dehydrogenase; DOPAC: 3,4-dihydroxyphenylacetic acid; HPAA: 4-hydroxyphenylacetic acid; MAO: Monoamine oxidase.*

Despite having verified this ethanol-induced hydroxytyrosol generation, the amount of hydroxytyrosol formed and recovered in urine was not high enough to explain the amounts of this compound recovered in urine after wine intake, evidencing the existence of additional sources of hydroxytyrosol formation behind wine.

OBJECTIVES AND HYPOTHESES

The **aim of the present work is to identify and study new mechanisms responsible for hydroxytyrosol *in vivo* generation.** These mechanisms would help to explain the apparent excessive urinary recovery of hydroxytyrosol following wine intake in humans and could contribute to the elucidation of the health beneficial effects associated with moderate red wine intake.

A **preliminary objective** is **to evaluate the *in vitro* antioxidant capacities of tyrosol and hydroxytyrosol**. We hypothesized that hydroxytyrosol would have a higher antioxidant capacity due to the additional hydroxyl group at the position 3 of the phenol ring, which provides this compound with *catechol* structure.

The **first objective** is **to assess the contribution of wine phenolic compounds to urinary hydroxytyrosol**. Bearing in mind the wine phenolic content and the chemical structure of hydroxytyrosol, three different hypotheses were considered based on three potential candidates that could be endogenously converted into hydroxytyrosol: (1) tyrosine, (2) tyrosol, and (3) tyramine.

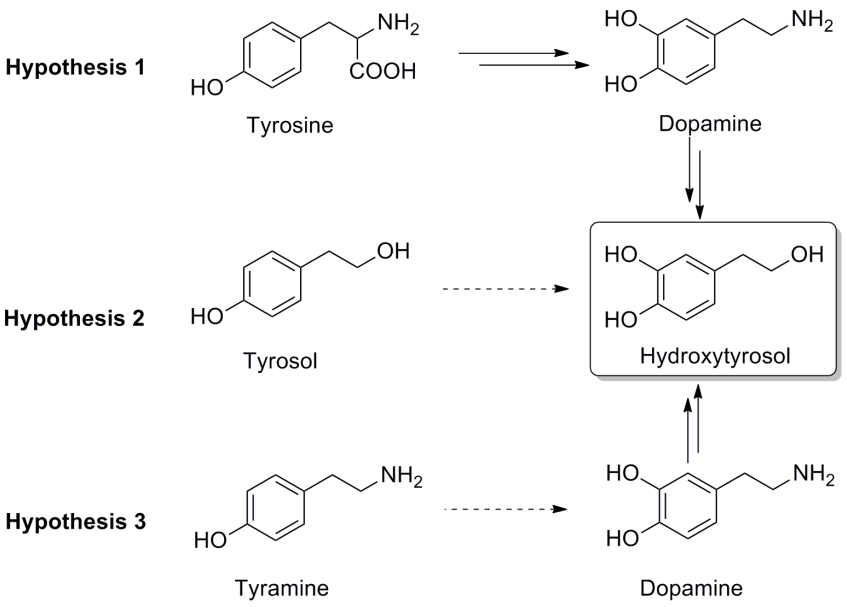


Figure 11. *Schematic representation of the postulated hypotheses: Three phenolic compounds (tyrosine, tyrosol and tyramine) are presented as potential precursors of hydroxytyrosol.*

(1) Tyrosine is one of the amino acids that link together to form proteins. Despite being synthesized in our organism by hydroxylation of phenylalanine, an important source of this amino acid is from dietary origin. Tyrosine plays an important role in the brain, as it is a key precursor in the synthesis of catecholamines (Fernstrom, 2000). Taking into account that tyrosine is a precursor of dopamine and that, in turn, hydroxytyrosol is formed as a by-product of dopamine oxidative metabolism, tyrosine was selected as a potential precursor of hydroxytyrosol.

(2) Tyrosol is an analogue of hydroxytyrosol which lacks the aromatic hydroxyl group in position 3 and, consequently, it has a lower antioxidant capacity (Carrasco-Pancorbo et al., 2005). It is found in wine and olive oil in important amounts. Taking into consideration that some enzymes are specialized in catalyzing *ortho*-hydroxylations (e.g. tyrosine hydroxylase) and that hydroxylation is widely used as a phase I reaction by hepatic enzymes (such as cytochrome P450; CYP), tyrosol was selected as a potential precursor of hydroxytyrosol.

(3) Tyramine is a naturally occurring monoamine with peripheral sympathomimetic activity that acts as a catecholamine releasing agent. It is produced endogenously by decarboxylation of tyrosine and it is consumed exogenously through different foods such as cheese, chocolate, alcoholic beverages, fish or processed meat (McCabe-Sellers et al., 2006). *In vitro* studies have shown that tyramine can be converted in dopamine via CYP2D6 (Bromek et al., 2010). Taking into account that tyramine is converted into dopamine, and that hydroxytyrosol is formed during dopamine oxidative metabolism, tyramine was selected as a potential precursor of hydroxytyrosol.

The **second objective** of the present work is **to evaluate the contribution of the three phenolic compounds selected (tyrosine, tyrosol or tyramine) alone or in combination with ethanol to urinary hydroxytyrosol**.

The **third objective** of this thesis is **to study the mechanism (chemical, enzymatic or mediated by microbiota) involved in the formation of hydroxytyrosol**. The corresponding hypotheses depend on which precursor (or precursors) generate(s) hydroxytyrosol, and would be explained in further detail later on.

The **fourth objective** is **to evaluate the pharmacogenomic impact of genetic polymorphisms on the formation of hydroxytyrosol from a phenolic precursor.**

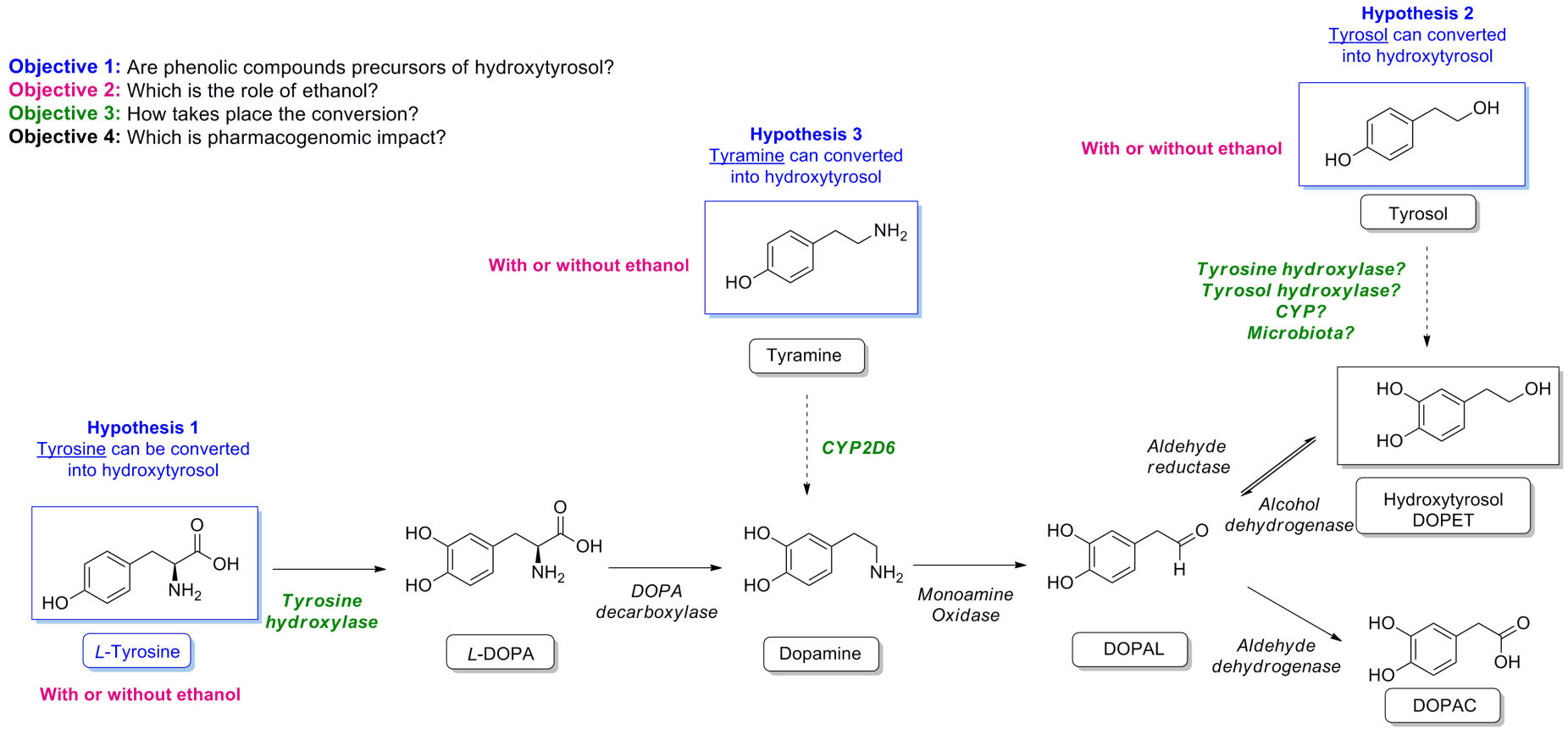


Figure 12. *Schematic representation of the objectives and hypotheses of the present work.*

METHODS AND RESULTS

A general overview of the experimental strategy designed to achieve the goals of the present thesis is summarized in **Figure 13**. Depending on the pursued objective, various *in vitro* and *in vivo* approaches were employed.

In order to evaluate the *in vitro* bioactions of tyrosol and hydroxytyrosol (preliminary objective), their antioxidant capacity were assessed using two antioxidant capacity assays (i.e. FRAP and ORAC). Moreover, the activity of both phenols was also evaluated in HepG2 cells treated with oxidative stress models (i.e. AAPH and H_2O_2) and evaluated using different assays (ROS, apoptosis, and necrosis).

The contribution of phenolic compounds alone or in combination with ethanol to urinary hydroxytyrosol (first and second objective) was evaluated in a cross-over, double-blind, randomized controlled clinical trial (n=28) and in an animal model (rats). Metabolites of dopamine oxidative metabolism were quantified in urine using GC-MS, whereas tyrosol, hydroxytyrosol and their corresponding metabolites were analyzed using LC-MS/MS.

In order to elucidate the mechanisms involved into hydroxytyrosol formation from phenolic precursors (third objective), specific enzymatic inhibitors were used in both rats and human liver microsomes. The evaluation of hydroxytyrosol formation and the inhibitory effect was assessed using LC-MS/MS.

Finally, the impact of genetic polymorphisms on hydroxytyrosol formation (fourth objective) was assessed in microsomes from human genotyped livers also using LC-MS/MS.

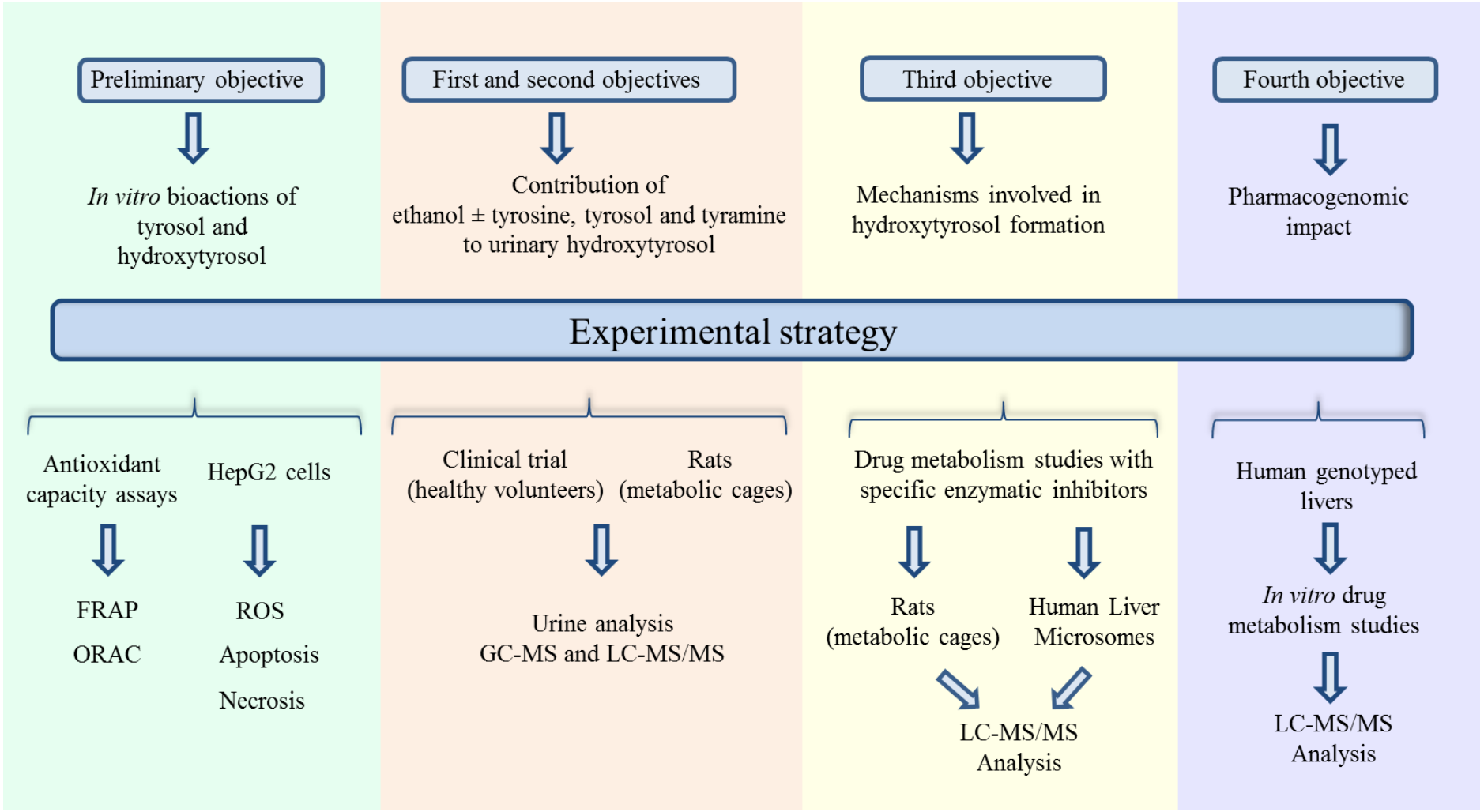


Figure 13. *Schematic representation of the experimental strategy employed in the present work.*

CHAPTER 1

Evaluation of the *in vitro* Bioactions of Tyrosol and Hydroxytyrosol

The work corresponding to the present chapter was performed in the Antioxidants Research Laboratory in the Jean Mayer USDA Human Nutrition Research Center on Aging (Boston, MA). All the research was done under the direct supervision of Drs. Oliver Chen and Jeffrey Blumberg during the period of time from March 2015 to June 2015.

1. Antioxidant Capacity Assays

1.1. Background, aim and hypothesis

During the last few decades, a great variety of antioxidant capacity assays has been developed. The differences between these assays lay on the oxidants, probes, reaction conditions, and quantitation methods. Depending on the chemical reactions involved, major antioxidant capacity assays can be divided into two categories (Huang et al., 2005):

- Hydrogen atom transfer reaction based assays
- Single electron transfer reaction based assays

Although some of these assays have been applied to determine the antioxidant capacity of tyrosol and hydroxytyrosol, it is difficult to compare results produced from different laboratories partially because of a lack of approved, standardized methods (Huang et al., 2005). Regarding the antioxidant capacity, inconsistent results have been obtained for different antioxidants due to the use of different assays and reference standards (Frankel and Meyer, 2000).

For the abovementioned reasons, prior to assessing the activity of tyrosol and hydroxytyrosol in HepG2 cells, their antioxidant capacity was evaluated using FRAP (as a representative assay based on single electron transfer reaction) and ORAC (as an assay based on hydrogen atom transfer). The main differences between these two assays are summarized in **Table 1**.

Table 1. *Comparison between FRAP and ORAC assays*

	FRAP	ORAC
Chemical reaction involved	Single electron transfer	Hydrogen atom transfer
Principle	Antioxidant reducing capacity of Fe^{3+} into Fe^{2+}	Competitive reaction preventing oxidation of fluorescent fluorescein
Initiation	-	AAPH-thermally generated peroxyl radicals
Quantitation methodology	One measurement of absorbance	Derived from reaction kinetic curves (area under the curve)
Measurement	Absorbance at 593 nm	Fluorescence (Fluorescein oxidation) (excitation 485 nm, emission 520 nm)
First description of the methodology	(Benzie and Strain, 1996)	(Cao et al., 1993)
Assays based on similar chemical reactions	DPPH TEAC Folin-Ciocalteau	TRAP Inhibition of LDL oxidation

Besides the possibility of analyzing the direct antioxidant capacity of a single compound, these assays can also been applied to evaluate the plasma antioxidant capacity (McKay et al., 2015), plant extracts (Chung et al., 2009), foods and drinks (Dávalos et al., 2004; Ninfali et al., 2005). Interestingly, Ninfali and coworkers evaluated ORAC in different vegetables and concluded that supplementation with olive oil and wine (both natural sources of tyrosol and hydroxytyrosol) led to the highest increases in antioxidant capacity in humans (Ninfali et al., 2005).

However, caution is required when interpreting these results as the *in vitro* values of antioxidant capacity do not always correlate with the biological activity that the antioxidants play on human health (USDA, 2010). The information provided by these assays itself cannot be used to predict the beneficial effects that compounds have in the human body. It should be interpreted together with the corresponding bioacessibility and bioavailability data of these compounds to see how the *in vitro* activity can be translated into *in vivo* beneficial effects.

The **aim of this chapter was to evaluate the *in vitro* antioxidant capacities of tyrosol and hydroxytyrosol** (preliminary objective of the thesis). It is worth remembering that, as explained in the introduction, these two phenolic compounds are known to be bioavailable (Miró-Casas et al., 2003b). **We hypothesized that hydroxytyrosol would have a higher antioxidant capacity than tyrosol due to the additional hydroxyl group at the position 3 of the phenol ring, which provides this compound with *catechol* structure** (**Figure 14**).

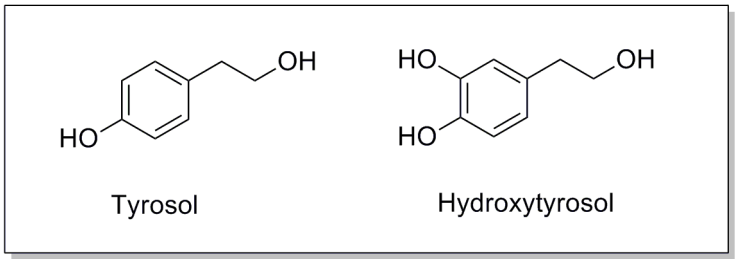


Figure 14. *Chemical structures of tyrosol and hydroxytyrosol.*

1.2. Methods

1.2.1. Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP values of tyrosol and hydroxytyrosol were determined by the spectrophotometric method of Benzie and Strain (Benzie and Strain, 1996). This assay measures directly the capacity of antioxidants to act as reductants in a redox colorimetric reaction of Fe^{3+}-2,4,6-tripyridyl-S-triazine (TPTZ) to a blue-colored Fe^{2+} complex under acidic conditions. Briefly, the FRAP reagent was freshly prepared and contained 3.5 mL of a 10 mM TPTZ solution in 40 mM HCl, 3.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$, and 35 mL of 300 mM acetate buffer. 900 µL of FRAP reagent were mixed with 90 µL of distilled water and 30 µL of either test samples, standard (Trolox solution), or blank (phosphate buffer). The absorbance was measured at 593 nm and was recorded after 1 hour (Chen and Blumberg, 2008). FRAP value of tyrosol and

hydroxytyrosol were calculated using standard curves established using Trolox at concentrations ranging 31.25-1000 µmol/L (**Figure 15**). Tyrosol and hydroxytyrosol concentrations were of 5, 50, and 500 µmol/L. The reducing power is expressed as µmol Trolox equivalents/L. All analyses were run in triplicate.

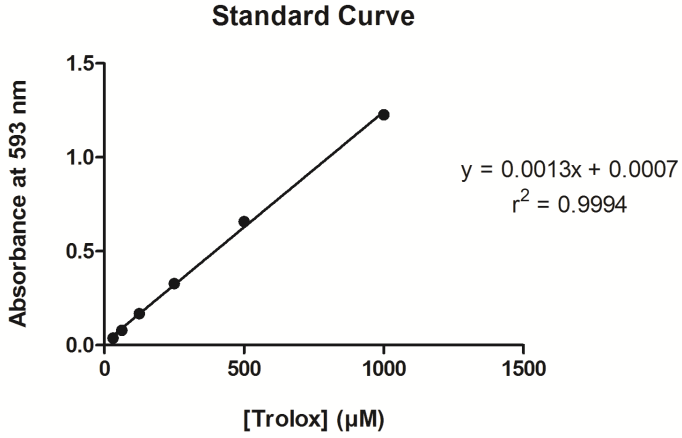


Figure 15. *Calibration curve for FRAP method related to Trolox concentrations*

1.2.2. Oxygen Radical Absorbance Capacity (ORAC) Assay

The ORAC value of tyrosol and hydroxytyrosol was determined according to the method of (Cao et al., 1993) and (Huang et al., 2002). The ORAC assay employs the area under the curve of the magnitude and time to the oxidation of fluorescein due to peroxyl radicals generated by the addition of AAPH. Twenty-five µL of either test sample, solvent (blank) or Trolox were added to a 96-well microplate followed by the addition of 150 µL of fluorescein work solution (200 nM) prepared in 75 mM phosphate buffer (pH 7.4). After the addition of 25 µL of AAPH (320 mM) prepared in 75 mM phosphate buffer, fluorescence was measured every 120 seconds. The assay was carried out at 37 ºC on a FLUOstar OPTIMA plate reader using fluorescence filters with an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Data reduction from the ORAC assay was achieved by calculating the area under the kinetic curve (AUC) and net AUC ($AUC_{sample} - AUC_{blank}$). ORAC values of tyrosol and

hydroxytyrosol were calculated on the basis of standard curves established using Trolox at 5-50 µmol/L (See **Figure 16**). Tyrosol and hydroxytyrosol were evaluated at 1.0, 2.5, 5.0, and 10 µmol/L. All data are expressed as µmol Trolox equivalents/L. All analyses were run in triplicate.

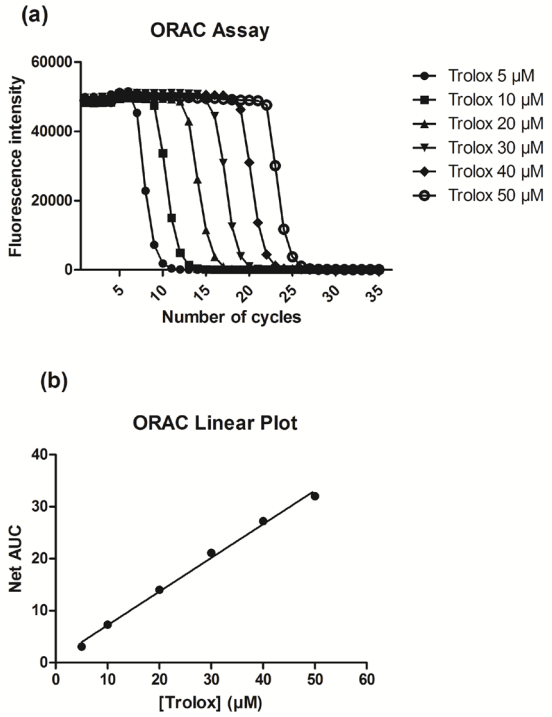


***Figure 16**. (a) Fluorescence decay curve of fluorescein in the presence of AAPH and increasing concentrations Trolox. (b) Linear plot of the net AUC versus Trolox concentration.*

1.3. Results

1.3.1. FRAP Assay

The results of the absorbance values and their corresponding Trolox equivalents are represented in **Table 2**. Following the FRAP assay, antioxidant capacity of hydroxytyrosol was 25-fold higher than tyrosol. FRAP value of tyrosol was only detected at the highest test concentration (500 µM). Antioxidant capacity of

hydroxytyrosol increased linearly with the test concentrations and was found to be around 80% of Trolox.

