Universidade do Minho
Escola de Ciências

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Synthesis and biological evaluation of olive oil polyphenol metabolites

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UMinho | 2019

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Synthesis and biological evaluation of olive oil polyphenol metabolites

Dissertação de Mestrado em
Química Medicinal

Trabalho efetuado sob a orientação do
Professor Doutor Luís Miguel Oliveira Sieuve Monteiro e da
Professora Doutora Maria de Fátima Azevedo Brandão Amaral Paiva Martins

outubro de 2019

Despacho RT - 31 /2019 - Anexo 3

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Agradecimentos

Queria começar por agradecer ao orientador deste projeto, Professor Doutor Luís Monteiro pela oportunidade que me deu de trabalhar consigo pela segunda vez e por toda ajuda e apoio durante o ano. À Professora Doutora Fátima Paiva-Martins, coorientadora deste projeto, queria agradecer por todo o apoio e disponibilidade total demonstrados ao longo deste último ano. Por toda a dedicação e paciência que apresentou para que o trabalho corresse da melhor forma e fossem obtidos os melhores resultados possíveis, um enorme obrigado.

Aos meus colegas de laboratório agradeço pelo apoio e pela força que me deram especialmente nos momentos mais difíceis. Agradeço à Doutora Marlene Costa pela ajuda que me forneceu, pelo apoio dado principalmente nos piores momentos, e por ter um auxílio permanente durante todo o ano. Agradeço também ao meu colega de bancada, Alfredo Esteves pelo companheirismo e por me fazer rir mesmo quando estava chateada, e por ter feito companhia nas horas mais tardias.

Ao doutor Ivo Dias agradeço pela paciência e pela ajuda que me deu quando mais precisei durante estes últimos meses. À mestre Sara Fernandes, obrigada pela dedicação para me fornecer resultados a tempo, e pelo stress passado.

Aos meus amigos, pela paciência compreensão que tiveram durante este ano. Um especial agradecimento à Ana Cláudia por me ter feito pensar em algo mais além da tese. Também lhe agradeço por me ter ajudado a espairecer. À Joana Ferreira por ter lido e relido a minha tese e por me ter ajudado na escrita. Pelas horas ao telefone, e por continuar a manter a esperança de que tudo é possível. À Ana Filipa agradeço pela paciência que teve por não estar sempre presente nos eventos e por ter tido sempre uma palavra amiga a me dar quando estava mais stressada.

Aos meus colegas de mestrado, agradeço por terem sido tão acolhedores durante o ano que estive em Braga, e por me terem feito sentir em casa.

À minha família, em especial à minha mãe que sempre me motivou e sempre acreditou que eu ia conseguir o mundo. Por ter me feito companhia nos dias de escrita e me ter apoiado todos os dias. Ao meu querido avô que sempre teve um orgulho enorme por tudo o que nós fazemos ao longo da nossa vida. À minha irmã que mais do que ninguém, teve sempre fé no que

eu fiz e sempre acreditou que este dia chegaria. À minha avó que foi a luz que guiou todo o meu percurso e que era a pessoa que mais queria ver este dia chegar. Obrigada por seres a minha estrelinha.

Agradeço a todos os voluntários a colaboração e a disponibilidade para colher um pouco de sangue, pois só assim foi possível realizar o estudo da hemólise. Agradeço à Universidade do Minho e à Faculdade de Ciências da Universidade do Porto, por permitirem que tudo isto se tenha concretizado.

O trabalho foi apoiado pela UID / QUI / 50006/2019 com financiamento da FCT / MCTES através de fundos nacionais e pelo PTDC / OCE-ETA / 32492/2017 - POCI-01-0145-FEDER-032492 – PHENOLIVA.

STATEMENT OF INTEGRITY

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Resumo

Estudos de biodisponibilidade têm demonstrado que os principais polifenóis encontrados no azeite, nomeadamente o hidroxitirosol e os seus ésteres, são eficientemente absorvidos no intestino delgado. Deste modo, os potenciais benefícios do hidroxitirosol e dos seus derivados para a saúde podem ser atribuídos tanto aos compostos parentais como aos seus metabolitos de fase I e de fase II. O crescente interesse na bioatividade de polifenóis naturais e dos seus metabolitos requer que estes existam puros para poderem ser usados em ensaios biológicos e como padrões em protocolos de investigação. Assim, pretendeu-se efetuar a síntese de alguns metabolitos sulfatados de polifenóis naturais provenientes do azeite e estudar a capacidade de proteção destes compostos contra a hemólise oxidativa.

Os eritrócitos estão particularmente expostos ao stress oxidativo, ocorrendo lesões na sua estrutura, uma vez que estes por não terem organelos têm mecanismos limitados de reparação e de biossíntese. Quando a hemoglobina é liberta dos eritrócitos, esta é particularmente perigosa para todos os componentes do sangue e têm um papel importante em muitas patologias, nomeadamente nas anemias hemolíticas.

Este trabalho teve como objetivo estudar o efeito protetor de metabolitos sulfatados do tirosol, do hidroxitorosol e do álcool homovanílico contra o stress oxidativo em eritrócitos. Para esse efeito foram sintetizados, com rendimentos acima dos 40%, alguns metabolitos sulfatados destes compostos, nomeadamente do hidroxitirosol (1-*O*-sulfato de hidroxitirosol; a mistura de 3-*O* e 4-*O*-sulfato de hidroxitirosol), do tirosol (4-*O*-sulfato de tirosol), do álcool homovanílico (4-*O*-sulfato de álcool homovanílico), do acetato de hidroxitirosol (misturas de 3-*O* e 4-*O*-sulfato de acetato de hidroxitirosol), do acetato de tirosol (4-*O*-sulfato de acetato de tirosol) e do acetato homovanílico (4-*O*-sulfato de acetato homovanílico). A capacidade de proteção destes compostos contra a hemólise oxidativa provocada pelo AAPH foi determinada. Também se tentou fazer a síntese enzimática do secoiridóide 4-HPEA-EDA e a síntese de sulfatação do secoiridóide 3,4-DHPEA-EDA.

Palavras-chave: AAPH; álcool homovanílico; azeite; eritrócitos; hidroxitirosol; metabolitos sulfatados; polifenóis; secoiridóide; stress oxidativo; tirosol.

Abstract

Bioavailability studies have shown that the main polyphenols found in olive oil, namely hydroxytyrosol and its esters, are efficiently absorbed in the small intestine. Therefore, the potential health benefits of hydroxytyrosol and its derivatives may be attributed to both the parental compounds and to their phase I and phase II metabolites. The growing interest in the bioactivity of natural polyphenols and of their metabolites requires pure metabolites to be used in bioassays and as standards in research protocols. Thus, we aimed to synthesize some sulfated metabolites of natural polyphenols from olive oil and to study their protection against oxidative haemolysis.

Red blood cells (RBC) are particularly exposed to oxidative damage, and damage to their structure occurs, as they lack organelles and have limited repair and biosynthesis mechanisms. Whenever hemoglobin is released from RBCs, it is potentially dangerous for all components of the blood and plays an important role in many pathologies, When hemoglobin is released from erythrocytes, it is particularly dangerous and plays an important role in many pathologies, notably hemolytic anemias.

This work aimed to study the protective effect of sulfated metabolites of tyrosol, hydroxytorosol and homovanillyl alcohol against oxidative stress in erythrocytes. For this purpose, some sulfated metabolites of these compounds were synthesized in yields above 40%, namely hydroxytyrosol (hydroxytyrosol 1-*O*-sulfate; the mixture of hydroxytyrosol 3-*O* and 4-*O*-sulfate), tyrosol (tyrosol 4-*O*-sulfate), homovanillyl alcohol (homovanillyl alcohol 4-*O*-sulfate), hydroxytyrosol acetate (mixtures of hydroxytyrosol acetate 3-*O* and 4-*O*-sulfate), tyrosol acetate (tyrosol acetate 4-*O*-sulfate) and homovanillyl acetate (homovanillyl acetate 4-*O*-sulfate).The ability of these compounds to protect against oxidative haemolysis caused by AAPH was determined. Enzymatic synthesis of secoiridoid 4-HPEA-EDA and sulfation synthesis of secoiridoid 3,4-DHPEA-EDA were also attempted.

Keywords: AAPH; erythrocytes; hidroxytyrosol; homovanillic alcohol; olive oil; oxidative stress; polyphenols; secoiridoid; sulfates metabolites; tyrosol.

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List of Abbreviations

ACT – *O*-Acetyltransferase

AD – Alzheimer's Disease

ADP – Adenosine Diphosphate

APPH – 2,2'-Azobis(2-amidinopropane) dihydrochloride

APS – Adenosine 5'-phosphosulfate

APT – Attached Proton Test

ATP – Adenosine triphosphate

CHD – Coronary heart disease

13C-NMR – Carbon nuclear magnetic resonance

COMT – Catechol methyl transferase

COSY – Homonuclear correlation spectroscopy

DEA – Diethylamine

3,4-DHPEA-EDA – 2-(3,4-Dihydroxyphenyl)ethyl (4*Z*)-4-formyl-3-(2-oxoethyl) hex-4-enoate

3,4-DHPEA-EA – Methyl (*E*)-4-(2-(3,4-dihydroxyphenethoxy)-2-oxoethyl)-3-ethylidene-2-hydroxy-3,4-dihydro-2*H*-pyran-5-carboxylate

DMC – Dimethyl carbonate

DNA – Deoxyribonucleic acid

DOPAC – 3,4-Dihydroxyphenylacetic acid

DOPAC-4'*O*-sulfate – 3,4-Dihydroxyphenylacetic acid 4'*O*-sulfate

DOPAC-3'O-sulfate – 3,4-Dihydroxyphenylacetic acid 3'O-sulfate

EVOO – Extra virgin olive oil

Hb – Haemoglobin

HMBC – Heteronuclear multiple bond correlation

^{1}H NMR – Proton nuclear magnetic resonance

4-HPEA-EDA – 2-(4-Hydroxyphenethyl)-(*Z*)-4-formyl-3-(2-oxoethyl)hex-4-enoate

HSQC – Heteronuclear single quantum correlation

Ht – Haematocrit

HVA – 2-(4-Hydroxy-3-methoxyphenyl)ethanol or homovanillyl alcohol

HVAAc – 2-(4-Hydroxy-3-methoxyphenyl)ethyl ethanoate or homovanillyl Acetate

HVAc – Homovanillyl acid

HVA-GlcA – Homovanillyl glucuronide

HVA sulfate – Homovanillyl sulfate

HVAAc sulfate – Homovanillyl acetate sulfate

HVAc sulfate – Homovanillyl acid sulfate

HT – 2-(3,4-Dihydroxyphenyl)etanol, 3,4-DHPEA or hydroxytyrosol

HTAc – 2-(3,4-diHydroxyphenyl)ethyl ethanoate or hydroxytyrosol acetate

HT-3'*O*-GlcA – Hydroxytyrosol 3'*O*-glucuronide

HT-4'*O*-GlcA – Hydroxytyrosol 4'*O*-glucuronide

HT-3'*O*-sulfate – Hydroxytyrosol 3'*O*-sulfate

HT-4'*O*-sulfate – Hydroxytyrosol 4'*O*-sulfate

HTAc-3'*O*-sulfate – Hydroxytyrosol acetate 3'*O*-sulfate

HTAc-4'O-sulfate – Hydroxytyrosol acetate 4'O-sulfate

LDL – Low-density lipoproteins

metHb – Methaemoglobin

NMR – Nuclear magnetic resonance

PAPS – 3'-Phosphoadenosine 5'-phosphosulfate

PBS – Phosphate-buffered saline

RBC – Red blood cells

ROS – Reactive species of oxygen

Sen – Sensitizers

^{1}Sen* – Excited Singlet State

ST – Sulfotransferase

TLC – Thin layer chromatography

Ty – 2-(4-Hydroxyphenyl)ethanol, 4-HPEA or tyrosol

UGT – Glucuronosyltransferase

UHPLC – Ultra-high efficiency liquid chromatography

UV – Ultra-violet

UV-Vis – Visible ultraviolet spectrophotometry

VOO – Virgin olive oil

I. Introduction

1. *Olea europaea* L.

The olive, *Olea europaea* L. (figure 1) is a small tree belonging to the family Oleaceae. *Olea europaea* is native to dry, subtropical climate, existing between latitudes 30° and 45°, and it is one of the oldest known cultivated plants.[1] Since ancient times, Mediterranean countries have cultivated *Olea europaea* to produce both oil and medicinal compounds. The olive tree is commercially essential in the Mediterranean region as source for the production of olive oil.[2-3] In the last decades, the positive effects of olive biophenols on human health have been scientifically demonstrated on several occasions.

The Oleaceae family, or the family of dicotyledons includes 30 genera or deciduous trees and shrubs including the olive tree and its relatives, numbering about 600 species. The genus *Olea* is comprised of 30 species but *Olea europaea* L. is the most popular member of the genus.[4]

Figure 1: Olive tree (*Olea europaea* L.).

1.1. Olive oil

Olive oil extractions is one of the most traditional agricultural industries in the Mediterranean region and is still of primary importance for the economy of most of the Mediterranean countries.[3, 5-6]

Olives are not used as a natural fruit because of their extremely bitter taste but are preferentially consumed either as olive oil or as table olives.[4] Olive oil is a transparent, yellowish,

and aromatic liquid extracted from the olive by simple pressure. It is a vegetal fat that is obtained from the fruit, whereas most of the edible oils are extracted from seeds. It is the only one consumed without refining.[7]

Figure 2: Olives, fruit from olive trees.

To manufacture olive oil, the fruits of the olive tree are crushed to create a pomace, which is homogenised and pressed to produce oil. The first oil extracted is the high quality extra virgin olive oil (EVOO), which is believed to exert biological beneficial effects through its antioxidant components.[8] Further extractions produce virgin or pomace olive oil. Virgin olive oil (VOO) is produced by direct pressing of olives. When the acidity of VOO exceeds 3.0 degrees, this oil is submitted to a refining process in which some components, mainly phenolic compounds and to a lesser degree squalenes are lost. By mixing virgin and refined olive oil, an ordinary olive oil is produced. After virgin olive oil production, the rest of the olive drupe and seed is processed, resulting in pomace olive oil, to which after refining, a certain quantity of virgin olive oil is added before marketing.[7-8]

1.2. Composition of virgin olive oil

VOO chemical composition consists of major and minor components. The major components make up the saponificable fraction which comprises 98-99 % of the total weight of the oil and is formed mainly by triacylglycerides (TAG). Oleic acid (18:1) is the main fatty acid found esterified (68-81,5 %) in these TAG, accounting for a greater portion than the other acids such as

palmitic (16:0), stearic (18:0), linoleic (18:2) and α-linolenic (18:3).[9-10] The minor components of VOO are classified into two types: the unsaponificable fraction, defined as the fraction extracted with solvents after saponification of the oil, and the soluble fraction, which includes the phenolic compounds. These minor compounds represent only around 2% of the total weight of the oil, although it includes more than 230 different chemical compounds.[7, 11, 9, 12-14]

The chemical composition of olives varies depending on several factors such as the cultivars, proportion of branches on the tree, storage conditions, climatic conditions and moisture content. There are also significant changes in the phenolic composition of fruits and leaves during the maturation period.[2] Therefore, it is expected to obtain different chemical compositions of oils obtained from different drupes.

2. Lipid Oxidation

Lipids are susceptible to oxidation in the presence of catalytic systems such as light, heat, enzymes, among other factors. This leads to complex processes of autoxidation, photooxidation or enzymatic oxidation, most of which involve free radicals and/or other reactive species as intermediates.[15] The oxidation products have an unpleasant flavour and odour and may adversely affect the nutritional and functional value of the oil.[16] Olive oil is quite resistant to autoxidation when compared with most edible oils, because of its low content of polyunsaturated fatty acids and presence of natural antioxidants. However, it is very sensitive to photooxidation, because of the presence of chlorophylls that act as photosensitizers.[1]

Oxidation normally proceeds slowly at the initial stage and then a sudden rise in the oxidation rate occurs. The main factors that affect oxidation processes are oxygen concentration, temperature and light.[17]

2.1. Autoxidation

The reaction of oxygen with unsaturated fatty acids constitutes the major means by which fatty substances undergo deterioration. Oxidation is frequently characterized as autoxidation because the rate of oxidation increases as the reaction proceeds.[1]

Lipid autoxidation occurs via a free radical chain mechanism that proceeds through three distinct stages, namely, initiation, propagation and termination, leading to a series of complex chemical changes (figure 3).

Unsaturated lipid molecules (RH), in the presence of initiators such as heat, light/ionizing radiation and metal ions/metalloproteins loose a hydrogen atom and produce free radicals (R•). The lipid radicals, formed at the initiation stage, then react with oxygen to form peroxyl radicals (ROO•). These act as the chain carriers of the rapid processing reaction by attacking a new lipid molecule, to produce the major initial reaction products of oxidation, hydroperoxides (ROOH). In this step, oxidation process is becoming more complicated since the peroxides formed, being unstable, are easily decomposed and form more free radicals that in turn participate in new reactions. This reaction may be repeated for several times during propagation before a hydrogen source is available or the chain is interrupted, for example by antioxidants.

Indeed, during propagation, lipid hydroperoxides are produced as primary products of oxidation. They are unstable and break down to form a wide range of secondary oxidation products, including, among others, aldehydes, ketones, alcohols, hydrocarbons, volatile organic acids and epoxy compounds, among others, some of which have undesirable odours with very low threshold values. Therefore, oxidation-related rancidity is generally related to the secondary oxidation products. In the meantime, alkoyl (RO•), peroxyl (ROO•), hydroxyl (HO•) and new lipid radicals (R•) are generated from decomposition of hydroperoxides, and further participate in the chain reaction of free radicals.

In the termination stage of oxidation, radicals neutralize each other through radical–radical coupling or radical–radical disproportionation to form stable non-radical products, including a variety of polymer products.[1, 15-16]

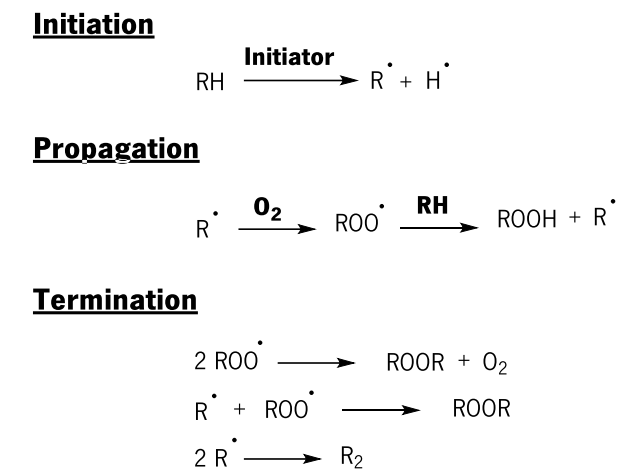


Figure 3: Lipid autoxidation pathways (adapted from Frankel [18]).

2.2. Photooxidation

Photooxidation causes serious deterioration of olive oil, due to the presence of chlorophylls and pheophytins. These pigments act as prooxidants under light and as antioxidants in the dark.

In addition to the normal or ground state (triplet) oxygen in autoxidation, the excited state (singlet) oxygen can be involved in lipid oxidation by directly reacting with the double bonds of unsaturated fatty acids through non-radical pathways, as commonly encountered in photooxidation. Singlet oxygen ($^1O_2$) is an excited stage of oxygen, which can be generated from the triplet state of oxygen by chemical, photochemical and enzymatic means, as well as by decomposition of hydroperoxides. Singlet oxygen is a major reactive species (ROS) that readily participates in the oxidation process (1500 times faster than triplet oxygen).[15]

Type I photooxidation proceeds via a free radical or free radical ion route, leading to the formation of hydroperoxides, whereas in type II photooxidation, the highly electrophilic singlet oxygen reacts directly with the double bonds of unsaturated fatty acids by addition rather than through free radical intermediates. Hydroperoxides are generated during singlet oxygen attack, accompanied by a shift of the double bonds in the molecule.[1, 15]

Type I

$$Sen \xrightarrow{h\nu} {}^1Sen^* \longrightarrow {}^3Sen^*$$

$${}^3Sen^* \xrightarrow{{}^3O_2} {}^1O_2{}^* + {}^1Sen$$

$${}^1O_2{}^* + RH \longrightarrow ROOH$$

Type II

$${}^3Sen^{*1} + {}^3O_2 \longrightarrow {}^1[Sen - O_2]$$

$${}^1[Sen - O_2] + RH \longrightarrow ROOH + {}^1Sen$$

Figure 4: Mechanisms of photosensitized oxidation (adapted from Frankel [12]).

2.3. Antioxidants

Among the many methods employed for controlling lipid oxidation, the use of antioxidant is the most effective, convenient and economical. Antioxidants are also used in health-related areas for disease risk reduction and health promotion due to their ability to protect the body against oxidative damage.

Antioxidants are substances that when present at low concentrations compared to that at an oxidizable substrate, markedly delay or prevent their oxidation. Antioxidants that fit in this definition include, among others, free radical scavengers, singlet oxygen quenchers, inactivators of prooxidative enzymes.

Antioxidants are broadly classified by their mechanisms of action as primary or secondary antioxidants. Primary antioxidants inhibit the oxidation chain reaction by acting as hydrogen donors or free radical acceptors and generating more stable radicals. The inhibition reaction is considered to be in competition with the propagation step of lipid oxidation *via* a chain reaction. They mainly act as chain breakers by donating hydrogen to alkylperoxyl radicals formed during the propagation step of lipid oxidation. Subsequently they form a stable radical (A•) through the well-known reaction:

$$ROO^{\bullet} + AH \longrightarrow ROOH + A^{\bullet}$$

Secondary antioxidants prevent or retard oxidation by suppressing the oxidation promoters, including metal ions, singlet oxygen, pro-oxidative enzymes and other oxidants. Reducing agents

can reduce lipid peroxides and related oxidants through redox reactions and are also referred to as oxygen scavengers.[15]

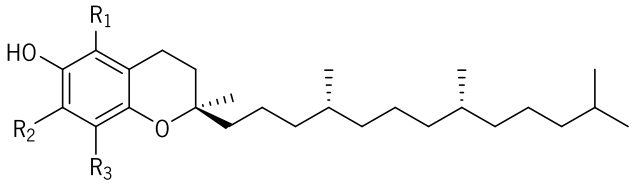
Antioxidants have received particular attention because of their potential to modulate oxidative stress associated with chronic disease. The lower incidence of coronary heart disease and of some cancers in the Mediterranean area led to the hypothesis that a diet rich in fruit, vegetables and grains has beneficial effects on health. The major fat component of the so-called "Mediterranean diet" is VOO, as has been stated previously. Many studies suggest that components of the minor fraction, such as, phenolic compounds in particular *ortho*-diphenols, may contribute to the healthy nature of this diet.[17, 19-23]

The antioxidant properties of *ortho*-diphenols can be related to hydrogen donation, i.e., their ability to improve radical stability by forming an intramolecular hydrogen bond between the free hydrogen of their hydroxyl group and their phenoxyl radical.[6, 22]

2.4. Olive oil phenolic compounds

Phenolic compounds present in VOO can be classified into a lipophilic group, like tocopherol, or a hydrophilic group, such as, phenolic acids and alcohols, flavonoids, secoiridoids and lignans.[24]

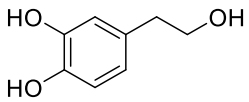
With respect to tocopherols (figure 5), α-tocopherol is the main tocopherol found in olive oil. β- and γ-tocopherol are only present in minor amounts. In virgin olive oils, they contribute together with polyphenols to virgin olive oil stability. Apart from their action as lipid radical scavengers, they also inhibit photooxidation by reacting with singlet oxygen. Thus, they contribute to an increase in the oxidative stability of oils during storage in the presence of light.[17] Furthermore, tocopherols, known as vitamin E, are considered one of the most important antioxidant *in vivo*.



