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# ESTERIFICATION OF NATURAL COMPOUNDS WITH FATTY ACIDS

CHIM 03/C1 (ex CHIM 06)

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## ABSTRACT

In this PhD project we dealt with β-sitosterol, resveratrol and quercetin, three natural substances featuring ascertained biological activities. These compounds are characterized by a limited bioavailability and a low stability: these features reduce their application in pharmaceutical, nutraceutical and dermocosmetic areas. In order to improve the above mentioned properties, we synthesized esters of these compounds, following either a chemical approach (resveratrol, quercetin, and β-sitosterol) and a enzymatic approach (resveratrol and β-sitosterol). The esterification was performed with saturated (palmitic and stearic) and unsaturated (oleic, linoleic and linolenic) fatty acids: this synthesis introduced in a single molecule (prodrug) two moieties both pharmacologically active.

Chemical synthesis was used to obtain resveratrol triesters, but failed with diesters: so these derivatives were synthesized by enzymatic approach. $^{1}$H-NMR and $^{13}$C-NMR analyses allowed to define the structure of these derivatives.

The synthesis of quercetin penta, tetra and triesters was obtained by modulating the acid/quercetin molar ratio; mono- and bi-dimensional NMR techniques were used to determine the structure of all quercetin esters, while for triester computational studies were needed.

In addition, for resveratrol and quercetin esters the antioxidant activity was evaluated: this property was not increased by esterification. Also the bioavailability was studied for resveratrol and its trioleoyl ester, but the resveratrol resulted more bioavailable in comparison with its ester.

In order to improve drug topical delivery, quercetin and its derivatives were also incorporated in liposome formulations.

# INDEX

# 1 - INTRODUCTION

In recent years, the interest for natural substances with beneficial activity in human, and in particular for the ones that are useful to counteract aging, has sharply risen. In fact, there is a significant increase in skin cosmetic, nutraceuticals and even pharmaceutical products, based on natural compounds or on their semi-synthetic derivatives. The main interest has been observed for natural substances with strong anti-oxidant activity, because oxidative stress induced by multiple factors is the main cause of many pathological conditions: inflammation, cancer, coronary heart disease and even skin aging.

In this PhD work we dealt with three natural compounds: β-sitosterol, resveratrol and quercetin, substances featuring biological activities which are ascertained and described in literature.

β-sitosterol is the most abundant phytosterol; plant sterols are structurally similar to cholesterol and a recent epidemiological study have shown an inverse relation between phytosterol consumption and serum cholesterol. In addition, growing evidence suggests that phytosterols have preventive effects against cancer of the lung, stomach, ovary and estrogen-dependent human breast cancer. Phytosterols consumption may also increase the activity of antioxidant enzymes and thereby reduce oxidative stress.

Resveratrol, a natural polyphenol present in several plants, especially in grapes, has been shown to have a number of physiological properties such as antioxidant, anti-inflammatory and antitumor activities. In addition, resveratrol was identified as the most potent activator of SIRT1, a protein possessing $NAD^+$-dependent deacetylase activity. SIRT1 plays a context-dependent role in health span regulation, for instance by mediating effects in metabolic stress situations, such as high-fat-diet-induced obesity, glucose intolerance and cancer. Resveratrol has been shown to have a preventive effect on photoaging and photocarcinogenesis as well, in fact the topical application of this polyphenol was found to inhibit skin edema, inflammation, peroxidation, protein expression induced by UVB irradiation.

Quercetin is a natural polyphenol, but unlike resveratrol, it belongs to the flavonoid family. Flavonoids are found in a wide variety of fruits and vegetables, either as glycosides (bound to a sugar moiety) or as aglycones (without sugar groups). Quercetin has been shown to possess several biological properties, such as antioxidant, antithrombotic, antihypertensive, antitumor and antiallergic activities. In addition, topical application of quercetin inhibits oxidative skin damage and the inflammatory process induced by solar UV radiation.

Although these compounds are present in significant amounts in foods, their dietary intake is generally characterized by a low bioavailability, either due to their low absorption and to the high rate with which they are metabolized, mainly by methylation, sulfation and glucuronidation of -OH groups. These factors reduce the potential preventive and therapeutic activities of these classes of molecules, hampering their

use in pharmaceutical and nutraceutical areas. Their dermocosmetic application is reduced by their low skin permeability and poor solubility in water media, making the development of pharmaceutical formulations difficult.