Table 2. *Tyrosol and hydroxytyrosol absorbance and Trolox equivalents following FRAP assay.*

Concentration (µM)	Trolox Equivalent Antioxidant Capacity (µM)	
	Tyrosol	**Hydroxytyrosol**
5	0	6
50	0	41
500	16	401

1.3.2. ORAC Assay

The antioxidant capacity of tyrosol and hydroxytyrosol assessed by ORAC followed a dose-response effect and presented higher values for hydroxytyrosol than that for tyrosol (**Figure 17 A**). The ORAC value of tyrosol was similar to that of Trolox, whereas the one from hydroxytyrosol was higher (330-370%). The fluorescence decay curves induced by AAPH in presence of hydroxytyrosol and tyrosol are depicted in **Figure 17 B** (5 µM) and **C** (10 µM).

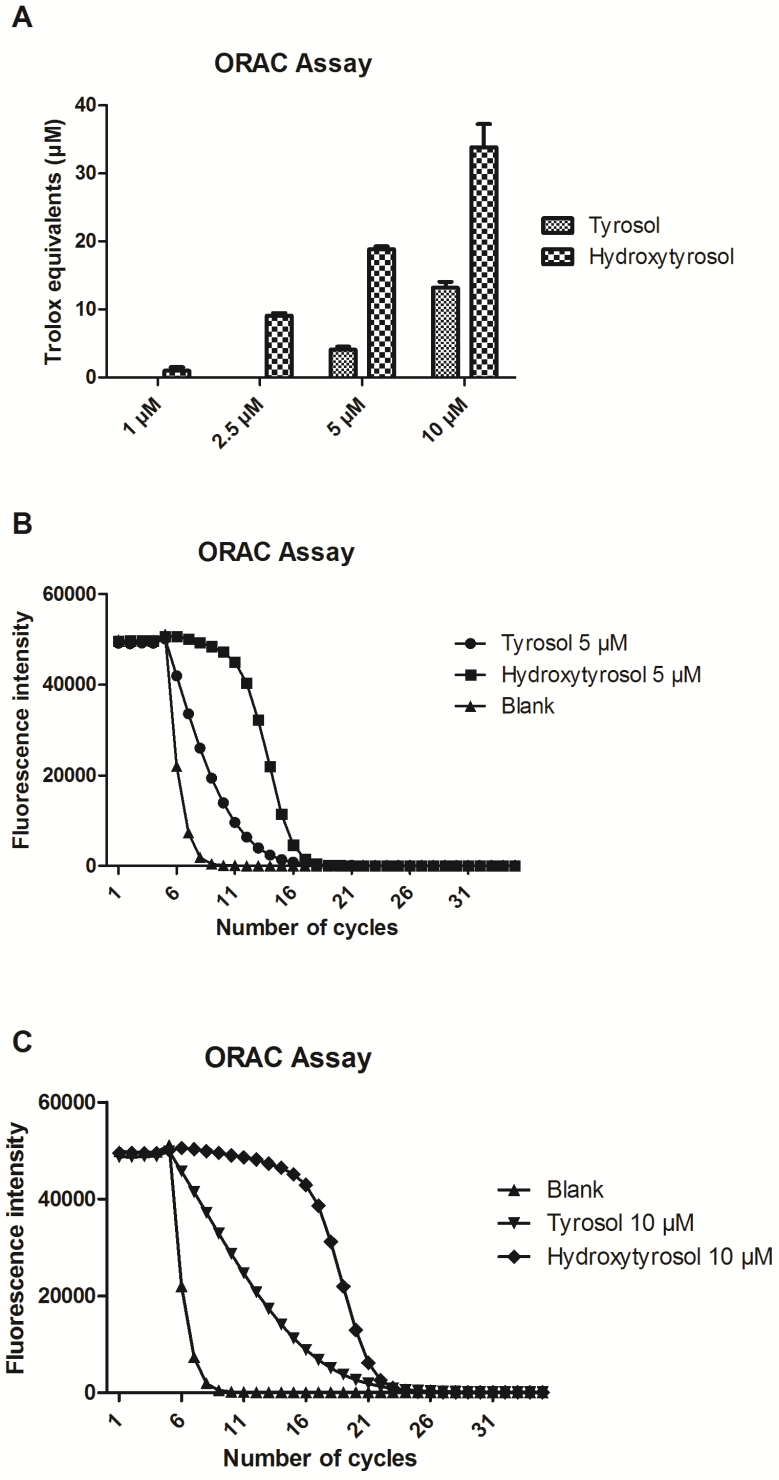


***Figure 17**. **(a)** Comparison of tyrosol and hydroxytyrosol ORAC values at different concentrations (1.0, 2.5, 5.0 and 10.0 µM). **(b)** Fluorescence decay curve induced by AAPH in absence and presence of tyrosol and hydroxytyrosol (5 µM). **(c)** Fluorescence decay curve induced by AAPH in absence and presence of tyrosol and hydroxytyrosol (10 µM). All data are expressed as µmol Trolox equivalents/L. All analyses were run in triplicate and expressed as mean ± SD.*

2. Experiments in HepG2 cells

2.1. Aim of the study

The aim of this second part of the *Chapter 1* was to evaluate the effect of tyrosol and hydroxytyrosol on preventing oxidant-induced damage in HepG2 cells.

2.2. Materials and methods

2.2.1. Materials

Methanol (MeOH), dimethylsulfoxide (DMSO), trypan blue dye and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). HepG2 cells, trypsin, phosphate-buffered saline (PBS, pH = 7.4), Eagle's Minimum Essential Medium (EMEM), Dulbecco's Modified Eagle's Medium (DMEM), Hank's Balanced Salt Solution (HBSS), and fetal bovine serum (FBS) were obtained from ATCC (Manassas, VA, USA). 5-(and-6)-chloromethyl-acetate acetyl ester (CM-H_2DCFDA), fluorescein isothiocyanate (FITC) annexin V/Dead Cell apoptosis Kit with FITC annexin V and propidium iodide (PI), were purchased from Molecular Probes, Inc. (Eugene, OR, USA).

2.2.2. Cell culture and treatments

HepG2 cells were grown in EMEM supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin and 100 µg/mL streptomycin in a humidified incubator containing 5% CO_2 and 95% air at 37ºC. Cells were subcultured every 6-7 days at 1:6 split ratio. Medium was changed every 2 days. Assays were carried out in FBS-free medium in order to avoid interference of serum factors on the results of the assays. Stock cells were routinely frozen and stored in liquid nitrogen. Cells were treated with

different concentrations of tyrosol and hydroxytyrosol (1-25 µM) diluted in serum-free culture medium or HBSS.

2.2.3. MTT

HepG2 cells were plated into 24-well plates at $5x10^5$ cells/well and the plate was incubated for 24 hours to assure attachment. After that, the media was aspirated off and replaced with new EMEM media (without FBS) containing tyrosol or hydroxytyrosol at different concentrations (0, 1, 5 and 25 µM). The plates were incubated at 37 ºC, 5% CO_2 for 24 hours. After incubation with compounds, MTT diluted with HBSS at final concentration of 0.75 mg/mL was added to each well. After 2.5 hours of incubation, MTT-containing medium was removed gently and the formazan precipitate was dissolved in 400 µL of DMSO. The plates were read on a plate reader at 570 nm. Each sample was assayed in triplicate. This assay is based on the conversion of MTT into formazan crystals by living cells, which determines mitochondrial activity. Total mitochondrial activity is related to the number of viable cells (**Figure 18**).

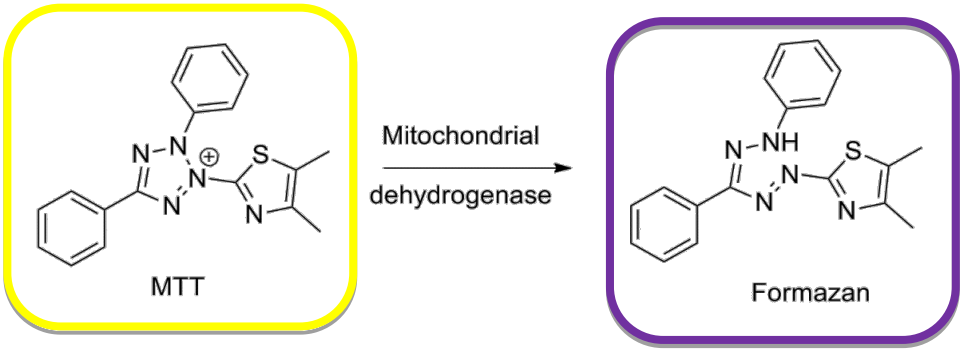


Figure 18. *Chemical reaction of the transformation of MTT into formazan.*

2.2.4. Quantification of reactive oxygen species

Reactive oxygen species were quantified by the dichlorofluorescein assay using the probe CM-H_2DCFDA. This compound passively diffuses into cells, where its acetate groups are enzymatically hydrolyzed by intracellular esterases to a non-fluorescent molecule. Subsequent oxidation by intracellular ROS yields a highly fluorescent product that is trapped inside the cell (See **Figure 19**).

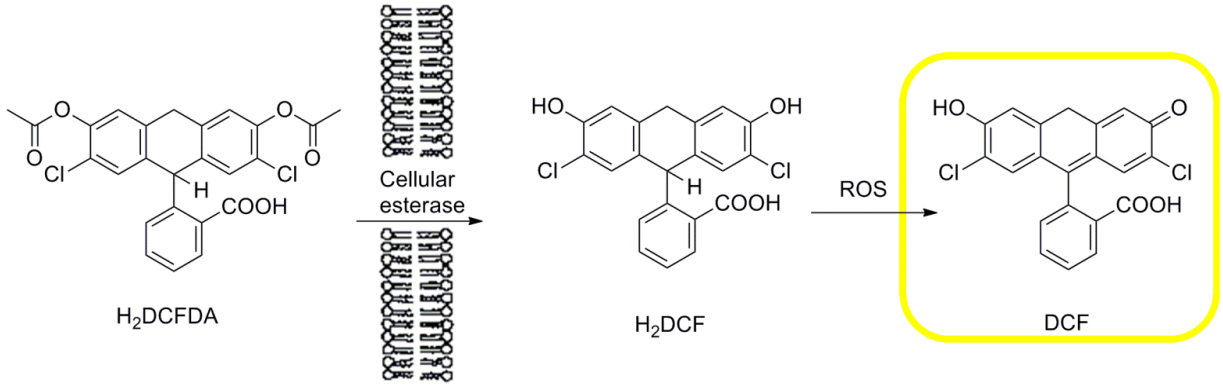


Figure 19. *Schematic representation of the ROS assay. H_2DCFDA is hydrolyzed by intracellular esterases to a non-fluorescent molecule (H_2DCF) which is further oxidized by ROS to a high fluorescent molecule (DCF).*

Therefore, the intensity of fluorescence detected reflects level of ROS formed. HepG2 cells were plated into 96-well plates at $5x10^4$ cells/well and the plate was incubated for 24 hours to assure attachment. After that, the media was aspirated off, and incubated with probe at a final concentration of 10 µM. After incubation for 30 min at 37ºC in darkness, the media was aspirated off, washed with PBS, and the cells were treated with the corresponding condition in HBSS (negative control) or an HBSS solution containing the different treatments. ROS were generated by the addition of high amounts of glucose (50 mM) (Model A), AAPH at a concentration of 50 µM (Model B) or H_2O_2 at 500 µM (Model C). Fluorescence was measured at the corresponding time

points at 37 ºC on a FLUOstar OPTIMA plate reader. The fluorescence filters used had an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Results were obtained from sextuplicate samples and are expressed as fluorescence units.

2.2.5. Determination of cell apoptosis and necrosis

HepG2 cells were plated into 12-well plates at $6x10^5$ cells/well and the plate was incubated for 24 hours to assure attachment. After that, the media was aspirated off and replaced with new EMEM media (without FBS) containing tyrosol or hydroxytyrosol at 0 or 5 μM in either normal glucose (5.5 mM) or higher glucose (50 mM) medium (model A) or EMEM media containing H_2O_2 250 μM (model B). The plates were then incubated at 37 ºC, 5% CO_2 for 24 hours (model B) or 72 hours (model A). Medium containing the respective treatment was replaced every 24 hours. At each corresponding time point, the cells were harvested, rinsed twice with ice-cold PBS, re-suspended in a binding buffer, stained with both Annexin V-FITC and PI, and incubated for 15 minutes in the dark. Cells were analyzed within 1 hour by the Accuri's C6 Flow Cytometer® System (BD Biosciences, San Jose, CA). A total number of 10,000 events were counted for each sample. Results were obtained from triplicate samples.

2.2.6. Data and statistical analyses

One-way ANOVA was performed to assess the differences between treatments within the same time points, followed by the Tukey's HSD multiple comparison test. In the case of apoptosis and necrosis studies, Student's T-test was performed. $P<0.05$ was considered statistically significant. Graph representation and statistical analyses were performed using GraphPad Prism (GraphPad Software, CA, USA, version 5.03 for Windows).

2.3. Results

2.3.1. MTT

HepG2 cells treatment with tyrosol and hydroxytyrosol (1.0, 5.0 and 25.0 μM) for 24 hours did not impair cell viability, as assessed by MTT test (**Figure 20**).

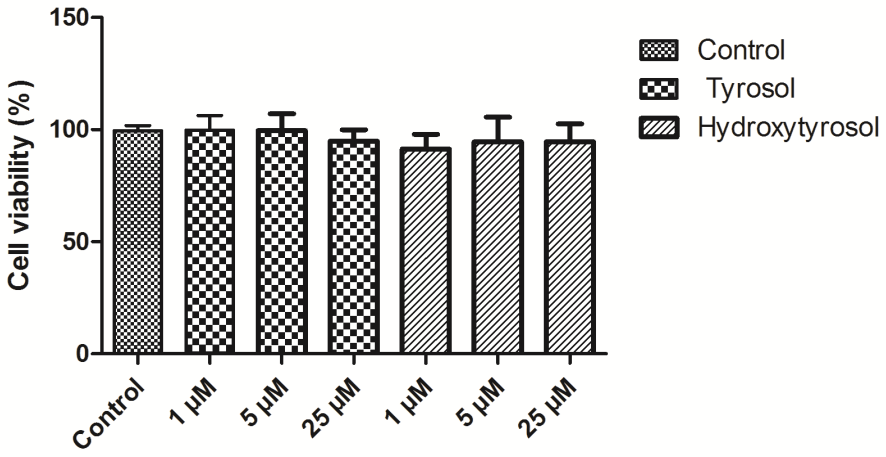


Figure 20. *Cytotoxicity of tyrosol and hydroxytyrosol on confluent HepG2 cells. Confluent HepG2 cells were treated with tyrosol and hydroxytyrosol (1.0, 5.0 and 25.0 μM) for 24 hours and cell viability was then determined using the MTT assay. Values represent mean ± SD (n=3).*

2.3.2. Reactive Oxygen Species

2.3.2.1. High glucose model

The formation of hydrogen peroxide, hydroxyl radicals, and peroxynitrite was assessed using CM-H_2DCFDA and measuring fluorescence at different time-points from 0.5 to 24 hours. Initial experiments were carried out with a high glucose-induced model. Although a dose-dependent decrease in fluorescence was observed when cells were treated with tyrosol and hydroxytyrosol, no differences between high glucose and low glucose were observed (**Figure 21**).

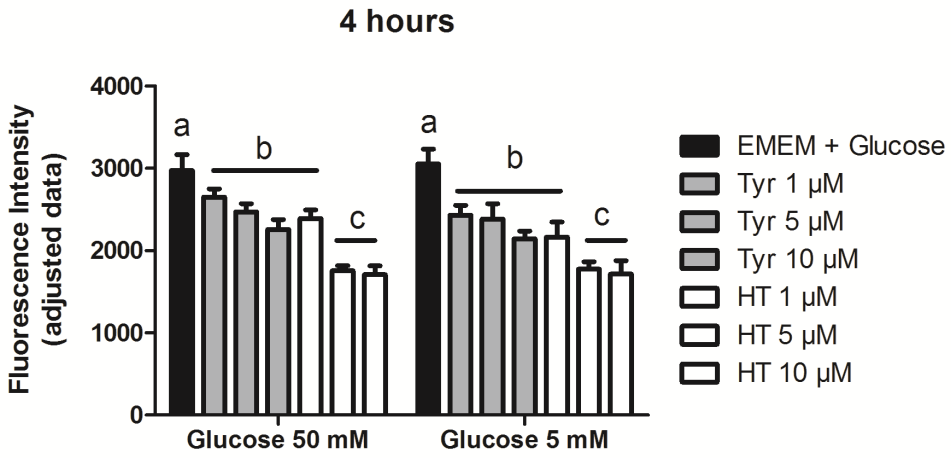


***Figure 21**. Effects of tyrosol (Tyr) and hydroxytyrosol (HT) (1.0, 5.0 and 10.0 µM) on ROS levels in either high glucose media (50 mM) or normal glucose media (5.5 mM). ROS production was estimated fluorometrically using a plate reader. Values represent mean ± SD (n=6).*

2.3.2.2. AAPH and H_2O_2 models

Alternatively, two different models using AAPH and H_2O_2 were evaluated. The treatment of HepG2 cells with AAPH (50 µM) resulted in a time-dependent increase of ROS production. This generation of reactive oxygen species was slightly increased by tyrosol (5 µM) but was significantly reduced by hydroxytyrosol (5 µM) (**Figure 22A**; Note: In order to simplify the data, only a few time points have been selected as representative examples). In a similar manner, when the cells were treated with H_2O_2 500 µM, an increase in ROS production was observed. This increase was not affected by the co-treatment with tyrosol (5 µM) but was significantly reduced by hydroxytyrosol (5 µM) (**Figure 22B**). The pattern remained the same for 10 µM tyrosol and hydroxytyrosol.

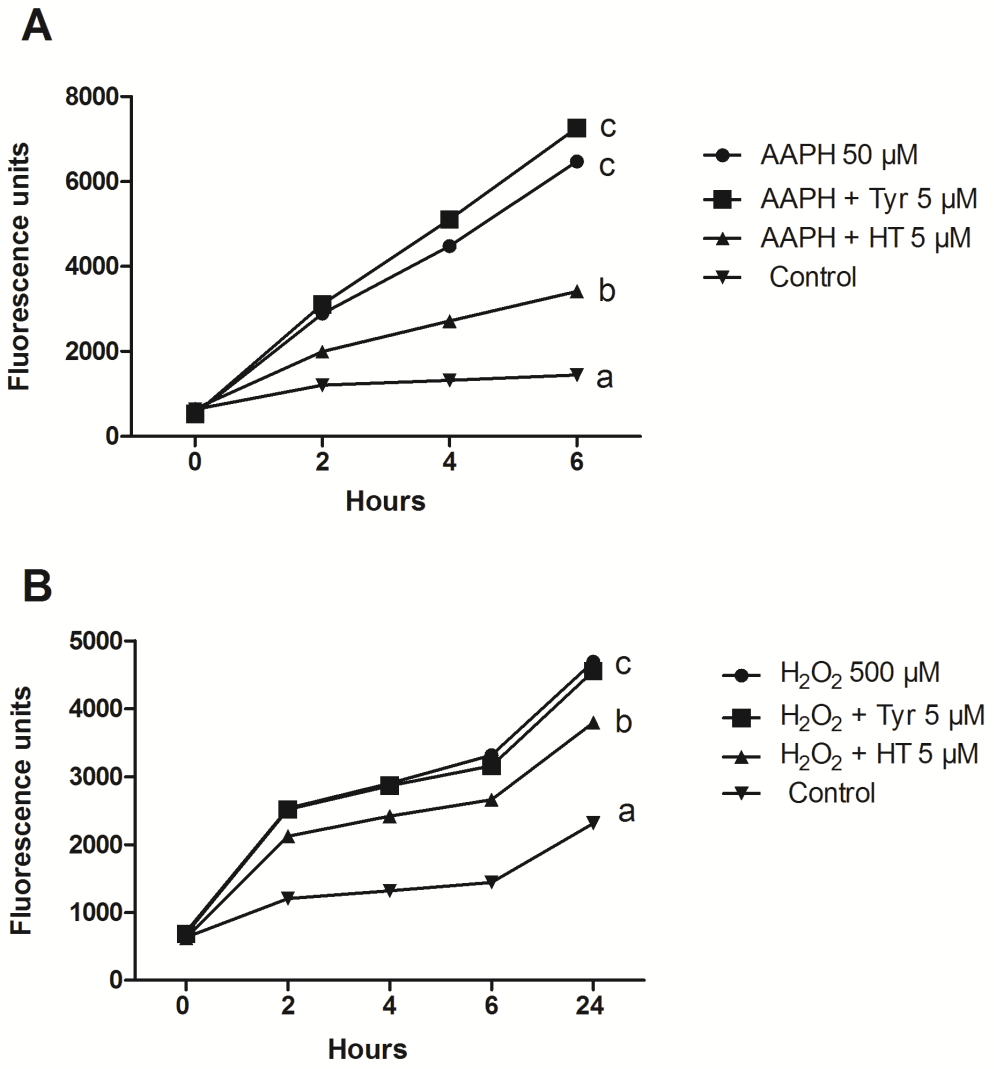


Figure 22. *Reactive oxygen species in HepG2 cells was induced by either AAPH (50 µM)* ***(A)*** *or H_2O_2 (500 µM)* ***(B)****. The effect of a cotreatment with tyrosol or hydroxytyrosol (5.0 µM) on ROS formation was evaluated. ROS production was estimated fluorometrically using a plate reader and is expressed as corrected DCF fluorescent intensity (Value – Blank). Values represent mean from sextuplicate samples.*

2.3.3. Cell apoptosis and necrosis

The percentage of necrotic cells slightly increased after 72 hours treatment with high glucose (50 mM), compared to control (5.5 mM). These differences disappeared after the treatment with tyrosol and hydroxytyrosol (5 µM) (**Figure 23A**).

When cells were treated with H_2O_2 (250 µM) for 24 hours, an increase in apoptotic cells was observed. The treatment with tyrosol and hydroxytyrosol were able to diminish the H_2O_2-induced apoptosis, hydroxytyrosol being more protective than tyrosol (**Figure 23B**).

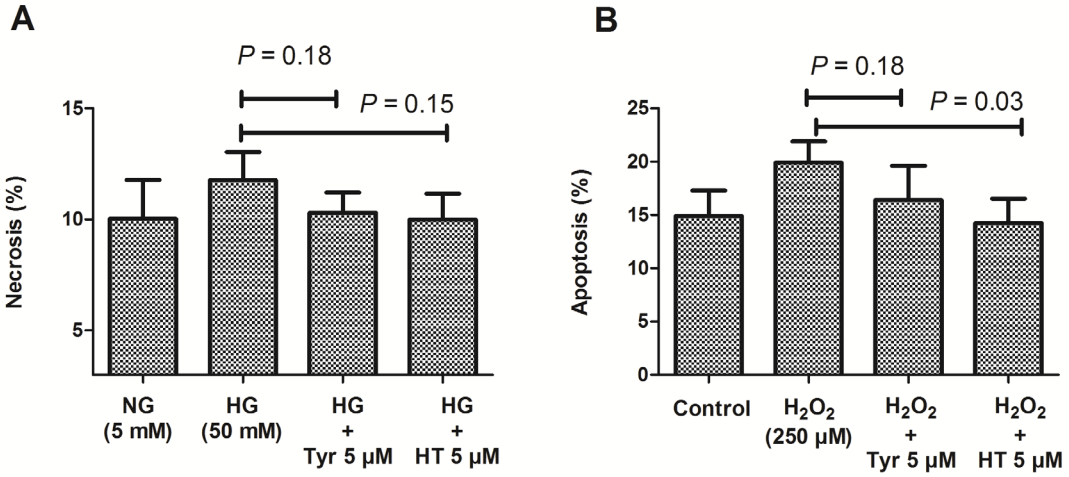


Figure 23. (A) *The treatment of HepG2 cells with high glucose (50 mM) during 72 hours caused a slight increase in dead cells. This effect was reverted by the treatment with tyrosol or hydroxytyrosol (5 µM).* ***(B)*** *The treatment of HepG2 cells with H_2O_2 (250 µM) for 24 hours resulted in an increase in apoptotic cells. This effect was diminished by tyrosol and completely reverted by hydroxytyrosol (5 µM). NG: Normal Glucose, HG: High Glucose; Tyr: Tyrosol; HT: Hydroxytyrosol.*

3. Discussion

In the present *Chapter*, we evaluated the *in vitro* bioactions of tyrosol and hydroxytyrosol by employing two different antioxidant capacity assays (FRAP and ORAC) and a HepG2 cell model. The results of the antioxidant capacity assays show that both compounds present antioxidant capacities, but hydroxytyrosol displays a higher antioxidant capacity than tyrosol. These results are in agreement with the predictable values taking into account additional hydroxyl group present in the phenol ring of hydroxytyrosol structure.