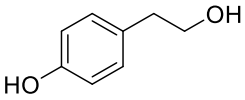
R_1	R_2	R_3	
CH_3	CH_3	CH_3	**α-tocopherol**
CH_3	H	CH_3	**β-tocopherol**
H	CH_3	CH_3	**γ-tocopherol**

Figure 5: α-, β- and γ-Tocopherol.

2-(3,4-diHydroxyphenyl)ethanol (3,4-DHPEA, hydroxytyrosol or HT), and 2-(4-hydroxyphenyl)ethanol (4-HPEA, tyrosol or Ty) are the most abundant phenolic alcohols in olive oil (figure 6).



Hydroxytyrosol



Tyrosol

Figure 6: Structures of phenolic alcohols present in olive oil.

The flavonoids include flavonol glycosides such as luteolin-7-glucoside and rutin as well as anthocyanins, cyanidun and delphinidin glycosides.[11-12, 25, 26] However, this class of phenols is found usually in very low concentrations in olive oil.

The secoiridoids are present in the olive fruit and are released into the oil during the mechanical extraction process. Secoiridoids are present exclusively in plants belonging to the family of Oleaceae that includes *Olea europaea* L. The olive drupe contains high concentrations of phenolic compounds being oleuropein, demethyloleuropein, ligstroside and nüzhenide the most abundant secoiridoid glucosides in olive fruits (figure 7).[27]

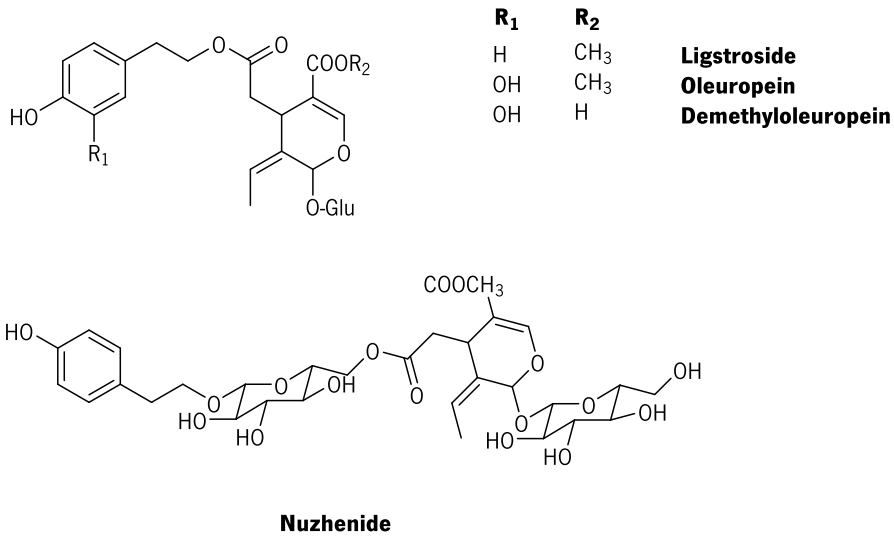


Figure 7: Structure of the major secoiridoids found in olive fruit.

However, the most abundant secoiridoids in VOO are the dialdehydic form of elenolic acid linked to hydroxytyrosol or tyrosol (3,4-DHPEA-EDA or 4-HPEA-EDA) and an isomer of the oleuropein aglycon (3,4-DHPEA-EA) (figure 8).[2, 4, 7-10]

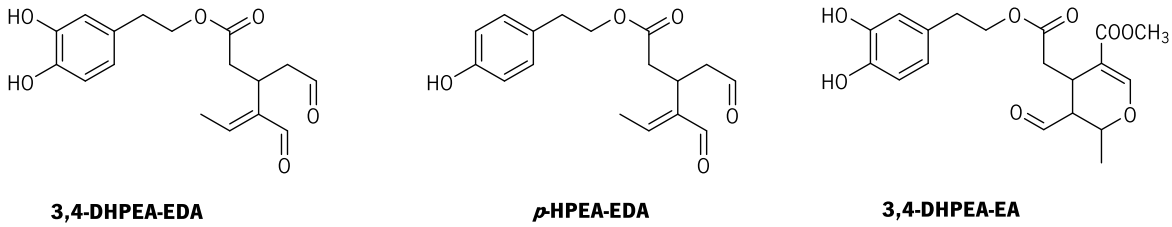


Figure 8: Structure of secoiridoids of VOO.

During olive oil extraction, specific glycosidases hydrolyse (figure 9) the glycosides, producing aglycones that are more lipophilic and therefore can be retained in the oil phase. After hydrolysis the secoiridoid aglicones undergo two main degradation pathways leading to secoiridoid derivatives (figure 9) or suffer further hydrolysis to HT, Ty and elenolic acid.[28-29]

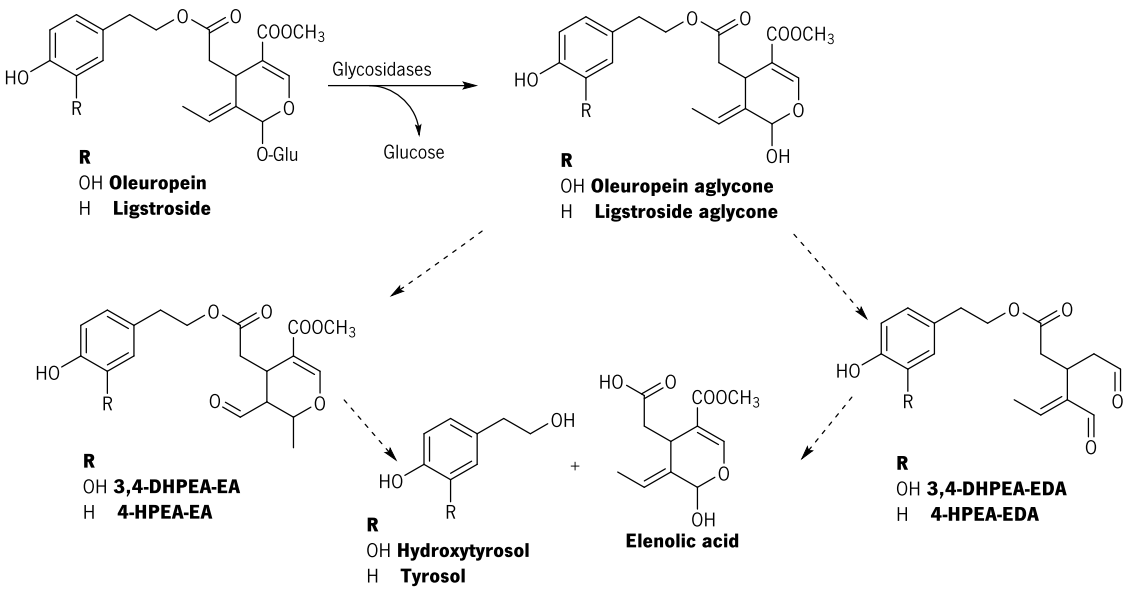


Figure 9: Structure of oleuropein and ligstroside aglycones and their derivatives, the major secoiridoids found in olive oil (adapted from Paiva-Martins & Kiritsakis [1]).

The amount of phenolic compounds in VOO is an important factor to bear in mind when the quality of virgin olive oils is evaluated. The natural phenols not only improve its resistance to oxidation, but there are also responsible for its sharp bitter taste and pungency.[30-31]

3. Health effects of olive oil and olive oil polyphenols

The Mediterranean diet, in which olive oil is the major fat component, has been associated with a low incidence of coronary heart disease and certain cancers. The beneficial effects of olive oil could be linked to both its monounsaturated fatty acid and its antioxidant content.[32-34]

The antioxidant activity of Ty and HT has received an increasing attention in the last years since it has been related to the protection against important chronic and degenerative diseases, such as, coronary heart diseases (CHD), ageing neuro-degenerative diseases and tumours at different localizations. They may also have antimicrobial, antidiabetic or anti-inflammatory properties.[8, 12, 35-38]

Atherosclerosis is a multifactorial disease that represents the primary cause of death worldwide. Elevated levels of circulating low-density lipoproteins (LDL), which are rich in cholesterol

and cholesterol esters, are a well-established risk factor for developing CHD.[39] The uptake of LDL by cells of the arterial wall – monocytes/macrophages – is generally mediated by a self-regulating, receptor, which limits cholesterol deposition when the required intracellular levels are reached. The discovery of a macrophagic "scavenger" receptor, which internalizes oxidatively modified forms of LDL (oxLDL) and is not down-regulated by increasing intracellular cholesterol levels, led to the hypothesis that oxLDLs play a crucial role in the onset of the atherosclerotic lesion. This can be assigned to the fact that their uptake causes infiltration and deposition of cholesterol loaded cells (foam cells) into the arterial wall.[22, 40]

In recent years, there has been much interest in antioxidants that retard oxidative modification of LDL. The LDL isolated from animals and humans that have been fed with VOO has shown increased stability, when compared with that of animals and humans fed with refined olive oil. This can be attributed to the phenolic compounds found in the oil.[21, 40-41]

Reactive oxygen species (ROS) responsible for oxidative stress are involved in the all above mentioned diseases through mechanisms that in part have been elucidated. ROS in fact, oxidize lipoproteins deposited on the arteries, leading to arteriosclerosis and, with regard to the carcinogenic process, they are able to produce DNA oxidative damage.[12, 40] On the other hand, some studies have shown that olive oil phenolic compounds reduce oxidative damage to both red blood cells and renal cells.[40]

Recently, *p*–HPEA–EDA has been demonstrated to have potential neuroprotective properties and contributes to preventing cognitive decline due to neurogenerative diseases. Different mechanisms have been proposed to describe the role that this compound plays in reducing the incidence of Alzheimer's disease (AD), a neurodegenerative disease that affects about 30 million people worldwide.[34]

4. Bioavailability of olive oil phenolic compounds

The biological properties of phenolic compounds present in olive oil depend on their bioavailability, this is their absorption rate and their metabolism. In fact, the potential health

benefits of olive polyphenols may be attributed to both parental compounds and their phase I and phase II metabolites.

Metabolization usually converts a molecule capable of crossing biological membranes into one more hydrophilic that can be excreted by urine. The metabolism is usually divided in two distinct phases: phase I and phase II, where each of these phases entails different transformations to the molecule that will be metabolized. Throughout the metabolism phases, the polarity of the molecules usually increases (and the lipophilicity decrease), thus determining whether this molecule goes to the kidneys in order to be excreted by the urine or proceeds to a next metabolic phase, which may enable or disable the bioactivity of the molecule. The metabolization products of a given phenol depend on its initial chemical structure.[42-45]

As stated before, there have been a large number of studies investigating the *in vitro* antioxidant properties of VOO phenol, as well as their protective effects against cell injury. The biological properties of these phenols *in vivo* depend on the extent to which they are absorbed and metabolized. Olive oil polyphenols undergo extensive metabolism in the intestines and liver and are thus mainly found in biological fluids as phase II metabolites.[33, 46-47]

A human study conducted in normal and ileostomy subjects fed with olive oil polyphenols has indicated that the major site for the absorption of olive oil polyphenols is the small intestine.[48] Following ingestion of VOO, the levels of HT and Ty increase rapidly achieving a peak concentration after 1 h in plasma and around 2 h in urine. These results support the evidence that the small intestine is the major site of absorption for these compounds. The analysis of human urine has also demonstrated that both HT and Ty are dose-dependently absorbed and are metabolized primarily to *O*-glucuronidated conjugates in the intestine. HT also undergoes *O*-methylation by the action of catechol-*O*-methyl-transferase (COMT), and homovanillyl alcohol has been detected in human and animal plasma and urine after oral administration of either VOO or pure HT and Ty.[32-33, 39, 46, 49-52]

The bioavailability of secoiridoids 3,4-DHPEA-EDA and 3,4-DHPEA-EA was studied and showed that both compounds are efficiently absorbed by Caco-2 cells and by rat intestine. The secoiridoids are hydrolyzed with production of hydroxytyrosol and its metabolites, as shown in figure 10.

In a study with the aim of identifying biomarkers for olive oil consumption after 3 weeks dietary intervention with phenol-enriched olive oils as part of a randomized, double-blind, crossover, and controlled nutrition intervention trial, it was found that hydroxytyrosol sulfate (compounds 3/4) and hydroxytyrosol acetate sulfate (compounds 5/6) and not glucoronides were the metabolites most suitable for monitoring EVOO intake.[47]

Figure 10 shows the metabolic pathways of secoiridoids and hydroxytyrosol leading to sulfate metabolites. A similar sequence can be drawn for glucuronide metabolites.

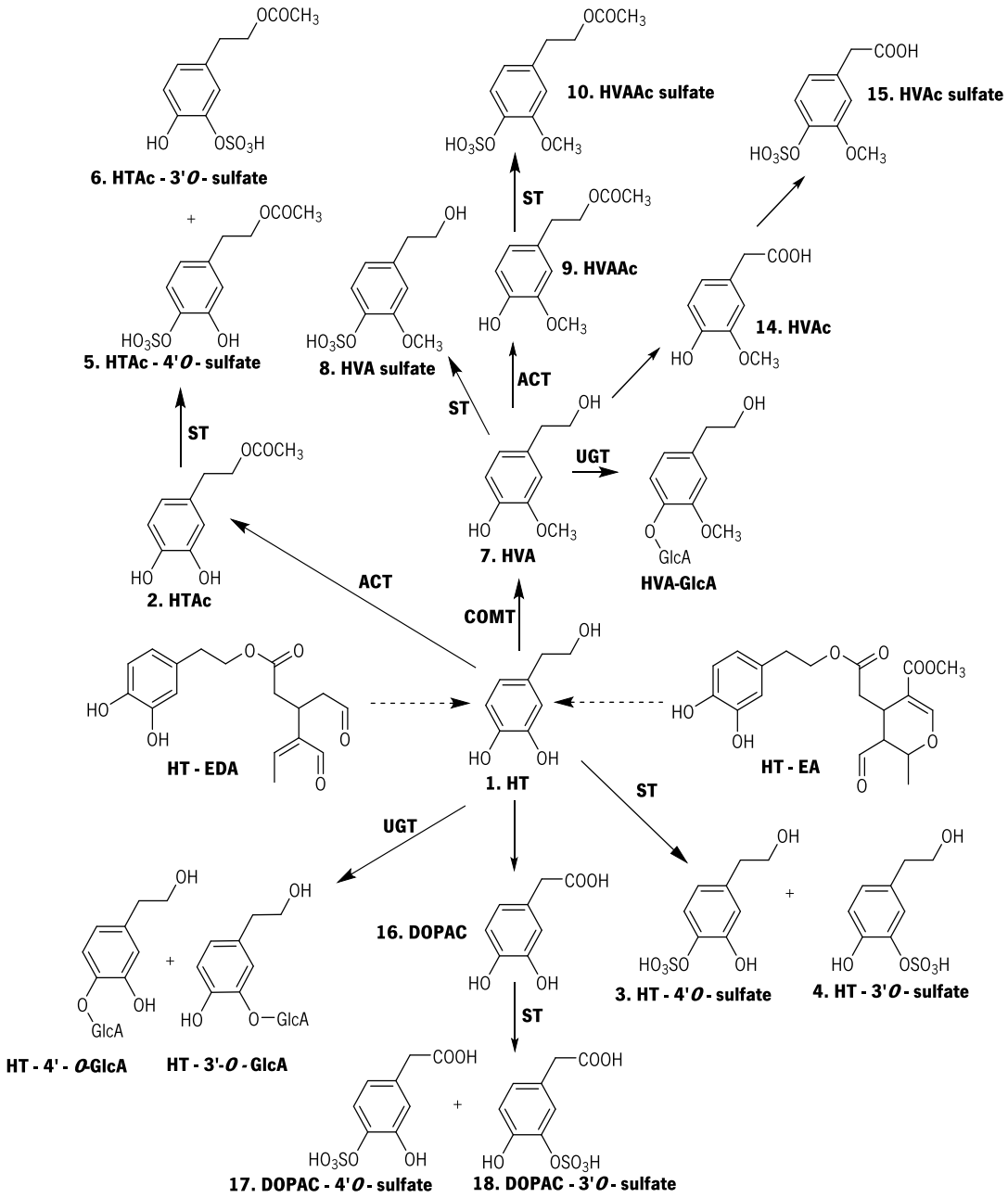


Figure 10: Metabolic pathways of HT found in olive oil (adapted from Gomes et al. [47]).

5. Red Blood Cell

Human red blood cells (RBC) are the most abundant blood cells and are in intime contact with metabolites and every xenobiotic. These cells enable the transport of sufficient O_2 between respiratory surfaces (lungs, gills) and metabolizing tissues, due to their high intracellular

concentration of haemoglobin (Hb) and the appropriate allosteric interaction between ligands (O_2, CO_2, H^+) and the binding sites in the Hb molecule.[53-56]

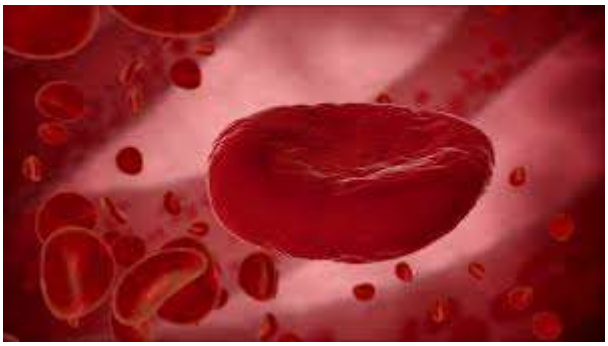


Figure 11: Human red blood cell.

Oxidative damage to cell components has been established as a factor in various pathological conditions. In circulation, RBCs are continuously exposed to both endogenous and exogenous sources of ROS that can damage them and impair their function.[54, 57-58] ROS are highly reactive, and many of them are released from neutrophils and macrophages into the plasma.[55-56, 59] Upon entry into the RBC cytoplasm, ROS are usually neutralized by the cytosolic antioxidant system. In fact, hydrogen peroxide added to RBCs rapidly reacts with catalase being converted to oxygen without any oxidation of Hb. In addition, endogenous ROS are continuously generated by the slow autoxidation of Hb, which produces methemoglobin (metHb) and superoxide anion (O_2^-) which rapidly dismutates to form hydrogen peroxide (H_2O_2).[54] In the presence of reduced metal ions, particularly iron, these compounds in turn form the highly reactive hydroxyl radical, which can damage both plasma membrane and cytosolic components, eventually leading to oxidative haemolysis.[54, 60] When haemoglobin is denatured, it links to the membrane at the cytoplasmic domain of band 3 protein, inducing its aggregation and the linkage of natural anti – band 3 antibodies and complement fixation on the erythrocyte surface, marking the cell for removal by the macrophages of the reticuloendothelial system. Whenever the haemoglobin is released from erythrocytes, it is potentially dangerous because it can be converted into oxidized forms, which are powerful promoters of oxidative processes in blood.[21]

Under physiological conditions there is a steady-state balance between the production of ROS and their destruction by the endogenous defence system, which include enzymatic and nonenzymatic antioxidants, such as vitamin E.[61] However, if ROS are overproduced or if the

endogenous defenses are impaired, the situation referred to as "oxidative stress" will result.[19, 21, 41, 54, 60]

A certain amount of oxidative stress is useful to the body for growth and cell signalling, but excess levels have deleterious effects on cell components including proteins, lipids and nucleic acids and alter the redox status of the cell.[55]

The use of antioxidants as preventive and/or as therapeutic agents in oxidative stress related diseases has generally been proposed. Consequently, the consumption of natural antioxidants may be of extreme health importance. As stated before, it is believed that some of the olive oil phenolic compounds bioactivity, may be due to their capacity in reducing oxidative damage in cells.

6. Synthesis of sulfates

In vivo, the most common sulfo group donor is 3'-phosphoadenosine-5'-phosphosulfate (PAPS). In the case of a hydroxyl group as acceptor, the product is a sulfate.

PAPS synthesis (figure 12) requires a ready supply of sulfate, which is available from the catabolism of proteins and sugar sulfates. The activation of inorganic sulfate to form PAPS results from the concerted action of two enzyme systems which in animal species and is carried out by a bifunctional protein. The first step is catalysed by ATP sulfurylase and involves the reaction of inorganic sulfate with ATP to form adenosine 5'-phosphosulfate (APS) and inorganic phosphate. The second step is catalysed by APS kinase and involves the reaction of APS with another molecule of ATP to form PAPS and ADP. Unlike ATP sulfurylase, APS kinase is not involved in the activation of sulfate.

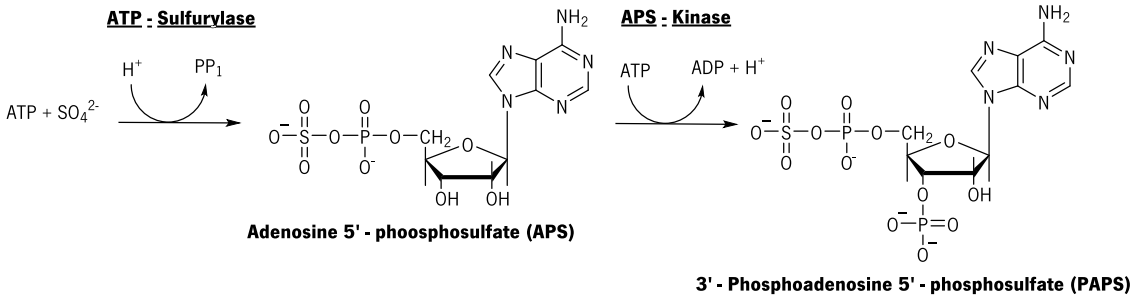


Figure 12: Catalytic reactions in the formation of PAPS (adapted from Srott [62]).