The aim of this PhD project is to modify the structure of the three investigated compounds in order to improve their solubility, bioavailability and stability, leading to the development of a number of semi-synthetic compounds. In drugs containing carboxylic and hydroxylic groups, esterification represents one of the main reactions in organic synthesis used to improve the above mentioned properties.

We decided to esterify these natural substances with saturated and unsaturated fatty acids, especially ω-3 and ω-6. This would introduce in a single molecule (prodrug) two moieties both pharmacologically active. Fatty acids are a class of compounds with interesting pharmacological properties, such as cardiovascular activity and structural function especially in the skin. In fact, palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), and (all-*cis*)-11,14,17-eicosatrienoic acid (ETA, C20:3n-3) were determined as major fatty acid components in human epidermis; the levels of saturated fatty acids (such as palmitic and stearic acids) and unsaturated fatty acids (such as linoleic acid and ETA) result to be decreased in aged skin compared with those in young skin.

In this PhD project we looked at the development of esterification processes of β-sitosterol, resveratrol and quercetin with fatty acids and the analytical characterization of the compounds obtained.

The esterification of β-sitosterol with fatty acids was performed either by chemical and enzymatic approach. The β-sitosterol esters chemically synthesized were used as reference standards for the determination of the esters enzymatically obtained.

Every attempt of chemical esterification of resveratrol led indistinctly to the synthesis of the triester, even by modulating the molar ratio of acid vs. resveratrol. To obtain the mono and di derivatives we went to a biosynthetic approach: starting from the triester, in appropriate conditions and using suitable enzymes, we were able to synthesize and to characterize partially hydrolyzed derivatives of resveratrol.

Following a similar approach, the chemical esterification of quercetin led to the formation of completely esterified quercetin. By modulating the molar ratios between quercetin and fatty acid, on the contrary, a mixture of tri, tetraester was obtained. To identify non-esterified groups of quercetin, mono- and bi-dimensional NMR techniques were used: this allowed us to determine the structure of tetraester, while the structure of triester was determined by computational studies.

In addition, for the esters obtained from resveratrol and quercetin some pharmacological properties such as antioxidant activity and bioavailability were assessed, and liposome formulation trials were carried out.

## 1.1 - β-SITOSTEROL

Sterols in plants have cellular functions analogous to those of cholesterol in animals. β-sitosterol (Fig. 1) is the most common sterol in plants; it differs from cholesterol for the ethyl group at C-21. Hydrogenation of the 6, 7 double bond of β-sitosterol converts it into sitostanol.

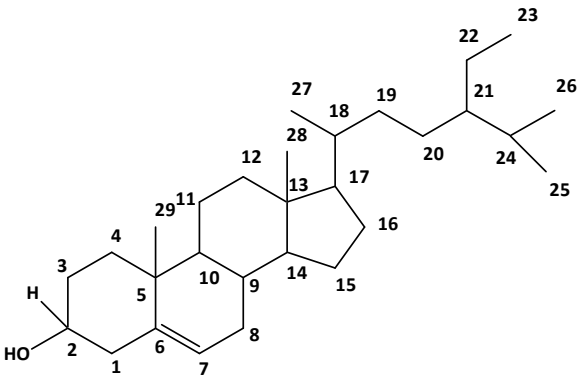



**Figure 1.**

In plants, sterols have both a structural function and a metabolic role. They are integral membrane components which regulate the membrane fluidity and permeability, affecting various functions such as simple diffusion, carrier-mediated diffusion and active transport across the membrane. They also modulate the activities of membrane-associated proteins including enzymes, receptors and signal transduction components. In addition, they are precursors of other bioactive steroids, such as the so-called brassinosteroids (a special class of growth substances), and substrates for the synthesis of numerous secondary plant metabolites **[1]**.

Vegetable oils and cereals are generally known to be the best natural sources of dietary plant sterols **[2]**.