Additionally, the results from the MTT assay in human hepatocytes show that neither tyrosol nor hydroxytyrosol is toxic to HepG2 when tested at concentrations ranging from 1 to 25 μM, which are compatible with the physiological levels that can be achieved after the consumption of foods containing these phenolic compounds.

Moreover, a higher activity of hydroxytyrosol (in comparison with tyrosol) has also been observed in HepG2 cells treated with different oxidative stress models (AAPH and H_2O_2) and evaluated using different assays (ROS, apoptosis, and necrosis).

Overall, these results show that both tyrosol and hydroxytyrosol present interesting *in vitro* bioactions, being hydroxytyrosol more active than tyrosol. Further research is needed in order to confirm if these *in vitro* activities can be translated into *in vivo* beneficial health effects.

CHAPTER 2

Effect of Wine Compounds on Hydroxytyrosol Disposition in Humans:

Impact on Dopamine and Tyramine Metabolism

1. Introduction

As already mentioned in the introduction, in a previous clinical trial of our research group it was observed that urinary hydroxytyrosol excretion after consumption of 250 mL of red wine was 1.4-fold higher in comparison with consumption of 25 mL of virgin olive oil, despite the hydroxytyrosol content being ~5-times lower (0.35 versus 1.70 mg). Moreover, urinary recovery of hydroxytyrosol following red wine administration was higher than the amount of this compound present in the wine itself, suggesting an endogenous hydroxytyrosol production (De la Torre et al., 2006).

The **objective** of this chapter was to **elucidate the contribution of wine phenolic compounds and ethanol (either alone or in combination) to the endogenous production of hydroxytyrosol.** To do so, hydroxytyrosol metabolic disposition was evaluated in the urine of healthy subjects that had received four different treatments: water, vodka, dealcoholized wine and red wine.

These treatments were chosen taking into account that ethanol can generate hydroxytyrosol via dopamine metabolism and that phenolic compounds could potentially be precursors of hydroxytyrosol. It is noteworthy that both wines (dealcoholized wine and red wine) had a similar content of phenolic compounds, whereas the two alcoholic beverages (vodka and wine) had the same ethanol content. Moreover, the doses of ethanol in alcoholic beverages tested were in the range of those reported as beneficial for human health.

The primary outcomes were urinary recoveries of total hydroxytyrosol and tyrosol (which were analyzed by LC-MS/MS) as well as dopamine and tyramine biosynthesis intermediates and metabolites (whose analysis was performed using GC/MS). A general diagram of dopamine and tyramine biosynthesis and metabolism is depicted in **Figure 24.**

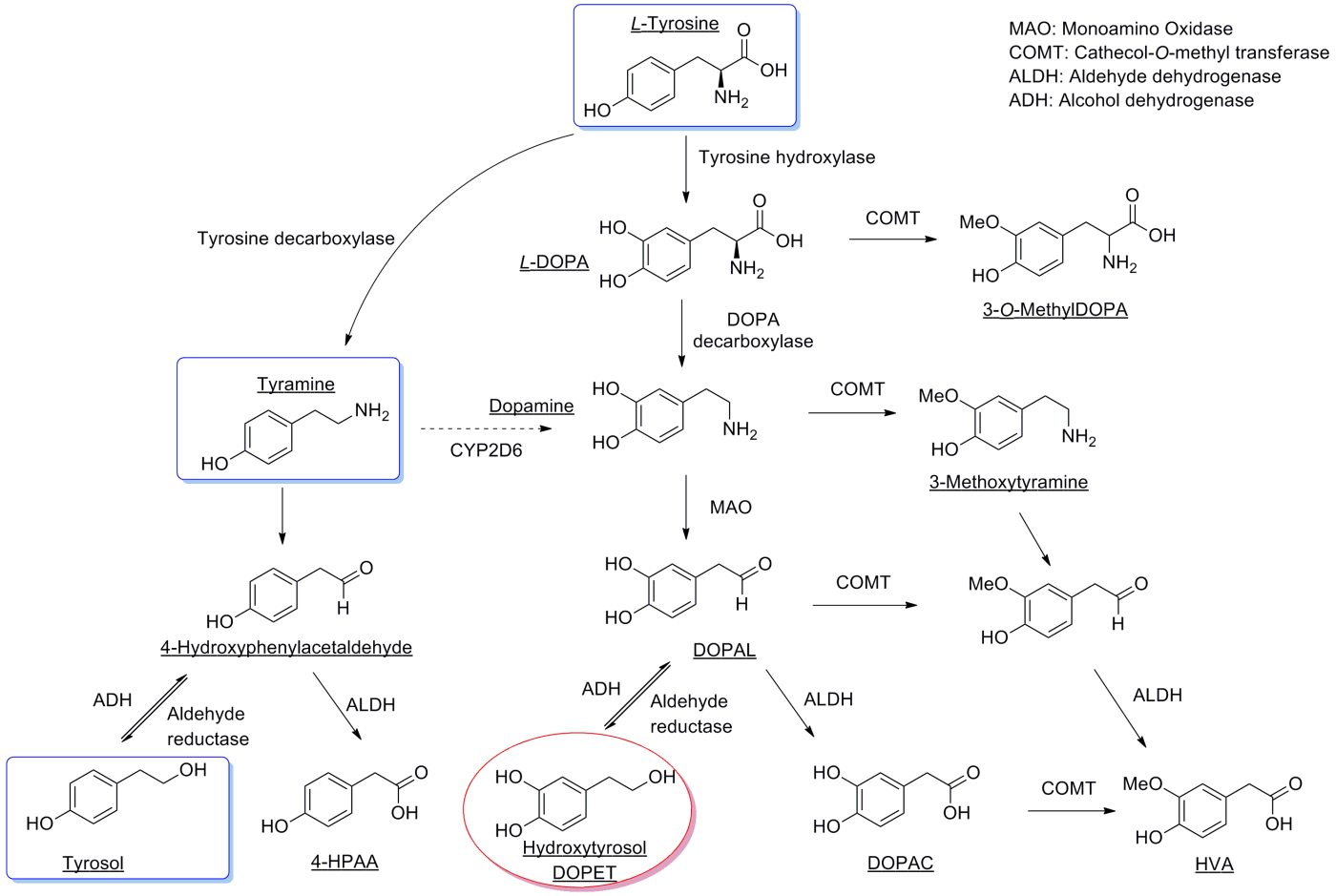


Figure 24. *General diagram of dopamine and tyramine biosynthesis and metabolism.* *DOPA: 3,4-dihydroxyphenylalanine; DOPAL: 3,4-dihydroxyphenylacetaldehyde; 4-HPAA: 4-hydroxyphenylacetic acid; DOPAC: 3,4-dihydroxyphenylacetic acid; HVA: homovanillic acid; DOPET: 3,4-dihydroxyphenylethanol.*

2. Materials and methods

2.1. Study design

Appendix III (which is the original published article and its supplementary material (Pérez-Mañá et al., 2015b)) contains a detailed description of the following features:

- ➔ Subjects (n=28)
- ➔ Dietary control
- ➔ Preparation of dealcoholized wine and characteristics
- ➔ Sample size calculation
- ➔ Statistical analyses

The clinical trial (NCT01790672) was designed and conducted by the members of the Clinical Research Unit of Hospital del Mar Medical Research Institute (IMIM, Barcelona, Spain) under the direction and supervision of Dr. Magí Farré. The statistical analysis of the data was performed by Dra. Clara Pérez-Mañá.

The dealcoholized wine preparation and wine analysis was performed by a research group from the French National Institute for Agricultural Research (INRA).

The author contribution to this project was collaborating in the analyses of hydroxytyrosol, tyrosol and their metabolites in urine using LC-MS/MS as well as the analysis of dopamine and tyramine precursors and metabolites using GC/MS.

2.2. Analysis of hydroxytyrosol, tyrosol and their metabolites in urine

Samples were analyzed by LC-MS/MS according to a previously reported method (Khymenets et al., 2010; Kotronoulas et al., 2013) with slight modifications as described below.

2.2.1. Instrumentation

Identification and quantification analysis was performed using an Agilent 1200 series HPLC system (Agilent technologies) coupled to a triple quadrupole (6410 Triple Quad LC/MS; Agilent) mass spectrometer with an electrospray interface. Gradient chromatographic separation of hydroxytyrosol and its metabolites was performed on an Acquity UPLC® BEH C_{18} column (100 mm x 3.0 mm i.d., 1.7 µm particle size) (Waters corporation) at 40 ºC using 0.01% ammonium acetate at p*H* 5 (mobile phase A) and 100% methanol (mobile phase B). Injection volume was 10 µL. The flow rate was 0.3 mL/min and run time was 25 min. All compounds were monitored in negative ionization using the multiple reaction mode (MRM). Hydroxytyrosol, tyrosol, homovanillic alcohol and hydroxytyrosol acetate were quantified by comparing their peak area ratios with hydroxyphenylpropanol (IS1); their corresponding glucuronides with hydroxyphenylpropanol-4-*O*-glucuronide (IS2); and hydroxytyrosol-3-*O*-sulfate and hydroxytyrosol acetate sulfate were quantified using hydroxytyrosol-1-*O*-sulfate as internal standard (IS3). Additional details are summarized in **Tables 3** and **4**.

Table 3. *LC-MS/MS instrumental conditions*

HPLC gradient program			**MS Tune settings**	
Time (min)	**Solvent A (%)**	**Solvent B (%)**	**Settings**	**Value**
0.0	91.0	9.0	Capillary	4000V
6.0	91.0	9.0	Gas Temp	300 ºC
7.0	70.0	30.0	Nebulizer	40 psi
12.0	70.0	30.0	Gas Flow	10 L/min
13.0	50.0	50.0		
14.0	30.0	70.0		
16.0	30.0	70.0		
21.0	91.0	9.0		
25.0	91.0	9.0		

Table 4. *MS/MS condition settings*

Compound	Retention Time (min)	Collision Energy (eV)	Precursor ion (m/Z^-)	Product ion (m/Z^-)	Dwell (s)	Fragmentor (V)
Hydroxytyrosol acetate	16.94	5	195	59	0.05	60
Hydroxytyrosol-3-*O*-sulfate acetate	13.23	15	275	195, 80, 59	0.05	100
Hydroxytyrosol-3-*O*-sulfate	3.75	10	233	153, 123, 80	0.05	100
Hydroxytyrosol-1-*O*-sulfate	3.08	5	233	97	0.2	100
Tyrosol	11.18	10	137	106	0.05	135
Hydroxyphenylpropanol	13.71	10	151	106	0.05	135
Hydroxytyrosol	6.18	10	153	123	0.05	135
Tyrosol-4-*O*-glucuronide	2.60	20	313	137	0.05	135
Hydroxyphenylpropanol-4-*O*-glucuronide	3.82	20	327	151	0.05	135
Homovanillyl alcohol	12.37	10	167	152	0.05	135
Hydroxytyrosol-4-*O*-glucuronide	2.62	10	329	153	0.05	135
Hydroxytyrosol-3-*O*-glucuronide	2.89	10	329	153	0.05	135
Homovanillyl alcohol-4-*O*-glucuronide	3.59	10	343	167	0.05	135

2.2.2. Sample extraction procedure

Briefly, 1 mL of urine was spiked with 20 µL of a solution (10 µg/mL) containing a mixture of the internal standards (IS1, IS2 and IS3) and diluted with 1 mL of 4% H_3PO_4. They were then submitted to a solid-phase extraction (SPE) procedure (Oasis HLB© 3cc, 60-mg cartridges; Waters Corporation, Dublin, Ireland). Cartridges were conditioned with 2 mL of methanol and equilibrated with 2 mL of water. After sample loading, interferences were washed away with 2 mL of water. The compounds of interest were then eluted with 3 mL of methanol. After the evaporation of the solvent (25ºC, 10-15 psi), they were reconstituted in 100 µL of solvent A of mobile phase, ultra-centrifuged at 10,000 rpm for 3 min and analyzed by LC-MS/MS. For every batch analysis, a calibration curve (20-500 ng/mL) was performed by adding known solutions of all analytes. Final concentrations for the calibration curves were 20, 100, 200, 300 and 500 ng/mL.

2.3. Analysis of tyrosine, DOPAC, HVA and 4-HPAA in urine

Tyrosine, DOPAC, homovanillic acid (HVA) and 4-hydroxyphenylacetic acid (4-HPAA) (See **Figure 24**) were detected and quantified in urine using an extractive derivatization together with CG-MS determination according to a previously described methodology (Hušek, 1991; Namera et al., 2002; Qiu et al., 2007) as detailed below.

2.3.1. Instrumentation

Identification and quantification analysis of tyrosine, DOPAC, HVA and 4-HPAA were carried out on a GC (HP 6890 Series Plus GC system, Hewlett-Packard, Palo Alto, CA, USA) equipped with a quadrupole MS (HP 5973 mass selective detector) and autosampler (Agilent 7683 series injector) using L-tyrosine-(*phenyl*-$^{13}C_6$)

as IS. Data acquisition and analysis were performed using standard software supplied by the manufacturer (Agilent Chemstation). Samples were injected in splitless mode and analytes separation was achieved using a 5%-phenyl-95%-dimethylpolysiloxane capillary column (ZB-5MS, 15.0 m x 0.25-mm i.d., 0.25-µm film thickness, Phenomenex, Inc.). The oven temperature was programmed at 100 ºC, followed by a 15 ºC/min rate to 255 ºC. Then, it was increased from 25ºC/min rate to 300ºC and held for 3 minutes. The injector and the interface were operated at 280ºC. Helium was used as carrier gas at a flow rate of 1.4 mL/min. The chromatographic separation of all analytes was achieved in 15.13 min. The injection volume was 1.0 µL, and a split ratio (10:1) was used.

The mass spectrometer (MS) was operated in electron-impact ionization mode at 70 eV. The electron-impact mass spectra of the analytes and IS were firstly recorded in scan mode (scan range 50-650 *m/z*) to determine retention times and characteristic mass fragments. For routine analysis, three characteristic mass fragments were monitored in the selected-ion-monitoring (SIM) mode. The retention times as well as the ions selected for substance identification and quantification are shown in **Table 5**.

Table 5. *Retention times and m/z ions selected for substances identification and quantification. (4-HPAA: 4-hydroxyphenylacetic acid, HVA: homovanillic acid, DOPAC: 3,4-dihydroxyphenylacetic acid).*

Compound	Retention time (min)	Target ion and qualifiers *(m/z)**		
4-HPAA	6.19	107	135	**180**
HVA	7.35	137	**210**	282
DOPAC	8.84	151	**196**	224
Tyrosine	9.89	107	**192**	264
L-tyrosine-(*phenyl*-$^{13}C_6$)	9.89	113	**198**	270

* Ions selected for quantification are in bold face.

2.3.2. Sample analysis

Briefly, 600 µL of human urine were added to an amber screw-top glass tube. After adding 50 µL of L-tyrosine-(*phenyl*-$^{13}C_6$), 400 µL of anhydrous ethanol and 100 µL of pyridine, the first derivatization was performed by adding 50 µL of ethyl chloroformate. The pooled mixtures were sonicated at 40 kHz and 20 ºC for 60 s. After having adjusted the aqueous layer p*H* to 9.0-10.0 using 100 µL of NaOH 7N, the extraction was performed using 300 µL of chloroform. The derivatization procedure was repeated adding 50 µL of ethyl chloroformate. The previous mixture was vortexed for 10 s and centrifuged for 3 min at 3000 rpm. The organic layer was then carefully transferred to a new glass tube, dried with anhydrous sodium sulfate, centrifuged for 3 min at 10,000 rpm and subsequently subjected to GC/MS analysis. For every batch analysis a calibration curve (0.4-12.5 µg/mL) was performed. A 1/10 dilution of the samples was necessary in order to determine 4-HPAA concentrations.

3. Results

A detailed description of the whole clinical trial and the corresponding results can be found in the original published article and its corresponding Supplementary Material (*Appendix III*). In the following subheadings there is a summary of the findings that are more relevant to understand mechanisms responsible for the endogenous hydroxytyrosol formation following red wine.

3.1. Doses of phenolic compounds administered with wines

The doses of phenolic compounds administered with wines are summarized in **Table 6.** The phenolic content of both beverages was very similar, with the only exception of tyrosine, which was present in higher amount in wine, compared to

dealcoholized wine. It is noteworthy that, in both beverages, the content of tyrosol was much higher (22-25 µmol) than the content of hydroxytyrosol (around 1 µmol).

Table 6. *Doses of hydroxytyrosol and three phenolic compounds (tyrosol, tyrosine and tyramine) that were administered with dealcoholized wine and wine treatments in the clinical trial.*

Condition	Dose mg (µmol)			
	Hydroxytyrosol mg (µmol)	Tyrosol mg (µmol)	Tyrosine mg (µmol)	Tyramine mg (µmol)
Dealcoholized wine	0.2 (1.1)	3.5 (25.1)	0.3 (1.5)	0.5 (3.9)
Wine 13º (15g)	0.2 (1.0)	3.0 (22.0)	0.6 (3.2)	0.4 (3.1)

3.2. Total hydroxytyrosol and tyrosol excretion

The excretion of total hydroxytyrosol from 0 to 6 hours after each intervention is depicted in **Figure 25**.

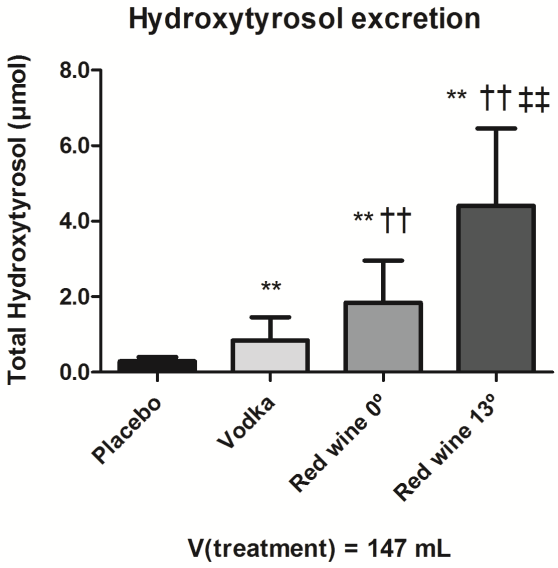


Figure 25. *Total hydroxytyrosol urinary excretion from 0 to 6 hours after beverages (n=28). Data are expressed as mean ± SD. *p < 0.05, **p < 0.01 versus placebo; †p < 0.05, ††p < 0.01 versus vodka; ‡p < 0.05, ‡‡p < 0.01 versus dealcoholized wine.*

Total hydroxytyrosol was calculated as the sum of hydroxytyrosol-3-*O*-glucuronide, hydroxytyrosol-4-*O*-glucuronide, hydroxytyrosol-3-*O*-sulfate, free hydroxytyrosol, and the hydroxytyrosol methylated metabolite homovanillyl alcohol (HVAlc free and as HVAlc-4-*O*-glucuronide). After the administration of the different alcoholic beverages and dealcoholized wine, hydroxytyrosol was mainly excreted conjugated with sulfate. Total hydroxytyrosol excretion after wine was higher than that after all treatments (P<0.01). Total hydroxytyrosol excretion after dealcoholized wine was higher than vodka and placebo, and that of vodka higher than placebo (P<0.01) (**Figure 25**).

The excretion of total tyrosol from 0 to 6 hours after each intervention is depicted in **Figure 26**.

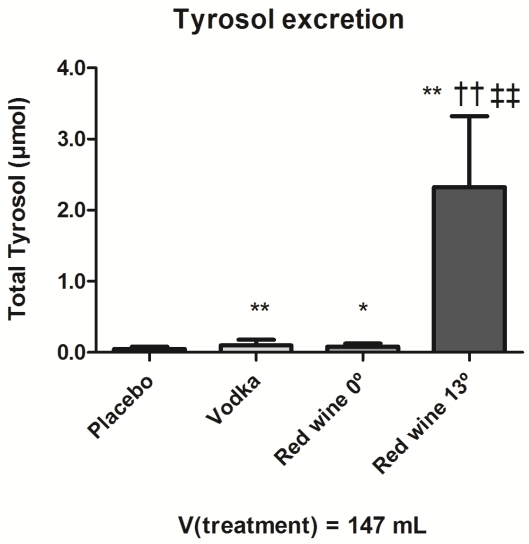


Figure 26. *Total tyrosol excretion from 0 to 6 hours after beverages (n=28). Data are expressed as mean ± SD. *p < 0.05, **p < 0.01 versus placebo; †p < 0.05, ††p < 0.01 versus vodka; ‡p < 0.05, ‡‡p < 0.01 versus dealcoholized wine.*

Total tyrosol in urine was calculated as the sum of free tyrosol and tyrosol-4-*O*-glucuronide. Total tyrosol excretion after wine ingestion was from around 24 to 46-fold

higher (P<0.01) than with the other treatments. Total tyrosol after vodka and dealcoholized wine was around 2-fold and 1.6-fold of that after placebo (p<0.01 and p<0.05 respectively).

3.3. Total hydroxytyrosol and tyrosol recoveries

Total hydroxytyrosol recoveries from 0 to 6 hours were 162% and 420% after dealcoholized wine and wine, respectively (considering the dose of hydroxytyrosol administered) (**Figure 27**).

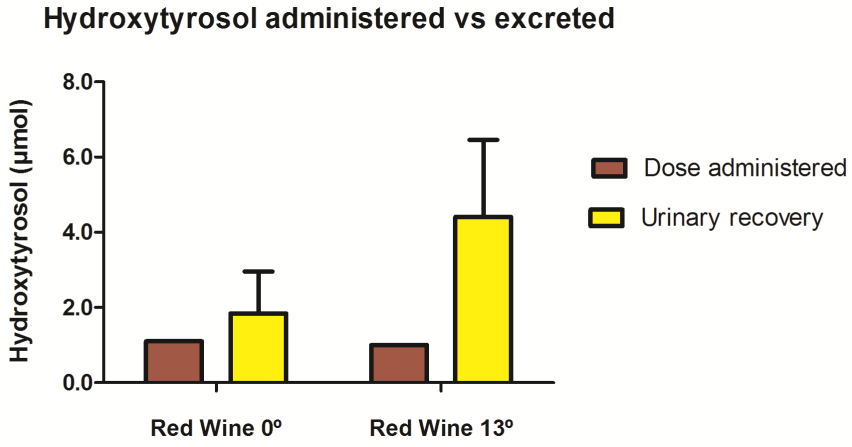


Figure 27. *Relationship between the dose of hydroxytyrosol administered and the amount of total hydroxytyrosol recovered in urine from 0 to 6 hours after treatments.*

Total tyrosol recoveries were 0.3% and 10.6% after dealcoholized wine and wine respectively (considering the dose of tyrosol administered). These low recoveries for phenolic compounds are in agreement with the results obtained from other clinical studies of our research group (e.g. recoveries after the intake of 50 mL of virgin olive oil for total hydroxytyrosol and tyrosol were 16.4% and 9.1%, respectively, considering the doses administered).