Sulfotransferases can be divided into two classes (cytosolic sulfotransferases and Golgi – associated sulfotransferases) based upon whether they are soluble or membrane – associated proteins. Cytosol sulfotransferases embodies an ever – enlarging superfamily of enzymes that catalyse the sulfonation of relatively small endobiotics and exobiotics. The biotransformation of endogenous substrates and xenobiotics by sulfonation is a major metabolic reaction that has two possible consequences, activation or inactivation of a biological effect. The other class, membrane – sulfotransferases are located in the *trans* – Golgi complex, where they are involved in the post – translational modification of macromolecules.[62]

Sulfation of xenobiotics is an important mechanism of removing potentially toxic agents. Metabolic sulfation of compounds occurs in the cytosol through the action of one of the sulfotransferases from PAPS, due to its capacity to donate an activated sulfonate group (SO_3) to an acceptor alcohol, phenol, or amine group. This introduces anionic character in the molecule, thereby enhancing its excretion properties, avoiding potential adverse effects. Therefore, phase II metabolites of the biotransformation of polyphenols are often sulfates.[63-68]

Sulfotransferases may be used for the *in vitro* synthesis of sulfate metabolites as an alternative to chemical synthesis. Enzymatic sulfation is more selective, but enzimes are difficult to obtain and expensive. Furthermore, the reaction yields obtained are generally lower than the ones obtained by chemical sulfations. Therefore, sulfated compounds are in general synthesised by the pharmaceutical/chemical industry using complexes of sulfur trioxide (SO_3) with tertiary amines, tertiary amides or pyridine (figure 13). [69] Some of the advantages in using these complexes are their solid nature at room temperature and their stability at high temperatures, being the most suitable for sulfation of the hydroxyl groups present in phenolic compounds. However, they can

promote hydrolysis reactions because they usually have an acidic character when dissolved in organic solvents.[64]

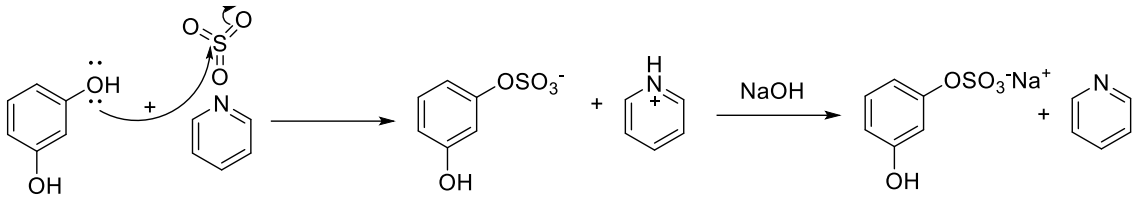


Figure 13: Reaction scheme for sulfation of hydroxyl groups of phenolic compounds by the sulphur trioxide complex method (adapted by Yan et al. [70]).

The main disadvantage is the difficulty in selective control of sulfation when several hydroxyl groups are present.

II. Aim

Many epidemiology studies show that the incidence of cardiovascular disease (CVD) in Mediterranean countries is low, suggesting a crucial protective role of diet in this area where virgin olive oil (VOO) is the principal source of fat. These studies also shown that VOO consumption could reduce oxidative damage in part due to the phenolic compounds, who have an important role against a range of pathologies associate to oxidative stress. Their antioxidant activity has shown a great impact on human health. However, the potential health benefits of these molecules in general may also be attributed to their metabolites.

The aim of this study is to understand how the properties of VOO phenols in vivo depends on the extent of their metabolism. A synthetic strategy is used in order to obtain VOO phenol metabolites in considerable yields in order to be possible to in their protective role against AAPH-induced oxidative stress in normal human RBCs.

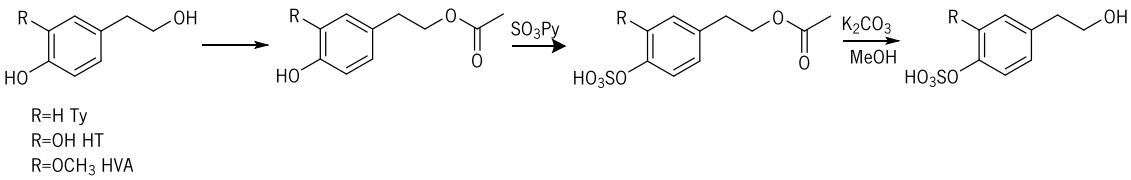


Figure 14: Proposal synyhesis of phenolic olive oil metabolites.

III. Materials and Methods

1. General methods

All reagents and solvents used in the synthetic procedures were at least pure quality. Solvents used in column chromatography were PA quality. When necessary, dry solvents were obtained by treatment with phosphorus pentoxide.

All reactions and purifications were monitored by thin layer chromatography (TLC) on precoated silica gel 60 plates F_{254}, and detected by:

- UV-Vis, at λ = 254 nm (lamp U.V.: CN-6 from Vilber Lourmal);
- Iodine;
- Mostain solution [100 mL H_2SO_4 10%; 5,0 g $(NH_4)_6Mo_7O_{24}.H_2O$; 0,22 g $Ce(SO_4)_2.4H_2O$].

Products were purified by flash chromatography through silica gel 60 (0,040-0,063 mm, Merck). NMR spectra were recorded on a 400 MHz equipment (Bruker Avance III 400) at room temperature for solutions in CD_3OD, CD_3COCD_3 and D_2O. Chemical shifts are referred to the solvent signal and are expressed in ppm. 2D NMR experiments (COSY, APT, HMBC and HMQC) were carried out when necessary to assign the corresponding signals.

The ultra-high performance liquid chromatography (UHPLC) system used was a Vanquish (Thermoscientific) chromatograph, with UV-Vis detector of the same model. Chromatography was carried out using a C18 LiChrospher ® column 100 RP-18, 5 μm (Merck) of dimensions 250 mm x 4 mm. The volume of injections was 10 μL and the mobile phase used was a mixture of water:acetic acid (99:1 v/v) (eluent A) and a mixture of methanol:acetonitrile (1:1 v/v) (eluent B) with a total analysis time of 70 min.

Table 1: Eluent gradient used in ultra-high performance liquid chromatography.

Time / min	Eluent A (%)	Eluent B (%)
Run		
0	95	5
25	70	30
35	65	35
40	60	40
50	30	70
55	0	100
70	0	100

2. Olive oil Phenolic Compounds

Hydroxytyrosol was purchased from Seprox Biotech, tyrosol was obtain from Acros Organics and homovanillyl alcohol was purchased from Sigma-Aldrich. Hydroxytyrosol acetate was obtained from hydroxytyrosol, by two different procedures. Tyrosol acetate and homovanillyl alcohol acetate were synthetized from tyrosol and homovanillyl alcohol, respectively by two different procedures.

The secoiridoids 3,4-DHPEA-EDA and 3,4-DHPEA-EA, were isolated from olive leaves according to a procedure described by Paiva-Martins.[71] An attempt to synthetize 4-HPEA-EDA was made using isolated 3,4-DHPEA-EDA as starting material.

2.1. Synthesis of 2-(3,4-dihydroxyphenyl)ethyl ethanoate (Hydroxytyrosol acetate)

Two different techniques were used to synthetize 2-(3,4-dihydroxyphenyl)ethyl ethanoate. Hydroxytyrosol was acetylated using acetyl chloride in dimethyl carbonate, following a procedure described by Bernini et al. [72], and by transesterification with ethyl acetate using Amberlite IR-120 as catalyst, based on a procedure from Begines et al. [73]

2.1.1. Synthesis of 2-(3,4-dihydroxyphenyl)ethyl ethanoate using acetyl chloride as acylating agent

The reaction of hydroxytyrysol with acetyl chloride in dimethyl carbonate (DMC), is shown in figure 15. The synthesis was done in a proportion of hydroxytyrosol to acetyl chloride of 1:1.2.

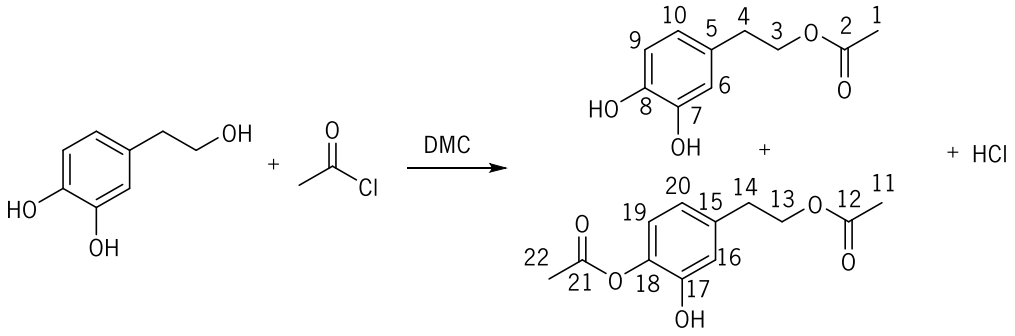


Figure 15: Synthesis of 2-(3,4-dihydroxyphenyl)ethyl ethanoate using acetyl chloride and dimethyl carbonate.

In a dry Schlenk tube, 0.50 g of hydroxytyrosol (3.24 mmol) was dissolved in 12 mL of DMC. Then, in an ice bath, 280 µL of acetyl chloride (3.93 mmol) was added dropwise. The mixture was left under argon, protected from light, with magnetic stirring for 2 days at room temperature. The reaction was monitored by TLC [ethyl acetate/petroleum ether (2:1)] and plates revealed by iodine.

The solvent was evaporated at reduced pressure and the crude mixture dissolved in 20 mL of ethyl acetate. The organic phase was washed with 20 mL of brine, and the aqueous phase was successively washed with ethyl acetate (4 x 20 mL). The combined organic layers were dried over anhydrous Na_2SO_4 and the solvent evaporated at reduced pressure to give a yellowish oil (0.38 g). The crude product was purified by flash chromatography [ethyl acetate/petroleum ether (2:1 v/v)] to give 0.33 g of a yellowish oil. ^{1}HNMR indicated that acetylation occurred both at the phenolic and the aliphatic hydroxyl groups, forming two products 2-(3,4-dihydroxyphenyl)ethyl ethanoate and 2-(4-acetoxy-3-hydroxyphenyl)ethyl ethanoate in a 1:8 ratio. Both compounds presented the same retention factor (rf = 0.27 [ethyl acetate/petroleum ether (2:1)]). ^{1}HNMR (400 MHz, CD_3OD)**:** δ = 6.91 (d, J = 8.0 Hz, 1H, 19 – H), 6.85 (dd, J = 8.0, J = 2.0 Hz, 20 – H), 6.82 (d, J = 2.0 Hz, 1H, 16 – H), 6.71 (d, J = 8.0 Hz, 1H, 9 – H), 6.69 (d, J = 2.0 Hz, 1H, 6 – H), 6.55 (dd, J =

8.0, J = 2.0 Hz, 1H, 10 – H), 4.24 (t, J= 6.8 Hz, 2H, 13 – H), 4.20 (t, J= 7.2 Hz, 2H, 3 – H), 2.85 (t, J= 6.8 Hz, 2H, 14 – H), 2.77 (t, J= 7.2 Hz, 2H, 4 – H), 2.28 (s, 3H, 22 – H), 2.02 (s, 3H, 11 – H), 2.01 (s, 3H, 11 – H) ppm.

2.1.2. Synthesis of 2-(3,4-dihydroxyphenyl)ethyl ethanoate by transesterification with ethyl acetate catalysed by amberlite IR – 120

The reaction between hydroxytyrosol and ethyl acetate catalysed by amberlite IR–120, is shown in figure 16.

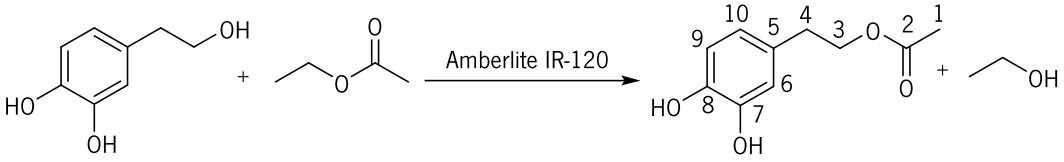


Figure 16: Synthesis of 2-(3,4-dihydroxyphenyl)ethyl ethanoate using amberlite IR-120 in ethyl acetate.

In a round bottom flask, 0.50 g of hydroxytyrosol (3.24 mmol) was dissolved in 25 mL of ethyl acetate. Then 1.05 g of amberlite IR-120 (3.37 mmol) was added and the mixture was left under reflux, protected from light and with magnetic stirring for 11 hours. The reaction was monitored by TLC [ethyl acetate/petroleum ether (2:1)] and the plates revealed by iodine.

The resin was filtered, and the solvent was evaporated at 30 °C. The crude extract (yellowish oil, 0.88 g) was purified by flash chromatography on silica gel [ethyl acetate/petroleum ether (2:1 v/v)], to give 0.63 g of a yellow compound corresponding to 98 % of yield. ^{1}HNMR (400 MHz, CD_3OD)**:** δ = 6.69 (d, J= 8.0 Hz, 1H, 9 – H), 6.66 (d, J= 2.0 Hz, 1H, 6 – H), 6.54 (dd, J= 8.0, J = 2.0 Hz, 1H, 10 – H), 4.19 (t, J= 7.2 Hz, 2H, 3 – H), 2.76 (t, J= 7.2 Hz, 2H, 4 – H), 2.01 (s, 3H, 1 – H) ppm.

2.2. Synthesis of 2-(4-hydroxyphenyl)ethyl ethanoate (Tyrosol acetate)

As for the synthesis of 2-(3,4-dihydroxyphenyl)ethyl ethanoate, two procedures to synthetize 2-(4-hydroxyphenyl)ethyl ethanoate were attempted. Tyrosol was acetylated using acetyl chloride in dry dichloromethane and by transesterification with ethyl acetate using amberlite IR-120 as catalyst.

2.2.1. Synthesis of 2-(4-hydroxyphenyl)ethyl ethanoate using acetyl chloride as acylating agent

The synthesis of 2-(4-hydroxyphenyl)ethyl ethanoate is shown is figure 17. Tyrosol reacts with acetyl chloride in dry dichloromethane. The synthesis was done in a proportion of tyrosol to acetyl chloride of 1:1.2.

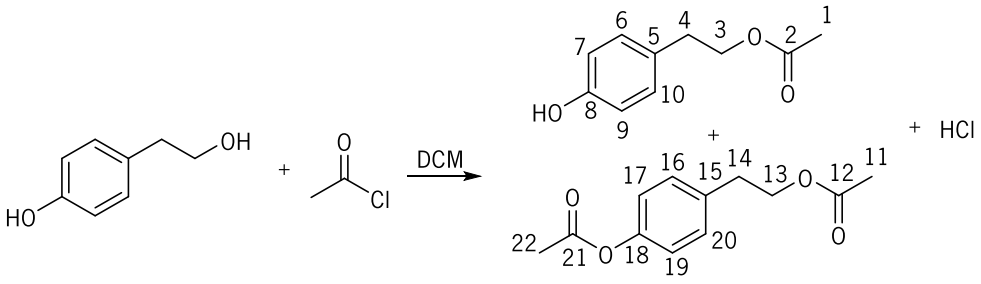


Figure 17: Synthesis of 2-(4-hydroxyphenyl)ethyl ethanoate using acetyl chloride in dry dichloromethane.

In a Schlenk tube, under argon 1.13 g of tyrosol (8.18 mmol) was dissolved in 15 mL of dry dichloromethane. Then, the mixture was cooled in an ice bath, and 700 µL of acetyl chloride (9.81 mmol) was added dropwise. The mixture was left stirring, under argon, protected from light, for 24 hours at room temperature. The reaction was monitored by TLC [ethyl acetate/petroleum ether (2:1)] and the plates were revealed by iodine.

The solvent was evaporated under vacuum, and 20 mL of ethyl acetate was added to the crude mixture. In a separatory funnel the organic layer was washed with 20 mL of brine and the aqueous phase was washed 3 times with 20 mL of ethyl acetate. The organic layer was extracted

and dried with Na_2SO_4 and ethyl acetate evaporated at reduced pressure, giving 1.19 g of whitish liquid. The crude residue obtained was purified by flash chromatography [ethyl acetate/petroleum ether (2:1 v/v)] to give 0.96 g of a white solid. ^{1}HNMR show that acetylation using acetyl chloride with dichloromethane occurred on both the aliphatic and the phenolic hydroxyl groups forming two products 2-(4-hydroxyphenyl)ethyl ethanoate and 2-(4-acetoxyphenyl)ethyl ethanoate in a 1:3 ratio. Both compounds presented the same retention factor (rf = 0.52 [ethyl acetate/petroleum ether (2:1)]). ^{1}HNMR (400 MHz, CD_3OD)**:** δ = 7.25 (d, J= 8.4 Hz, 2H, 16 and 17 – H), 7.02 (d, J= 8.4 Hz, 2H, 19 and 20 – H), 7.04 (d, J= 8.4 Hz, 2H, 6 and 10 – H), 6.72 (d, J= 8.4 Hz, 2H, 7 and 9 – H), 4.25 (t, J= 7.2 Hz, 2H, 13 – H), 4.19 (t, J= 7.2 Hz, 2H, 3 – H), 2.92 (t, J= 7.2 Hz, 2H, 14 – H), 2.81 (t, J= 7.2 Hz, 2H, 4 – H), 2.25 (s, 3H, 22 – H), 1.99 (s, 2H, 1 and 11 – H) ppm.

2.2.2. Synthesis of 2-(4-hydroxyphenyl)ethyl ethanoate by transesterification with ethyl acetate catalysed by amberlite IR – 120

Figure 18 shows the reaction of tyrosol with ethyl acetate using amberlite IR-120 as catalyst.

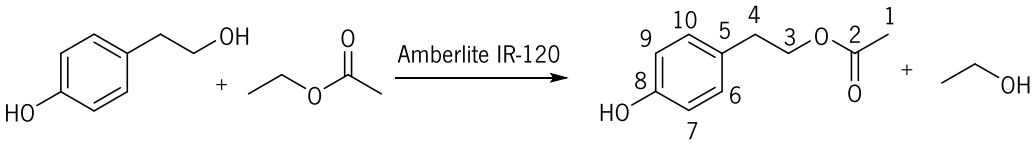


Figure 18: Synthesis of 2-(4-hydroxyphenyl)ethyl ethanoate by transesterification with ethyl acetate catalysed by amberlite IR–120.

In a round bottom flask, 1.01 g of tyrosol (7.31 mmol) was dissolved in 25 mL of ethyl acetate and 2.04 g of amberlite IR-120 (6.54 mmol) was added. The mixture was left under reflux, protected from light, for 11 hours with magnetic stirring. The reaction was monitored by TLC [ethyl acetate/petroleum ether (2:1)] and the plates were revealed by iodine. Then the resin was filtered under vacuum, and the solvent evaporated at 30 °C giving a yellowish oil (1.63 g).

Then the crude mixture was purified by flash chromatography [ethyl acetate/petroleum ether (2:1 v/v)] giving a 0.98 g of a white solid (75 % of yield). ^{1}HNMR (400 MHz, CD_3OD)**:** δ =

7.04 (d, *J* = 8.4 Hz, 2H, 6 and 10 – H), 6.72 (d, *J* = 8.4 Hz, 2H, 7 and 9 – H), 4.19 (t, *J* = 7.2 Hz, 2H, 3 – H), 2.82 (t, *J* = 7.2 Hz, 2H, 4 – H), 2.00 (s, 3H, 1 – H) ppm.

2.3. Synthesis of 2-(4-hydroxy-3-methoxyphenyl)ethyl ethanoate (homovanillyl acetate) by transesterification with ethyl acetate catalysed by amberlite IR – 120

The transesterification of homovanillyl alcohol was done using ethyl acetate and amberlite IR-120 as catalyst. Figure 19 shows the synthesis of 2-(4-hydroxy-3-methoxyphenyl)ethyl ethanoate.

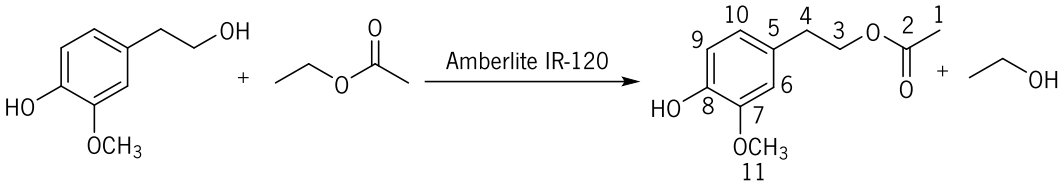


Figure 19: Synthesis of 2-(4-hydroxy-3-methoxyphenyl)ethyl ethanoate by transesterification with ethyl acetate catalysed by amberlite IR–120.

In a round bottom flask, 1.00 g of homovanillyl alcohol (5.95 mmol) was dissolved in 25 mL of ethyl acetate and 2.00 g of amberlite IR-120 (6.42 mmol) was added. The mixture was left under reflux, protected from light, for 11 hours with magnetic stirring. The reaction was monitored by TLC [ethyl acetate/petroleum ether (2:1)] and the plates were revealed by iodine. Then the resin was filtered under vacuum and the solvent evaporated at 30 °C.

The crude extract (pink oil, 1.94 g) was purified by flash chromatography [ethyl acetate/petroleum ether (2:1 v/v)], to give 1.01 g (80 % of yield) of a whitish compound (rf = 0.53). ^{1}HNMR (400 MHz, CD_3OD)**:** δ = 6.82 (d, *J* = 2.0 Hz, 1H, 6 – H), 6.76 (d, *J* = 8.0 Hz, 1H, 9 – H), 6.68 (dd, *J* = 8.0, *J* = 2.0 Hz, 1H, 10 – H), 4.23 (t, *J* = 7.2 Hz, 2H, 3 – H), 3.85 (s, 3H, 11 – H), 2.85 (t, *J* = 7.2 Hz, 2H, 4 – H), 2.02 (s, 3H, 1 – H) ppm.

2.4. Isolation of secoiridoids, 3,4-DHPEA-EDA and 3,4-DHPEA-EA

Fresh olive tree leaves (300 g), from Cobrançosa cultivars, harvested in October from the Trás-os-Montes region of Portugal, were stored in bags (12,5x12,5 cm). Bags were pressed manually to reduce the air inside before they were closed and kept at 37 °C in an oven protected from light for 24 hours.

Leave samples were macerated in 2 L of methanol for 5 days in the dark at room temperature. The extract was separated by filtration, and the solvent was evaporated under vacuum. The residue was taken up in 300 mL of acetone/water (1:1 v/v). The aqueous mixture was successively extracted with *n*-hexane (6 x 150 mL) followed by chloroform (6 x 150 mL). The chloroform extract was then dried by anhydrous Na_2SO_4, filtered and evaporated under reduced pressure at room temperature. After solvent evaporation, the extract (5,0 g) was purified 3 times by column chromatography using silica gel 60 eluted with diethyl ether/methanol (35:1). The solvent was evaporated, and the fractions containing the two products were dissolved in deuterated chloroform and identified by ^{1}H NMR.

To characterize 3,4-DHPEA-EDA e 3,4-DHPEA-EA compounds by UHPLC, two solutions with a concentration of 0.4 mM in methanol were prepared and 2 mL of each one was filtered to vails and injected in UHPLC.

2.5. Enzymatic synthesis of 4-HPEA-EDA

The reaction between 3,4-DHPEA-EDA and tyrosol catalysed by novozym 435 in THF to give 4-HPEA-EDA is shown in figure 20. The synthesis was done in a proportion of tyrosol to 3,4-DHPEA-EDA of 3:1.

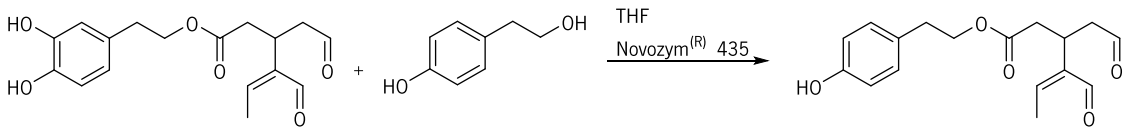


Figure 20: Synthesis of 4-HPEA-EDA from 3,4-DHPEA-EDA and tyrosol in THF.