Plant sterols occur both as free sterols and as bound conjugates, i.e., fatty acid esters (mainly C-16 and C-18 fatty acids), esters of phenolic acids, glycosides (most commonly with β-D-glucose) and acylated glycosides (esterified at the 6-hydroxy group of the sugar moiety) **[3]**. The sterol content in a given plant may vary depending on many factors, such as genetic background, growing conditions, tissue maturity and postharvest changes **[2]**.

### 1.1.1 - BIOLOGICAL ACTIVITIES

Cardiovascular activity

There is a scientific consensus that reduction of low density lipoprotein (LDL) cholesterol is important for coronary risk decrease. Dietary cholesterol intake is variable but is often less than 300 mg/day and currently 200 mg/day is recommended. Approximately 25% of the plasma cholesterol is due to dietary

intake, while 75% is accounted by endogenous synthesis **[4]**. However, dietary cholesterol appears to be quite important because it is inversely correlated with endogenously synthesized cholesterol, suggesting that they are co-regulated **[5]**. Phytosterols are thought to act primarily in the intestinal lumen. As cholesterol analogs, they compete with it in absorptive micelles resulting in a reduced solubility of cholesterol **[6]**. The affinity of plant sterols for micelles is greater than that of cholesterol. Human physiological studies showed that the inclusion of phytosterols in a test meal resulted in the reduction of absorbable micellar cholesterol in duodenal aspirates **[7]**. Even if phytosterols have a primary mechanism of action in the intestine, their effects cause indirect changes in circulating LDL cholesterol. Reduced transportation of absorbed cholesterol to the liver results in increased tissue LDL receptors **[8]**. Further evidence of relative cholesterol deficiency after phytosterol treatment is the measured increase in whole body cholesterol biosynthesis of 38–53% **[9]**. These results also demonstrates the rationale for using phytosterols with statin drugs.

Then, phytosterols are recognized as an important component of healthy diets and diets designed to reduce hypercholesterolemia. The United States National Cholesterol Education Program recommends dietary phytosterol supplementation of 2 g/day as a lifestyle modification for cholesterol reduction.

Antioxidant and anti-inflammatory activity

Reactive oxygen species (ROS) produced by stressed cells can damage DNA. Vivancos **[10]** reported that β-sitosterol increases the activities of antioxidant enzymes, superoxide dismutase and glutathione peroxidase in cultured macrophage cells with oxidative stress induced by phorbol 12-myristate 13-acetate, indicating that phytosterols can protect cells from ROS damage. In another study **[11]**, cultured lipopolysaccharide-activated macrophage cells were treated with β-sitosterol and campesterol: the results showed a decreased production of prostaglandin E and prostaglandin I of series 2, by 68% and 67% (for sitosterol), and by 55% and 52% (for campesterol), respectively. These studies suggest that phytosterols are able to counteract the oxidative and inflammatory processes.

Anticancerogenic activity

Phytosterols seem to inhibit the development of various cancers mainly by inhibiting growth and promoting apoptosis of cancer cells by the activation of caspase enzymes. The increased activity of caspase enzymes could be attributed to the incorporation of phytosterols into cell membranes, which results in changes in membrane structure and function; furthermore, these changes increase the activities of proteins involved in extra- and intracellular signal-transduction pathways that activate caspase enzymes. Phytosterols could also inhibit cancer development by lowering blood cholesterol, as high blood cholesterol levels, and hence

the concentration of cholesterol in lipid rafts of cell membranes, are associated with reduced apoptosis of cancer cells. This combined evidence strongly supports an anticarcinogenic action of phytosterols and hence advocates their dietary inclusion as an important strategy in prevention and treatment of cancer **[12]**.