3.4. Changes in dopamine and tyramine metabolism

Figure 28 shows DOPAC, HVA, 4-HPAA excretion from 0-6h after treatments**.**

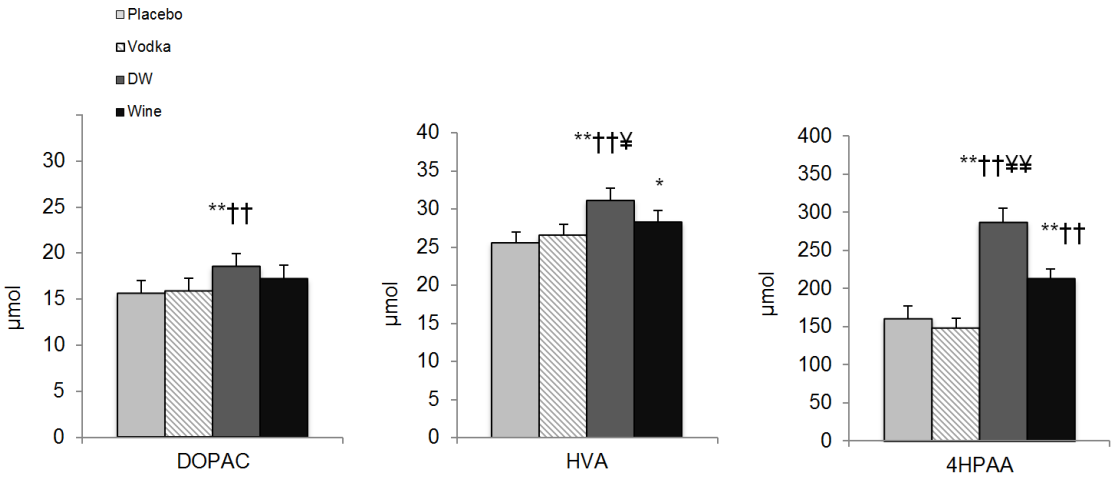


Figure 28. *Urinary excretion 0-6 h of 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 4-hydroxyphenylacetic acid (4-HPAA) after beverages (n=28). Data are expressed as mean ± SEM.* * *P<0.05,* ** *P<0.01 versus placebo;* † *P<0.05,* ††*P<0.01 versus vodka;* $^{¥}$ *P<0.05,* $^{¥¥}$ *P<0.01 versus wine. DW: Dealcoholized wine.*

The highest DOPAC levels were observed after dealcoholized wine in comparison to placebo and vodka. In a similar way, HVA levels (a DOPAC metabolite), were higher in dealcoholized versus the other treatments. 4-HPAA values were significantly higher in wine and dealcoholized wine versus placebo and vodka. Values after dealcoholized wine, however, were significantly higher than those of wine. No significant differences were observed in tyrosine excretion among treatments. The interpretation of all these results is discussed below.

4. Discussion

In this chapter, the contribution of wine phenolic compounds and ethanol (either alone or in combination) to the endogenous production of hydroxytyrosol was evaluated. To do so, hydroxytyrosol metabolic disposition was evaluated in the urine of 28 healthy subjects that had received four different treatments: water, vodka, dealcoholized wine and red wine. The two alcoholic beverages (vodka and wine) had the same ethanol content, and both wines (dealcoholized wine and wine) also had a similar content of phenolic compounds.

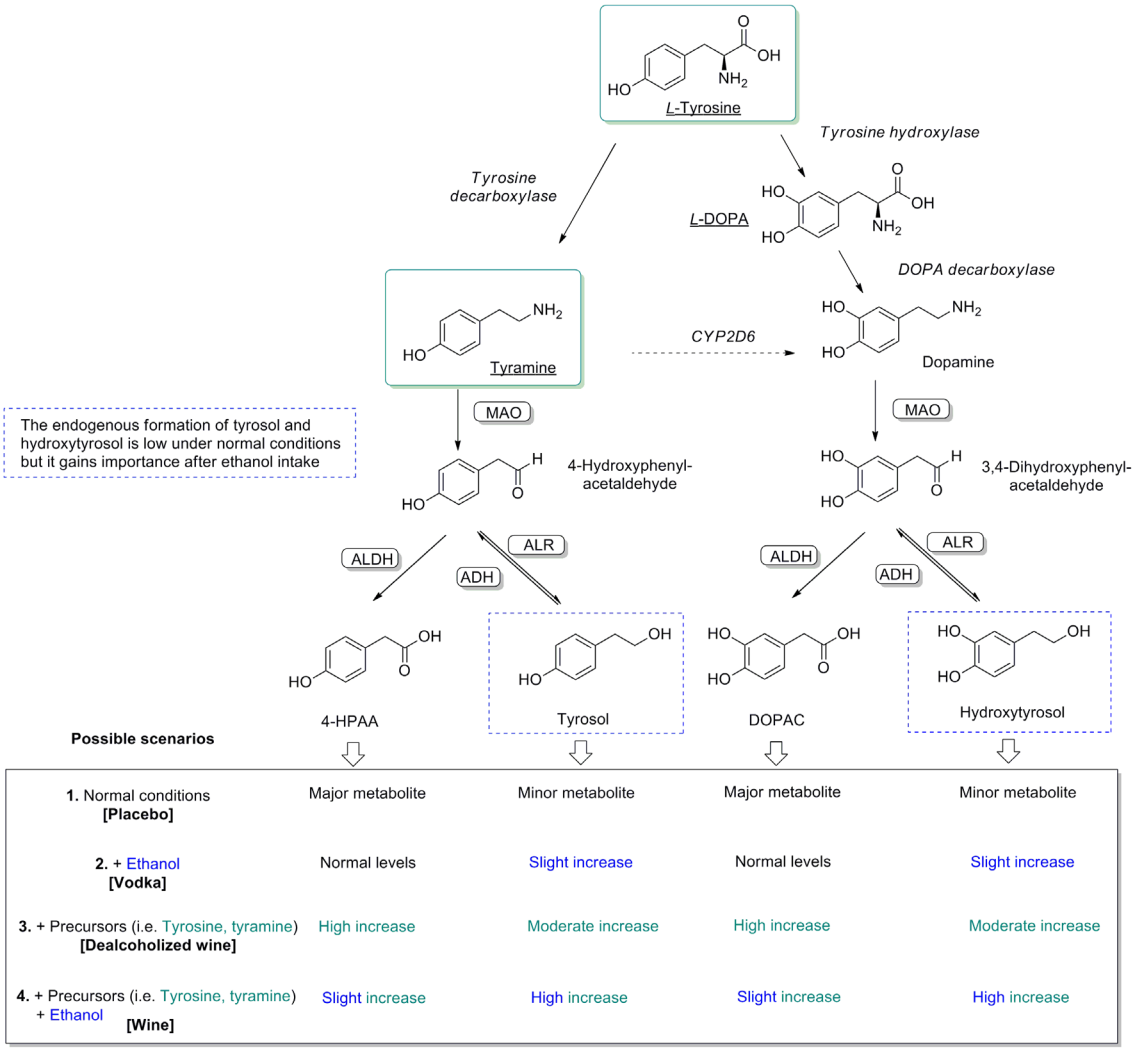
The rate of total hydroxytyrosol excretion was wine > dealcoholized wine > vodka > placebo ($p<0.01$). The highest total tyrosol excretion was after wine ingestion and the lowest after placebo. Recoveries for hydroxytyrosol from dealcoholized wine and wine were higher than expected and enhanced by the ethanol present in wine. Total hydroxytyrosol recoveries (regarding the dose of hydroxytyrosol administered) from 0 to 6 h were 161 and 420% after dealcoholized wine and wine, respectively. Total tyrosol recoveries were in all cases below 11%.

In order to delve into the mechanisms responsible for the generation of hydroxytyrosol, dopamine and tyramine biosynthesis intermediates and metabolites were analyzed. The changes in dopamine and tyramine metabolism observed supported a *de novo* endogenous hydroxytyrosol formation. The highest DOPAC levels were observed after dealcoholized wine ($p<0.01$). This increase could be attributed to the ingestion of tyrosine and tyramine present in the dealcoholized wine. In agreement with this, HVA, a metabolite of DOPAC, was higher in dealcoholized wine versus the other treatments. The fact that after wine there was not the same increase in DOPAC that after dealcoholized wine could be attributed to the ethanol effects shifting the dopamine metabolism to a reductive pathway, with an increase in hydroxytyrosol versus DOPAC,

as has previously observed in experimental studies with rats. In that study, the DOPAC/DOPET ratio produced in liver slices was close to 10 in the absence of ethanol, while it was 0.25 in the presence of ethanol (Tank and Weiner, 1979). In agreement with these data, in our *in vivo* study in humans, the DOPAC/DOPET ratio after vodka and wine was around 60 and 92% lower, respectively, compared with values after placebo. The ratio DOPAC/DOPET decreased in all treatments versus placebo ($p<0.01$). The rate of values was from placebo (65.9 ± 7.8)>Vodka (25.8 ± 4.5)>dealcoholized wine (13.7 ± 2.1)>Wine (5.2 ± 0.80).

These results were compatible with a *de novo* hydroxytyrosol generation via tyramine and tyrosine present in dealcoholized wine and wine, as well as the above referred shift in dopamine metabolism due to the presence of ethanol in vodka and wine.

4-HPAA values were significantly higher in wine and dealcoholized wine versus placebo and vodka ($P<0.01$). This increase could also be attributed to the ingestion of tyrosine and tyramine present in wines. Values after dealcoholized wine, however, were significantly higher than those of wine ($P<0.01$). This fact is compatible with a shift in tyramine metabolism from oxidative to a reductive pathway, due to the presence of ethanol in wine, thus enhancing tyrosol formation versus that of 4-HPAA, as has been described in animal models (Tacker et al., 1970). A general summary of the compatible mechanisms that would explain all these reactions is depicted in **Figure 29**.



Possible scenarios	4-HPAA	Tyrosol	DOPAC	Hydroxytyrosol
1. Normal conditions **[Placebo]**	Major metabolite	Minor metabolite	Major metabolite	Minor metabolite
2. + Ethanol **[Vodka]**	Normal levels	Slight increase	Normal levels	Slight increase
3. + Precursors (i.e. Tyrosine, tyramine) **[Dealcoholized wine]**	High increase	Moderate increase	High increase	Moderate increase
4. + Precursors (i.e. Tyrosine, tyramine) + Ethanol **[Wine]**	Slight increase	High increase	Slight increase	High increase

Figure 29. *Contribution of wine phenolic compounds and ethanol (either alone or in combination) to the endogenous production of hydroxytyrosol and tyrosol via dopamine and tyramine metabolism, respectively. The effects of ethanol are indicated in blue, whereas the effects related to wine phenolic compounds appear in green.*

CHAPTER 3

Evaluation of Tyrosine, Tyrosol and Tyramine as Potential Precursors of Hydroxytyrosol

1. Introduction

The results of the previous chapter evidenced that ethanol (administered as vodka), dealcoholized wine, and particularly wine promoted a *de novo* hydroxytyrosol generation *in vivo* in humans. The increase on hydroxytyrosol urinary excretion following vodka intake could be explained by the already described alteration in dopamine oxidative metabolism. However, the higher hydroxytyrosol urinary recoveries following dealcoholized wine and red wine ingestion could not be explained neither by this mechanism, nor by the amount of hydroxytyrosol present in wine (as in both cases the recovery exceeded the dose administered). Consequently, at least one additional endogenous mechanism capable of generating hydroxytyrosol remained to be elucidated. As the vodka and the wine administered in the study had the same ethanol content, the higher levels of hydroxytyrosol following wine could hypothetically be explained by the phenolic compounds present in wine and absent in vodka. As the dealcoholized wine and the wine administered in the study had the same phenolic content, the higher levels of hydroxytyrosol following wine could be explained by an ethanol-mediated effect.

The **objective** of this chapter was to assess the **contribution of wine phenolic compounds** alone or in combination with **ethanol** to **hydroxytyrosol formation**. In order to identify additional mechanisms responsible for hydroxytyrosol formation, three wine phenolic compounds were selected as potential precursors of hydroxytyrosol: tyrosine, tyrosol and tyramine. Additionally, and in order to evaluate the role of ethanol, the three selected compounds were administered with and without ethanol to rats, and hydroxytyrosol urinary recovery was assessed.

2. Materials and methods

2.1. Animal studies

Ninety-six male Wistar rats (Charles River) were used in the experiments. Rats were housed two per cage in a temperature (22 ± 2ºC) and humidity (55 ± 15%) controlled room with a 12-h light/dark cycle (lights on at 08:00h). The experiments were performed during the light period. Food and water were given *ad libitum* in the home cages, but only water was available in the metabolic cages (Harvard Apparatus, 48 cm x 28 cm x 36 cm) during the four hours of the experiment. Each cage was provided with a support grid for the animals, separate urine and feces collection funnels, and a drinking tube. One day prior to the experimental session, rats were habituated to the metabolic cages for two hours. The rats were assigned to fourteen different groups (n = 6-7/group) as shown in **Table 7**. They were first injected with either ethanol (1 g/kg as a 30% v/v in saline) or vehicle (saline). Thirty minutes later, rats received 10 or 20 mg/kg of either tyrosine, tyrosol, tyramine (prepared in saline), or vehicle (i.p.), and were immediately placed in the cages during 4 h. Urine produced during this period was collected. At the end of the experiment, rats were euthanized under isofluorane anesthesia. Urine samples were weighted, identified, preserved with HCl 6N (20 µl/mL urine) and stored at -20ºC until analysis. Animal procedures were in accordance with the guidelines of the European Communities Directive 86/609/EEC regulating animal research, and approved by the local ethical committee (CEEA-PRBB; ref. PRM-13-1525).

Table 7

Design of the different groups (n=6-7) in which the 96 rats were distributed.

	First administration	Second administration
Group 1	Ethanol 1 g/kg	Vehicle
Group 2	Ethanol 1 g/kg	Tyrosine 10 mg/kg
Group 3	Ethanol 1 g/kg	Tyrosine 20 mg/kg
Group 4	Ethanol 1 g/kg	Tyrosol 10 mg/kg
Group 5	Ethanol 1 g/kg	Tyrosol 20 mg/kg
Group 6	Ethanol 1 g/kg	Tyramine 10 mg/kg
Group 7	Ethanol 1 g/kg	Tyramine 20 mg/kg
Group 8	Vehicle	Vehicle
Group 9	Vehicle	Tyrosine 10 mg/kg
Group 10	Vehicle	Tyrosine 20 mg/kg
Group 11	Vehicle	Tyrosol 10 mg/kg
Group 12	Vehicle	Tyrosol 20 mg/kg
Group 13	Vehicle	Tyramine 10 mg/kg
Group 14	Vehicle	Tyramine 20 mg/kg

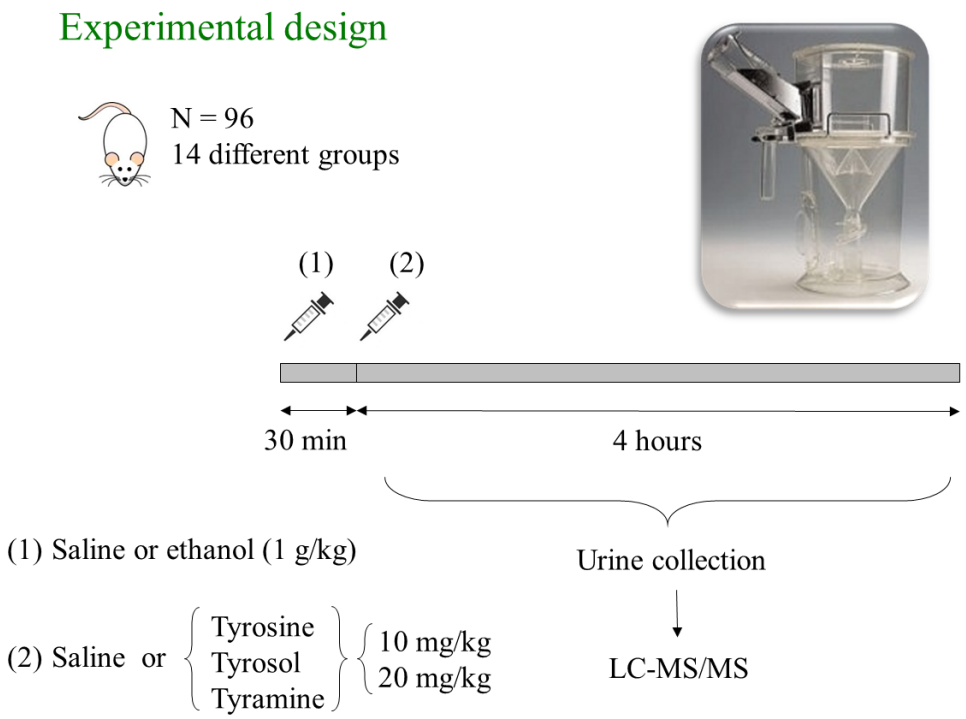


Figure 30. *Schematic representation of the experimental design.*

2.2. Analysis of hydroxytyrosol, tyrosol and their metabolites in urine

Phenolic compounds quantification was performed as described in *Chapter 2, subheading 2.2.2.* with the only modification that 200 µL of rat urine were diluted with 800 µL of human hydroxytyrosol-free urine.

3. Results

3.1. Quantification of total hydroxytyrosol in urine

Total hydroxytyrosol was calculated as already explained in *Chapter 2*. Total hydroxytyrosol urinary recoveries after the different treatments are depicted in **Figure 31**.

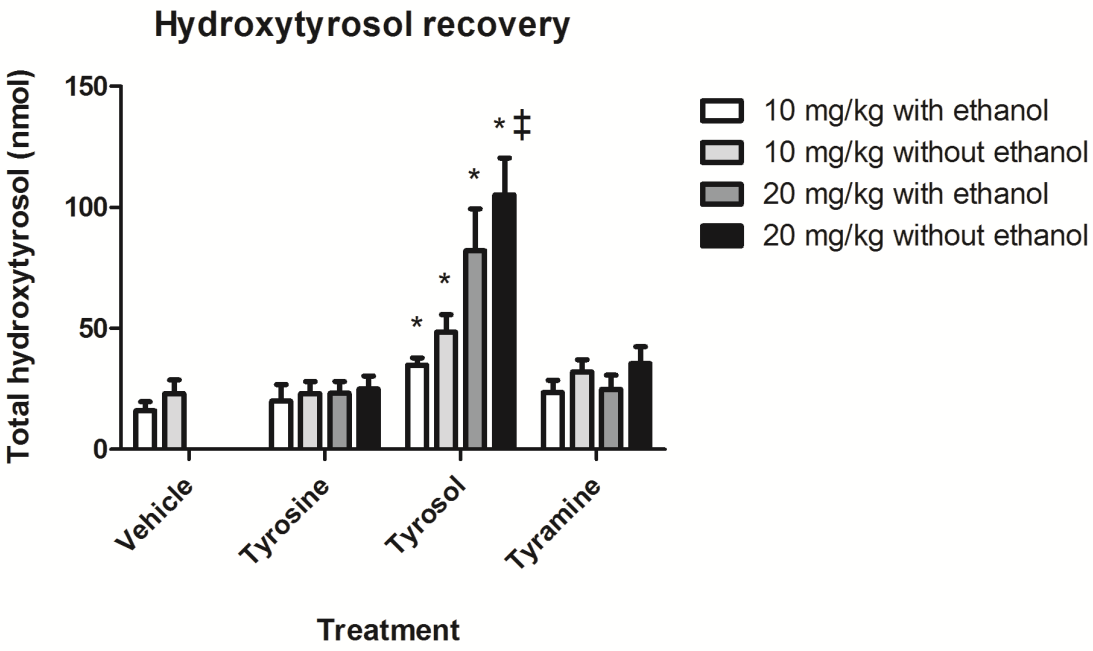


Figure 31. *Urinary recoveries (nmol, 0-4 h) for total hydroxytyrosol following administration of tyrosine, tyrosol, tyramine (10 and 20 mg/kg), and vehicle, with and without ethanol pretreatment (1 g/kg). Data are expressed as mean ± SEM. *p>0.005 versus vehicle; ‡p<0.005 versus 10 mg/kg.*

Ethanol by itself did not significantly increase hydroxytyrosol excretion. No differences were found after tyrosine and tyramine administration, with or without having administered ethanol previously. However, following tyrosol administration, a dose-dependent increase in hydroxytyrosol was found. This increase differed (although not significantly) in presence or absence of ethanol, being higher when no ethanol had been administered. Hydroxytyrosol was mainly present in urine as hydroxytyrosol-3-*O*-sulfate.

3.2. Quantification of total tyrosol in urine

Total tyrosol urinary recoveries (calculated as described in *Chapter 2*) after treatments are depicted in **Figure 32**.

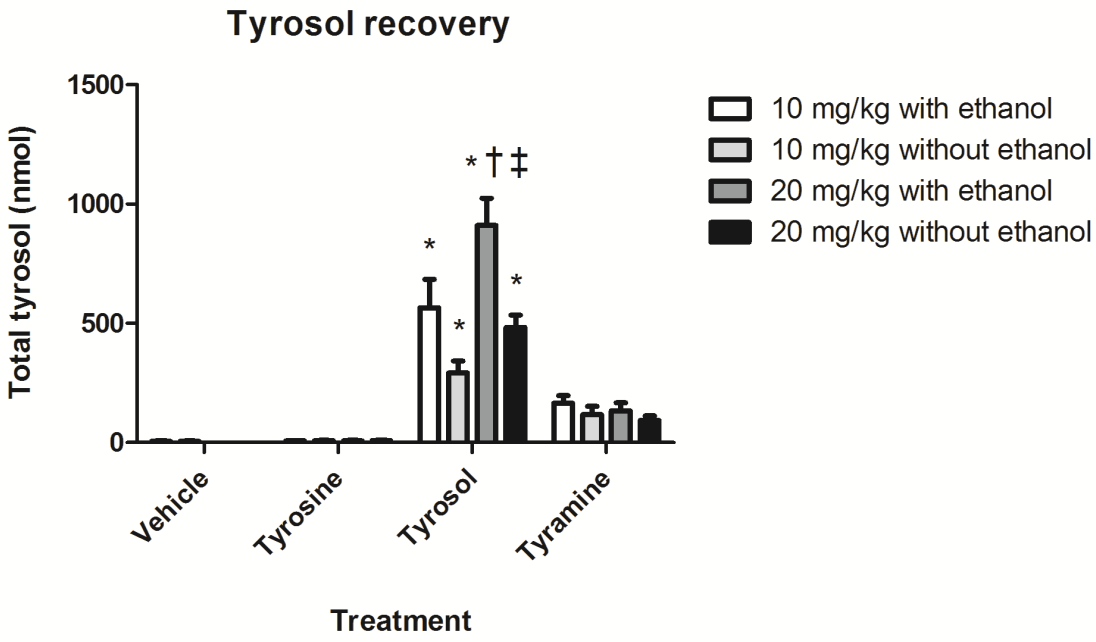


Figure 32. *Urinary recoveries (nmol, 0-4 h) for total tyrosol following administration of tyrosine, tyrosol, tyramine (10 and 20 mg/kg), and vehicle, with and without ethanol pretreatment (1 g/kg). Data are expressed as mean ± SEM. *p<0.005 versus vehicle; †p<0.005 versus same group without ethanol, ‡p<0.005 versus 10 mg/kg.*

The main results observed are:

- The administration of tyrosine had no effect on tyrosol urinary levels.
- The administration of tyramine resulted in an increased urinary excretion of tyrosol, although significance was not achieved. It is worth bearing in mind that tyrosol had already been described as a metabolite of tyramine (Tacker et al., 1970).
- The administration of tyrosol resulted in a remarkable dose-dependent increase of total tyrosol urinary recovery. Tyrosol urinary levels were higher when this compound was administered with ethanol versus saline ($p<0.005$), supporting the fact that tyrosol bioavailability could be enhanced by ethanol.

4. Discussion

In spite of having evidenced in the ethanol-induced alteration of dopamine and tyramine metabolism in humans *(Chapter 2)*, the origin from the abnormally high hydroxytyrosol urinary recoveries following dealcoholized wine (161%) and wine (420%) remained unsolved. In this chapter, we hypothesized that wine phenolic compounds could be endogenously transformed into hydroxytyrosol. Consequently, we assessed the contribution of wine phenolic compounds to urinary hydroxytyrosol. Three wine phenolic compounds were selected as potential precursors of hydroxytyrosol: tyrosine, tyrosol and tyramine. Additionally, and in order to evaluate the role of ethanol, the three selected compounds were administered with and without ethanol to rats, and hydroxytyrosol urinary recovery was assessed.