In a hydrolysis tube, 0.16 g of tyrosol (1.16 mmol) and 0.12 g of 3,4-DHPEA-EDA (0.37 mmol) were dissolved in 4 mL of THF. Then, 0.20 g of novozym® 435 were added. The mixture was left for 1 month protected from light, under magnetic stirring at 37 °C. After some days, the temperature was increased to 50 °C for 2 days. The reaction was monitored by TLC [diethyl ether/methanol (35:1); dichloromethane/methanol (5:1)]. When TLC showed the formation of a new product and the decrease in the size of the spots of the initial reagents, the reaction mixture was filtered under vacuum, and the enzyme was washed with 10 mL of dichloromethane. Then, a solution of approximately 0.4 mM (in oleocanthal) in methanol was made and 1 mL was filtered into a vial and analyzed by UHPLC.

3. Synthesis of the metabolites of phenolic compounds of olive oil

The synthesis of the metabolites of phenolic compounds olive oil was performed according to the procedure described by Gomes et al.[47] Sulfation was carried out on the esters synthetized in last chapter using a sulfur trioxide-pyridine complex in a proportion of ester to complex of 1:2. In order to remove the ester group, the sulfated compounds, were subject to solvolysis with potassium carbonate, in a proportion of sulfated to potassium carbonate of 1:2 to give sulfated alcohols.

3.1. Sulfation Reactions

3.1.1. Synthesis of the sodium salts of 4-(2-ethanoyloxyethyl)-2-hydroxyphenyl sulfate and of 5-(2-ethanoyloxyethyl)-2-hydroxyphenyl sulfate

The reaction of hydroxytyrosol acetate with sulfur trioxide-pyridine complex was carried out to give 4-(2-ethanoyloxyethyl)-2-hydroxyphenyl hydrogenosulfate and 5-(2-ethanoyloxyethyl)-2-hydroxyphenyl hydrogenosulfate as their sodium salts, as shown in figure 21.

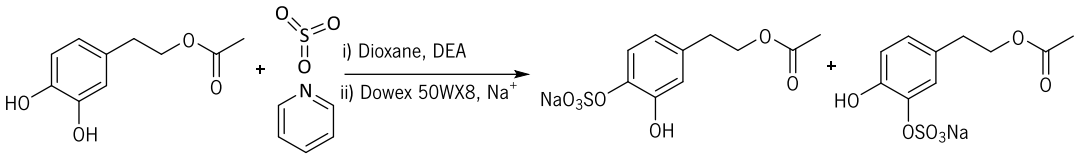


Figure 21: Synthesis of the sodium salts of 4-(2-ethanoyloxyethyl)-2-hydroxyphenyl sulfate and of 5-(2-ethanoyloxyethyl)-2-hydroxyphenyl sulfate.

In a Schlenk tube, under argon, 0,51 g of 2-(3,4-dihydroxyphenyl)ethyl ethanoate (2.60 mmol) was dissolved in 10 mL of dry dioxane. The mixture was cooled in an ice bath and 0.81 g of sulfur trioxide-pyridine complex (5.09 mmol) was added. The reaction was monitored by TLC [ethyl acetate/methanol (9:1)] and the plates were revealed by iodine. The mixture was left under magnetic stirring for 15 min at 0 °C under argon, and then closed and stored at -20 °C for 5 days.

Then the mixture was defrosted, and 3 mL of cooled water was added. The mixture was immediately neutralized with ~40 drops of diethylamine (DEA) (pH ≈ 7). Then the aqueous phase was washed with diethyl ether (3 x 10 mL) and the organic phase was washed with 2 mL of water. The aqueous layer was evaporated at 30 °C, given a yellow oil (1.29 g). The crude extract was purified by flash chromatography on silica gel [ethyl acetate/methanol (9:1 v/v)], giving a mixture of two isomers (0.38 g).

The mixture of DEA salts was then dissolved in 2 mL of water, applied to a column filled with a cation-exchange resin (Dowex 50WX8, Na^+ form, 10 g), and eluted with water. Fractions containing the desired product were lyophilized to obtain a mixture of the sodium salts of 4-(2-ethanoyloxyethyl)-2-hydroxyphenyl sulfate and of 5-(2-ethanoyloxyethyl)-2-hydroxyphenyl sulfate as a white solid (0.16 g).

3.1.2. Synthesis of the sodium salt of 4-(2-ethanoyloxyethyl)-phenyl sulfate

Compound 4-(2-ethanoyloxyethyl)-phenyl hydrogenosulfate and its sodium salt were synthesized by reacting 2-(4-hydroxyphenyl)ethyl ethanoate and sulfur trioxide-pyridine in dry dioxane, as shown in figure 22.

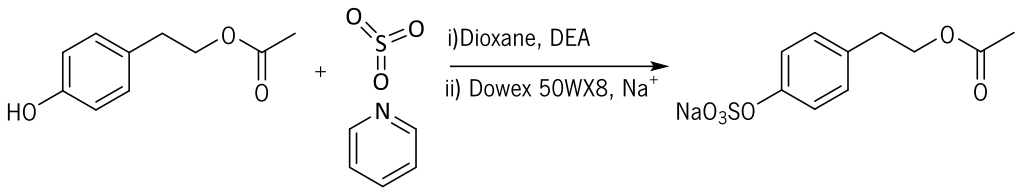


Figure 22: Synthesis of the sodium salt of 4-(2-ethanoyloxyethyl)-phenyl sulfate.

In a Schenck tube, under argon, 0.38 g of 4-hydroxyphenyl ethanoate (2.11 mmol) was dissolved with 10 mL of dry dioxane. Then, the mixture was cooled in an ice bath and 0.66 g of sulfur trioxide-pyridine complex (4.15 mmol) was added to the solution. The mixture was left under magnetic stirring for 15 minutes at 0 °C under argon and then closed and stored at -20 °C for 24 hours. The reaction was monitored by TLC [ethyl acetate/methanol (9:1)] and the plates were revealed with a Mostain solution.

When TLC showed a decrease of initial reagents and an increase of product, the mixture was defrosted, and 3 mL of cooled water was added. Then, the mixture was immediately neutralized with ~80 drops of DEA ($pH \approx 7-8$). Then, in a decantation funnel the aqueous phase was washed several times with diethyl ether (3 x 10 mL) and the organic phase was washed with 2 mL of deionized water. The aqueous layer was evaporated, giving a yellow oil (1.10 g). The crude extract was purified by flash chromatography on silica gel [ethyl acetate/methanol (9:1 v/v)] to give a white solid (0.25 g).

The mixture of DEA salts was then dissolved in 2 mL of water, applied to a column of cation-exchange resin (Dowex 50WX8, Na^+ form, 10 g), and eluted with water. Fractions containing the wanted product were lyophilized to obtain the sodium salts of 4-(2-ethanoyloxyethyl)-phenyl sulfate as a white solid (0.12 g).

3.1.3. Synthesis of the sodium salt of 4-(2-ethanoyloxyethyl)-2-methoxyphenyl sulfate

The synthesis of the sodium salt of 4-(2-ethanoyloxyethyl)-2-methoxyphenyl sulfate was carried out by reacting homovanillyl acetate with sulfur trioxide-pyridine complex is shown in figure 23.

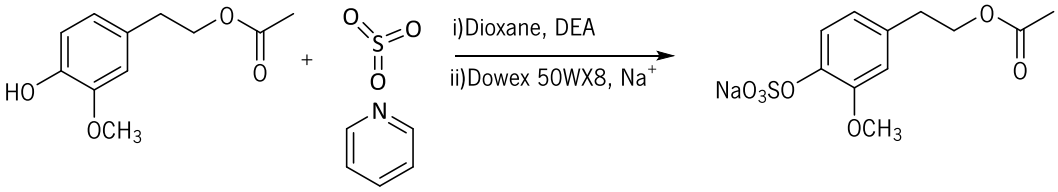


Figure 23: Synthesis of the sodium salt of 4-(2-ethanoyloxyethyl)-2-methoxyphenyl sulfate.

In a round bottom flask, under argon, 0.57 g of 2-(4-hydroxy-2-methoxyphenyl)ethyl ethanoate (2.71 mmol) was dissolved in 15 mL of dry dioxane and the mixture cooled in an ice bath. Then, 0.86 g of sulfur trioxide-pyridine complex (5.40 mmol) was added to the solution. The mixture was left under magnetic stirring for 15 minutes at 0 °C under argon and then closed and stored at -20 °C for 10 days. The reaction was monitored by TLC, [ethyl acetate/methanol (9:1)] and the plates were revealed with a Mostain solution.

When TLC showed a decrease of initial reagents and an increase of product, the mixture was defrosted and 3 mL of cooled water was added. Subsequently, ~80 drops of DEA was immediately added to neutralize the mixture (pH ≈ 7). Then the aqueous phase was washed with diethyl ether (3 x 10 mL) and the organic phase was washed 3 times with 2 mL of water. The aqueous layer was evaporated at 30 °C, giving a yellow oil (1.39 g). The crude extract was purified by flash chromatography on silica gel by elution with ethyl acetate/methanol (9:1 v/v).

The mixture of DEA salts was then dissolved in 2 mL of water, applied to a column of cation-exchange resin (Dowex 50WX8, Na^+ form, 10 g), and eluted with water. Fractions containing the desired product were lyophilized to obtain sodium salts of 4-(2-ethanoyloxyethyl)-2-methoxyphenyl sulfate as a white solid (0.007 g).

3.1.4. Synthesis of the diethylammonium salt of 4-(2-ethanoyloxyethyl)-2-hydroxyphenyl sulfate

The synthesis of the diethylammonium salt of 4-(2-ethanoyloxyehyl)-2-hydroxyphenyl sulfate was carried out following a procedure described by Teixeira, et al.[74] Figure 24 shows the reaction of the DEA salt of diethylammonium 4-(2-ethanoyloxyethyl)-2-methoxyphenyl sulfate and boron tribromide in dry dichloromethane. The synthesis was carried out with a proportion of diethylammonium 4-(2-ethanoyloxyethyl)-2-methoxyphenyl sulfate to boron tribromide of 1:2.

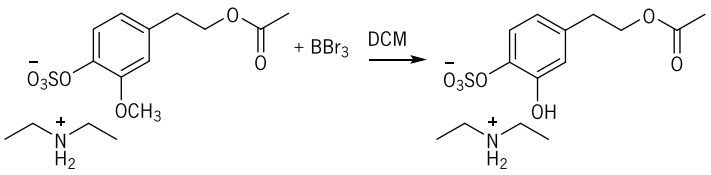


Figure 24: Synthesis of diethylammonium 4-(2-ethanoyloxyethyl)-2-hydroxyphenyl sulfate.

In a hydrolysis tube, 0.10 g of the DEA salt of diethylammonium 4-(2-ethanoyloxyethyl)-2-methoxyphenyl sulfate (0.275 mmol) was dissolved in 4 mL of dry dichloromethane, under argon at 80 ° C. To this solution, 540 µL of boron tribromide (0.534 mmol) was added, and the reaction was kept at 80 °C for 10 minutes and then allowed to reach room temperature. The reaction was monitored by TLC [dichloromethane/methanol (9:1)] and the plates were revealed by iodine.

After 12 hours, the reaction was quenched by cautious addition of 10 mL of methanol and the solvent evaporated. The crude product was purified by flash chromatography on silica gel [dichloromethane/methanol (9:1 v/v)] to give 7.90 mg of the diethylammonium salt of 4-(2-ethanoyloxyethyl)-2-hydroxyphenyl sulfate.

3.1.5. Synthesis of the sodium salts of (*Z*)-5-(2-((4-formyl-3-(2-oxoethyl)hex-4-enoyl)oxy)ethyl)-2-hydroxyphenyl sulfate and of (*Z*)-4-(2-((4-formyl-3-(2-oxoethyl)hex-4-enoyl)oxy)ethyl)-2-hydroxyphenyl sulfate

The reaction of 3,4-DHPEA-EDA with sulfur trioxide-pyridine complex in dry dioxane was carried out in order to obtain a mixture of hydrogenosulfate (*Z*)-5-(2-((4-formyl-3-(2-oxoethyl)hex-4-enoyl)oxy)ethyl)-2-hydroxyphenyl and hydrogenosulfate (*Z*)-4-(2-((4-formyl-3-(2-oxoethyl)hex-4-enoyl)oxy)ethyl)-2-hydroxyphenyl and subsequently, their corresponding sodium salts, as shown in figure 25. This would be in agreement with the synthesis of 4-(2-ethanoyloxyethyl)-2-hydroxyphenyl hydrogenosulfate and 5-(2-ethanoyloxyethyl)-2-hydroxyphenyl hydrogenosulfate and their corresponding sodium salts previously described.

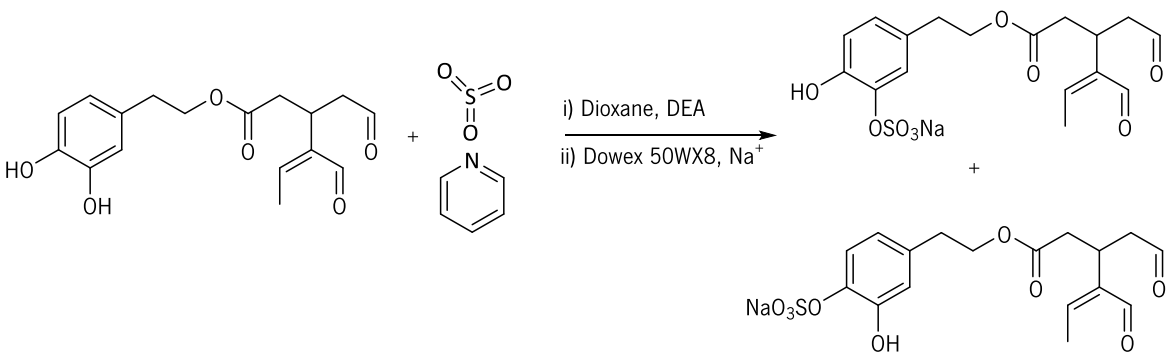


Figure 25: Synthesis of the sodium salts of (*Z*)-5-(2-((4-formyl-3-(2-oxoethyl)hex-4-enoyl)oxy)ethyl)-2-hydroxyphenyl sulfate and of (*Z*)-4-(2-((4-formyl-3-(2-oxoethyl)hex-4-enoyl)oxy)ethyl)-2-hydroxyphenyl sulfate.

In a round bottom flask, under argon, 0.26 g of 3,4-DHPEA-EDA (0.812 mmol) was dissolved in 5 mL of dry dioxane. Then the mixture was cooled in an ice bath and 0.26 g of sulfur trioxide-pyridine complex (1.63 mmol) was added to the solution. The mixture was left under magnetic stirring for 15 minutes at 0 °C under argon and then closed and stored at -20 °C for 5 days. The reaction was monitored by TLC [ethyl acetate/methanol (9:1)] and the plates were revealed on iodine.

When TLC showed a decrease of initial reagents and an increase of product, the mixture was defrosted, and 3 mL of cooled water was added. Immediately the mixture was neutralized with

~25 drops of DEA (pH ≈ 7). After this step, the aqueous phase was washed with diethyl ether (3 x 10 mL) and the organic phase was washed 2 times with 2 mL of deionized water. The aqueous layer was evaporated, giving a yellow oil (0.33 g). The crude extract was purified by flash chromatography [ethyl acetate/methanol (9:1 v/v)] to give 0.16 g of a yellow oil. As the ^{1}HNMR was inconclusive, it was decided to pass the product through the column of cation-exchange resin (Dowex 50WX8, Na^{+} form, 10 g), eluting with water. Only 0.0074 g were obtained as purified compound therefore the NMR spectra obtained after this procedure was inconclusive.

3.2. Solvolysis Reactions

3.2.1. Synthesis of the sodium salts of 2-hydroxy-4-(2-hydroxyethyl)phenyl sulfate and of 2-hydroxy-5-(2-hydroxyethyl)phenyl sulfate

The reaction of diethylammonium 4-(2-ethanoyloxyethyl)-2-hydroxyphenyl sulfate and diethylammonium 5-(2-ethanoyloxyethyl)-2-hydroxyphenyl sulfate with methanol and potassium carbonate, is shown in figure 26. Solvolysis of the ester group occurred, to give, after elution through cation-exchange resin (Dowex 50WX8, Na^{+} form), the sodium salts of 2-hydroxy-4-(2-hydroxyethyl)phenyl sulfate and of 2-hydroxy-5-(2-hydroxyethyl)phenyl sulfate.

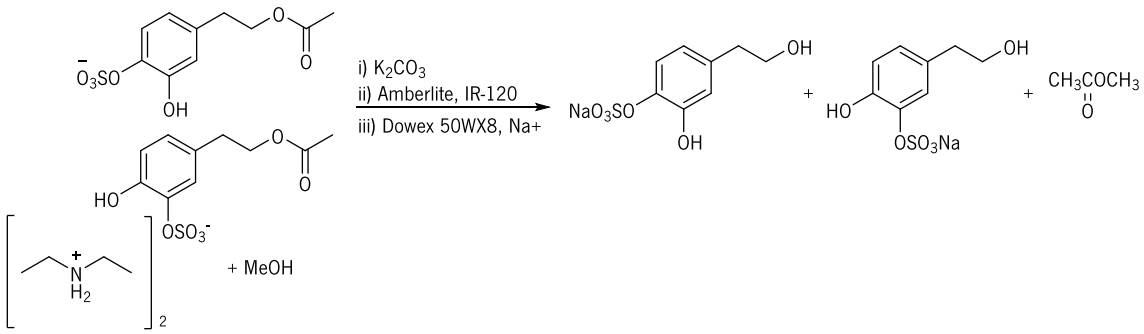


Figure 26: Synthesis of the sodium salts of 2-hydroxy-4-(2-hydroxyethyl)phenyl sulfate and of 2-hydroxy-5-(2-hydroxyethyl)phenyl sulfate.

In a round bottom flask, 0.23 g of the mixture of the sodium salts of 2-hydroxy-4-(2-hydroxyethyl)phenyl sulfate and of 2-hydroxy-5-(2-hydroxyethyl)phenyl sulfate were dissolved in 3

mL of methanol. Then 0.23 g of potassium carbonate was added. The mixture was left at room temperature and protected from light, under stirring for 24 hours. The reaction was monitored by TLC [ethyl acetate/methanol (9:1)] and plates were revealed by iodine.

After 24 hours, the solvent was evaporated, and 10 mL of deionized water was added and acidified with amberlite IR-120 resin, until bubbles started to evolve. Then the resin was filtrated under vacuum and the aqueous phase was washed with diethyl ether (6 x 5 mL) and immediately neutralized with ~4 drops of DEA (pH ≈ 7). The aqueous layer was extracted and evaporated at 30 °C.

The DEA salts solution was concentrated to 2 mL of water and applied to a column of cation-exchange resin (Dowex 50WX8, Na^+ form, 10 g) and eluted with water, to give a mixture of the sodium salts of 2-hydroxy-4-(2-hydroxyethyl)phenyl sulfate and of 2-hydroxy-5-(2-hydroxyethyl)phenyl sulfate as a cream-colored solid (0.11 g).

3.2.2. Synthesis of the sodium salt of 4-(2-hydroxyethyl)phenyl sulfate

The reaction of the diethylammonium salt of 4-(2-ethanoyloxyethyl)-phenyl sulfate with potassium carbonate in methanol is shown in figure 27. The product, sodium 4-(2-hydroxyethyl)phenyl sulfate results from solvolysis of the ester group.

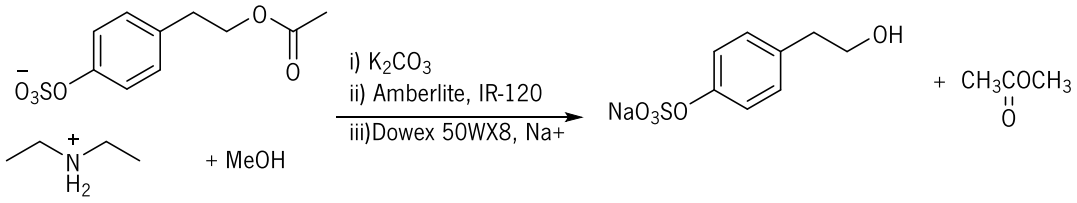


Figure 27: Synthesis of the sodium salt of 4-(2-hydroxyethyl)phenyl sulfate.

To a round bottom flask, 0.25 g of the diethylammonium salt of 4-(2-ethanoyloxyethyl)-phenyl sulfate (0.961 mmol) was added and dissolved in 3 mL of methanol. Then, 0.27 g of potassium carbonate (1.95 mmol) was added. The mixture was left at room temperature, protected from light, under magnetic stirring for 24 hours. The reaction was monitored by TLC [ethyl acetate/methanol (9:1)] and the plates revealed by Mostain solution.

After 24 hours, the solvent was evaporated, and 10 mL of water was added to the crude residue and then acidified with Amberlite IR-120 resin, until bubbles started to evolve. The resin was filtrated, and the aqueous phase was washed with diethyl ether (7 x 5 mL) and immediately neutralized with ~5 drops of DEA (pH ≈ 7). DEA salts were diluted in 2 mL and applied to a column of cation-exchange resin (Dowex 50WX8, Na^+ form, 10 g) eluted with water, to give the sodium salt of 4-(2-hydroxyethyl)phenyl sulfate as white a solid (0.09 g).

3.2.3. Synthesis of the sodium salt of 4-(2-hydroxyethyl)-2-methoxyphenyl sulfate

Figure 28 shows the reaction of the diethylammonium salt of 4-(2-ethanoyloxyethyl)-2-methoxyphenyl sulfate with methanol and potassium carbonate, to give the sodium salt of 4-(2-hydroxyethyl)-2-methoxyphenyl sulfate.

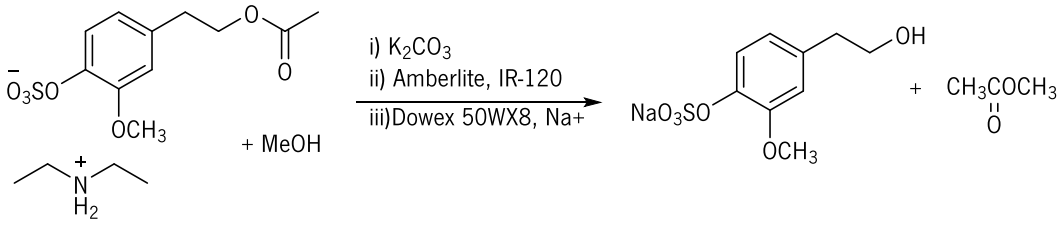


Figure 28: Synthesis of the sodium salt of 4-(2-hydroxyethyl)-2-methoxyphenyl sulfate.

In a round bottom flask, 0.10 g (0.320 mmol) of the diethylammonium salt of 4-(2-ethanoyloxyethyl)-2-methoxyphenyl sulfate was added and dissolved in 2 mL of methanol. Then 0.10 g (0.724 mmol) of potassium carbonate was added. The mixture was left at room temperature and protected from light, under magnetic stirring for 24 hours. The reaction was monitored by TLC [ethyl acetate/methanol (9:1)] and the plates were revealed by Mostain solution.