### 1.1.2 - METABOLISM AND BIOAVAILABILITY

After ingestion, phytosterols, like cholesterol and other lipids, are emulsified by bile salts secreted into the small intestine to form micelles for digestion. After micelle formation, the esterified phytosterols are hydrolyzed to free phytosterols probably by cholesterol esterase and pancreatic lipase **[13]**. Free phytosterols are then absorbed into enterocytes by ATP-binding cassette transporters which are also involved in cholesterol absorption. In the enterocytes these compounds are esterified with fatty acids by acyl-CoA cholesterol acyltransferases, and combined with cholesterol, triacylglycerol and apolipoproteins to form chylomicrons **[14]**. The chylomicrons are secreted into the lymph and then transferred to the bloodstream, where they are transformed to chylomicron remnants after the uptake of triacylglycerol by cells, and transported to the liver. In the liver, the phytosterols may either be used for synthesis of bile salts or be incorporated into very low-density lipoproteins and be secreted into the blood, from where they are converted to low-density lipoproteins and presented to cells for uptake **[15]**. In the tissues, phytosterols are incorporated into the cell membranes **[16]** and have been found to be highly concentrated in the lungs, adrenal cortex, intestinal epithelia and ovaries **[17]**. Phytosterols that are either not taken up by cells or secreted back into the blood are transported to the liver, from where they are excreted into the bile.

## 1.2-RESVERATROL

Resveratrol is a polyphenolic phytoalexin which is present in grapes, grape juice, red wine and other plant extracts **[18]**. In the grape species, this polyphenol reaches concentrations of 50-400 μg/g of fresh weight in the leaves. Fresh grape skin contains about 50-100 μg of resveratrol for gram **[19].** Consequently, the amount of this compound varies considerably in different types of grape juice and wine depending on the grape variety, environmental factors in the vineyard, juice extraction and wine processing techniques. In grapes and wine, resveratrol occurs both as free form and resveratrol 3β-glucoside **[20]**. In red wine the concentration of resveratrol is in the range of 1.5-3 mg/L **[21].**

This phytoalexin, produced in response to environmental stress or pathogenic attack, is an antifungal conferring disease resistance in plant kingdom **[22]**. It is a hydroxyl substituted stilbene (fig.2).

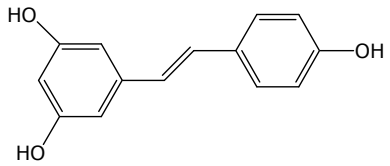



**Figure 2.**

Resveratrol exists as *trans* and *cis* isomers. In the Vitaceae fungal infection or UV light stimulates the production of stilbene synthase and catalyzes the reaction of 4-hydroxycinnamoyl-CoA and malonyl-CoA to produce *trans*-resveratrol. In the grape berry, *trans*-resveratrol production is stimulated by UV light exposure, fungal infection, or injury **[23]**. In red wine a small amount of *cis* isomer has been detected and it is supposed that the *cis* form is derived by isomerization from the *trans* isomer during the fermentation of grapes **[24]**. The balance between *trans* and *cis* isomer is obtained after exposure to the daylight and more than half of the *trans* isoform may change to its *cis* isomer. Although both *cis* and *trans* forms exhibit anticancer activity **[25]**, it is not clear whether the pharmacokinetic properties of the *cis* isomer are identical to those of the *trans* .

Resveratrol is characterized by a number of bioactivities, such as antioxidant, anti-inflammatory, antitumor, cardioprotective, neuroprotective and immunomodulatory; it has been also recognized to have a delaying effect on aging.

### 1.1.1 - BIOLOGICAL ACTIVITIES

Antioxidant activity

The antioxidant activities of polyphenols are due to the ability of the hydrogen of phenolic compounds to be donated, to neutralize free radicals produced by oxidative processes such as phospholipid peroxidation via UV radiation or by biologically-mediated events. It has been demonstrated that resveratrol may serve both as a primary antioxidant (free radical scavenger), which can directly react with free radicals and convert them into less active products **[26,27]**, and as a secondary (preventive) antioxidant, which can lower the rate of oxidation by inhibiting enzyme activities. For example, resveratrol has been shown to inhibit the activities of cyclooxygenase, lipooxygenase and xanthine oxidase where increased levels of these enzymes lead to variety of diseases **[28]** such as cancer, arteriosclerosis or heart ischemia.