The major finding from the study in rats described in this chapter was that **tyrosol was converted *in vivo* to hydroxytyrosol in a dose-dependent manner**. Moreover, tyrosol urinary excretion was augmented by ethanol ($p<0.005$), supporting the fact that tyrosol bioavailability is enhanced by ethanol. Another observation from this study was that tyramine increased urinary excretion of tyrosol. This fact is in agreement with the fact that tyramine after oxidative deamination (via MAO) and reduction (via ALR) gives rise to tyrosol.

CHAPTER 4

Evaluation of Tyrosine Hydroxylase as the Potential Enzyme Catalyzing the Conversion of Tyrosol to Hydroxytyrosol

1. Introduction

The major findings of the rat study described in the previous chapter were (1) that tyrosol was converted *in vivo* to hydroxytyrosol, and (2) that tyrosol urinary excretion was augmented by ethanol. Despite having identified tyrosol as the substrate for hydroxytyrosol production, the enzyme responsible for this biotransformation remained unknown. The present chapter and the following one describe the elucidation of the pathways involved in the endogenous conversion of tyrosol to hydroxytyrosol.

The **objective** of this chapter was to **evaluate if tyrosine hydroxylase was the enzyme responsible for the conversion of tyrosol to hydroxytyrosol**. Tyrosine hydroxylase is the rate-limiting enzyme in catecholamine biosynthesis. This enzyme catalyzes the aromatic hydroxylation that converts *L*-tyrosine to *L*-DOPA (Nagatsu et al., 1964). Taking into account that the structural difference between tyrosol and hydroxytyrosol is an aromatic hydroxylation, we hypothesized that tyrosine hydroxylase could be involved in the conversion of tyrosol to hydroxytyrosol. To evaluate the candidacy of this enzyme, we treated Wistar rats with the tyrosine hydroxylase inhibitor α-methyl-*L*-tyrosine (αMT). Then, we evaluated the effects of a combined administration of ethanol and tyrosol on the urinary recovery of both tyrosol and hydroxytyrosol.

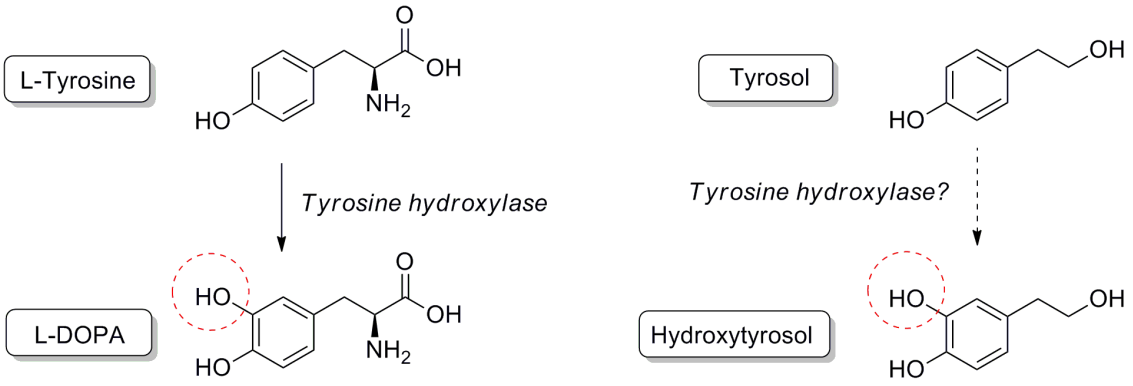


Figure 33. *The hypothesis of this chapter was that tyrosine hydroxylase catalyzes the hydroxylation of tyrosol in the same way as it catalyzes the hydroxylation of L-tyrosine.*

2. Materials and methods

2.1. Animal studies

Seventy-two male Wistar rats (Charles River) weighing 140-200 g were used in the experiments. Rats were housed two per cage in a temperature (22 ± 2 ºC) and humidity (55 ± 15%) controlled room with a 12-h light/dark cycle (lights on at 08:00 h). The experiments were performed during the light period. Food and water were given *ad libitum* in the home cages, but only water was available in the metabolic cages (Harvard Apparatus, 48 cm x 28 cm x 36 cm) during the four hours of the experiment. Each cage was provided with a support grid for the animals, separate urine and feces collection funnels, and a drinking tube. On the day prior to the experimental session, rats were habituated to the metabolic cages for two hours. The rats were assigned to twelve treatments with n=6/group (See **Table 8**).

They were first injected with either αMT (50 mg/kg) or saline, and thirty min. later rats received either 0.5 g/kg ethanol (30% ethanol in saline, v/v) or vehicle (saline). One hour later, rats were injected with 10 or 20 mg/kg of tyrosol (prepared in saline) or vehicle. All treatments were administered intraperitoneally (i.p.) Following the last administration, rats were immediately placed in the cages for 4 h. Urine produced during this period was collected. At the end of the experiment, rats were euthanized under isoflurane anesthesia. Urine samples were weighted and preserved with 6N HCl (20 µL/mL urine) and stored at -20ºC until analysis. Animal procedures were approved by the local ethical committee (CEEA-PRBB; ref. PRM-13-1525) and performed in accordance with the guidelines of the European Communities Directive 86/609/EEC regulating animal research.

Table 8

Design of the different groups (n=6) in which the 72 rats were distributed.

	First administration	Second administration	Third administration
Group 15	αMT 50 mg/kg	Ethanol 0.5 g/kg	Vehicle
Group 16	αMT 50 mg/kg	Ethanol 0.5 g/kg	Tyrosol 10 mg/kg
Group 17	αMT 50 mg/kg	Ethanol 0.5 g/kg	Tyrosol 20 mg/kg
Group 18	αMT 50 mg/kg	Vehicle	Vehicle
Group 19	αMT 50 mg/kg	Vehicle	Tyrosol 10 mg/kg
Group 20	αMT 50 mg/kg	Vehicle	Tyrosol 20 mg/kg
Group 21	Vehicle	Ethanol 0.5 g/kg	Vehicle
Group 22	Vehicle	Ethanol 0.5 g/kg	Tyrosol 10 mg/kg
Group 23	Vehicle	Ethanol 0.5 g/kg	Tyrosol 20 mg/kg
Group 24	Vehicle	Vehicle	Vehicle
Group 25	Vehicle	Vehicle	Tyrosol 10 mg/kg
Group 26	Vehicle	Vehicle	Tyrosol 20 mg/kg

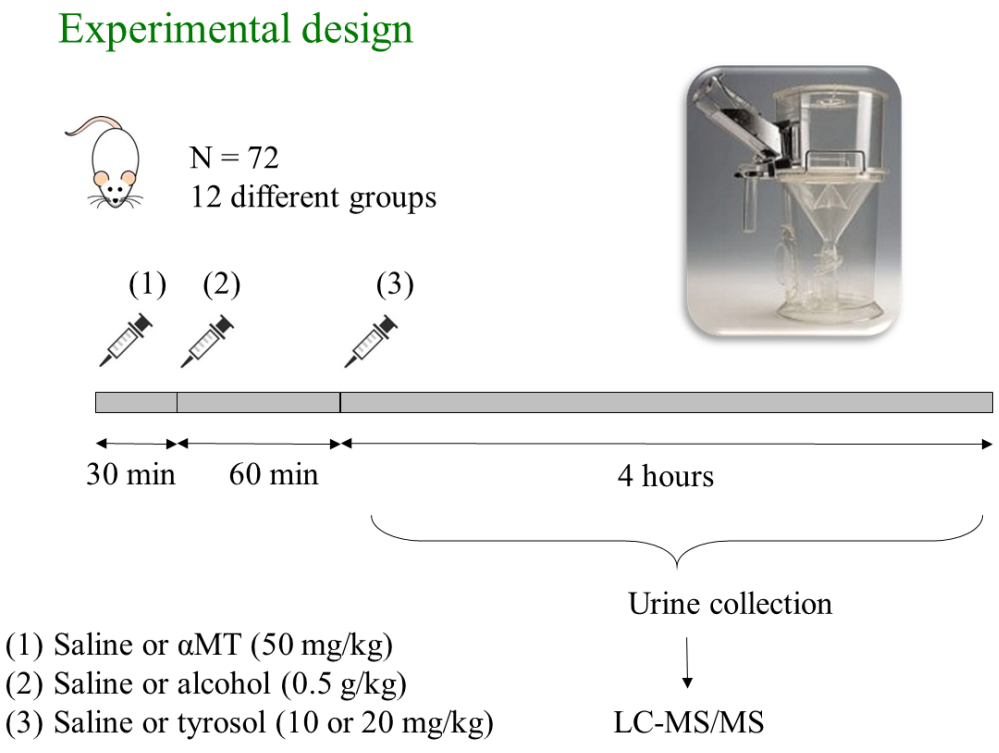


Figure 34. *Schematic representation of the experimental design.*

2.2. Analysis of hydroxytyrosol, tyrosol and their metabolites in urine

Phenolic compounds quantification was performed as described in *Chapter 2, subheading 2.2.2.* with the only modification that 200 μL of rat urine were diluted with 800 μL of human hydroxytyrosol-free urine.

2.3. Statistical analysis

Data analyses of animal studies and chemical inhibition studies were performed using a three-way ANOVA model (to evaluate effects of tyrosol, ethanol and αMT on hydroxytyrosol urinary excretion) as well as a two-way ANOVA (to evaluate the effects of tyrosol and ethanol on tyrosol urinary excretion). When significance was obtained in global analyses, *post hoc* multiple comparisons were performed with the LSD test. Statistical analyses were performed using SPSS Statistics for Windows (Version 21.0; SPSS Inc. Chicago, IL, USA). The level of statistical significance was defined as $P<0.05$. The results in rats are expressed as mean ± S.E.M.

3. Results

3.1. Quantification of total tyrosol in urine

A dose-dependent increase in total tyrosol urinary recovery was found following tyrosol administration (**Figure 35**). A two-way ANOVA revealed a main effect of tyrosol ($F_{(2,28)} = 92.2$, $p<0.001$), but no significant effect of ethanol or interaction between factors were observed, indicating that ethanol does not modify the excretion of tyrosol (**Figure 35**).

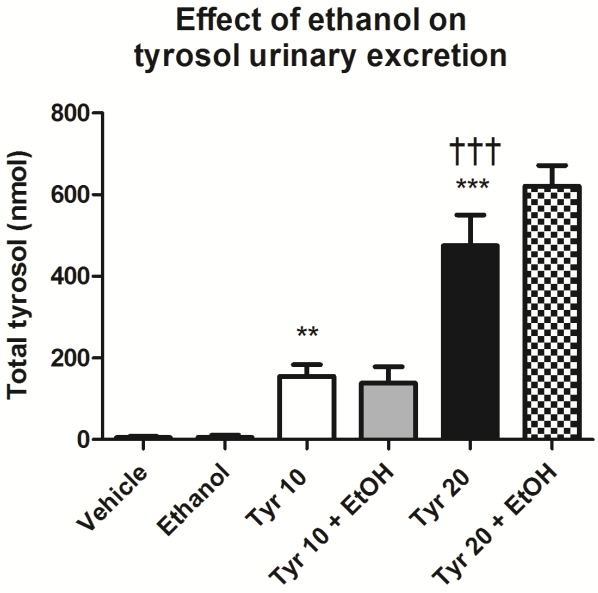


Figure 35. *Tyrosol urinary recoveries following tyrosol (Tyr) administration (10 and 20 mg/kg) with and without ethanol (0.5 g/kg). Data expressed as mean ± SEM. **P < 0.005, versus vehicle; ***P < 0.001, versus vehicle; ††† P < 0.05, versus Tyr 10 mg/kg.*

3.2. Quantification of total hydroxytyrosol in urine

A three-way ANOVA evaluating the effect of tyrosol, ethanol and αMT on total hydroxytyrosol urinary recovery showed a main effect of tyrosol ($F_{(2,42)}$ = 23.7, p<0.001), a main effect of ethanol ($F_{(1,42)}$ = 11.1, p<0.001) and no effect of αMT. No interactions between factors were observed. However, the results on **Figure 36** show that tyrosol increased total urinary hydroxytyrosol recovery in a dose-dependent fashion, while αMT did not significantly inhibit this effect at the dose studied. Ethanol slightly inhibited the conversion of tyrosol into hydroxytyrosol although this inhibition did not reach statistical significance. The combination of αMT and ethanol also decreased the formation of hydroxytyrosol, but this effect was mainly due to the inhibitory effect of ethanol (**Figure 36**).

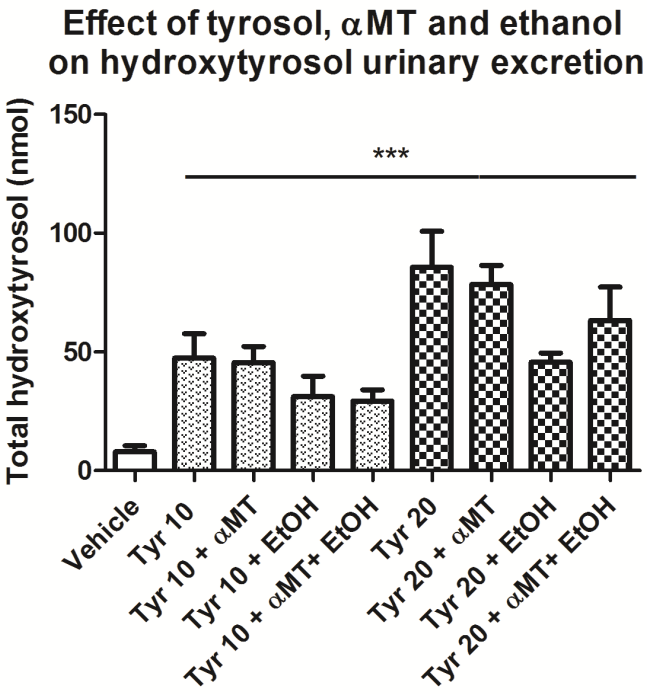


Figure 36. *Hydroxytyrosol urinary excretion following the administration of tyrosol (Tyr; 10 and 20 mg/kg), α-methyl-L-tyrosine (αMT; 50 mg/kg) and ethanol (EtOH; 0.5 g/kg). Data expressed as mean ± SEM. **P<0.005, versus vehicle; ***P<0.001, versus vehicle;* ††† *P<0.05, versus Tyr 10 mg/kg.*

4. Discussion

In *Chapter 3*, we identified that hydroxytyrosol was endogenously formed from tyrosol. However, the enzymes that mediated the conversion remained to be characterized. In the present *Chapter,* we hypothesized that this conversion was mediated by tyrosine hydroxylase. In order to examine the candidacy of this enzyme in the formation of hydroxytyrosol from tyrosol, we pretreated rats with αMT (a tyrosine hydroxylase inhibitor) before the administration of tyrosol. The unchanged urinary hydroxytyrosol excretion provided strong evidence that tyrosine hydroxylase was not involved in the conversion of tyrosol to hydroxytyrosol. In the same rat study, we confirmed that urinary hydroxytyrosol excretion was in parallel with the administered tyrosol doses, while 0.5 g/kg ethanol slightly (not significantly) inhibited this effect. It is noteworthy that ethanol plays a dual role: on the one hand, it alters dopamine oxidative metabolism generating small amounts of hydroxytyrosol, whereas on the other hand it appears to slightly inhibit the conversion of tyrosol to hydroxytyrosol.

CHAPTER 5

Evaluation of Cytochrome P450 as the Enzymatic Machinery Responsible for Catalyzing Tyrosol-to-Hydroxytyrosol Conversion

1. Introduction

In 1963, Julius Axelrod described for the first time a non-specific enzyme able to hydroxylate occurring and foreign monophenols to catechols and catecholamines. The enzyme was present in liver microsomes and was able to catalyze the oxidation of tyramine to dopamine (Axelrod, 1963). Thirty-five years later, Hiroi and coworkers identified cytochrome P450 (CYP) (and specifically CYP2D6) as the enzyme responsible for that reaction (Hiroi et al., 1998). After discarding the involvement of tyrosine hydroxylase in the hydroxylation of tyrosol, and inspired by the article of Julius Axelrod, we considered a new hypothesis focused on cytochrome CYP.

CYPs represent a family of isoenzymes responsible for catalyzing the oxidation of a wide variety of xenobiotic chemicals as well as various endogenous substrates. The most important regulators of drug metabolism are liver CYPs 1A2, 2C8, 2C9, 2C19, 2D6 and 3A4/5, which metabolize approximately 90% of therapeutic drugs (McGraw and Waller, 2012).

The **aim** of the present *Chapter* was to **evaluate if CYP isoenzymes regulate the *in vivo* hydroxylation of tyrosol**. The involvement of CYPs in this process was evaluated using human liver microsomes (HLM) as well as human recombinant proteins (baculosomes).

In this chapter, the *Results* and the *Discussion* will be presented under the same section in order to provide a better understanding of the interpretation of the results.

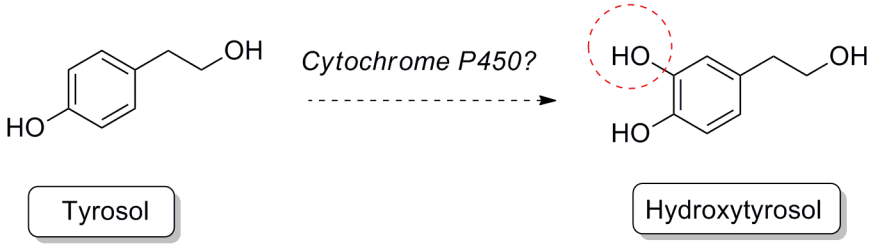


Figure 37. *The hypothesis of this chapter was that cytochrome P450 mediates the hydroxylation of tyrosol to hydroxytyrosol.*

2. Materials and Methods

Appendix IV (which is the submitted manuscript to *Food Chemistry* and its supplementary material) contains a detailed description of the following features:

- ➔ Experiments in Human Liver Microsomes
- ➔ Enzyme Kinetics Experiments
- ➔ Chemical Inhibition Analyses
- ➔ Hydroxytyrosol Formation in cDNA-Expressing CYP microsomes
- ➔ Statistical Analyses

3. Results and Discussion

3.1. Tyrosol hydroxylase activity in HLM

Hydroxytyrosol formation from tyrosol took place in human hepatic microsomes. The reaction was NADPH-dependent. Hydroxytyrosol formation was increased in a linear fashion (r^2 >0.98) with the reaction time from 0 to 60 min. This hydroxylation activity occurred in a substrate concentration-dependent manner (**Figure 38A**).

3.2. Kinetic analyses of Tyrosol *ortho*-hydroxylation in HLM

Figure 38 shows the Michaelis-Menten, Lineweaver-Burk, and Eadie-Hofstee plots for the HLM-mediated hydroxytyrosol formation from tyrosol. The values represent the mean of two separate experiments. The apparent K_m value was 709 ± 49 μM and the V_{max} value was 1294 ± 31 pmol/min/mg.

These initial *in vitro* HLM experiments confirmed at least one CYP isoform was involved in the conversion of tyrosol to hydroxytyrosol via hydroxylation with a typical Michaelis-Menten kinetic profile. This combination of high K_m and V_{max} values

indicate that, despite presenting a low specificity, tyrosol-3-hydroxylation occurs at a high velocity.

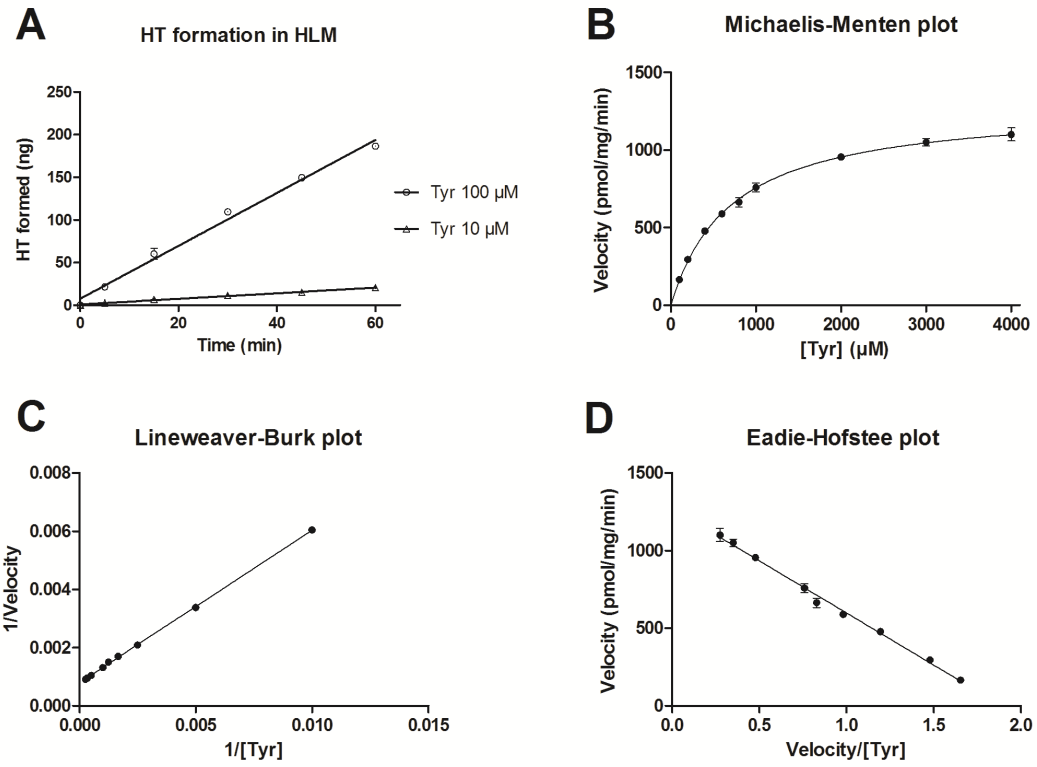


Figure 38. *Hydroxytyrosol (HT) formation from tyrosol in human liver microsomes (HLM).* ***A****, Time- and dose-dependent formation;* ***B****, Michaelis-Menten plot;* ***C****, Lineweaver-Burk plot;* ***D****, Eadie-Hofstee plot.*

3.3. Chemical inhibition studies with specific CYP inhibitors

After having demonstrated that tyrosol hydroxylation occurred in HLM, we sought to identify the specific CYP isoenzyme(s) that catalyze(s) the reaction. To do so, we used HLM and selective inhibitors. The effect of 8 CYP inhibitors on hydroxytyrosol formation from tyrosol was evaluated. Firstly, a primary screening using 8 specific inhibitors at 100 μM was performed. **Figure 39** shows the mean activities (from duplicate determinations) in the presence of these inhibitors. The major inhibitory effect was caused by coumarin (a CYP2A6 substrate), followed by quinidine (a

CYP2D6 inhibitor). The presence of coumarin reduced hydroxytyrosol formation from tyrosol (100 μM) by 60%. CYP2D6, CYP3A4, CYP2B6 and CYP2C9 selective inhibitors slightly diminished (>10% but < 20%) hydroxytyrosol formation (**Figure 39**).

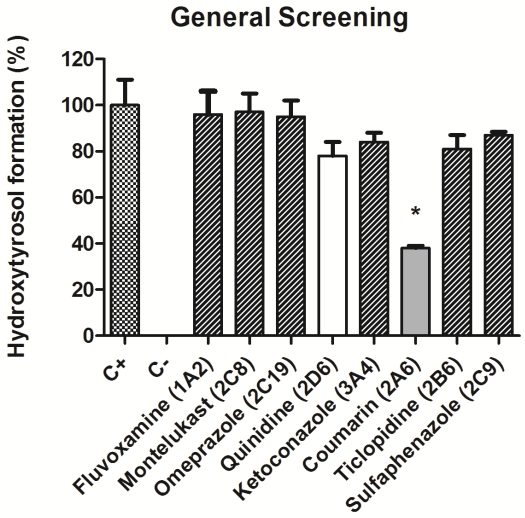


Figure 39. *Primary screening including selective inhibitors of 8 different isoenzymes*

The role of CYP2A6 was then studied in further detail. Additional incubations using nicotine (a CYP2A6 substrate), and tryptamine, methoxsalen and tranylcypromine (CYP2A6 inhibitors) (Zhang et al., 2001) were performed. Although nicotine was not found to decrease hydroxytyrosol formation, three CYP2A6 specific inhibitors reduced the biotransformation to a larger degree than coumarin (74%, 77% and 85%, respectively), with tranylcypromine being the strongest (**Figure 40, Left**). It is worth noting that the rate of coumarin metabolism by CYP2A6 is relatively fast, compared to the other substrates/inhibitors tested.