After 24 hours, the solvent was evaporated, and 5 mL of water was added to the crude residue and then acidified with Amberlite IR-120 resin, until bubbles started to evolve. The resin was then filtrated, and the aqueous phase was washed with diethyl ether (5 x 5 mL) and immediately neutralized with ~3 drops of DEA (pH ≈ 7). DEA salts were diluted in 2 mL and

applied to a column of cation-exchange resin (Dowex 50WX8, Na^+ form, 10 g) eluted with water, to give the sodium salt of 4-(2-hydroxyethyl)-2-methoxyphenyl sulfate as a white solid (0.15 g).

4. Haemolysis Assay

4.1. Preparation of RBC suspension

Blood was obtained from healthy volunteers by venepuncture. Blood samples (about 9 mL each) were collected into tubes containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant. Blood samples were centrifuged (Gyrozen Multi-Purpose High Speed Centrifuge 1580R) at 4 °C, 700 g for 10 min; plasma and buffy coat were carefully removed by aspiration and discarded. RBCs were washed three times with phosphate-buffered saline solution (PBS) pH 7.4 and centrifuged at 4 °C, 700 g for 7 minutes. Supernatant was carefully removed after each wash. RBCs were resuspended in PBS pH 7.4 after the final wash to obtain 2.0% haematocrit.

4.2. AAPH-induced haemolysis

In order to evaluate the protection of the different phenolic compounds and its metabolites against the APPH-induced haemolysis of RBCs, *in vitro* assays ($n \geq 4$) were prepared according to table 2 and 3.

Table 2: Assay conditions in the study of AAPH-induced haemolysis in the presence of phenolic compounds and its metabolites.

		PBS pH 7.4 (µL)	DMSO 18.1 % (µL)	Compound 800 µM (µL)	RBCs suspension Ht=2.6 % (µL)	AAPH 600mM (µL)
Positive control	Without DMSO	85	-	-	500	65
	DMSO 1.13 %	44.4	40.6	-		
Negative control	Without DMSO	150	-	-		-
	DMSO 1.13%	109.4	40.6	-		
Olive oil phenols and its metabolites	2.5 µM	44.4	38.57	2.03		65
	5 µM		36.54	4.06		
	10 µM					
	20 µM					
	40 µM		24.35	16.25		
	80 µM		-	40.6		

RBCs suspensions were prepared at 2.0% haematocrit (Ht), and the assays were performed using phenolic compounds at final concentrations of 2.5, 5, 10, 20, 40 and 80 µM, and AAPH (Sigma Aldrich Chemistry) at final concentration of 60 mM (chosen in agreement to previous work[41]). Sample tests were run in duplicate. Negative controls (RBCs in PBS with and without DMSO 1.13%) and positive controls (RBCs in PBS with AAPH, and with or without DMSO 1.13%) were run in triplicate. RBCs suspensions were incubated at 37 °C for 10 min with each phenolic compound before addition of AAPH. Subsequently, AAPH was added and the incubations of RBCs suspensions were carried out at 37 °C for 4 hours, under gentle shaking. Tubes were shaken by inversion at the end of each hour.

Haemolysis was determined spectrophotometrically according to the method described by Ko et al.[75] After 4 hours of incubation, an aliquot of each tube was diluted with PBS and an aliquot

with the same volume was diluted with distilled water to yield complete haemolysis. All Eppendorfs were centrifuged (Haraeus™ Fresco™ 21 Centriguge, Thermo Scientific) at room temperature, 700 g for 10 minutes. The supernatant (300 µL) from each Eppendorf was collected and the absorvance was read at 540 nm (ref. 690 nm) (Synergy HTX multi-mode reader, Biotek). The percentage of haemolysis was calculated by the following formula:

$$\%\, Hemolysis = \frac{A}{B} \times 100$$

were A represents the absorption of the supernatant from the aliquot diluted in PBS, while B represents the absorption of the supernatant from the aliquot diluted in distilled water.

IV. Results and Discussion

1. Olive oil Phenolic Compounds

1.1. Acetylation of Phenolic Compounds

In order to obtain selective sulfation of the phenolic hydroxyl groups, the aliphatic hydroxyl group needed to be protected. Therefore, the tentative selective acetylation of hydroxytyrosol, tyrosol and homovanillyl alcohol was performed using two different method, namely, using acetyl chloride or ethyl acetate and amberlite IR-120..

After two days of reaction, TLC analysis indicated that all phenolic reactant had been consumed. However, the esterification of phenolic alcohols with acetyl chloride was not selective. Even using different reaction conditions, the nucleophilic attack occurred both at the phenolic and aliphatic hydroxyl group, giving two products with the same retention factor (figure 29).

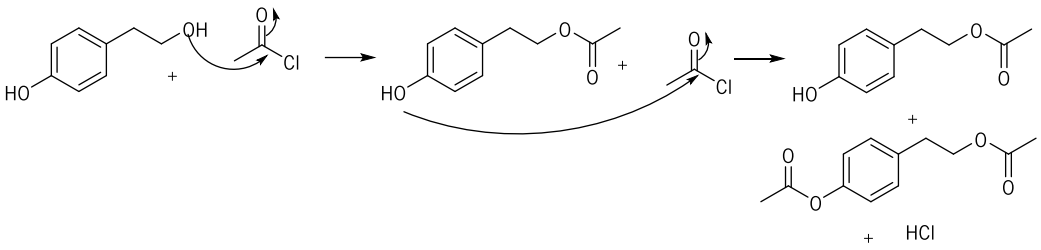


Figure 29: Proposed sequence for acetylation of tyrosol using acetyl chloride.

The method that was more effective and more selective was the reaction of the phenolic compounds with ethyl acetate and amberlite IR-120. The resin can be easily removed from the reaction medium by filtration and reused, thus constituting a green, economical and effective method for the chemoselective monoacetylation of phenolic alcohols (figure 30).

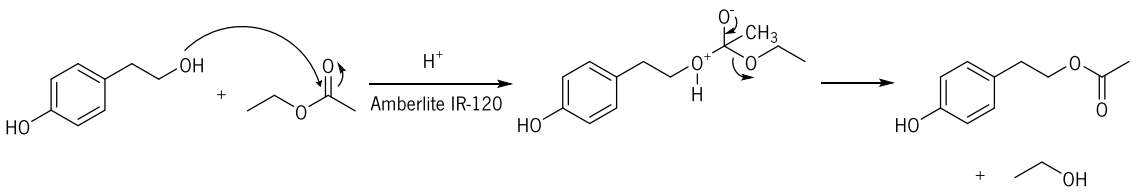


Figure 30: Proposed sequence for transesterification with ethyl acetate using amberlite IR-120 as catalyst.

1.1.1. Synthesis of 2-(3,4-dihydroxyphenyl)ethyl ethanoate

The initial attempt to synthesis 2-(3,4-dihydroxyphenyl)ethyl ethanoate, was carried out using acetyl chloride in dimethyl carbonate. The reaction was left under argon for 2 days and monitored by TLC. After this period, two spots could be detected, one we believed to be the wanted product and the other the starting material. NMR showed that the reaction formed two products, 2-(4-acetyloxi-3-hydroxyphenyl)ethyl ethanoate and 2-(3,4-dihydroxyphenyl)ethyl ethanoate in a proportion of 1:8, as shown in figure 31.

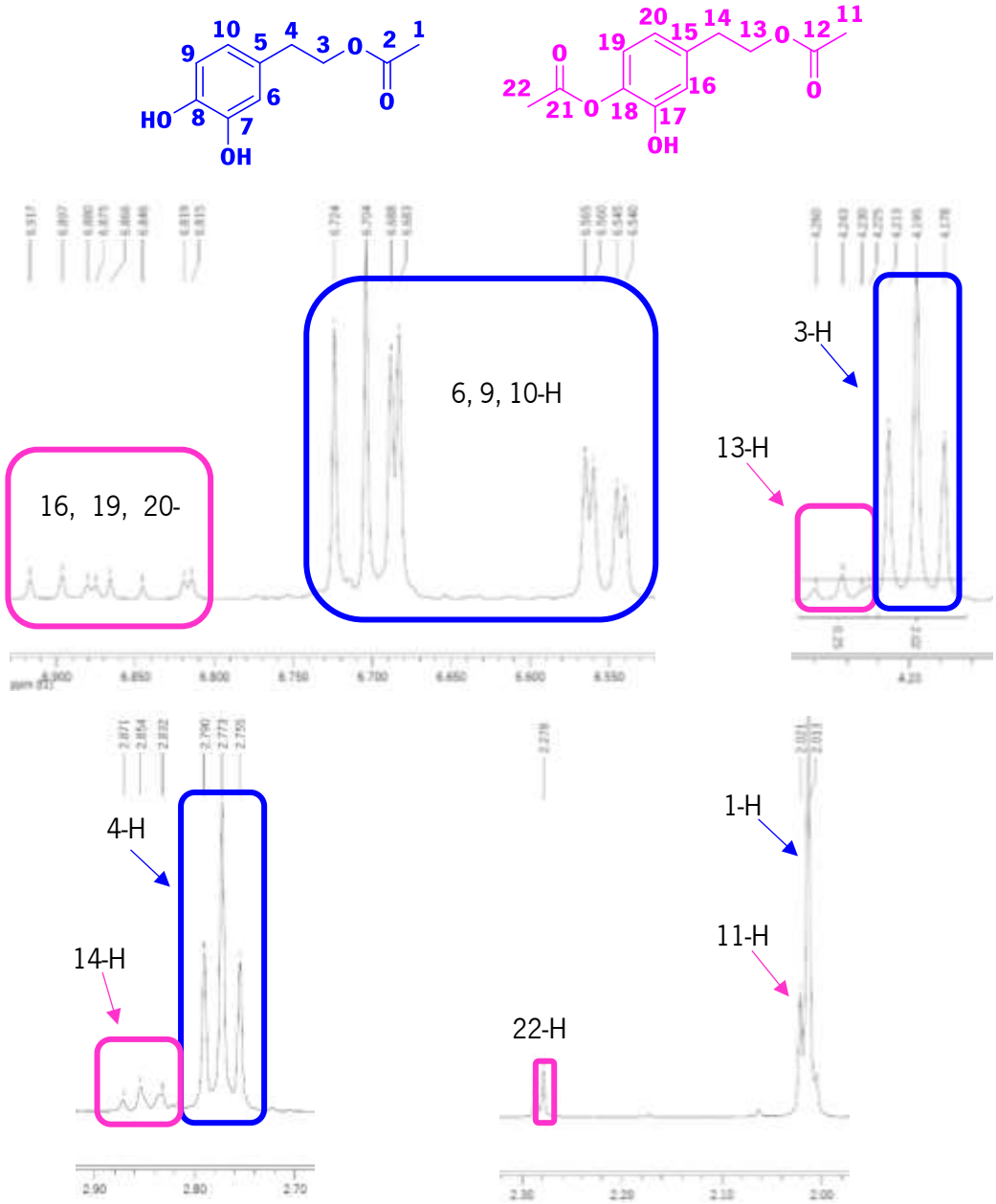


Figure 31: NMR in deuterated methanol of the mixture of 2-(4-acetyloxi-3-hydroxyphenyl)ethyl ethanoate and 2-(3,4-dihydroxyphenyl)ethyl ethanoate.

The hydroxytyrosol ring has an additional hydroxyl group at the *meta* position and is therefore a catechol. Acetylation of the hydroxyl group in *para* position occurs preferentially over that in *meta*, due to the greater activation of this hydroxyl that results from the electron-donor effect of the alkyl group. The two compounds, 2-(4-acetyloxi-3-hydroxyphenyl)ethyl ethanoate and 2-(3,4-dihydroxyphenyl)ethyl ethanoate, were separated by flash chromatography [chloroform/ethyl acetate (7:3 v/v)], but because of the similar retention time of both compounds only 0.05 g of 2-(3,4-dihydroxyphenyl)ethyl ethanoate was isolated (yield = 7.4 %).

Using amberlite as catalyst and ethyl acetate, the transesterification of the aliphatic hydroxyl group was more selective, and only the 2-(3,4-dihydroxyphenyl)ethyl ethanoate was formed with a yield of 98 %. Figure 32 shows selected NMR peaks in methanol of the compound obtained.

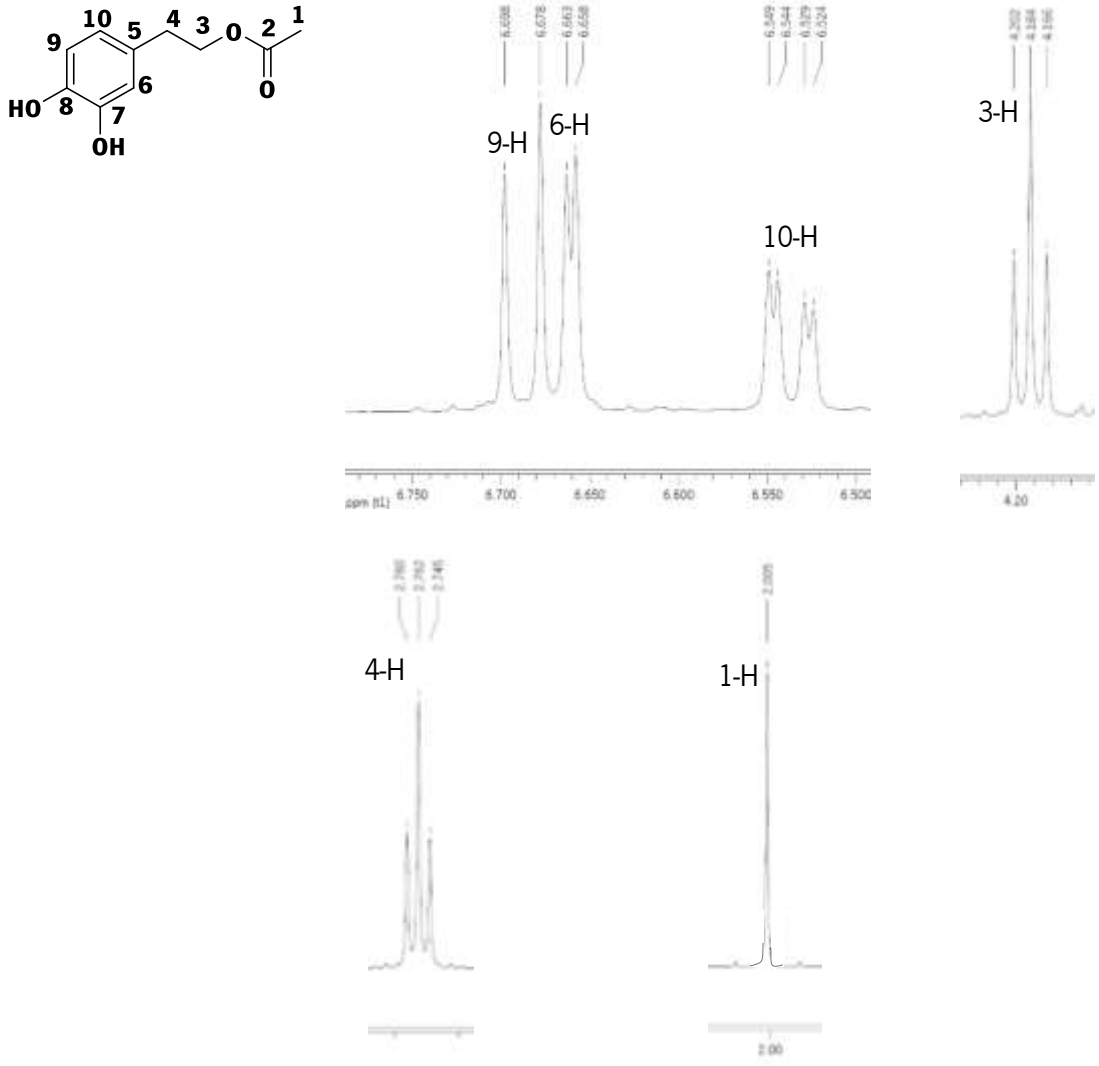


Figure 32: NMR in deuterated methanol of 2-(3,4-dihydroxyphenyl)ethyl ethanoate.

1.1.2. Synthesis of 2-(4-hydroxyphenyl)ethyl ethanoate

The synthesis of 2-(4-hydroxyphenyl)ethyl ethanoate was carried out by reacting tyrosol with acetyl chloride in anhydrous dichloromethane. A mixture of two products was again obtained, which were identified by NMR (figure 33). Acetylation occurred at both aliphatic and phenolic hydroxyl groups, forming a mixture of 2-(4-hydroxyphenyl)ethyl ethanoate and 2-(4-acetyloxyphenyl)ethyl ethanoate. Esterification occurs more favourably at the aliphatic hydroxyl group, due to lower electron density at this position. Modification of the reaction conditions, such as, temperature and reaction time, were tested. The reaction was carried out at 40 °C for 36 hours, and at room temperature for 24 and 22 hours. However, formation of both products could not be avoided.

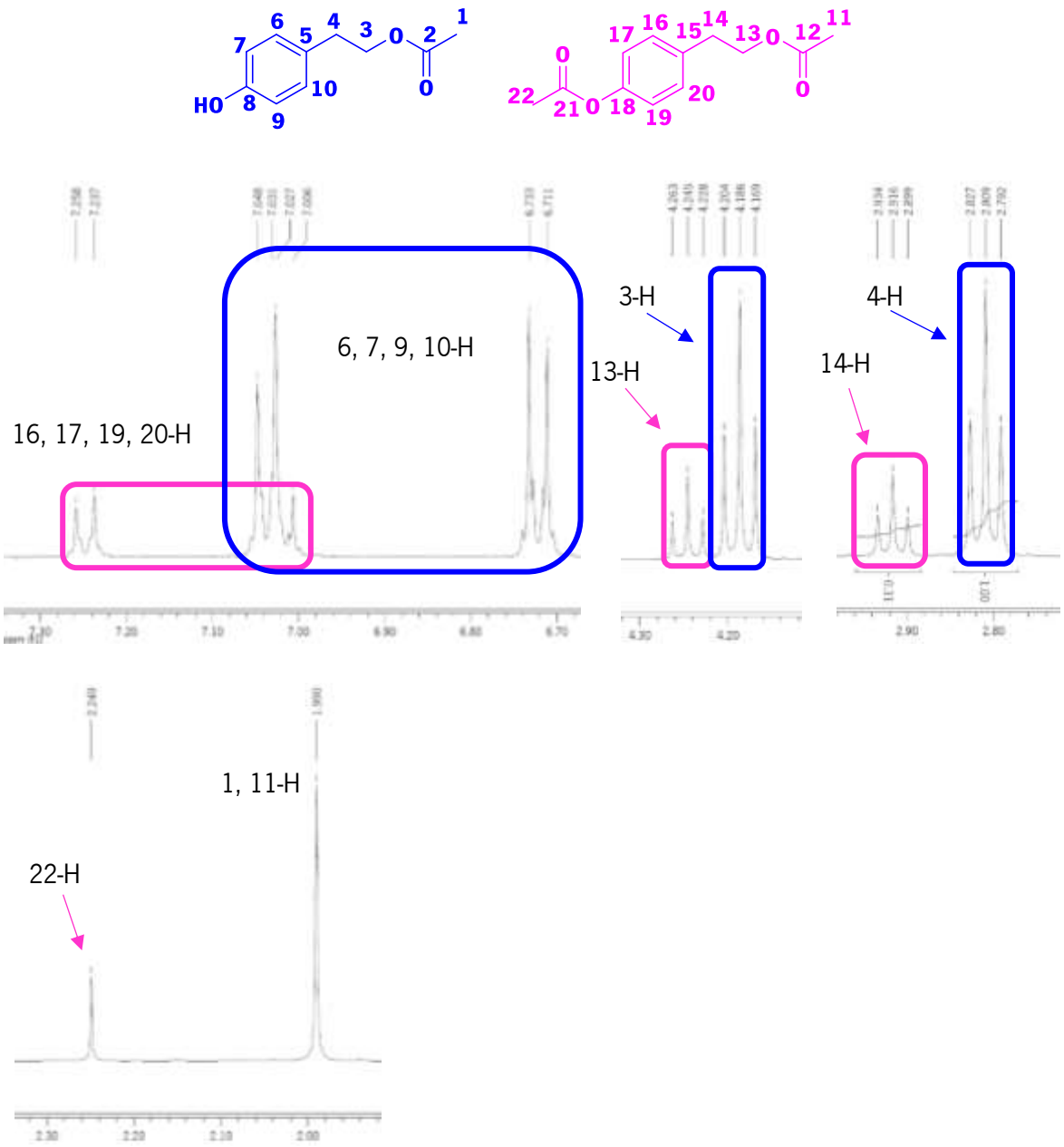


Figure 33: NMR in deuterated methanol of the mixture of 2-(4-hydroxyphenyl)ethyl ethanoate and 2-(4-acetyloxyphenyl)ethanoate.

As the two products could not be separated by chromatography, an attempt to separate them by liquid-liquid extraction using NaOH and HCl was carried out. In an initial attempt, the mixture was dissolved in 20 mL of ethyl acetate, and the organic layer extracted with NaOH 1 M (2 x 15 mL). The aqueous layers were collected and immediately acidified with HCl 0.74 M (pH ≈ 1). This phase was then extracted with ethyl acetate (2 x 15 mL), and finally the organic phase was dried with anhydrous Na_2SO_4, and the solvent evaporated. TLC of the extract gave two spots, one

of them corresponding to tyrosol (starting reagent) and another corresponding to 2-(4-hydroxyphenyl)ethyl ethanoate (figure 34). This suggests that by using NaOH 1M hydrolysis of the product occurred.

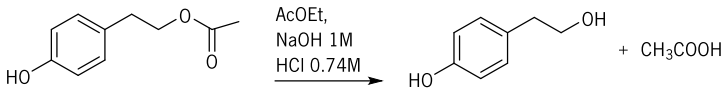


Figure 34: Result obtained in liquid-liquid extraction of the reaction mixture using NaOH 1M and HCl 0.74M.

Therefore, the synthesis of 2-(4-hydroxyphenyl)ethyl ethanoate by transterification using tyrosol and ethyl acetate, catalyzed by amberlite IR-120 was attempted. In these conditions the yield in the wanted product was 75%. Figure 35 shows the NMR spectra obtained for 2-(4-hydroxyphenyl)ethyl ethanoate.