Cardiovascular activity

Coronary artery disease remains the most deadly illness in western countries. Atherosclerosis, hypertension, obesity, endothelial dysfunction, alteration in platelet function and oxidative stress all contribute to the onset and progression of coronary artery disease. Many of the risk factors for this pathology such as age, sex and family history are uncontrollable; hence, emphasis must be placed on the modifiable risk factors. In particular, interest in dietary modification and even supplementation is at an all-time high: examples of compounds that can be supplemented are omega-3 fatty acids, natural antioxidants and plant sterols, all recognized as "heart-healthy" foods **[29]**. Red wine was found to be one of these heart-healthy foods. In fact, the term "French paradox" refers to the observation that mortality due to coronary diseases in France, where red wine consumption is considerable, is significantly lower compared to that in other countries despite similarities in other risk factors such as dietary fat intake, obesity and cigarette smoking: resveratrol contributes to the antioxidant potential of red wine, and may be related with the decrease in coronary heart disease among wine drinkers.

Atherosclerosis lies at the root of coronary artery disease. The generation of atherosclerotic plaque can be thought of as a stepwise process, initiated by the concentration-dependent transport of LDLs into the arterial wall followed by modification of lipids and proteins by oxidation and inflammation invasion of magrophages and their transformation into foam cells, which infiltrate smooth muscle cells with subsequent production of fibrin and extracellular matrix **[30]**. Resveratrol has been shown to attenuate each of these processes.

LDL and HDL play quite different roles in the genesis of atherosclerosis. LDL is the cholesterol compound that is responsible for mural deposition and initiating atherosclerotic lesion, whereas HDL transports cholesterol back to the liver where it is metabolized. Treatment with resveratrol has been shown repeatedly to produce an anti-atherogenic serum lipid profile. It had been demonstrated that apolipoprotein E-deficient mice treated with daily oral resveratrol for 20 weeks had significantly lower circulating LDL and higher HDL compared to untreated controls **[31]**. Moreover, most studies show that this compound, administered at a wide range of doses and for even short periods of time, exerts a favorable effect on lipids profile in hypercholesterolemic animals. One possible explanation for this beneficial effect of resveratrol on lipids was proposed by Cho et al **[32]**, who showed that HGM-CoA mRNA was up-regulated by resveratrol supplementation in hamsters fed with a high-fat diet.

Intramural lipid accumulation is followed by LDL oxidation, which promotes macrophage migration into the vessel wall. Macrophages in turn take up oxidized LDL in large quantities and transform it into the cholesterol-rich foam cells, that characterize early atherosclerotic lesion **[33]**. Resveratrol by scavenging free radicals, decreases the oxidation of LDL and increases serum HDL, which not only has antioxidant activity but also promotes the excretion of excess cholesterol from macrophages, transporting it back to the liver **[34].** Resveratrol has also been shown to inhibit monocyte recruitment and macrophage activation. Studies by Deng et al. **[35]** showed that this compound inhibits tumor necrosis factor-α (TNF-α)-mediated adhesion of monocytes to endothelial cells and reduces protein and gene expression of the intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), by inhibiting activation of NF-kB; ICAM-1 and VCAM-1 are essential to the localization of monocytes to early atherosclerotic lesion. Other studies also demonstrated that resveratrol down-regulates the production of other cytokines that contribute to the inflammatory environment, that is essential for the progression of atherosclerosis **[36].**

Later in the course of atherosclerosis, smooth muscle cells, lured by the cytokines and growth factors secreted by macrophages, migrate into the atherosclerotic plaque, proliferate, and begin to secrete components of extracellular matrix, maturing the plaque into a capped, fibrous lesion. This process is mediated by various chemotactic factor proteins and receptors, and resveratrol may oppose multiple steps in this process. In fact, the estrogen receptor has also been shown to play a role in smooth muscle cells proliferation, and resveratrol has been found to modulate this receptor, leading to increased inducible nitric oxide synthase activity and subsequent decrease in smooth muscle cell proliferation **[37].**

The fibrotic plaque formed by migrating smooth muscle cells predisposes to the final step in atherosclerosis, which is thrombogenesis. Several hypotheses have been proposed to explain the inhibitory effect of resveratrol on platelet activation. The initial step in platelet activation is adhesion to type I collagen. Resveratrol decreased adhesion to collagen and fibrinogen after activation with lipopolysaccharide or thrombin **[38]**. Moreover, the protein kinase C (PKC) plays a key role in platelet activation, and resveratrol has been shown to depress the activity of PKC in platelet membranes **[39].** Each of these mechanisms may contribute to the antithrombotic action of resveratrol.