In order to delve into the potential role of CYP2D6 and to confirm the role of CYP2A6 on hydroxytyrosol formation, additional inhibitors of CYP2D6 (quinidine, fluoxetine, paroxetine) and CYP2A6 (tranylcypromine, methoxsalen) were evaluated. These inhibitors have different mechanisms of action and include mechanism-based inactivators (paroxetine, methoxsalen). Mechanism-based inactivators are defined as

substrates for a CYP enzyme that, during catalysis of the enzyme, are converted to one or more products, which immediately and irreversibly inactivate the enzyme and do not leave the active site. To explore the possibility of mechanism-based inhibition (MBI), then enzymes were pre-incubated with NADPH and microsomes for 30 min and, after the addition of tyrosol, the reactions took place for 30 additional min.

The pre-incubation with the CYP2D6 inhibitors did not lead to a >20% inhibition, whereas the CYP2A6 inhibitors surpassed 90%. These results are in agreement with the initial screening, which evidenced a major role of CYP2A6, compared to CYP2D6. Interestingly, the combination of two mechanism-based inhibitors [paroxetine (CYP2D6) and methoxsalen (CYP2A6)] inhibited hydroxytyrosol formation by 97% (**Figure 40, Right**). The inhibitory effect of methoxsalen and tranylcypromine were very similar with both procedures (normal versus MBI procedure) No inhibitory effect was observed when a deuterated analog of hydroxytyrosol (hydroxytyrosol-D_4) was added at 1, 10 and 100 μM to the reaction mixture, indicating there was no product initiated inhibition of the reaction.

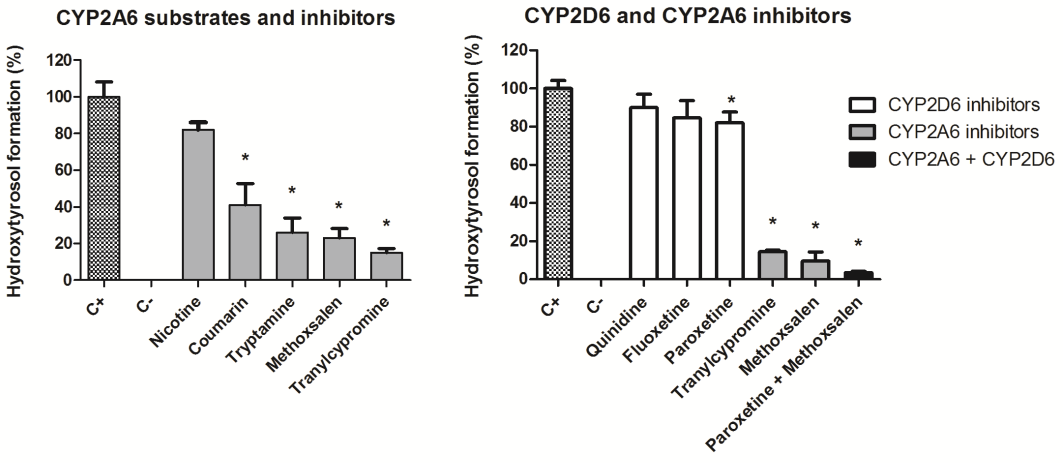


Figure 40. ***Left***, *Confirmatory incubations using nicotine as a CYP2A6 substrate and selective CYP2A6 inhibitors (tryptamine, methoxsalen and tranylcypromine);* ***Right***, *Additional experiments to evaluate the*

potential effect of different inhibitors of CYP2A6 and CYP2D6. In this case, microsomes were pre-incubated with inhibitors for 30 min.

Overall, these results from the HLM experiments using specific CYP inhibitors suggest that CYP2A6 and CYP2D6 isoforms react toward tyrosol in a cooperative manner with CYP2A6 being the major contributor and CYP2D6 the minor one.

3.4. Experiments using human recombinant CYP enzymes (Baculosomes)

On the previous subheadings, it has been described that the conversion of tyrosol to hydroxytyrosol takes place in HLM and that the conversion can be diminished by CYP2A6 and, to a lesser extent, by CYP2D6 inhibitors. Microsomes are subcellular fractions derived from the endoplasmic reticulum of liver which contain a variety of metabolic enzymes. Consequently, in addition to CYP2A6 and CYP2D6, microsomes contain all liver microsomal CYP enzymes with physiological levels of cytochrome b5 and NADPH-CYP reductase. These other enzymes can potentially contribute also to the transformation of tyrosol to hydroxytyrosol. In order to rule out this possibility, a different approach using human recombinant enzymes (baculosomes) was taken. Baculosomes are microsomes prepared from insect cells infected with recombinant baculovirus containing cDNA for a human CYP isoforms. The main advantage of using baculosomes is that they prevent interfering metabolism by other CYP isoforms. A disadvantage is that baculosomes are not expressed in cells at concentrations that reflect their levels in HLM. Consequently, an evaluation of metabolism by several recombinant human CYP enzymes only establishes that a particular CYP enzyme can metabolize a drug, but it does not provides information about the extent to which it contributes to its metabolism.

Hydroxytyrosol formation from tyrosol was evaluated in baculosomes. CYP2A6 capacity for hydroxylating tyrosol was confirmed, and hydroxytyrosol was produced in a linear fashion (r^2 >0.99) along with the amount of administered protein and concentration of tyrosol (**Figure 41**).

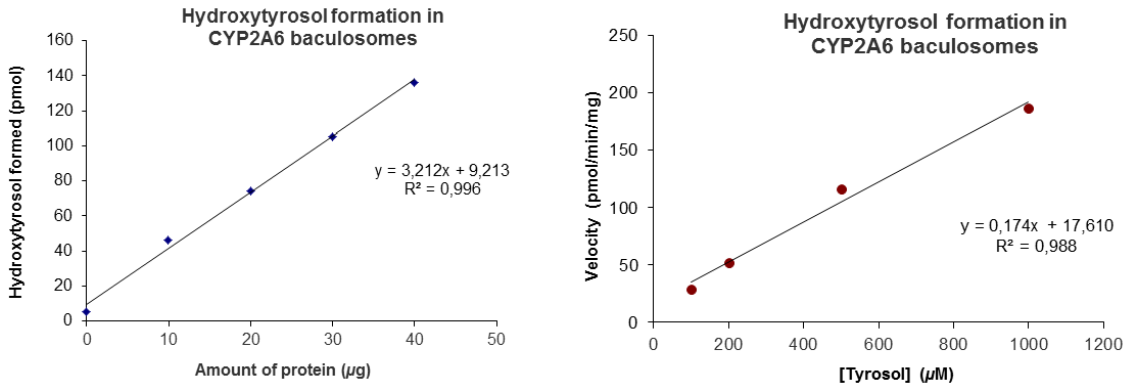


Figure 41. Left, *Linear formation of hydroxytyrosol using increasing amounts of CYP2A6 protein;* ***Right,*** *hydroxytyrosol formation velocity mediated by CYP2A6 baculosomes is increased along with tyrosol concentrations.*

In order to evaluate if other CYP isoforms could contribute to hydroxytyrosol production, human recombinant CYP3A4, CYP2B6, CYP2C9, and CYP2D6 (as well as CYP2A6) were tested. These isoforms were chosen based on the results of the chemical inhibition assays, in which the treatment with specific inhibitors of these isoenzymes decreased at least 10% hydroxytyrosol formation. All the five human recombinant CYP isoforms tested were capable of transforming tyrosol into hydroxytyrosol with CYP2D6, CYP3A4, and CYP2A6 being more reactive than CYP2B6 and CYP2C9 (**Figure 42**). In all the cases, hydroxytyrosol formation was NADPH-dependent.

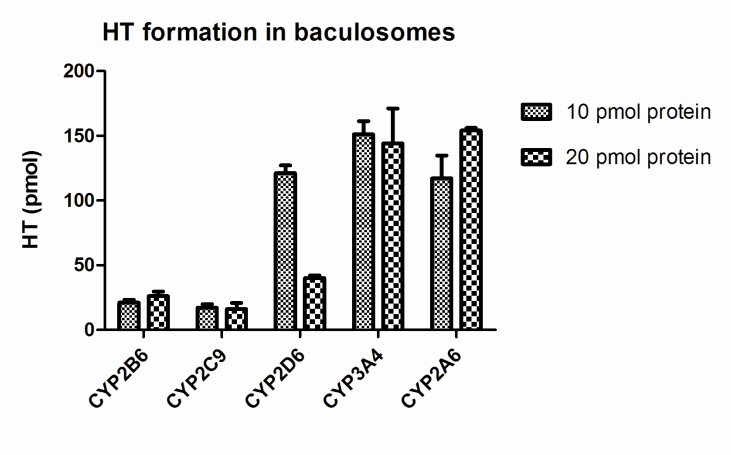


***Figure 42**. Hydroxytyrosol (HT) formation from tyrosol using different CYP isoforms. Based on previous studies in microsomes using specific inhibitors, five different baculosomes were used (CYP2B6, CYP2C9, CYP2D6, CYP3A4 and CYP2A6). For each baculosome, two different amounts of protein were used (10 and 20 pmol). All the experiments were performed in duplicate and are expressed as mean ± SD.*

Enzyme kinetics of CYP2D6, CYP3A4, and CYP2A6 (3 major contributors) were evaluated individually at 6 time points (0, 5, 15, 30, 45 and 60 min). Different kinetics were noted for each CYP isoenzyme. CYP2A6-mediated hydroxytyrosol formation occurred in a linear time-dependent manner from 0 to 60 min. The linearity in hydroxytyrosol formation mediated by CYP3A4 and CYP2D6 was noted only in the first 15 min, and there was a time-dependent reduction in hydroxytyrosol content from 15 to 60 min.

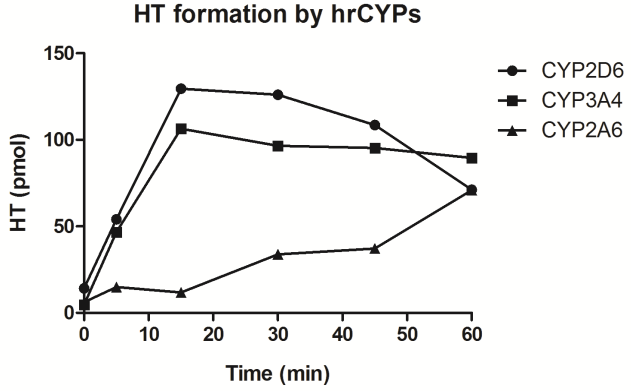


***Figure 43**. Time-course formation of hydroxytyrosol (HT) in three different human recombinant CYP isoforms (CYP2D6, CYP3A4 and CYP2A6). Linearity with CYP2D6 and CYP3A4 takes place only from 0 to 15 min, whereas HT formation in CYP2A6 presents a broader range of linearity over time (0-60 min).*

The use of baculosomes allowed us to confirm the capability of CYP2A6, CYP2D6, and CYP3A4 in the biotransformation of tyrosol to hydroxytyrosol. The capability of CYP3A4 baculosomes to carry out this conversion is not surprising, as it is the most abundant isoform in the liver, and it metabolizes the greatest number of drugs and other xenobiotics (Pelkonen et al., 2008). However, it shall be noted that, according to the inhibition experiments with ketoconazole in pooled HLM, CYP3A4 is not a strong contributor to *in vivo* hydroxytyrosol formation. More interesting seems the role of CYP2A6 and CYP2D6 in the conversion of tyrosol to hydroxytyrosol. For this reason, the relationship between tyrosol concentration and hydroxytyrosol formation rate was evaluated in CYP2A6 and CYP2D6 baculosomes with the aim of comparing the kinetic parameters with those obtained using HLM. Hydroxytyrosol formation kinetics in baculosomes did not follow a typical Michaelis-Menten hyperbolic pattern. In the case of CYP2A6, a biphasic kinetic profile was observed (**Figure 44, Left**). This enzymatic behavior has already been described (Hutzler and Tracy, 2002) and is characterized as a non-asymptotic profile that becomes linear with increasing substrate concentration. In the case of CYP2D6, the kinetics followed a sigmoidal autoactivation profile (**Figure 44, Right**). These atypical kinetic profiles have been previously described *in vitro*, especially for CYP3A4 (Hutzler and Tracy, 2002), suggesting that it might result from the simultaneous binding of multiple ligands to a single active site (Atkins, 2005).

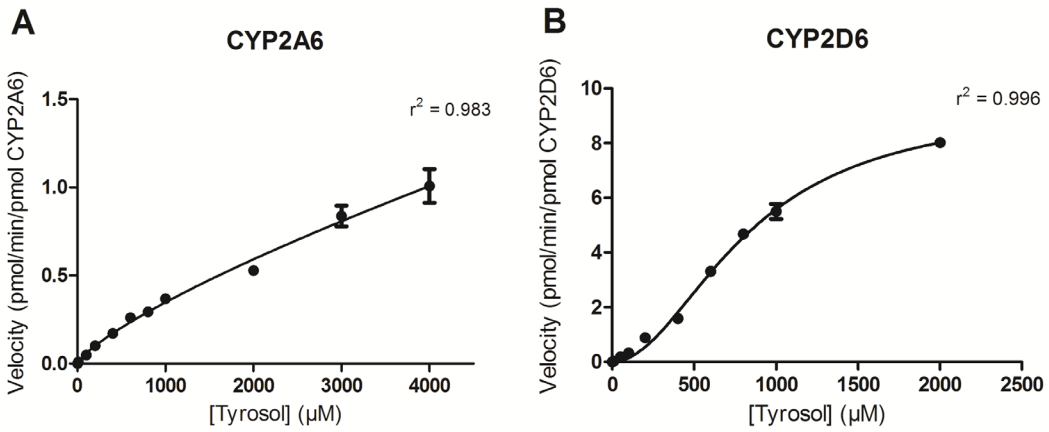


***Figure 44. Left**, Kinetic plot of hydroxytyrosol formation in CYP2A6 baculosomes followed a biphasic kinetic profile; **Right**, Kinetic plot of hydroxytyrosol formation in CYP2D6 baculosomes followed a sigmoidal autoactivation profile. Values are given as mean ± SD of two independent experiments.*

In our study, the lack of Michaelis-Menten kinetic profiles in baculosomes made difficult the comparison of the individual contribution of CYP2A6 and CYP2D6 to hydroxytyrosol formation, although it can be observed that, at equimolar concentrations, CYP2D6 presented a higher velocity of hydroxytyrosol formation than CYP2A6. A reason that could explain the discrepancy between the kinetic profile observed in microsomes and baculosomes could be the fact that recombinant CYP enzymes are usually expressed with much higher levels of NADPH and CYP reductase than those presented in HLMs. This non-physiological levels have been identified as a cause of artifacts (Rodrigues, 2008). Moreover, tyrosol hydroxylation in a mix of 50 HLMs (presenting a more physiological condition than baculosomes) perfectly followed a typical Michaelis-Menten kinetic profile. Taking into account the previous observations, we thus suggest that the *in vivo* tyrosol hydroxylation shall follow a Michaelis-Menten kinetic profile catalyzed by CYP2A6 and also by CYP2D6.

Both CYP2A6 and CYP2D6 activities present high interindividual variability due to genetic polymorphisms (Yokoi and Kamataki, 1998). This fact opens the door to

evaluate whether these polymorphisms lead to different rates of tyrosol-to-hydroxytyrosol conversion, a fact that could ultimately have an impact on hydroxytyrosol-associated beneficial health effects.

CHAPTER 6

Nutrigenomics: Impact of CYP2A6 and CYP2D6

Genetic Polymorphisms on Tyrosol-to-Hydroxytyrosol Conversion

The work corresponding to the present chapter was performed in collaboration with a Canadian research group headed by Professor Rachel. F. Tyndale, who kindly provided us with characterized livers and useful advice.

1. Introduction

Pharmacogenetics has been defined as the study of variability in drug response due to heredity. Its history dates back to 510 B.C. when Pythagoras noted that ingestion of fava beans resulted in a potentially fatal reaction only in some individuals (later characterized to be due to deficiency of glucose-6-phosphate dehydrogenase) (Pirmohamed, 2001). Nowadays, the pharmacogenetic impact on drug metabolism is well appreciated to the extent that the American and European regulatory agencies (FDA and EMA) have developed guidelines for industry concerning pharmacogenomics data submission with new drugs (Samer et al., 2013). In a similar way, nutrigenomics studies whether the interaction between genes and food bioactive compounds has an impact (either positive or negative) on human health (Sales et al., 2014)..

As described in *Chapter 5*, we found out that CYP was responsible for the conversion of tyrosol to hydroxytyrosol. CYP2A6 and CYP2D6 (two isoforms involved in this biotransformation) are highly polymorphic and are important for the metabolism of drugs (Johansson and Ingelman-Sundberg, 2011). In the view of these considerations, the **aim of this chapter** was to **evaluate if hydroxytyrosol production presents interindividual differences due to genetic polymorphisms of CYP2A6 and CYP2D6**. To do so, we collaborated with a Canadian research group headed by Dr. Rachel F. Tyndale who provided us with 15 selected livers exhibiting a wide range of CYP2A6 and CYP2D6 activities (according to genotype-predicted activities, as well as the capacity for coumarin-to-7-hydroxycoumarin conversion and dextromethorphan-to-dextrorphan conversion, respectively).

1. Materials and Methods

Appendix IV (which is the submitted manuscript to *Food Chemistry* and its supplementary material) contains a detailed description of the following features:

- ➔ Human Hepatic Microsomes Preparation and Genotyping
- ➔ Experiments in Human Liver Microsomes
    - o Tyrosol metabolism to hydroxytyrosol
    - o Nicotine metabolism to cotinine
    - o Coumarin metabolism to 7-hydroxycoumarin
    - o Dextromethorphan metabolism to dextrorphan
- ➔ Statistical Analyses

2. Results

2.1. Genotype and predicted CYP2A6/CYP2D6 activity

Table 9 shows the individual genotypes for CYP2D6 and CYP2A6 and the predicted activities of both isoforms according to the genotype.

***Table 9**. Genotype of 15 individual livers and predicted CYP2A6 and CYP2D6 activities according to the genotype.*

Liver ID	***CYP2A6***		***CYP2D6***	
	Genotype	**Predicted Activity**	**Genotype**	**Predicted Activity**
K10	*1/*1	Normal	*1/*1	Normal
K12	*1/*1	Normal	*1/*1	Normal
K14	*1/*1	Normal	*1/*4	Reduced
K15	*1/*1x2	Normal	*1/*1	Normal
K16	*1/*1	Normal	*1/*4	Reduced
K18	*1/*1	Normal	*1/*4	Reduced
K19	*1/*2	Reduced	*1/*4	Reduced
K20	*1/*12	Reduced	*1/*1	Normal
K22	*1/*1	Normal	*1/*1	Normal
K23	*1/*1	Normal	*1/*3	Reduced
K26	*1/*1	Normal	*1/*3	Reduced
K29	*1/*2	Reduced	*1/*1	Normal
M17	*1/*12	Reduced	*4, *10/*10	Reduced
M18	*1/*1	Normal	*1/*10	Reduced
M21	*1/*1	Normal	*4, *10/*10	Reduced

2.2. Correlation of Tyrosol Hydroxylation by Individual HLM

The correlation between hydroxytyrosol formation and CYP2A6 and CYP2D6 activities (determined using the velocity of coumarin metabolism to 7-hydroxycoumarin and dextromethorphan metabolism to dextrorphan, respectively) was not significant (**Figure 45A** and **B**). The impact of genotype on the association between hydroxytyrosol formation and *CYP2A6* and *CYP2D6* genotypes was not significant (**Figure 45C** and **D**).

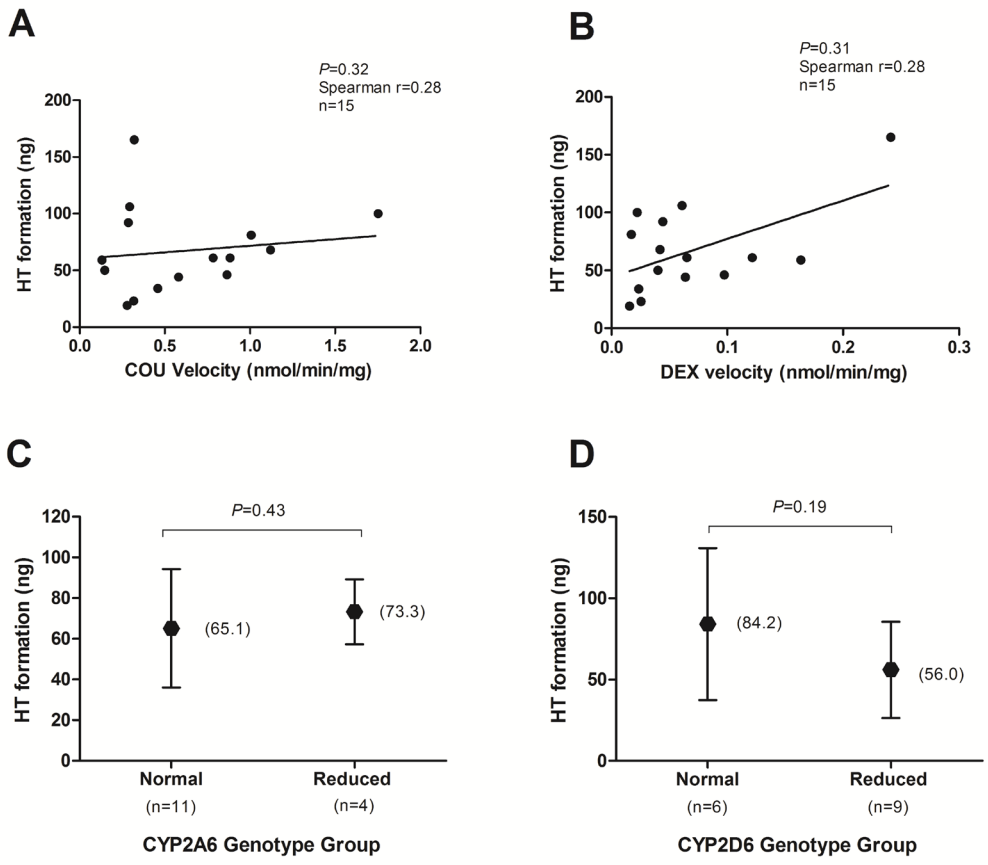


Figure 45. ***A.*** *Correlation between hydroxytyrosol (HT) formation and CYP2A6 activity (determined using the velocity of coumarin (COU) metabolism to 7-hydroxycoumarin).* ***B.*** *Correlation between HT formation and CYP2D6 activity (determined using the velocity of dextromethorphan (DEX) metabolism to dextrorphan). P and r values are based on Spearman correlation test.* ***C.*** *Association between CYP2A6 genotype and HT formation (ng). Normal metabolizers are those with *1/*1, *1/*1X2, and *14/*14 CYP2A6 genotypes. Reduced metabolizers are those with CYP2A6 *1/*2 and *1/*12 genotypes.* ***D.*** *Association between CYP2D6 genotype and HT formation (ng). Normal metabolizers are those with *1/*1 CYP2D6 genotype. Reduced metabolizers are those with CYP2D6 *1/*3, *1/*4, and *10/*10 genotypes. P values are based on Mann-Whitney tests.*

In order to evaluate the concomitant involvement of both isoforms on hydroxytyrosol formation, we performed a multivariate regression analysis. A three-dimensional scatter plot for the sample-to-sample variation in tyrosol hydroxylation of the 15 individual HLMs and their correlations with the individual CYP2D6 and

CYP2A6 activities is depicted in **Figure 46**. The equation of the adjusted surface that describes the velocity of tyrosol hydroxylation is:

$$\textbf{Tyrosol hydroxylation} = 0.01263 + 0.52778 \cdot X_1 + 0.044483 \cdot X_2 - 0.57453 \cdot X_1 \cdot X_2$$

Where X_1 is dextromethorphan velocity and X_2 is coumarin velocity. The corresponding correlation coefficient (r^2) is 0.50 (F = 3.69; P = 0.047). It shall be noted that the regression coefficient values were comparable when CYP2A6 velocity toward either coumarin or nicotine was included in the analysis.