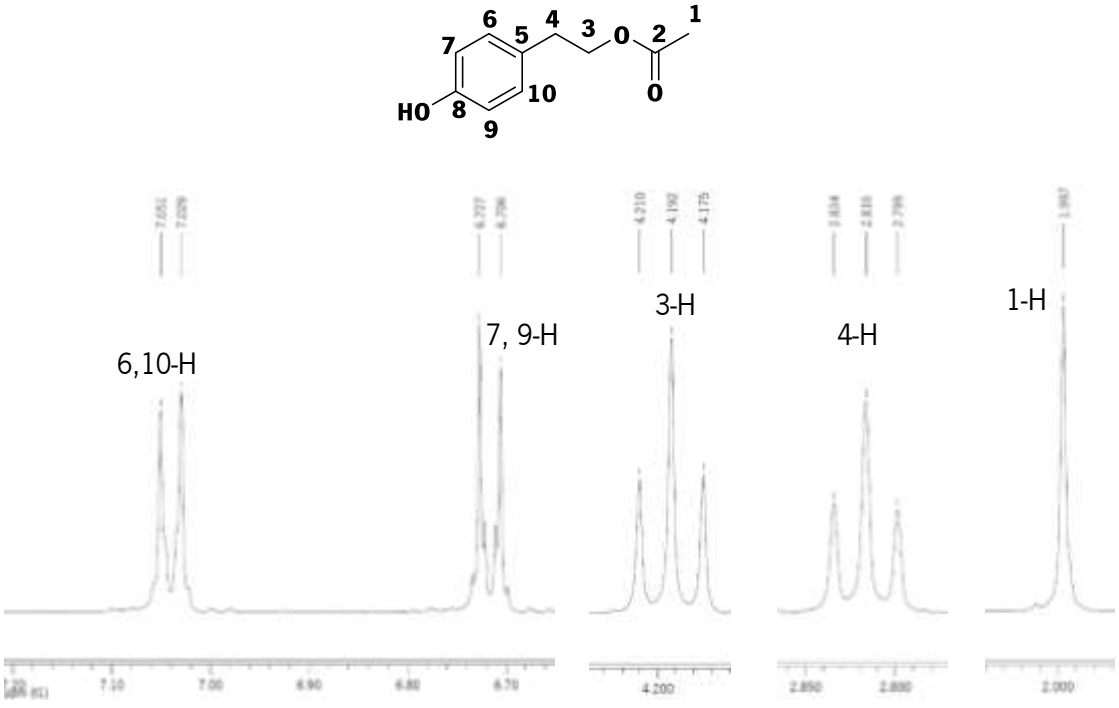


Figure 35: NMR in deuterated methanol of 2-(4-hydroxyphenyl)ethyl ethanoate.

1.1.3. Synthesis of 2-(4-hydroxy-3-methoxyphenyl)ethyl ethanoate

2-(4-Hydroxy-3-methoxyphenyl)ethyl ethanoate was synthesized, using homovanillyl alcohol by transterification with ethyl acetate and amberlite as catalyst. In these conditions the yield in wanted product was 80 %.

1.2. Isolation of secoiridoids from olive tree leaves

It has been reported that secoiridoids, 3,4-DHPEA-EDA and 3,4-DHPEA-EA, can be derived from oleuropein aglycone through a process that results from storing the leaves in bags at 37 °C. This process induces oleuropein to degrade into these two secoiridoids. Thus, fresh olive tree leaves were processed at 40 °C in a plastic bag for 24 hours and then macerated in ethanol. After solvent evaporation, a crude extract was obtained and after purification of this extract by liquid-liquid extraction and column chromatography, 3,4-DHPEA-EDA and 3,4-DHPEA-EA were obtained and identified by UHPLC, by comparison with standards of 3,4-DHPEA-EDA and 3,4-DHPEA-EA and also by NMR.

1.2.1. Isolation of 3,4-DHPEA-EDA and 3,4-DHPEA-EA

After a preliminary purification by liquid-liquid extraction, a crude extract was obtained and further purified by column chromatography. UHPLC analysis of the mixture before purification and comparison with standards, showed the presence of two phenolic compounds in the secoiridoid region of the chromatogram, which were identified as 3,4-DHPEA-EDA and 3,4-DHPEA-EA (figure 36).

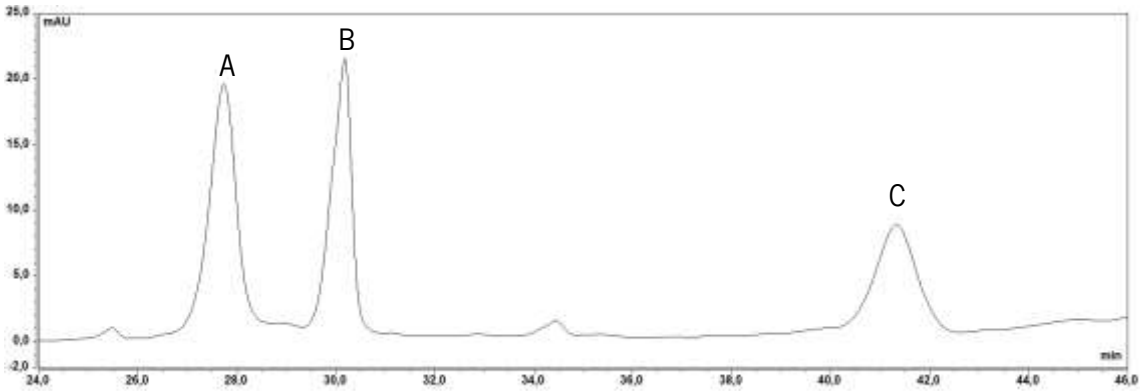


Figure 36: Chromatogram of standards (A=3,4-DHPEA-EDA; B=Oleuropein; C=3,4-DHPEA-EA).

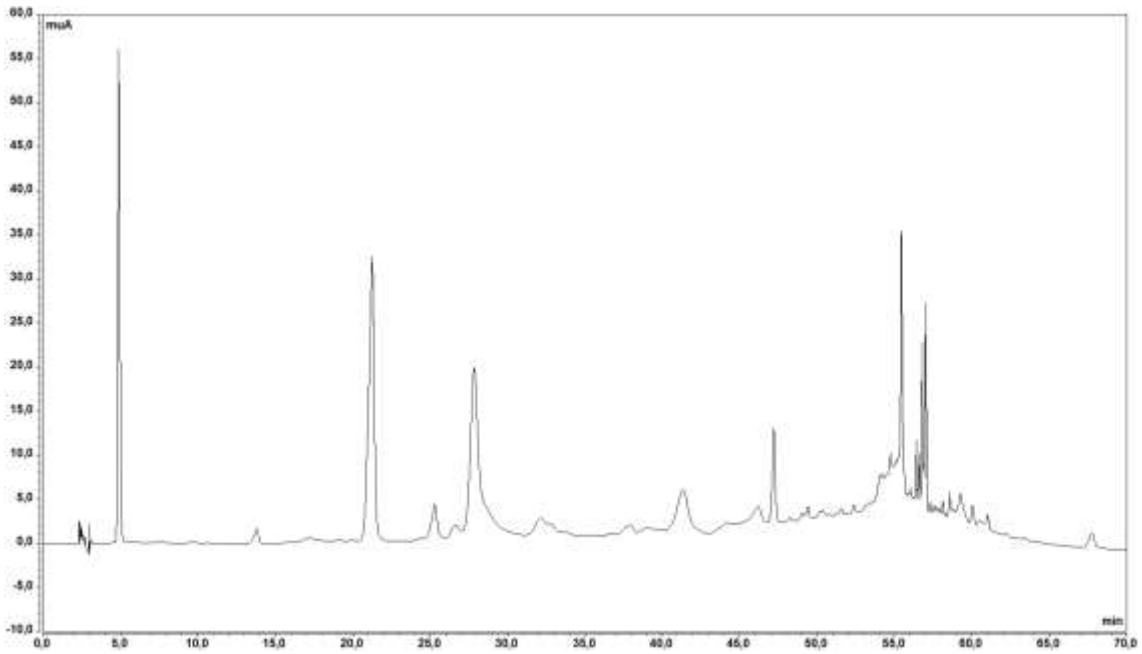


Figure 37: Chromatogram of mixture before purification.

Further purification by column chromatography allowed separation of these two compounds giving two fractions, one containing 3,4-DHPEA-EDA (figure 38) and another containing 3,4-DHPEA-EA (figure 39).

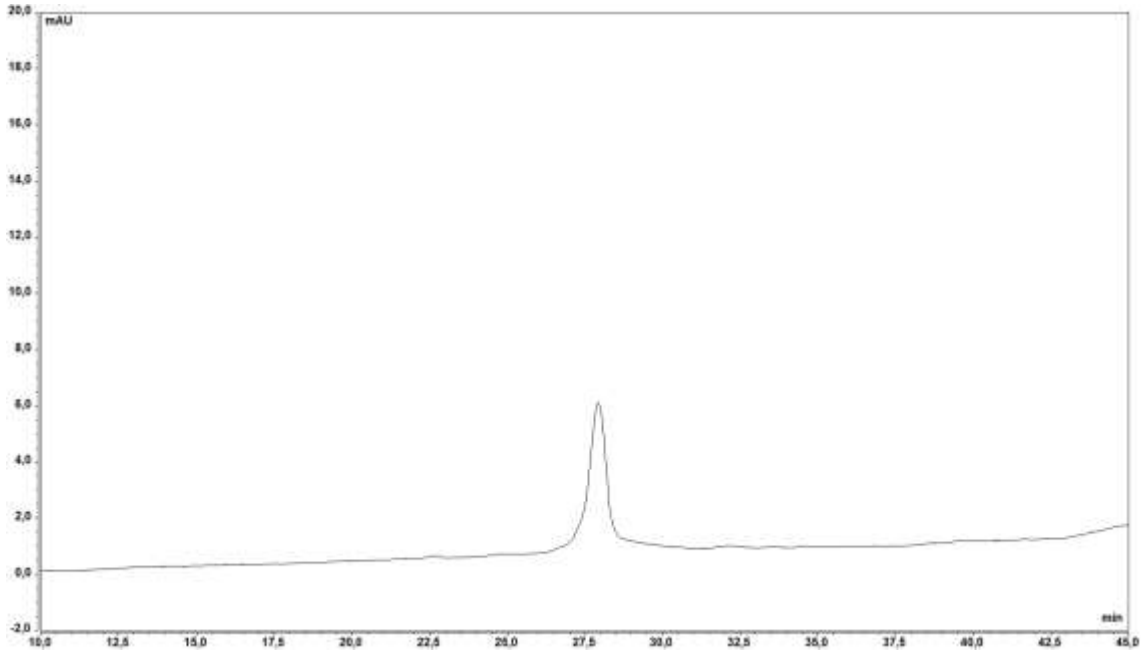
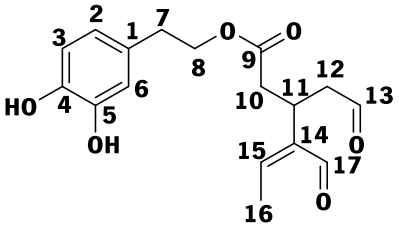


Figure 36: Chromatogram of sample with 3,4-DHPEA-EDA.

After UHPLC analysis, it was found that in the fraction containing 3,4-DHPEA-EDA, only one peak with a retention time of 27.9 min could be observed in the chromatogram, as it shown in figure 36. After solvent evaporation, 0.28 g of compound was obtained as a cream coloured oil.

Through analysis of the NMR spectra it was found that the compound did not present any contamination and thus could be considered as pure. It was important the extract be pure, since it was to be used later in synthetic processes.

Table 3: NMR chemical shifts in deuterated chloroform for secoiridoid 3,4-DHPEA-EDA.



	^{1}HNMR			^{13}CNMR
C Number	δ (ppm)	Signal type	*J* (Hz)	δ (ppm)
1	———	———	———	130.11
2	6.59	dd	8.0 2.0	120.82
3	6.78	d	8.0	114.85
4	———	———	———	142.79
5	———	———	———	143.17
6	6.71	d	2.0	115.83
7	2.78	m	———	33.92
8a	4.22	m	———	64.86
8b	4.17	m	———	
9	———	———	———	171.58
10a	2.70	dd	15.6	36.61
10b	2.60	dd	15.6	
11	3.62	m	———	26.85
12a	2.76	m	———	45.88
12b	2.93	dd	18.4	
13	9.19	d	2.0	200.61
14	———	———	———	142.50
15	6.64	q	6.8	154.91
16	2.05	d	7.2	14.96
17	9.63	s	———	195.47

In contrast, the chromatogram obtained from the fraction containing 3,4-DHPEA-EA (figure 39), showed two additional peaks, meaning the extract was not pure and would require further purification. In fact, one peak corresponding to 3,4-DHPEA-EDA at 29.1 min, another at 34.3 min and another peak corresponding to 3,4DHPEA-EA at 42.4 min could be observed.

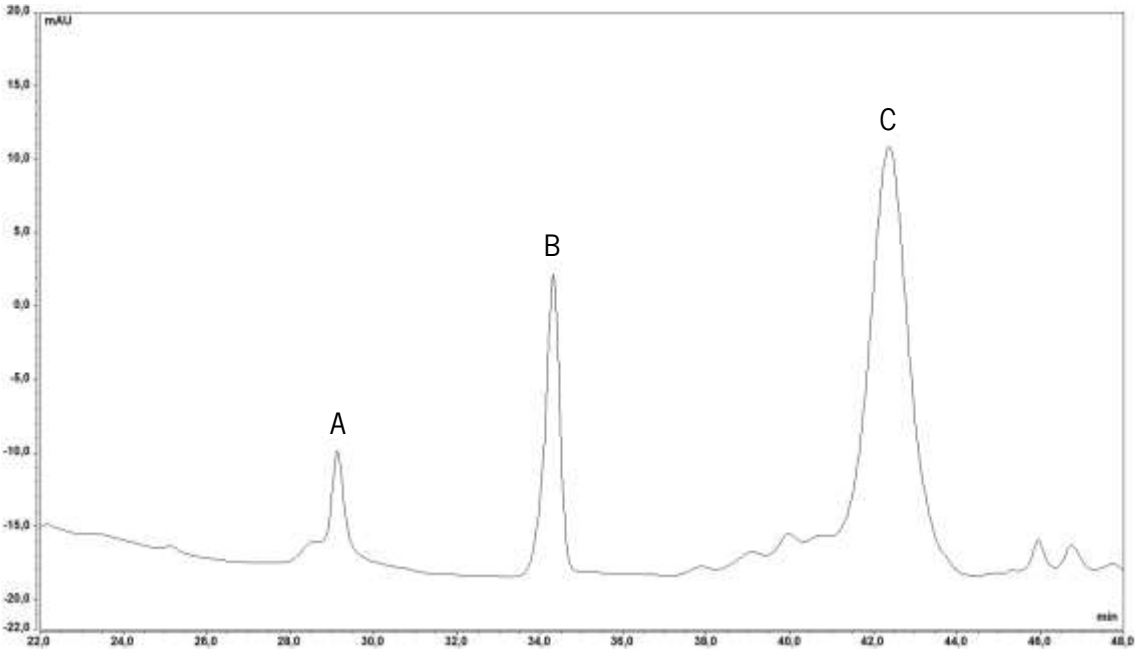
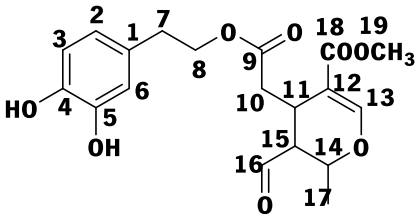


Figure 37: Chromatogram of a sample containing 3,4-DHPEA-EDA (A), oleocanthal (B) and 3,4-DHPEA-EA (C).

In view of the retention time of 34.3 min, the peak could be attributed to oleocanthal. After solvent evaporation 0.25 g of impure 3,4-DHPEA-EA (74%) was obtained. The NMR analysis confirmed the presence of 3,4-DHPEA-EA as the major compound in the extract.

Table 4: Chemical shifts in deuterated chloroform for secoiridoid 3,4-DHPEA-EDA.



	^{1}HNMR			^{13}CNMR
C Number	δ (ppm)	Signal type	*J* (Hz)	δ (ppm)
1	———	———	———	130.20
2	6.57	dd	8.0 1.9	120.80
3	6.74	d	8.0	116.70
4	———	———	———	144.90
5	———	———	———	144.60
6	6.73	d	1.9	116.00
7	2.76	t	7.1	34.90
8	4.20	m	———	65.80
9	———	———	———	172.10
10a	2.84	dd	15.9	37.50
10b	2.35	m	———	
11	3.37	m	———	27.20
12	———	———	———	107.50
13a	7.55	s	———	155.40
13b	7.50	d	1.2	
14	4.57	qd	6.7 5.7	71.40
15	2.65	m	———	54.70
16a	9.67	s	———	200.80
16b	9.54	d	1.4	
17a	1.55	d	6.8	19.40
17b	1.38	d	6.7	
18	———	———	———	167.50
19	3.66	s	———	51.40

1.3. Tentative of synthesis of 4-HPEA-EDA

From 3,4-DHPEA-EDA extracted from olive tree leaves, an attempt to synthetize 4-HPEA-EDA by transesterification of tyrosol was undertaken. For this propose, an enzymatic catalyst, novozym 435, was used.

The reaction was left for 1 month at 37°C. Every day, a TLC in dichloromethane/methanol (5:1) and diethyl ether/methanol (35:1) was carried out in order to accompany the evolution of stains and the emergence of new ones. A spot of hydroxytyrosol started to appear, which led to think that transesterification could actually be happening, but the evolution was slow and so, more enzyme was added to accelerate the process. In the last week, the temperature was increased to 50°C. Subsequently, the hydrotyrosol spot increased and another spot appeared with a very similar retention factor to that of tyrosol. It was decided to stop the reaction when the new stain no longer increased. The enzyme was then filtered and washed with 10 mL of dichloromethane. TLC of the filtrated mixture in dichloromethane/methanol (5:1) and diethyl ether/methanol (35:1) showed the presence of tyrosol, hydroxytyrosol, 3,4-DHPEA-EDA and a further compound over tyrosol spot. Several eluents were tested in order to separate the compound formed from tyrosol. However, no satisfactory solvent was identified. Since we knew where oleocanthal should appears in the UHPLC chromatogram, we decided to determine by UHPLC if the new spot formed was 4-HPEA-EDA before proceeding with separation by column chromatography.

Figure 40 shows the chromatogram obtained. Only four peaks appear related to hydroxytyrosol, tyrosol, 3,4-DHPEA-EDA and unknown compound. The chromatogram allowed us to verify that the appearance of hydroxytyrosol had nothing to do with the transesterification because the pick expected for oleocanthal did not appeared in the chromatogram.

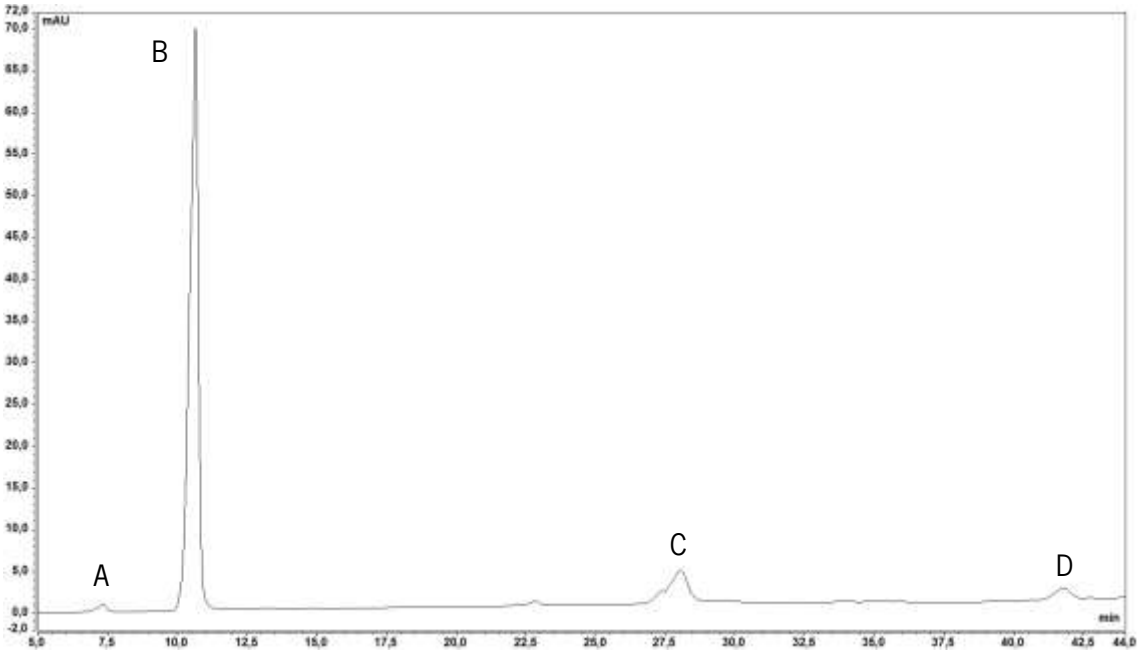


Figure 38: Chromatogram of reaction sample (A=hydroxytyrosol; B=tyrosol; C=3,4-DHPEA-EDA; D=Unknown compound).

2. Synthesis of sulfate metabolites

The sulfation of olive oil phenolic compounds was done using sulfur trioxide-pyridine complex in dioxane. To avoid reaction of the aliphatic hydroxyl groups with sulfur trioxide, this group needs to be protected prior to sulfation by an acetyl group. The sulfation reaction occurs by the nucleophilic attack of the phenolic hydroxyl group, at the sulfur as shows in figure 41.

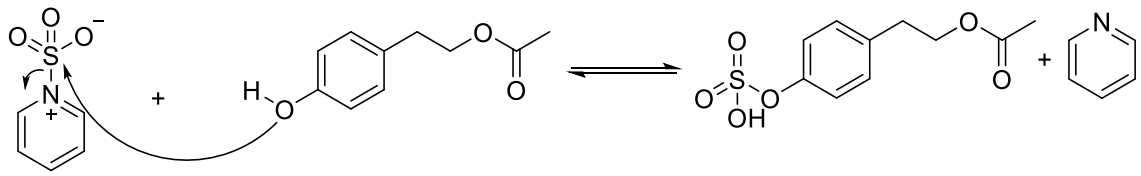


Figure 39: Proposed mechanism of sulfation of tyrosol.

The acidic mixture obtained from the reaction was neutralized with DEA to avoid decomposition of sulfate during evaporation of the solvent. At pH<4, a rapid acid-catalyzed reaction

occurs, in which the sulfate group is protonated, leading to a unimolecular sulfur-oxygen bond fission with elimination of sulfur trioxide (figure 42). This decomposition is avoided with a base stronger than pyridine. Thus, DEA was chosen, since its excess can be easily removed by evaporation.

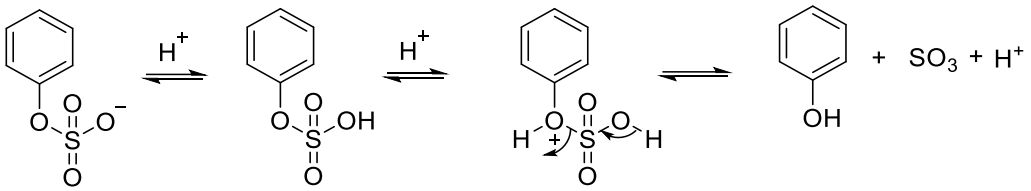


Figure 40: Decomposition of phenylsulfate.

After purification by column chromatography, the solvent was evaporated, dissolved in water and applied to a cation-exchange resin column, allowing the exchange between DEA and the physiological cation Na^+.

When necessary acetyl deprotection was carried out before cation-exchange using the DEA salts (figure 43). The reaction was carried out using methanol and potassium carbonate, to obtain the monosulfated alcohols derivatives.