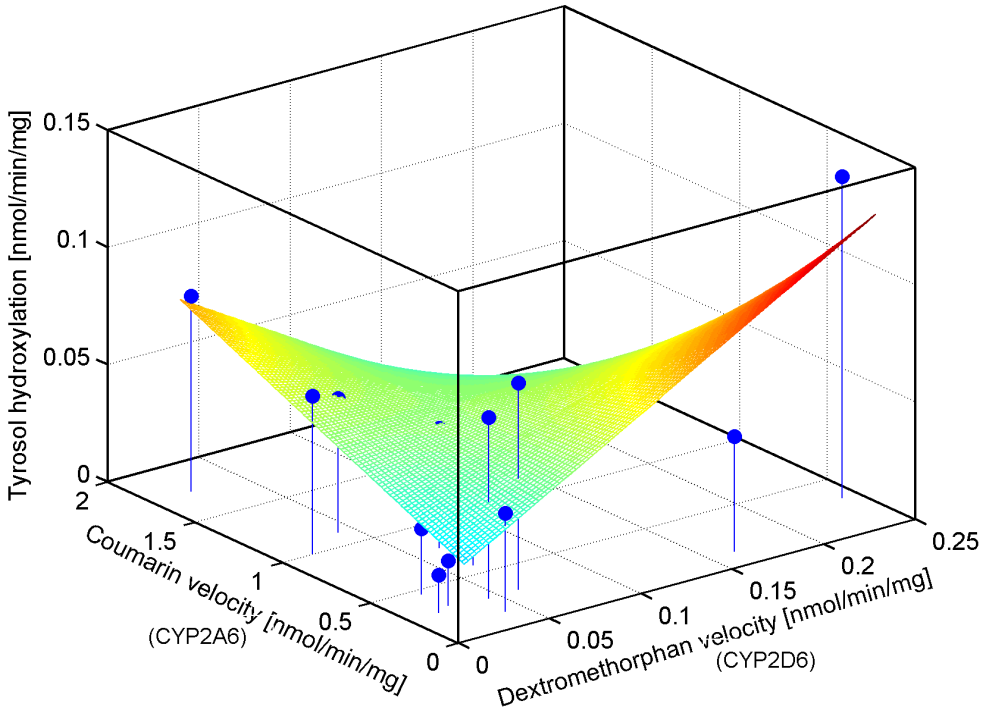


Figure 46. *Multivariate correlation analysis of sample-to-sample variation (n = 15) in tyrosol-3-hydroxylation with CYP2D6 and CYPA6 activities.*

3. Discussion

In the previous chapter, we identified CYPAD6 and CYP2A6 as the main isoforms responsible for the conversion of tyrosol to hydroxytyrosol. Both isoforms present high interindividual variability in their activities due to genetic polymorphisms (Yokoi and Kamataki, 1998). CYP2D6, which catalyzes more than 25% of commercial drugs, has a gene locus with more than 80 allelic variants, resulting in high variability associated with increased or reduced enzymatic activity and clinical consequences (Teh and Bertilsson, 2012). Similarly, the *CYP2A6* gene is polymorphic, resulting in high interindividual (Rautio et al., 1992) and interethnic differences in enzyme activity (Raunio et al., 2001; Piliguian et al., 2014). Indeed, 20% of Asians are CYP2A6 poor metabolizers, whereas this prevalence is lower in Caucasians (< 1%) (Raunio et al., 2001). Regarding the relative abundance of protein in the liver, CYP2D6 accounts for less than 5% whereas CYP2A6 represents approximately 10% of total hepatic CYP protein (Pelkonen et al., 2008).

Specificity of each CYP isoform toward its substrates is another issue that must be taken into account. CYP2D6 (together with CYP3A4) is responsible for the metabolism of hundreds of therapeutic drugs (Pelkonen et al., 2008). However, relatively few therapeutic drugs are metabolized by CYP2A6, which is the primary enzyme responsible for nicotine metabolism (Messina et al., 1997).

The pharmacogenomic impact on drug metabolism is well appreciated in the literature. In this study, we classified CYP2A6 and CYP2D6 of the selected 15 livers, according to genotype-predicted activities, as well as the capacity for nicotine-to-cotinine conversion and dextromethorphan-to-dextrorphan conversion, respectively. As expected, we found there was a wide interindividual variability of the tyrosol-to-hydroxytyrosol conversion in these 15 liver samples. We also noted that the correlations

between hydroxytyrosol formation and coumarin (a CYP2A6 substrate) or dextromethorphan (a CYP2D6 substrate) velocity were weak (Spearman r = 0.28 in both cases), and there were modest associations between hydroxytyrosol formation and CYP2A6 or CYP2D6 activities. These results and the previously observed (in *Chapter 5*) from baculosomes (showing a role of CYP2D6 and CYP2A6) and inhibition studies with microsomes (evidencing a role of CYP2A6), indicate that both CYP2D6 and CYP2A6 are actively involved in hydroxytyrosol formation from tyrosol. This implication is further upheld by a larger regression coefficient generated in the multivariate regression analysis with CYP2D6 and CYP2A6 velocities as two independent variables.

One limitation of the present chapter is the relatively low number of human livers used due to the difficulty in obtaining these samples. One strength is the good translation between the different techniques employed. Indeed, the conversion of tyrosol to hydroxytyrosol takes place in human livers as shown in this chapter, and these results are in line with the *in vivo* experiments with rats (*Chapter 3* and *Chapter 4*) as well as with the *in vitro* experiments with microsomes and baculosomes (*Chapter 5*).

In conclusion, we have described for the first time that hydroxytyrosol (a potent bioactive molecule with multiple health benefits) is formed from dietary tyrosol via CYP2A6 and CYP2D6, both working in a cooperative manner. The involvement of two CYP isoenzymes in the production of hydroxytyrosol is relevant since only a few known substrates of CYP2A6 have been reported so far. Moreover, genetic polymorphisms of both *CYP2D6* and *CYP2A6* will potentially have an impact on the magnitude of health benefits associated with tyrosol-containing food consumption between individuals and ethnicities.

FINAL CHAPTER

Contribution of Tyrosol and Ethanol to the *In Vivo* Endogenous Formation of Hydroxytyrosol in Humans: a Proof-of-Concept Study

1. Introduction

Throughout the previous *Chapters*, it has been shown that tyrosol can be converted to hydroxytyrosol. In *Chapters 3 and 4* it was described that the administration of tyrosol to rats results in a dose-dependent increase in hydroxytyrosol urinary recovery. In *Chapter 5* it was confirmed that this reaction takes place in human liver microsomes and baculosomes. In *Chapter 6* it was shown that the hydroxylation of tyrosol also takes place in microsomes from human genotyped livers. On the other hand, the results form *Chapters 2, 3 and 4* (as well as other published works (Pérez-Mañá et al., 2015a)) provided evidence that ethanol was interfering on the final levels of hydroxytyrosol. However, the *in vivo* contribution of exogenous tyrosol and ethanol to the endogenous levels of hydroxytyrosol in humans remained to be clarified.

The **aim** of this *Chapter* was to **assess the contribution of pure tyrosol and/or ethanol on hydroxytyrosol recovery in the urine of healthy volunteers**. The effect of white wine (a source of ethanol with low phenolic content) and/or pure tyrosol on the urinary excretion of total hydroxytyrosol was evaluated. It is worth mentioning that this was a proof-of-concept study which was performed only in two healthy volunteers to verify if the formation of hydroxytyrosol from tyrosol observed in animal models and *in vitro* tests could be translated to humans.

2. Material and methods

2.1. Hydroxytyrosol urinary excretion studies

Excretion studies were performed on two healthy male volunteers (age: 27 and 57 years) who gave informed consent prior to the studies. Urine was collected 2 hours after treatment administration and from 0 to 6 hours after treatments. In the 72 hours prior to each intervention and during the 6 hours that followed the treatment, both subjects had to follow a diet poor in phenolic compounds and alcoholic beverages were also restricted. This diet excluded olive oil, olives and wine. In the first study, 200 mg of pure tyrosol (Sigma-Aldrich, Inc. St. Louis, MO, USA) were administered in two gelatin capsules (*Study 1*). In the second study, the effect of the administration of 160 mL of white wine (Crin Roja Macabeo, 12.5%) was assessed (*Study 2*). In the third study, the combination of pure tyrosol (200 mg) and white wine (160 mL) was evaluated (*Study 3*). Finally, one additional study with lower doses of tyrosol (50 mg) and lower doses of white wine (100 mL) was performed (*Study 4*).

2.2. Analysis of hydroxytyrosol and their metabolites in urine

Total hydroxytyrosol excretion in urine after each treatment was assessed. Hydroxytyrosol and it corresponding metabolites were determined by LC-MS/MS as detailed in *Chapter 2*. Total hydroxytyrosol was calculated as detailed in *Chapter 2*.

3. Results and Discussion

3.1. Quantification of total hydroxytyrosol in urine: Studies 1-4

The results form *Study 1* show that the oral administration of pure tyrosol (200 mg) to humans was followed by in an increase in total hydroxytyrosol in urine. These results allow confirming that tyrosol conversion to hydroxytyrosol takes place *in vivo* in humans.

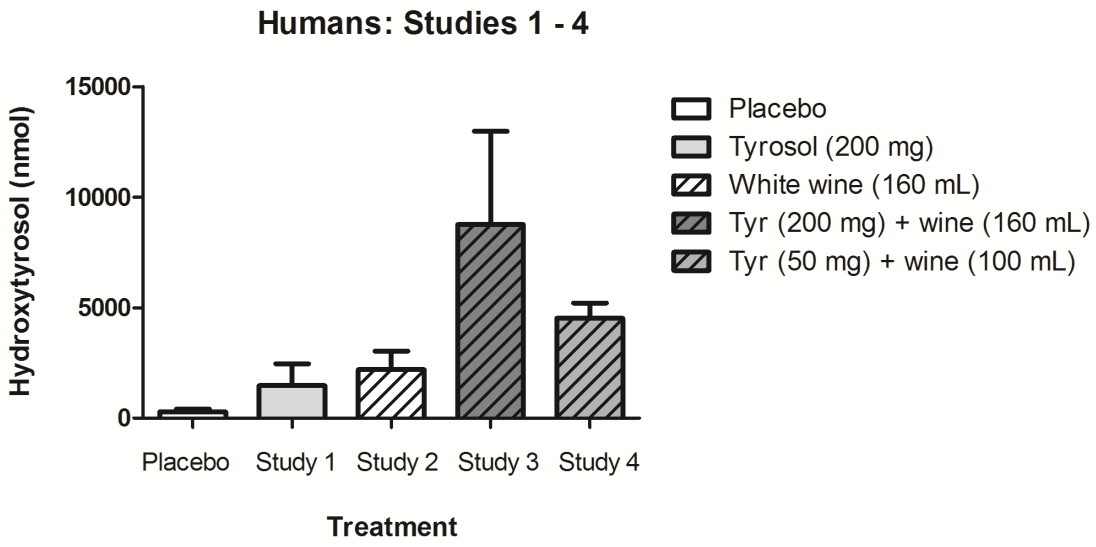


Figure 47. *Total hydroxytyrosol urinary recovery (0-6 hours) from human studies 1-4 (n=2).*

In *Study 2*, the effects of 160 mL of white wine on hydroxytyrosol urinary excretion were assessed. This volume of wine was compatible with dietary doses. The amount of ethanol administered with the treatment (16 g) is within the range considered protective against cardiovascular and total mortality (Costanzo et al., 2010). White wine was chosen due to its low content in phenolic compounds, compared to red wine. The results showed an increase in hydroxytyrosol urinary excretion higher to that observed after pure tyrosol administration (*Study 1*= 1.5 µmol; *Study 2* = 2.2 µmol, 46% difference). Taking into account that white wine has a very low phenolic content, the hydroxytyrosol urinary increase is compatible with an ethanol-induced endogenous production via dopamine metabolism. However, the absorption of the low amounts of tyrosol and hydroxytyrosol present in white wine cannot be discarded.

Interestingly, the *Study 3* provides further evidence of the synergistic effects on hydroxytyrosol recovery that take place when phenolic compounds and ethanol are combined. Indeed, the concomitant administration of tyrosol (200 mg) and white wine (160 mL) resulted not only on an additive effect but a synergistic one, in hydroxytyrosol

urinary levels (total hydroxytyrosol excretion = 8.7 μmol, 580% increase in bioavailability from 200 mg tyrosol alone). These high levels of hydroxytyrosol may be explained by several mechanisms: (i) the potential ethanol-induced increase in tyrosol bioavailability, (ii) the conversion of tyrosol to hydroxytyrosol via CYP, and (iii) the ethanol-induced increase on endogenous tyrosol and hydroxytyrosol levels via tyramine and dopamine metabolisms. As explained in *Chapter 2*, a similar effect on hydroxytyrosol urinary excretion has been observed following the administration of red wine. In that case, the amount of hydroxytyrosol found in urine was higher than the arithmetic sum of the amount of hydroxytyrosol induced by ethanol and that found following dealcoholized wine intake.

As the total recovery of hydroxytyrosol from *Study 3* was very high, an additional experiment (*Study 4*) administering lower doses of tyrosol (50 mg) and white wine (100 mL) was performed. The results of *Study 4* showed a total hydroxytyrosol excretion of 4.5 μmol., which is very similar to the levels observed following dietary doses of red wine or olive oil. In fact, hydroxytyrosol urinary recovery following white wine (100 mL) + tyrosol (50 mg) was similar to the one that followed red wine (146 mL). It is worth noting that the amount of ethanol administered with 100 mL of white wine (12.5 g) is 35% lower than the ethanol administered with 146 mL of red wine (19 g). Consequently, the supplementation of wine with tyrosol opens the possibility of reducing the ethanol intake and maintaining the levels of hydroxytyrosol.

For comparative purposes, the results from these 4 studies are represented in **Figure 48** together with (1) previous studies in humans following different doses of ethanol (Pérez-Mañá et al., 2015a) and (2) treatments with vodka, dealcoholized wine, and red wines (Pérez-Mañá et al., 2015b).

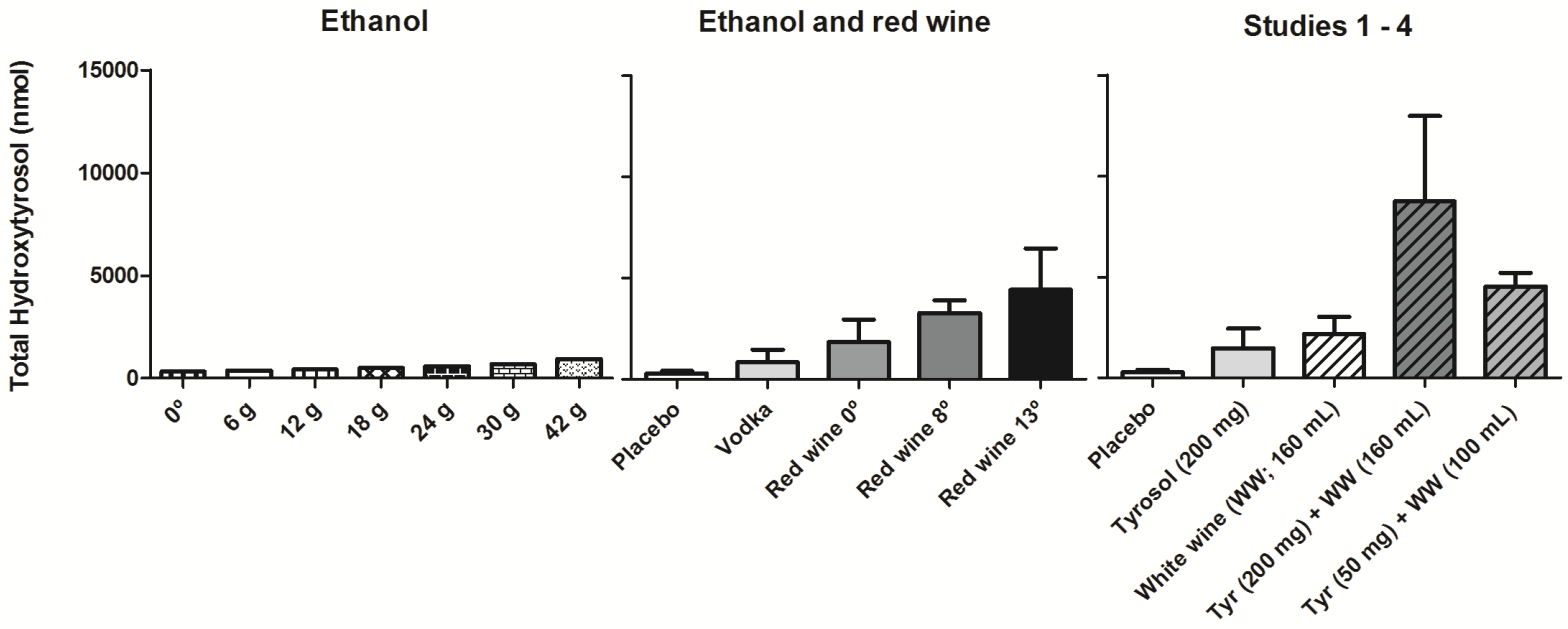


Figure 48. *Comparison of total hydroxytyrosol urinary excretion following different studies in humans (Tyr: Tyrosol; WW: White wine).*

The main observations that stem out from the studies on hydroxytyrosol excretion are the following:

(1) The administration of pure ethanol promotes a slight dose-dependent increase in hydroxytyrosol urinary levels. This fact is compatible with the already-known alteration of dopamine oxidative metabolism (Tank and Weiner, 1979; Pérez-Mañá et al., 2015a).

(2) The administration of dealcoholized red wine (rich in phenolic compounds but without alcohol) also results in an increase in hydroxytyrosol urinary levels. The amount of hydroxytyrosol excreted is higher (162%) than the amount present in the wine ingested. This fact underpins the idea that, besides ethanol, phenolic compounds present in wine are able to generate hydroxytyrosol (Pérez-Mañá et al., 2015b).

(3) The administration of red wine (a source of ethanol rich in phenolic compounds) is followed by a high hydroxytyrosol urinary excretion. Taking into account the dose administered, the abnormally high recovery (420%) evidences a synergistic effect between the phenolic compounds and the ethanol content, and suggests the existence of additional sources of hydroxytyrosol (Pérez-Mañá et al., 2015b).

(4) The oral administration of pure tyrosol at high doses (200 mg) promotes an increase on hydroxytyrosol urinary levels. This fact evidences that tyrosol is firstly absorbed following oral intake and then it is endogenously transformed to give rise to hydroxytyrosol. The percentage of hydroxytyrosol found in urine (1.645 μmol) represents around 1% of the total tyrosol administered (1448 μmol) with 200 mg.

(5) The administration of 160 mL of white wine (a source of ethanol with a low content in phenolic compounds) resulted in a hydroxytyrosol urinary recovery of 2.2 μmol. The origin of this hydroxytyrosol is compatible with an endogenous origin via dopamine metabolism (enhanced by ethanol) but the absorption of exogenous tyrosol and hydroxytyrosol present in white wine can potentially be minor contributors.

(6) The administration of pure tyrosol in combination with white wine results in a dramatic increase in the levels of hydroxytyrosol. In a similar way to what happened with red wine, the increase following pure tyrosol together with white wine has a synergistic effect that leads to very high hydroxytyrosol amounts.

(7) Following the an oral intake of 100 mL of white wine together with 50 mg of pure tyrosol, the levels of hydroxytyrosol in urine are 4.5 μmol. Interestingly, these amounts are very similar to those obtained following the administration of dietary doses of red wine (146 mL) or olive oil (50 mL) (**Figure 49**).

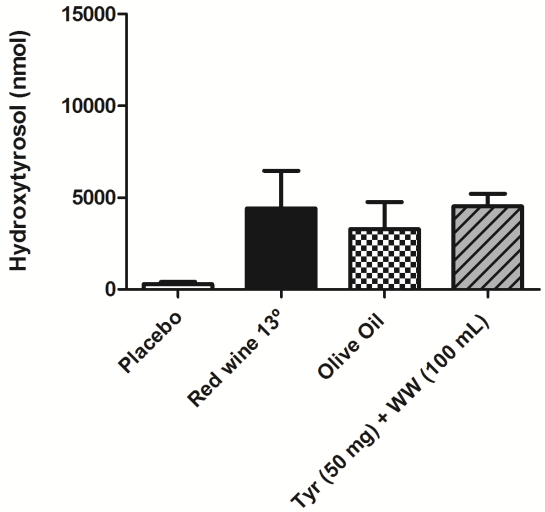


Figure 49. *Hydroxytyrosol urinary recoveries from human intervention studies (Tyr: Tyrosol; WW: White wine).*

These results open a new venue in our understanding of health beneficial effects associated to the intake of moderate doses of wine. It can be hypothesized that the administration of 100 mL of white wine supplemented with 50 mg of tyrosol could display beneficial health effects, although future studies are needed to confirm this hypothesis.

The main limitation of the results of this chapter is the low sample size of the excretion studies 1-4 (n=2). As already mentioned, these studies were performed only in two healthy volunteers as a proof-of-concept, in order to evaluate if the results from preclinical studies described in the previous chapters could be translated to humans. Future studies (already on-going) with a larger sample size will provide more robust data regarding hydroxytyrosol disposition in humans. Another limitation is that only urine was analyzed. The analysis of urine, although appropriate for excretion studies, does not provide direct information about the circulating levels of hydroxytyrosol. In order to overcome this limitation, we have recently contributed to the development of a method to detect free hydroxytyrosol in human plasma using LC-MS/MS (Pastor et al., 2016). In the near future, the development of additional methods to detect and quantify hydroxytyrosol phase II metabolites in human plasma will allow the measurement of circulating levels of hydroxytyrosol and its metabolites in incoming intervention studies.

GENERAL DISCUSSION

The present PhD thesis is aimed at investigating new mechanisms involved in the *in vivo* formation of hydroxytyrosol. This phenolic compound is one of the most potent natural antioxidants and is present in two characteristic constituents of the Mediterranean Diet (MD): virgin olive oil and wine. Following observations that the inhabitants of Mediterranean countries have longer life expectancy and a lower risk of suffering certain chronic diseases, there has been growing scientific interest focused on identifying the characteristic components of this diet that could be responsible for its associated health benefits (Ortega, 2006). On the one hand, a large number of studies have assigned most of its beneficial effects on human health to virgin olive oil (Huang and Sumpio, 2008), particularly preventive effects towards cardiovascular diseases (Covas et al., 2006b; Estruch et al., 2013). On the other hand, some of the beneficial effects of the MD have been attributed to the polyphenols in red wine (Ortega, 2006).

Hydroxytyrosol [2-(3,4-dihydroxyphenyl)ethanol] displays strong anti-proliferative, pro-apoptotic, anti-microbial, anti-platelet aggregation, antioxidant, and anti-inflammatory properties (Granados-Principal et al., 2010). These biological activities have led to considerable preclinical research (*in vitro, ex vivo,* and animal model studies) on the protective potential of hydroxytyrosol with respect to cardiovascular diseases and metabolic syndrome (Bulotta et al., 2014). In fact, human intervention clinical trials with virgin olive oil rich in hydroxytyrosol have confirmed that this phenolic compound has biological activities related to cardiovascular prevention (Covas et al., 2015). There is growing evidence that hydroxytyrosol protective effects are not only limited to the field of cardiovascular diseases, but could also be of relevance in the secondary prevention of neurodegenerative diseases. We have reviewed current scientific knowledge supporting this information (Rodríguez-Morató et al., 2015) (See *Appendix I* for the published article).

Tyrosol [2-(4-hydroxyphenyl)ethanol)] is a phenolic compound also known to be present in extra-virgin olive oil and wine. Despite presenting antioxidant properties (García-Padial et al., 2013), it is considered to have a lower antioxidant activity than hydroxytyrosol (Di Benedetto et al., 2007).

A preliminary objective of this thesis was to **evaluate and compare the *in vitro* bioactions of tyrosol and hydroxytyrosol**. We hypothesized that hydroxytyrosol would have a higher antioxidant capacity due to the additional hydroxyl group at the position 3 of the phenol ring, which provides this compound with *catechol* structure. In this regard, the *in vitro* bioactions of tyrosol and hydroxytyrosol were evaluated by two different strategies: (1) two antioxidant capacity assays strictly based on chemical reactions *in vitro* (FRAP and ORAC), and (2) a human hepatocyte cell model (HepG2 cells). HepG2 cells were treated with different oxidative stress models (AAPH and H_2O_2) and tyrosol and hydroxytyrosol activities were evaluated using various assays (ROS, apoptosis, and necrosis). Prior to these experiments, the cell viability of HepG2 cells in the presence of tyrosol and hydroxytyrosol was assessed. The tested concentrations, ranging from 1 to 25 μM, are compatible with the physiological levels achieved after the consumption of foods containing these phenolic compounds. Neither tyrosol nor hydroxytyrosol was found to be toxic to HepG2 cells at the doses tested. Overall, the chemical-based antioxidant capacity assays, and the experiments in HepG2 cells, showed that tyrosol and hydroxytyrosol present interesting *in vitro* bioactions, hydroxytyrosol being more active than tyrosol, thus confirming our initial hypothesis.

It is noteworthy that for a considerable period of time the beneficial effects of antioxidants were attributed to their ability to reduce reactive oxygen species (ROS) and fight oxidative stress. Nevertheless, it is now known that the *in vivo* antioxidant mechanism of action is far more complex (Holst and Williamson, 2008) and may be

independent of its intrinsic antioxidant capacity (e.g. by stimulating cell signaling pathways that result in the modulation of gene expression or by interaction with nuclear receptors (Virgili and Marino, 2008)). The simple information obtained from *in vitro* antioxidant capacity assays does not guarantee that the compounds tested have an *in vivo* antioxidant effect on the complexity of the human body. Indeed, several factors determine the amount of unaltered antioxidant that reaches human plasma. These mainly include processes of absorption, metabolism, distribution, and excretion. Moreover, a molecule can have beneficial effects through mechanisms of action which may be independent of any direct antioxidant property (Sies, 2007). Consequently, antioxidant capacity data gathered from *in vitro* assays (as in *Chapter 1*), although useful, should be complemented with information regarding bioavailability and *in vivo* effects. Throughout the chapters of the thesis, several aspects regarding the metabolism of tyrosol and hydroxytyrosol have been studied. In particular, some information comes from clinical trials in which hydroxytyrosol- and tyrosol-containing foods were administered to human volunteers, and their corresponding concentrations were measured in biological fluids.

The **origin of the core research of the present thesis** stems from the observations obtained from two similar clinical trials designed to assess the short-term and postprandial effects of moderate doses of wine (250 mL/day) and virgin olive oil (25 mL/day). When the pharmacokinetics of hydroxytyrosol from both clinical trials were compared, it was found by chance that: (1) AUCs were quite similar even though the dose of hydroxytyrosol in red wine was much lower (2.3 μmol) than that in olive oil (11 μmol), and (2) hydroxytyrosol urinary recovery was two-fold with respect to the dose administered (De la Torre et al., 2006). It is noteworthy that in the literature normal values for hydroxytyrosol bioavailability are lower than 10% when it is

administered as pure hydroxytyrosol (González-Santiago et al., 2010) and in the range of 30-60% in an olive oil matrix (Visioli et al., 2000).

The endogenous formation of hydroxytyrosol as a by-product of dopamine oxidative metabolism is augmented by ethanol (**Figure 50 Right**) (Tank and Weiner, 1979). It was initially hypothesized, therefore, that ethanol present in wine could be responsible, via dopamine metabolism, for the abnormally high recovery of hydroxytyrosol observed after wine intake. In a similar way, tyrosol is endogenously produced as a by-product of tyramine oxidative metabolism and this formation is increased by ethanol (**Figure 50 Left**).

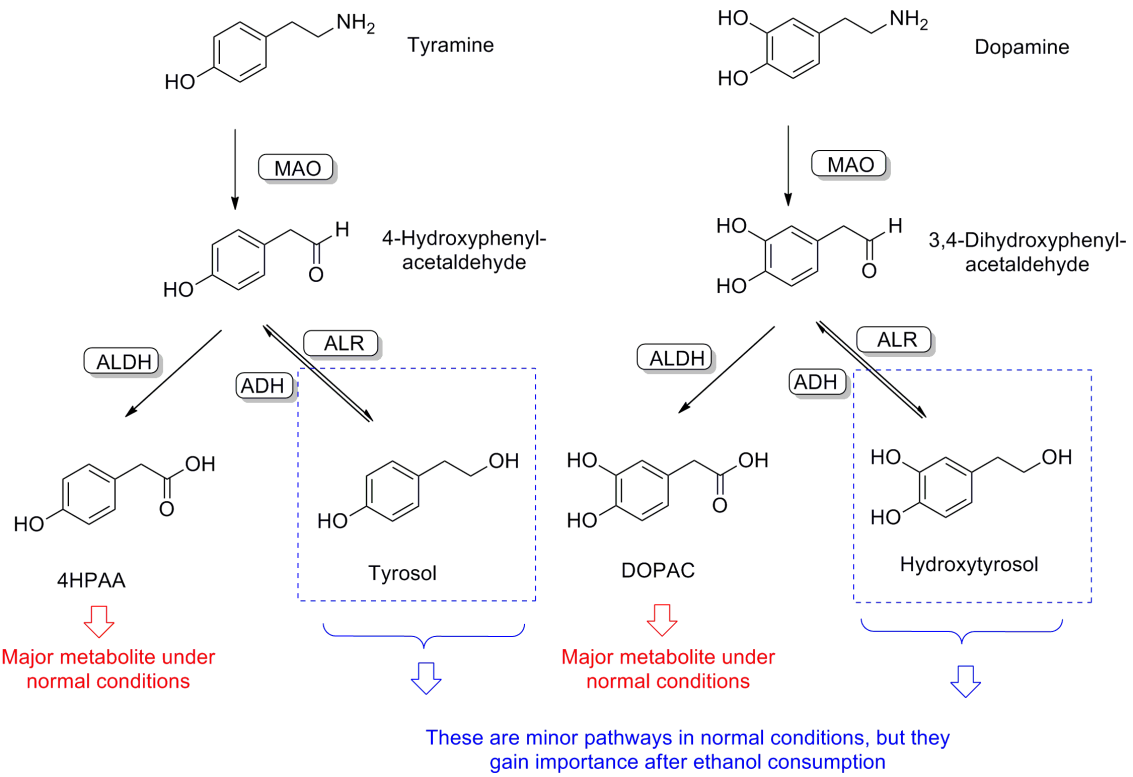


***Figure 50**. Tyramine and dopamine metabolism. Both biogenic amines are deaminated by monoamine oxidase (MAO) forming an aldehyde intermediate which can be either oxidized by aldehyde dehydrogenase (ALR) generating carboxylic acids or reduced by alcohol dehydrogenase (ADH) generating aliphatic alcohols. Ethanol causes an increase in the production of alcohols and a decrease in the production of carboxylic acids.*

In a subsequent intervention study in which twenty-four male volunteers received different doses of ethanol (6-42 g) or placebo, it was found that urinary excretion of hydroxytyrosol (and also of tyrosol) increased with the dose of ethanol administered which was compatible with a shift in dopamine (and tyramine) oxidative metabolism. However, the amount of hydroxytyrosol formed and recovered in urine was not high enough to explain the amounts of this compound recovered in urine after wine intake (**Figure 51**), pointing to the existence of additional sources of hydroxytyrosol formation coming from wine (Pérez-Mañá et al., 2015a).

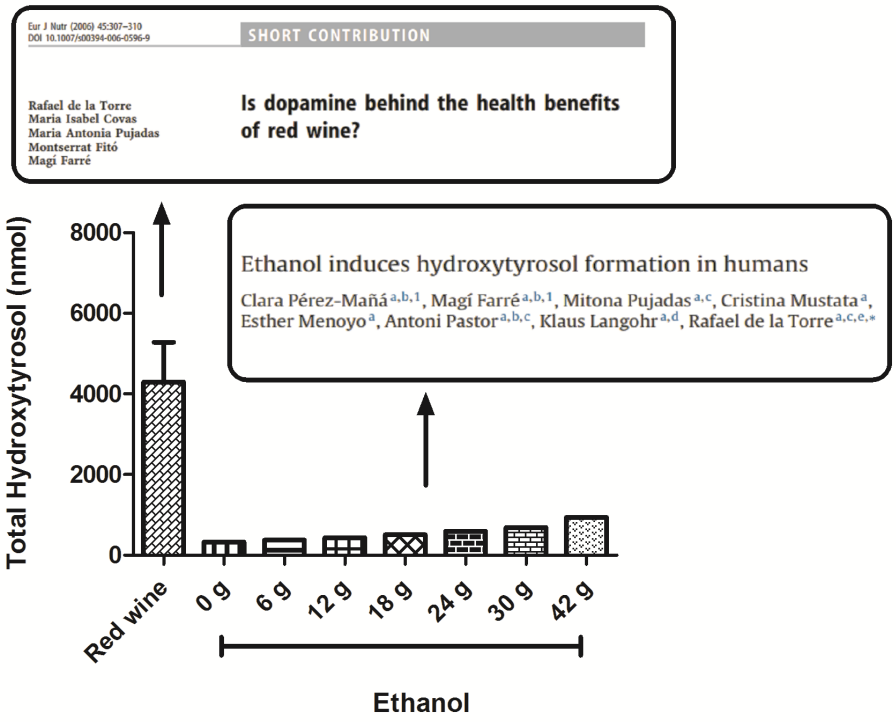


Figure 51. *Effects of red wine (15 g ethanol) and ethanol (6-42 g) administration on total hydroxytyrosol urinary excretion in humans.*

Thus, **the objective of the present thesis was to study the underlying mechanisms responsible for the formation of hydroxytyrosol following wine intake.** As described in *Chapter 2*, a randomized controlled clinical trial was performed in 28 volunteers who received four different treatments: placebo, vodka, dealcoholized wine,

and red wine. The rate of hydroxytyrosol excretion was red wine>dealcoholized wine>vodka>placebo. Total hydroxytyrosol recoveries following dealcoholized wine and red wine were 161% and 420%, respectively. On the one hand, the evaluation of tyramine and dopamine metabolism would support a *di novo* formation of hydroxytyrosol following dealcoholized wine and wine intake. On the other hand, the results were compatible with an ethanol-induced alteration of dopamine metabolism leading to an increase in hydroxytyrosol formation following vodka and red wine administration.

A synergistic effect between phenolic compounds and ethanol was postulated to be responsible for the amounts of hydroxytyrosol recovered. In order to examine in depth the mechanisms of this endogenous formation of hydroxytyrosol, studies in rats (n=96) were performed (*Chapter 3*). Three potential phenolic precursors were evaluated: tyrosine, tyrosol, and tyramine. The effect of these phenolic compounds (either alone or in combination with ethanol) on the urinary excretion of hydroxytyrosol was assessed. The two major findings of those experiments in rats were: (1) tyrosol was converted *in vivo* to hydroxytyrosol in a dose-dependent manner, and (2) urinary recoveries of tyrosol were higher when this compound was administered with ethanol versus saline.

The conversion of tyrosol to hydroxytyrosol and the ethanol-mediated increase in tyrosol bioavailability (findings from *Chapter 3*) are key mechanisms compatible with the high recoveries (>100%) reported in humans after the administration of wines as described in *Chapter 2* (**Figure 52 Left**). This information makes even more sense when we take into account that the amount of tyrosol administered with wine (22-25 μmol) was much higher than that of hydroxytyrosol (around 1 μmol), tyramine, and tyrosine (**Figure 52 Right**).

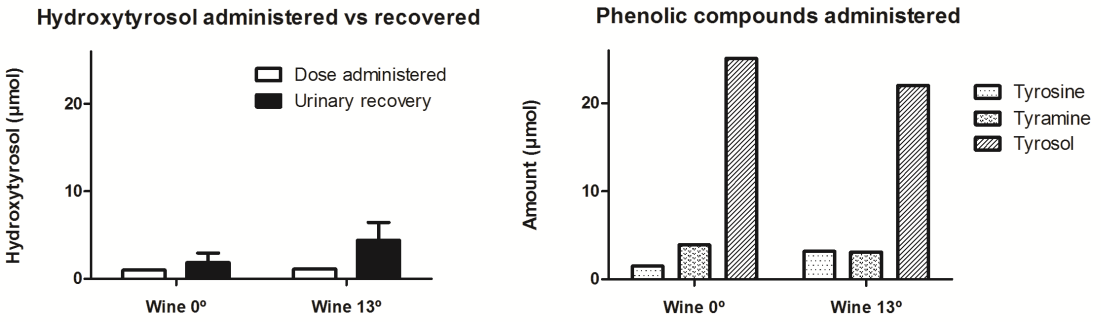


***Figure 52. Left**, Comparison between the dose of hydroxytyrosol administered and the amount of hydroxytyrosol recovered in urine following dealcoholized (0º) and red wine (13º) intake (147 mL). **Right**, Amounts of tyrosine, tyramine and tyrosol administered, in wines (147 mL).*

The conversion of tyrosol to hydroxytyrosol would, therefore, explain the recoveries of hydroxytyrosol being higher than the dose administered in both wines. Furthermore, the increase in tyrosol absorption mediated by ethanol would account for the higher hydroxytyrosol recoveries in 13º wine compared to dealcoholized wine.

Despite having established that hydroxytyrosol was endogenously formed from tyrosol, the enzymes that mediated the conversion remained to be characterized. We hypothesized that either tyrosine hydroxylase (*Chapter 4*) or CYPs (*Chapter 5*) were involved. Additional experiments in rats (n=72) discarded the involvement of the former. Subsequent experiments using human liver microsomes, human recombinant proteins, and specific enzymatic inhibitors allowed us to identify CYP2A6 and CYP2D6 as the main isoforms responsible for the conversion of tyrosol to hydroxytyrosol. As both isoforms have genetic polymorphisms, the pharmacogenetic impact was assessed in 15 selected human livers (*Chapter 6*). These livers displayed a wide range of CYP2A6 and CYP2D6 activities, according to their capacity for nicotine-to-cotinine conversion and dextromethorphan-to-dextrorphan conversion, respectively. A wide interindividual variability of the tyrosol-to-hydroxytyrosol conversion in the

liver samples was reported, and the involvement of both CYP2A6 and CYP2D6 in hydroxytyrosol formation from tyrosol was confirmed. Such observations open the way to a promising research field: the evaluation of health benefits associated with tyrosol-containing foods (e.g. olive oil, wine) in the light of CYP genetic polymorphisms.

A final set of pilot experiments was performed in order to establish wether the tyrosol-to-hydroxytyrosol conversion takes place *in vivo* in humans (*Final Chapter*). The effect of white wine and/or pure tyrosol on hydroxytyrosol urinary excretion was evaluated in 2 healthy volunteers. Oral administration of pure tyrosol was found to promote an increase in hydroxytyrosol urinary levels, demonstrating that tyrosol is absorbed and transformed to hydroxytyrosol in humans. White wine intake (a source of ethanol with a low phenolic content) also resulted in increased hydroxytyrosol levels. Moreover, in a similar way to red wine, the administration of pure tyrosol in combination with white wine resulted in a synergistic effect that led to the formation of very high amounts of hydroxytyrosol. This is compatible with an ethanol-mediated increase in tyrosol absorption that could, once absorbed, be endogenously converted to hydroxytyrosol.

Overall, two major mechanisms (the conversion of tyrosol to hydroxytyrosol via CYP2A6/2D6, and the increase in tyrosol absorption due to ethanol) and two minor ones (the generation of hydroxytyrosol via dopamine oxidative metabolism, and the conversion of tyramine to tyrosol) explain the abnormally high hydroxytyrosol recoveries found in urine after red wine intake. The biological relevance of these hydroxytyrosol levels remains to be elucidated.

Future Perspectives: on-going experiments and upcoming projects

The future perspectives related to this PhD thesis are summarized in the following points:

1) Use of tyrosol as a precursor of hydroxytyrosol

The potential use of pure hydroxytyrosol as a nutraceutical, although feasible, presents some limitations. These include low chemical stability, rapid oxidation, high cost of production, and poor bioavailability in humans. The tyrosol-to-hydroxytyrosol endogenous conversion opens up the possibility of employing tyrosol in nutraceuticals as a precursor of hydroxytyrosol. Following this line of thought, the findings from the pilot study (also described in the *Final Chapter*) should be confirmed in a randomized, controlled clinical trial.

2) Biological relevance of the tyrosol-to-hydroxytyrosol conversion

2.1. Clinical trials

Future studies with higher sample size should assess the long-term beneficial effects of tyrosol-enriched wine on atherosclerosis and neurodegeneration. We postulate that tyrosol-enriched wine will be better than wine alone on promoting beneficial effects on vascular risk and cognitive decline in humans in a dose-dependent manner. The study should include individuals of both genders at high cardiovascular disease risk.

As an example, future clinical trials could assess the long-term effects of sustained consumption of low-alcohol-tyrosol-enriched wine versus the same wine alone or placebo, on cognitive decline and cardiovascular risk protection. An on-going clinical trial is already assessing the effects of ethanol ingestion from moderate wine consumption plus tyrosol, versus the same wine alone or placebo, on endogenous hydroxytyrosol generation, endothelial function, and vascular risk factors (FIS ref. PI14/00072).

2.2. Studies in animal models

Due to the lengthy progression of some of the diseases in which the beneficial effects of moderate tyrosol-enriched wine consumption is evaluated, and the relatively short duration of funded grants (3-5 years), the integration in the project of animal disease models is necessary. In this context, two potential approaches could be in:

- An *ApoE3-TR mouse model*, examining the effect of combining tyrosol with ethanol in the prevention of atherosclerosis development.
- An *ApoE4-TR and Parkinson mice disease models*, evaluating the effect of alcohol alone and with tyrosol on disease end-points such as atherosclerosis development and brain neurodegenerative diseases.

3) Pharmacogenetics

The nutrigenetic and nutrigenomic impact should be evaluated by assessing the genetic polymorphisms related to tyrosol biotransformation (CYP2A6 and CYP2D6 genotyping) and ethanol metabolism (ADH1B), and the gene expression of mononuclear cells from selected genes involved in endothelial function and inflammation. Data regarding endothelial function and vascular risk factors, together with genetic polymorphisms, will provide information with respect to the relationship between the differences in the DNA sequence and the health benefits associated with tyrosol-containing foods or supplements. Gene expression experiments will provide knowledge concerning the mechanisms underlying health benefits.

4) Biological activity of sulfates

Another issue that deserves further study is the evaluation of tyrosol and hydroxytyrosol mechanisms of action. In spite of recent advances of research into the metabolisms of hydroxytyrosol and tyrosol, the specific mechanisms of action of these compounds needs to be fully elucidated. According to present data it seems that the

conjugated forms may display biological activities related to the free forms, probably through different mechanisms of actions. The possibility of deconjugation represents an additional challenge when trying to credit a particular biological effect to a metabolite. Besides their typical direct antioxidant properties, phytochemicals are thought to contribute to beneficial effects through mechanisms independent of such capacity (Virgili and Marino, 2008). In this regard, future studies should take into account the assessment of indirect antioxidant mechanisms of action.

The hydroxyl group at position 3 seems to be a key factor for the antioxidant capacity of hydroxytyrosol. Paradoxically, the extensive metabolism that this compound undergoes in the human body inactivates this hydroxyl group to the extent that the circulating levels of unaltered free hydroxytyrosol are very low (<2%) and, until recently, were deemed undetectable (Miró-Casas et al., 2003a; Pastor et al., 2016). It is striking that most of the reported activities of hydroxytyrosol and tyrosol are focused on the free forms (very minor ones in plasma) whereas only a few recent studies have evaluated the activity of the metabolites (major forms in plasma) (for further details, see the review in *Appendix II*). For instance, to date, only one article has focused on the activity of hydroxytyrosol and tyrosol sulfates (Atzeri et al., 2016) when these compounds constitute around 80-90% of the total hydroxytyrosol and tyrosol forms found in urine after wine intake (Pérez-Mañá et al., 2015b). In contrast, a considerable variety of studies have evaluated the activity of free tyrosol and hydroxytyrosol even though they only represent 1-6% of the total urinary hydroxytyrosol and tyrosol (Pérez-Mañá et al., 2015b). As a consequence, future studies on hydroxytyrosol and tyrosol activities (especially those performed *in vitro*) should give more weight to the phase I/II metabolites (particularly to sulfates).

CONCLUSIONS

The main objective of the present research project was to identify and to study mechanisms responsible for hydroxytyrosol *in vivo* generation. On the basis of the results obtained, the major conclusions are listed below:

1. In humans, tyrosol is endogenously converted to hydroxytyrosol via CYP2A6/CYP2D6. As tyrosol is normally present in wine in higher amounts than hydroxytyrosol, this reaction is of relevance and opens the possibility to employ tyrosol as a pro-drug of hydroxytyrosol in nutraceutical preparations or functional foods.
2. Exogenous tyrosol presents a higher excretion when it is administered in combination with ethanol, suggesting that ethanol increases its bioavailability.
3. The concomitant administration of tyrosol and ethanol results in an exponential increase of hydroxytyrosol urinary levels. This fact can be explained by the combination of the two previously discussed mechanisms.
4. The administration of ethanol alters the metabolism of dopamine (and tyramine) generating a slight increase of hydroxytyrosol (and tyrosol) at the expense of DOPAC (and 4-HPAA). Nevertheless, this mechanism of hydroxytyrosol generation is not relevant enough in terms of the amounts formed.
5. The tyrosol-to-hydroxytyrosol conversion presents a wide interindividual variability that can be explained by the genetic polymorphisms displayed by CYP2A6 and CYP2D6.
6. According to *in vitro* studies, neither tyrosol nor hydroxytyrosol are toxic to HepG2 when tested at concentrations ranging from 1 to 25 μM. A higher activity of hydroxytyrosol (in comparison with tyrosol) has been observed in chemical-based assays and HepG2 cells treated with different oxidative stress

models (AAPH and H_2O_2) and evaluated using various assays (ROS, apoptosis, and necrosis).

Taken together, these results point at hydroxytyrosol as candidate molecule that may contribute significantly to the health effects derived from moderate wine intake.

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APPENDICES

Appendix I

Potential Role of Olive Oil Phenolic Compounds in the Prevention of Neurodegenerative Diseases

Rodríguez-Morató J, Xicota L, Fitó M, Farré M, Dierssen M, de la Torre R. Potential role of olive oil phenolic compounds in the prevention of neurodegenerative diseases. Molecules. 2015 Mar 13;20(3):4655-80. doi: 10.3390/molecules20034655

Appendix II

Metabolic disposition and biological significance of simple phenols of dietary origin: hydroxytyrosol and tyrosol

Rodríguez-Morató J, Boronat A, Kotronoulas A, Pujadas M, Pastor A, Olesti E, Pérez-Mañá C, Khymenets O, Fitó M, Farré M, de la Torre R. Metabolic disposition and biological significance of simple phenols of dietary origin: hydroxytyrosol and tyrosol. Drug Metab Rev. 2016 May;48(2):218-36. doi: 10.1080/03602532.2016.1179754

Appendix III

Moderate consumption of wine, through both its phenolic compounds and alcohol content, promotes hydroxytyrosol endogenous generation in humans. A randomized controlled trial

Pérez-Mañá C, Farré M, Rodríguez-Morató J, Papaseit E, Pujadas M, Fitó M, Robledo P, Covas MI, Cheynier V, Meudec E, Escudier JL, de la Torre R. Moderate consumption of wine, through both its phenolic compounds and alcohol content, promotes hydroxytyrosol endogenous generation in humans. A randomized controlled trial. Mol Nutr Food Res. 2015 Jun;59(6):1213-6. doi: 10.1002/mnfr.201400842.

Appendix IV

CYP2D6 and CYP2A6 biotransform dietary tyrosol into hydroxytyrosol

Rodríguez-Morató J, Robledo P, Tanner JA, Boronat A, Pérez-Mañá C, Oliver Chen CY, Tyndale RF, de la Torre R. CYP2D6 and CYP2A6 biotransform dietary tyrosol into hydroxytyrosol. Food Chem. 2017 Feb 15;217:716-25. doi: 10.1016/j.foodchem.2016.09.026