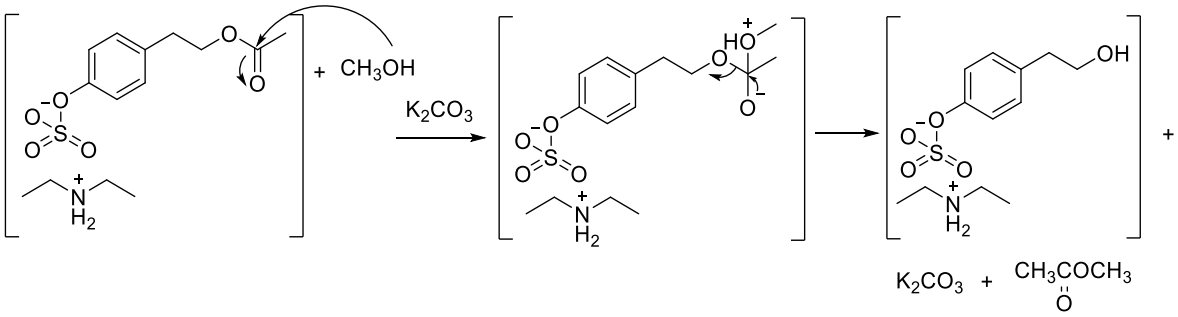


Figure 41: Proposed mechanism of solvation of tyrosol.

An acid resin, amberlite IR-120 was added to neutralize the mixture. This process allows removal of the excess of potassium carbonate excess by formation of CO_2. After resin filtration, the aqueous layer was washed with diethyl ether to remove the methyl acetate formed and the mixture

was immediately neutralized with DEA to prevent decomposition of the sulfate. Once more, application on a cation-exchange resin column allowed the exchange between DEA and the physiological cation Na^+.

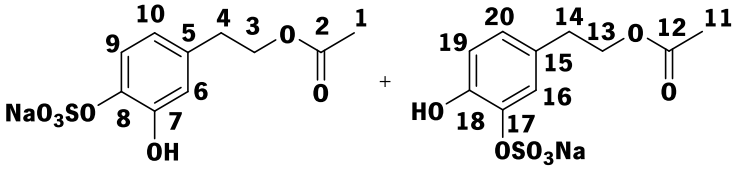
With this two-step reaction, it was possible to synthetize monosulfates derivatives of olive oil polyphenols.

2.1. Synthesis of the sodium salts of 4-(2-ethanoyloxyethyl)-2-hydroxyphenyl sulfate and of 5-(2-ethanoyloxyethyl)-2-hydroxyphenyl sulfate

The synthesis of a mixture of the sodium salts of 4-(2-ethanoyloxyethyl)-2-hydroxyphenyl sulfate and 5-(2-ethanoyloxyethyl)-2-hydroxyphenyl sulfate was done by reacting 2-(3,4-dihydroxyphenyl)ethyl ethanoate with sulf ur trioxide-pyridine in dry dioxane. After 5 days, the complete disappearance of the polyphenol was observed by TLC analysis. The yield of this reaction was 50% after purification.

The NMR spectra of the product obtained (figure 39) shows the presence of two isomers in a proportion of 0.69 to 1.00. Since 2-(3,4-dihydroxyphenyl)ethyl ethanoate has two phenolic hydroxyls, nucleophilic attack occurs on both hydroxyl groups. The sodium salt of the 4-(2-ethanoyloxyethyl)-2-hydroxyphenyl sulfate isomer is the major product, since nucleophilic attack occurs preferentially at the more activated hydroxyl in *para* position. This activation is due to the electron donor effect of the alkyl group. Table 6 shows the NMR chemical shifts for the isomers obtained.

Table 5: NMR chemical shifts in deuterated methanol of the sodium salt of 4-(2-ethanoyloxyethyl)-2-hydroxyphenyl sulfate and sodium 5-(2-ethanoyloxyethyl)-2-hydroxyphenyl sulfate.



^{1}HRMN				^{13}CRMN
Number of C	δ (ppm)	Signal type	*J* (Hz)	δ (ppm)
1	2.02	s	———	21.10
2	———	———	———	173.55
3	4.23	t	6.8	66.58
4	2.84	t	6.8	35.61
5	———	———	———	137.81
6	6.78	d	2.0	118.91
7	———	———	———	150.39
8	———	———	———	140.13
9	7.19	d	8.4	124.27
10	6.69	dd	8.4 2.0	121.58
11	2.01	s	———	21.07
12	———	———	———	173.45
13	4.21	t	6.8	66.47
14	2.83	t	6.8	35.23
15	———	———	———	131.19
16	7.18	d	2.0	124.73
17	———	———	———	141.28
18	———	———	———	149.05
19	6.82	d	8.4	118.40
20	6.91	dd	8.4 2.0	127.76

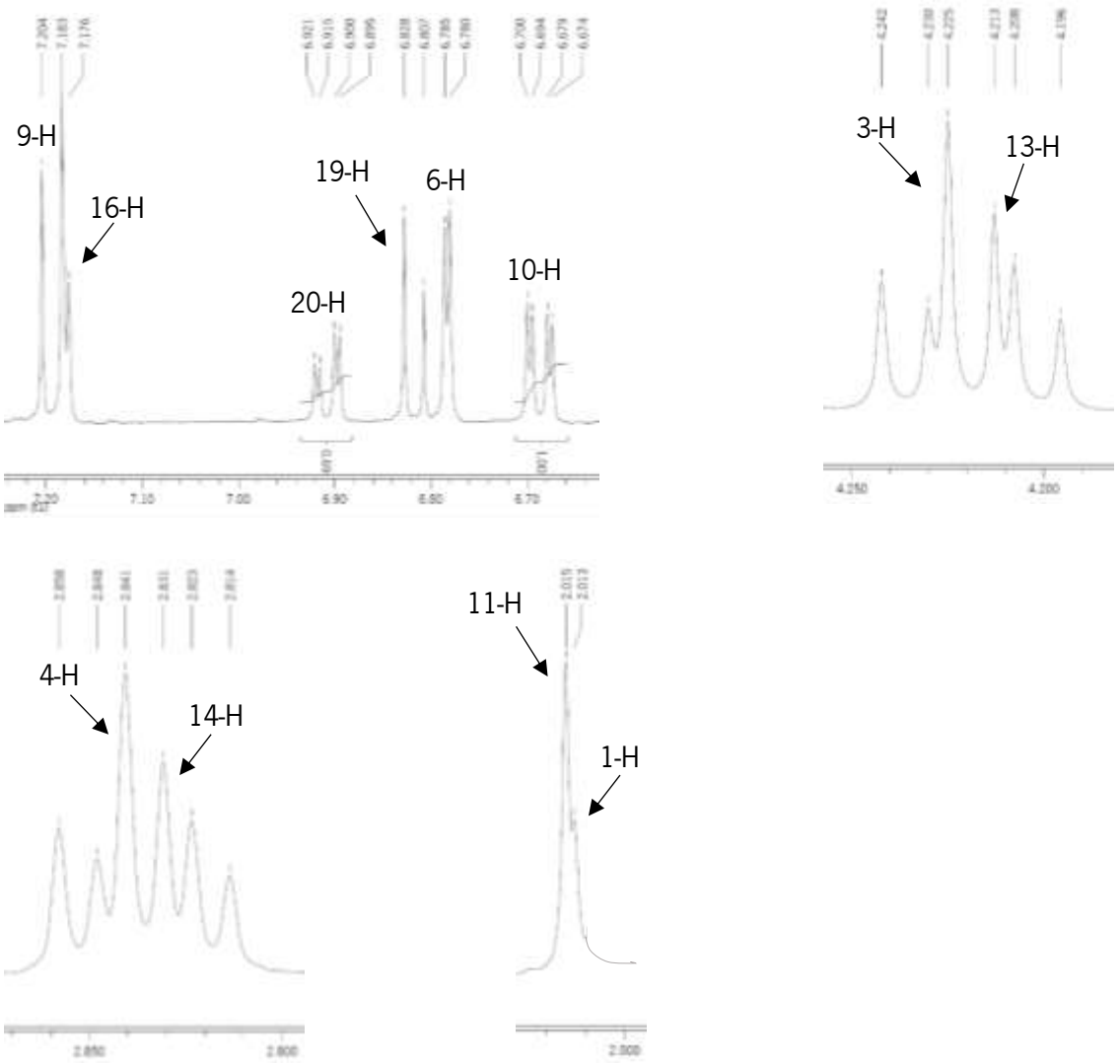
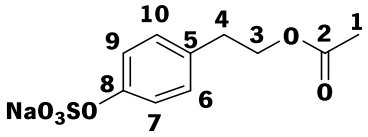


Figure 42: NMR of the sodium salt of 4-(2-ethanoyloxyethyl)-2-hydroxyphenyl sulfate and the sodium salt of 5-(2-ethanoyloxyethyl)-2-hydroxyphenyl sulfate.

2.2. Synthesis of the sodium salt of 4-(2-ethanoyloxyethyl)phenyl sulfate

Table 7 shows the NMR chemical shifts for the sodium salt of 4-(2-ethanoyloxyethyl)-phenyl sulfate. This product is formed from nucleophilic attack of the phenolic hydroxyl group of 2-(4-hydroxyphenyl)ethyl ethanoate on the sulfur trioxide pyridine complex. Once more, after 24 hours, the disappearance of the phenolic reagent was observed by TLC analysis using ethyl acetate/methanol (9:1) as eluent. The yield of this reaction was 40%.

Table 6: NMR chemical shifts in deuterated methanol of the sodium salt of 4-(2-ethanoyloxyethyl)-phenyl sulfate.

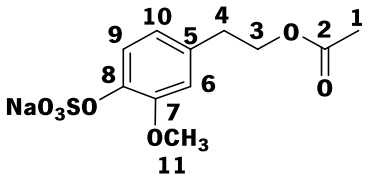


	^{1}HNMR			^{13}CNMR
Number of C	δ (ppm)	Signal type	*J* (Hz)	δ (ppm)
1	2.04	s	———	20.84
2	———	———	———	173.03
3	4.28	t	6.8	66.31
4	2.95	t	6.8	35.33
5	———	———	———	135.96
6	7.28	d	8.8	130.63
7	7.24	d	8.8	122.60
8	———	———	———	152.63
9	7.24	d	8.8	122.60
10	7.28	d	8.8	130.63

2.3. Synthesis of the sodium salt of 4-(2-ethanoyloxyethyl)-2-methoxyphenyl sulfate

The sodium salt of 4-(2-ethanoyloethyl)-2-methoxyphenyl sulfate was obtained from 2-(4-hydroxy-2-methoxyphenyl)ethyl ethanoate by reaction with the sulfur trioxide-pyridine complex in dry dioxane in 45% yield after purification. Table 7 shows NMR chemical shifts for this compound.

Table 7: NMR chemical shifts in deuterated methanol of the sodium salt of 4-(2-ethanoyloethyl)-2-methoxyphenyl sulfate.



^{1}HNMR				^{13}CNMR
Number of C	δ (ppm)	Signal type	*J* (Hz)	δ (ppm)
1	2.00	S	———	20.35
2	———	———	———	174.43
3	4.26	T	6.8	65.52
4	2.90	T	6.8	33.97
5	———	———	———	137.59
6	6.91	D	2.0	114.06
7	———	———	———	137.59
8	———	———	———	138.58
9	7.37	D	8.0	121.39
10	6.77	Dd	8.0 2.0	122.70
11	3.84	S	———	56.05

2.4. Tentative synthesis of 3-hydroxy-4-(sulfoxy)phenylethyl (*Z*)-4-formyl-3-(2-oxoethyl)hex-4-enoate and 4-hydroxy-3-(sulfoxy)phenylethyl (*Z*)-4-formyl-3-(2-oxoethyl)hex-4-enoate

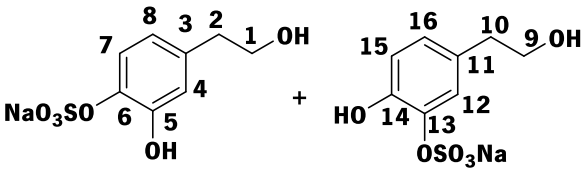
Synthetize of 3-hydroxy-4-(sulfoxy)phenylethyl (*Z*)-4-formyl-3-(2-oxoethyl)hex-4-enoate and 4-hydroxy-3-(sulfoxy)phenylethyl (*Z*)-4-formyl-3-(2-oxoethyl)hex-4-enoate by reacting the secoiridoid 3,4-DHPEA-EDA with the sulfur trioxide-pyridine complex was attempted.

Since the secoiridoid 3,4-DHPEA-EDA is a catechol, the reaction was left for 5 days in similar condition to those used with the catechol hydroxytyrosol acetate. After this time, TLC analysis showed a spot with Rf=0.12 [ethyl acetate/methanol (9:1)]. Purification was carried out in order to isolate the product and it was obtained as a yellowish oil (0.16 g). After ionic purification, NMR analysis of the product was not conclusive of the formation of the secoiridoid 3,4-DHPEA-EDA sulfate.

2.5. Synthesis of the sodium salts of 2-hydroxy-4-(2-hydroxyethyl)phenyl sulfate and 2-hydroxy-5-(2-hydroxyethyl)phenyl sulfate

Solvolysis of diethylammonium 4-(2-ethanoyloxyethyl)-2-hydroxyphenyl sulfate and diethylammonium 5-(2-ethanoyloxyethyl)-2-hydroxyphenyl sulfate was carried out by reacting these compounds with methanol. Table 8 shows the NMR chemical shifts of the isomeric mixture obtained after cation exchange. The yield was 64%.

Table 8: NMR chemical shifts in deuterated water of the sodium salts of 2-hydroxy-4-(2-hydroxyethyl)phenyl sulfate and 2-hydroxy-5-(2-hydroxyethyl)phenyl sulfate.



^{1}HNMR				^{13}CNMR
Number of C	δ (ppm)	Signal type	*J* (Hz)	δ (ppm)
1	3.72	t	6.4	62.51
2	2.70	t	6.4	37.34
3	———	———	———	137.23
4	6.81	d	2.0	117.68
5	———	———	———	147.74
6	———	———	———	138.52
7	7.18	d	8.4	122.80
8	6.74	dd	8.4 2.0	121.18
9	3.70	t	6.4	62.35
10	2.69	t	6.4	36.89
11	———	———	———	131.64
12	7.15	d	2.0	123.19
13	———	———	———	138.63
14	———	———	———	146.19
15	6.86	d	8.4	117.22
16	6.97	dd	8.4 2.0	127.67

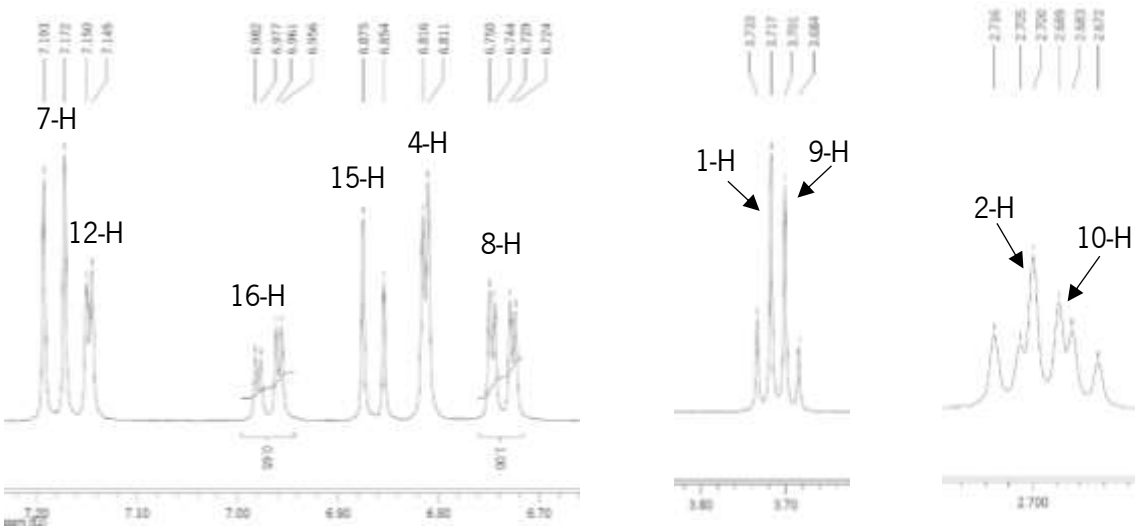


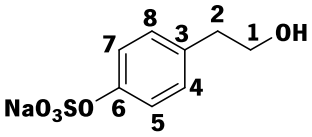
Figure 43: NMR of the sodium salts of 2-hydroxy-4-(2-hydroxyethyl)phenyl sulfate and 2-hydroxy-5-(2-hydroxyethyl)phenyl sulfate.

The proton NMR spectrum (figure 45) shows that the solvolysis reaction was successful due to the absence of the ester methyl singlets (2.02, 2.01 ppm). Additionally, the chemical shifts of the triplets of hydrogens 1 and 9 (3.72 and 3.70 ppm) are lower than the chemical shifts of the triplets of hydrogens 1 and 9 (4.23 and 4.21 ppm) of the sodium salt of 4-(2-ethanoyloxyethyl)-2-hydroxyphenyl sulfate and sodium 5-(2-ethanoyloxyethyl)-2-hydroxyphenyl sulfate. This results from an increased shielding effect by the hydroxyl groups formed.

2.6. Synthesis of the sodium salt of 4-(2-hydroxyethyl)phenyl sulfate

The deprotection of the ester of diethylammonium 4-(2-ethanoyloxyethyl)-phenyl sulfate was carried out by reaction with methanol. Table 9 shows the NMR chemical shifts of the sodium salt of 4-(2-hydroxyethyl)phenyl sulfate obtained after cation-exchange. The yield was 87%.

Table 9: NMR chemical shifts in deuterated water of the sodium salt of 4-(2-hydroxyethyl)phenyl sulfate.



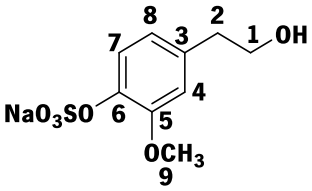
	^{1}HNMR			^{13}CNMR
Number of C	δ (ppm)	Signal type	*J* (Hz)	δ (ppm)
1	3.77	T	6.4	63.34
2	2.80	T	6.4	38.08
3	———	———	———	137.97
4	7.26	D	8.8	131.09
5	7.19	D	8.8	122.37
6	———	———	———	150.46
7	7.19	D	8.8	122.37
8	7.26	D	8.8	131.09

The disappearance of the singlet at 1.90 ppm and the lower chemical shift of the triplet at 3.77 ppm, due to the increased shielding effect caused by the hydroxyl group, shows that solvolysis occurred successfully.

2.7. Synthesis of the sodium salt of 4-(2-hydroxyethyl)-2-methoxyphenyl sulfate

The reaction of diethylammonium 4-(2-ethanoyloethyl)-2-methoxyphenyl sulfate with methanol was carried out to give the sodium salt of 4-(2-hydroxyethyl)-2-methoxyphenyl sulfate after cation-exchange. The yield of this reaction was 89%.

Table 10: NMR chemical shifts in deuterated water of the sodium salt of 4-(2-hydroxyethyl)-2-methoxyphenyl sulfate.



	^{1}HNMR			^{13}CNMR
Number of C	δ (ppm)	Signal type	*J* (Hz)	δ (ppm)
1	3.76	t	6.4	63.31
2	2.78	t	6.4	38.56
3	———	———	———	139.19
4	6.79	d	1.6	114.99
5	———	———	———	151.97
6	———	———	———	139.38
7	7.23	d	8.0	122.37
8	6.82	dd	8.0 1.6	123.53
9	3.80	s	———	56.89

Table 10 shows that the singlet at 2.00 ppm disappeared and the chemical shift of the triplet of hydrogen 1 (3.76 ppm) is lower than chemical shift of the triplet of hydrogen 3 (4.26 ppm) of the sodium salt 4-(2-ethanoyloethyl)-2-methoxyphenyl sulfate.

2.8. Tentative synthesis of diethylammonium 4-(2-ethanoyloxyethyl)-3-hydroxyphenyl sulfate

Since a mixture of monossulfates are obtained by the direct reaction of the cathecol with SO_3, an attemp to obtain only one isomer was performed. For that, diethylammonium 4-(2-ethanoyloxyethyl)-3-methoxyphenyl sulfate was used as initial reagent and made to reacted with boron tribromide. A possible mechanism for this reaction is shown in figure 46.

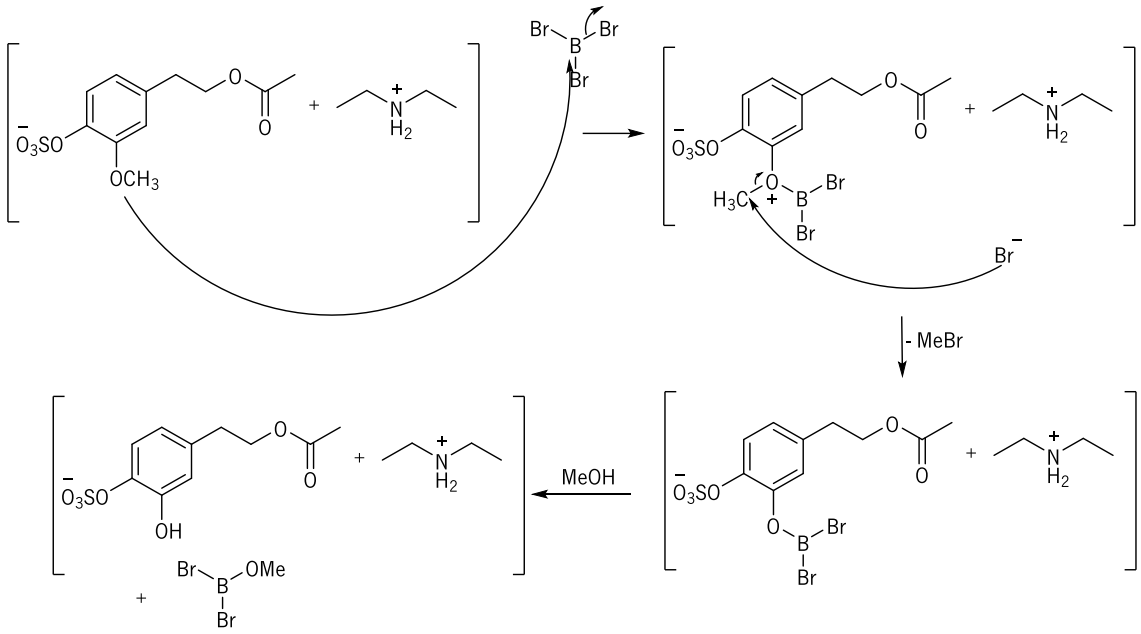
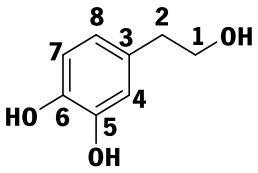


Figure 44: Proposed mechanism for the demethylation of diethylammonium 4-(2-ethanoyloxyethyl)-3-methoxyphenyl sulfate with boron tribromide.

The reaction was left for 24 hours and then 10 mL of methanol were added dropwise to destroy BBr_3. After purification, proton NMR gave a spectrum with the peaks and chemical shifts of hydroxytyrosol and not of diethylammonium 4-(2-ethanoyloxyethyl)-3-hydroxyphenyl sulfate.

Table 11: NMR chemical shifts in deuterated methanol of hydroxytyrosol.



^{1}HNMR				^{13}CNMR
Number of C	δ (ppm)	Signal type	*J* (Hz)	δ (ppm)
1	3.68	t	7.2	63.88
2	2.67	t	7.2	38.91
3	———	———	———	129.29
4	6.68	d	8.0	116.37
5	———	———	———	145.36
6	———	———	———	145.31
7	6.66	d	2.0	115.61
8	6.53	dd	8.0 2.0	120.52

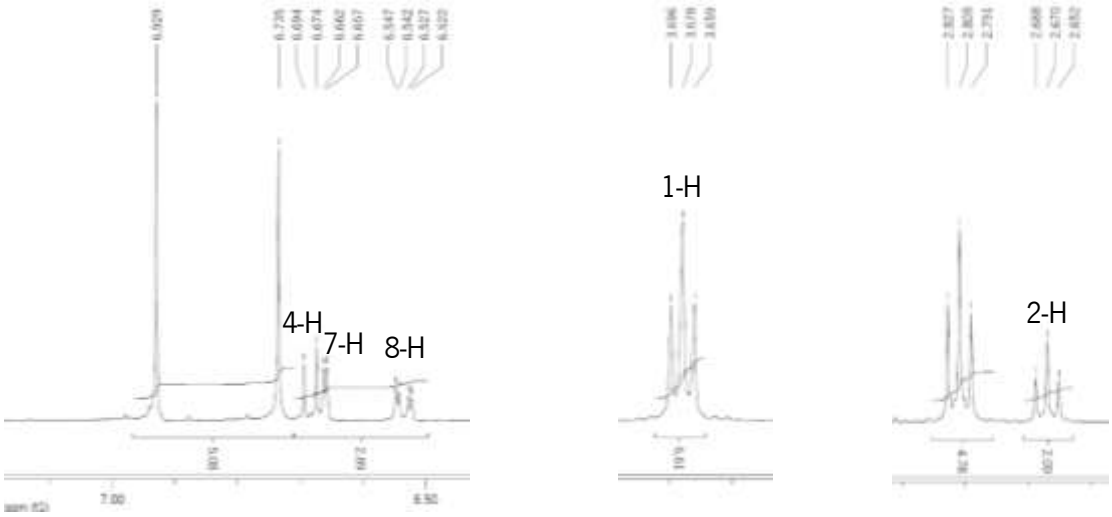


Figure 45: MR of mixtures of two compounds. Compound that is identified is hydroxytyrosol.

The chemical shifts of hydrogens 4, 7 and 8 are below those obtained for diethylammonium 4-(2-ethanoyloxyethyl)-3–hydroxyphenyl sulfate, which indicate that there is a greater shielding effect that would not occur if the hydroxyl at position 6 was sulfated. The chemical shifts of carbons 1 and 2 are also lower, which confirms that solvolysis of the ester occurred. In addition to these peaks, there are also two singlets in the aromatic zone and one additional triplet (figure 47). The hydrogen 1 triplet is integrated into 6 protons, leading to the conclusion that another product has been formed. Cosy, APT and HMQC analysis (see annexes) were performed to analyse the correlation of the protons with the carbons. However, it was not possible to determine the chemical structure of the secondary product.

3. Preliminar *in vitro* bioactivity evaluation

The protection against AAPH-induced haemolysis in human RBCs of tyrosol (Ty), hydroxytyrosol (HT), homovanillyc alcohol (HVA), 2-(3,4-dihydroxyphenyl)ethyl hydrogenosulfate (1-SO_4-HT), the mixture of the sodium salts of 2-hydroxy-4-(2-hydroxyethyl)phenyl sulfate and 2-hydroxy-5-(2-hydroxyethyl)phenyl sulfate (3,4-SO_4-HT), 4-(2-hydroxyethyl)phenyl sulfate (3-SO_4-Ty), hydroxytyrosol acetate (HTAc), homovanillyc acetate (HVAAc), the mixture of the sodium salts of 4-(2-ethanoyloxyethyl)-2-hydroxyphenyl sulfate and 5-(2-ethanoyloxyethyl)-2-hydroxyphenyl sulfate (3,4-SO_4-HTAc), and the sodium salt of 4-(2-ethanoyloethyl)-2-methoxyphenyl sulfate (4-SO_4-HVAAc)

was studied. Different concentrations of compounds were used (2.5, 5, 10, 20, 40 and 80 μM) with incubation at 37°C for 4 hours. A high percentage of inhibition of haemolysis reflects a high protection by the compound in study. The percentage of inhibition of haemolysis was measured by comparing the spectrophotometric absorbance values of aliquots of the supernatant of RBC suspension in PBS (pH=7.4 at λ=540 nm) and other aliquots in water. In this way, it was possible to conclude which compounds, and the respective concentration, confer more protection against haemolysis.

Figure 48 shows that in the presence of AAPH, parental compounds protected RBCs from oxidative-induced haemolysis in a concentration dependent manner, but only HT and HTAc showed important activity at 5 μM. In fact, HTAc showed better activity probably because of its higher lipossolubility that may allow a better location of this compound at the RBC membrane.

Although with a lower activity, the same rank is observed for HVA and its acetate, with the acetate showing a better protective activity.

It is interesting to note that, Ty shows a significantly activity against haemolysis inhibition of RBCs although it is a monophenol. It was able to protect RBC to an extent similar to that obtained by HVA. Most monophenols do not show radical scavenging activity against radicals used for radical scavenging activity evaluation studies such, as DPPH• or ABTS•. However, in vivo, highly reactive radicals can be formed during oxidative injury and they can actually be trapped by these monophenols, and therefore protect cells. This is the case of tyrosine, a monophenolic amino acid present in all membrane proteins.

In the case of metabolites, all sulfates showed a much lower protective activity than the parental compound. Only the 1-*O*-sulfate of HT retained much of the parental activity, showing that the two hydroxyl groups at the aromatic ring are important for the bioactivity of these compounds. In fact, the sulfation of one aromatic hydroxyl groups decreased drastically the protective activity of all compounds, in particular in the case of monophenols (Ty and HVA) that did not show any protection at any concentration.

Moreover, the increase of hydrophilicity of compounds by the conjugation with the sulfate group also contribute for a lower interaction of these metabolites with RBC membranes, deviating them from the place of action.

Nevertheless, it is important to point out that the HT and HTAc sulfates still show some protective activity. Although modest, in a nutritional context, this protection can be important since the consumption of olive oil takes place on a regular basis throughout life. It has also been described that conjugated metabolites may behave as carriers of bioactivity compounds in plasma, which may deconjugate in situ in target tissues realising the parental compound, which is the final effector.[76-77]

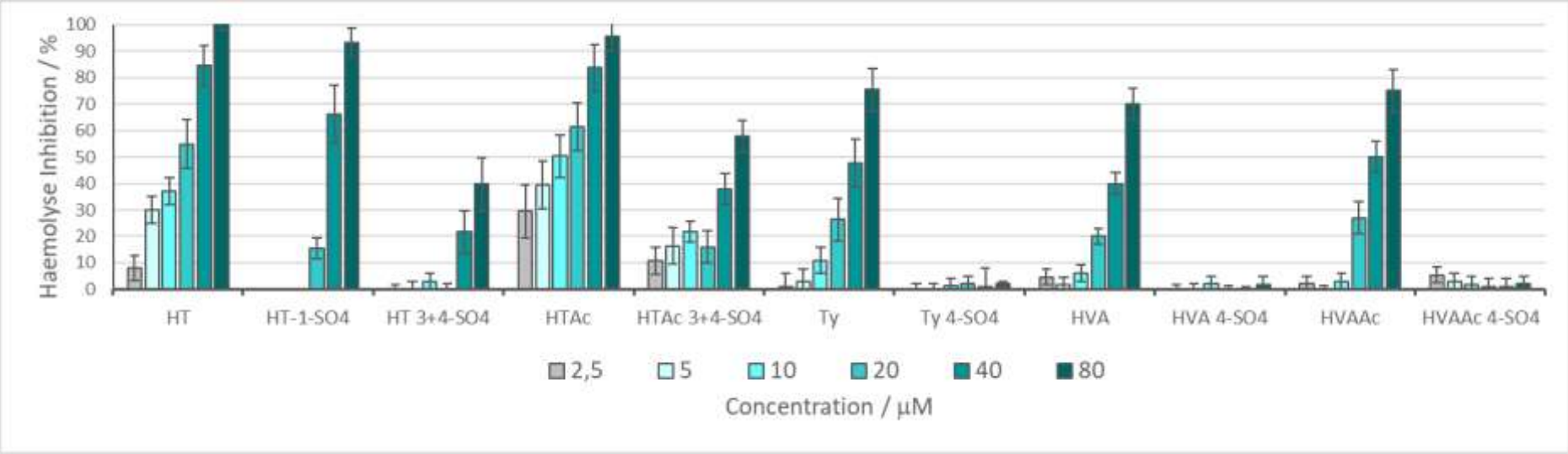


Figure 48: Percentage of inhibition of haemolysis of RBCs at 2% haematocrit incubated with olive oil phenolic compounds (2.5, 5, 10, 20, 40 and 80 µM and AAPH (60 mM) for 4 hours at 37 °C. Hydroxytyrosol (HT, 2-(3,4-dihydroxyphenyl)ethyl hydrogenosulfate (HT-1-SO_4), soium salt of 2-hydroxy-4-(2-hydroxyethyl)phenyl sulfate and odium salt of 2-hydroxy-5-(2-hydroxyethyl)phenyl sulfate (H 3+4 SO_4), hydroxytyrosol acetate (HTAc, sodium salts of 4-(2-ethanoyloxyethyl)-2-hydroxyphenyl sulfate and 5-(2-ethanoyloxyethyl)-2-hydroyphenyl sulfate (HTAc 3+4 SO_4), tyrosol (Ty), sodium salt of 4-(2-hydroxyethyl)phenyl sulfate (Ty 4-SO_4), homovanillyc alcohol (HVA), sodium salt of 4-(2-hydroxyethyl)-2-methoxyphenyl sulfate (HVA 4-SO_4), homovanillyc acetate (HVAAc) and sodium salt of 4-(2-ethanoyloethyl)-2-methoxyphenyl sulfate (HVAAc 4-SO_4).

V. Conclusion

VOO is the major source of fat in the Mediterranean diet and many studies indicate that minor constituents of VOO, the phenolic compounds, contribute to the healthy Mediterranean diet. The most common phenols in olives include phenolic alcohols, such as HT and Ty, secoiridoids, flavonoids and lignans. The major class of phenols found in VOO are secoiridoids. Secoiridoid glycosides such as oleuropein are hydrolysed during VOO extraction by β-glucosidases. Therefore, the major phenolic compounds found in VOO are the dialdehydic form of elenolic acid linked to HT (3,4-DHPEA-EDA), the oleuropein aglycon (3,4-DHPEA-EA), and the dialdehydic form of elenolic acid linked to Ty (4-HPEA-EDA).

Many studies investigating the in vitro antioxidant properties of VOO phenolics, as well as their protective effects against cell injury, indicate that the biological properties of these phenols *in vivo* depend on the extent to which they are absorbed and metabolized. Olive oil phenols undergo extensive metabolism in the intestines and liver and are thus chiefly found in biological fluids as phase II metabolites. Bioavailability studies have shown that HT is efficiently absorbed in the small intestine and, together with its acetate, glucuronide and sulfate conjugates, can be found in human urine and plasma after olive oil consumption.

RBCs are the most abundant blood cells in the human organism. These cells enable the transport of O_2 between respiratory surfaces and metabolizing tissues. Oxidative damage to cell components has been established as a factor in various pathological conditions. In circulation, RBCs are continuously exposed to both endogenous and exogenous sources of ROS that can damage them and impair their function.

In this work, synthetic strategy has been used in the preparation of considerable amounts of sulfate metabolites. For this purpose, the parental phenolic compounds HT, Ty and HVA needed to be protected at the alkyl hydroxy groups by esterification. In the synthesis of these esters, it was found that the best process in order to obtain the monoesters was the transesterification with the ethyl acetate catalysed by the amberlite IR-120. The yields obtained were between 75% to 98%. Then, six monosulfates (HT 3,4-SO_4, HTAc 3,4-SO_4, Ty 4-SO_4, TyAc 4-SO_4, HVA 4-SO_4, HVAAc 4-SO_4) have been synthetized in 1 to 2 steps in a good yield and using simple, cheap, and fast procedures.

Isolation of 3,4-DHPEA-EDA and 3,4-DHPEA-EA was also carried out. The secoiridoid 3,4-DHPEA-EDA was isolated and identified by NMR and UHPLC leading to the conclusion that the compound obtained was pure. However, the extracted containing the isolated compound 3,4-

DHPEA-EA was not pure, and containing 74% of 3,4-DHPEA-EA, 9% of 3,4-DHPEA-EDA and 17% of oleocanthal. The synthesis of 4-HPEA-EDA was attempted by transesterification with no success.

The synthesis of 3,4-DHPEA-EDA sulfate metabolite was also attempted but this attempted also failed.

Finally, the tentative synthesis of only one monosulfate of HT, the 4-*O*-sulfate, by the demethylation of the homovanillyc acetate 4-*O*-sulfate was attempted. However, only hydroxytyrosol was obtained at the end of this reaction.

The protection against AAPH-induced haemolysis in human RBCs of hydroxytyrosol (HT), 2-(3,4-dihydroxyphenyl)ethyl hydrogenosulfate (1-SO_4-HT), the mixture of sodium 2-hydroxy-4-(2-hydroxyethyl)phenyl sulfate and sodium 2-hydroxy-5-(2-hydroxyethyl)phenyl sulfate (3,4-SO_4-HT), the mixture of the sodium 4-(2-ethanoyloxyethyl)-2-hydroxyphenyl sulfate and 5-(2-ethanoyloxyethyl)-2-hydroxyphenyl sulfate (3,4-SO_4-HTAc), tyrosol (Ty), sodium 4-(2-hydroxyethyl)phenyl sulfate (4-SO_4-Ty), sodium 4-(2-ethanoyloethyl)-2-methoxyphenyl sulfate (4-SO_4-HVA) and sodium 4-(2-ethanoyloethyl)-2-methoxyphenyl sulfate (4-SO_4-HVAAc) were evaluated. All compounds except 4-SO_4-Ty protected RBCs from haemolysis in a concentration-dependent manner, which means that when concentration increased, haemolysis inhibition also increased. We could also conclude that the sulfation of aromatic hydroxyl groups leads to a significant decrease in the protective activity of the compounds. The compound Ty, although being a monophenol, showed a significant activity against the induced haemolysis inhibition of RBCs.

As future work, we intend to obtain each of the hydroxytyrosol monosulfates sodium salts separately and the 3,4-DHPEA-EDA sulfates. We also intend to achieve the synthesis of oleocanthal since it is reported to have high health benefits.

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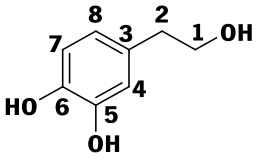
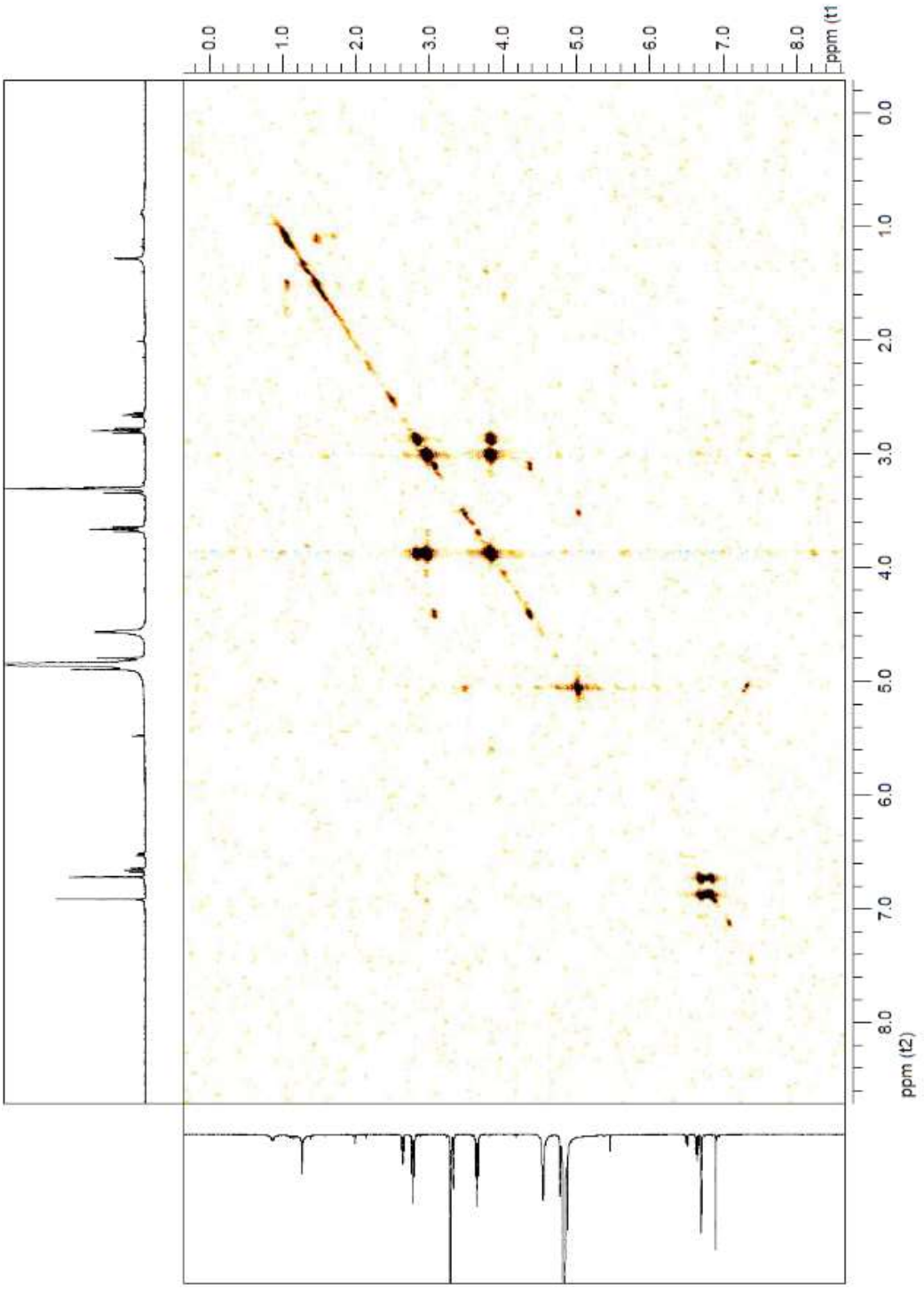
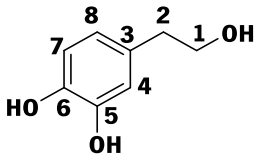
VII. Annexes

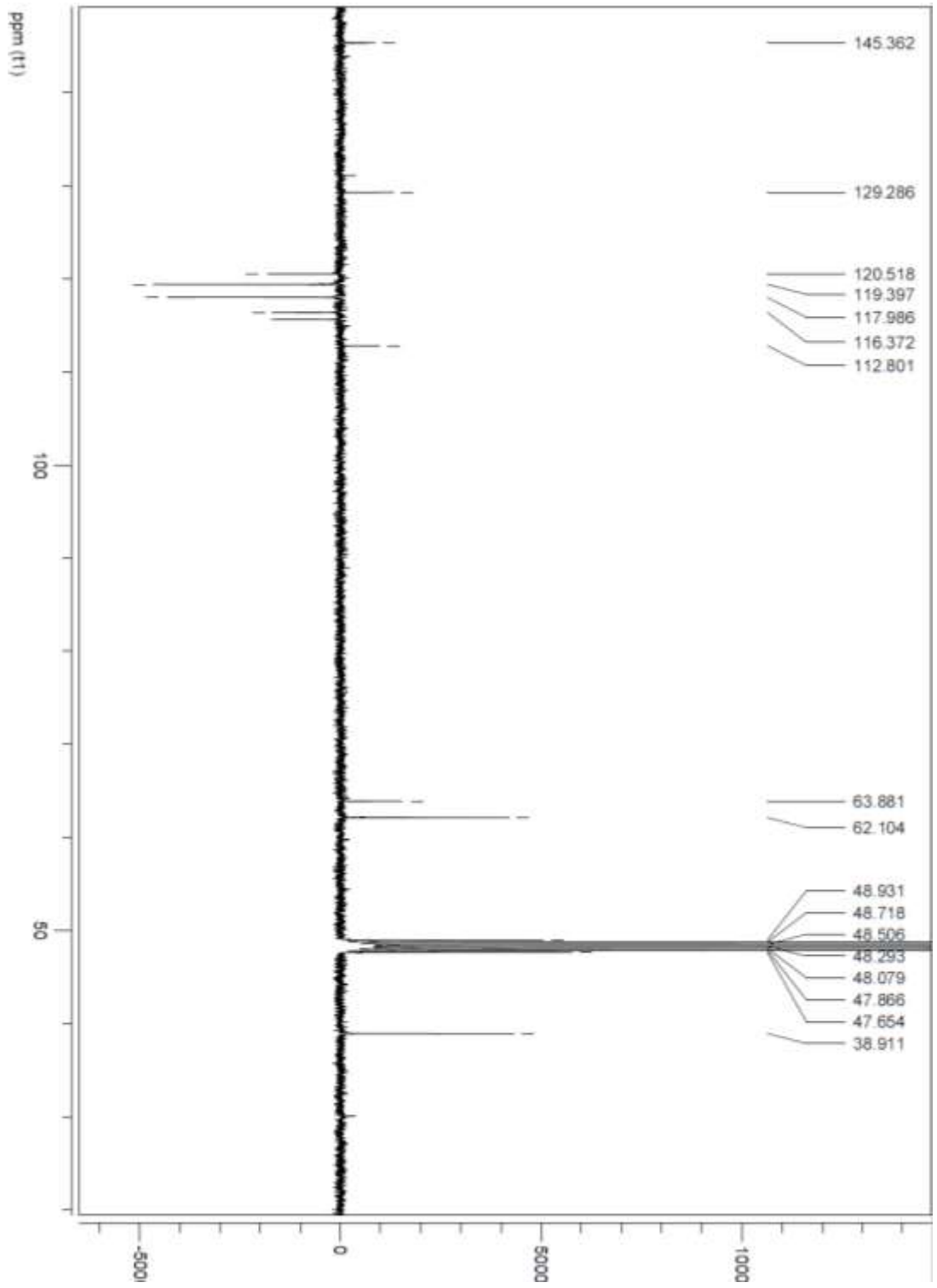
Annex A - Cosy spectrum of hydroxytyrosol in deuterated methanol.





Annex B - APT spectrum of hydroxytyrosol in deuterated methanol.


8
2
3
1
OH
7
4
HO
6
5
OH


ppm (t1)
145.362
129.286
120.518
119.397
117.986
116.372
112.801
63.881
62.104
48.931
48.718
48.506
48.293
48.079
47.866
47.654
38.911
100
50
-500
0
5000
1000

Annex B - HMQC spectrum of hydroxytyrosol in deuterated methanol.