Antitumor activity

Recent studies have documented that resveratrol has cancer-preventive properties **[40]**, and this compound is currently at the stage of preclinical studies for human cancer prevention **[41].** Its potential

chemopreventive and chemotherapeutic activities have been demonstrated at all three steps of carcinogenesis : initiation, promotion, progression.

Both its m-hydroxyquinone and 4-hydroxylstyryl moieties have been shown to be important for determination of resveratrol inhibitory properties against various enzymes: these include lipoxygenases and cyclooxygeneses that synthesize pro-inflammatory mediators from arachidonic acid, protein kinases (PKCs and PKD), and receptor tyrosine kinases **[42]**. Resveratrol in addition regulates apoptosis and cell proliferation: it induces growth arrest followed by apoptotic cell death and interferes with cell survival by up-regulating the expression of pro-apoptotic genes while simultaneously down-regulates the expression of antiapoptotic genes **[43].**

Resveratrol has been shown to be an effective chemopreventing agent in multiple murine models of human cancers.

Skin cancer is the most common type of human malignancy. Topical administration of resveratrol has been tested for its efficacy against the development of several cutaneous disorders, including skin cancer. The chemoprotective effect of this compound was assessed on UVB-mediated skin tumorigenesis in the SKH-1 hairless mouse model. Topical application of resveratrol either pre- or post-UVB significantly inhibited tumor incidence and delayed the onset of tumorigenesis **[44]** .

Resveratrol is considered to be a phytoestrogen, given its structural similarity to diethylstilbestrol, a synthetic estrogen. It can bind to both alpha and beta estrogen receptors, and activates estrogen receptor-dependent transcription in human breast cancer cells. Moreover, resveratrol inhibition of P450/Aromatase, by limiting the amount of available estrogens and consequently the activity of estrogen receptors, has been proposed to contribute to its activity against several types of cancer, including breast cancer . Despite a number of studies performed using both hormone-sensitive and hormone-resistant breast cancer cells, the estrogen modulatory effects of resveratrol remain controversial **[45]**.

Furthermore, growth inhibitory effect of resveratrol has been demonstrated in various cultured prostate cancer cells, both hormone-sensitive and hormone-refractory, which mimic the initial or advanced stages of prostate carcinoma, respectively **[46].** These studies have shown that resveratrol substantially modulates the growth of these cells and alters the expression of more than one set of functionally related molecular targets. In fact, this polyphenol can repress different classes of androgen-responsive genes, including prostate-specific antigen (PSA) **[47].**

The primary etiological determinants for gastric cancer are thought to be exposure to chemical carcinogens and/ or chronic infection with *Helicobacter pylori*. Resveratrol was found to be effective in inhibiting the

replication of *H. Pylori* **[48]**: this provides a reason for the intervention studies using resveratrol to counteract gastric cancer **[49]**.

A number of in vitro studies have also demonstrated the antiproliferative effect of resveratrol in various leukemic cell lines **[50]**.

Anti-inflammatory activity

One of the possible protective activities of antioxidants is the down-regulation of inflammatory responses, which includes inhibition of synthesis and release of pro-inflammatory mediators, modification of eicosanoid synthesis, inhibition of enzymes such as ciclooxygenase-1 (COX-1) and ciclooxygenase-2 (COX-2) **[51]**. In mouse macrophages, resveratrol also has antioxidant activity, decreasing the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and inhibiting nitric oxygen synthetase (NOS)-2 and COX-2 synthesis as well as prostaglandin $E_2$ production **[52]**. Furthermore, this compound inhibits the production of TNF-induced monocyte chemo-attractant protein which plays an essential role in early events during macrophage infiltration into adipose tissue, which results in chronic low-grade inflammation, a key feature of obesity in type 2 diabetes characterized by adipose tissue macrophage infiltration and abnormal cytokine production **[53]**. Finally, it is also established that some anti-inflammatory effects of resveratrol arise from its capability to up-regulate histone deacetylase sirtuin (SIRT1) activity, that also shows antitumor and anti-inflammatory effects **[54]**. Altogether, it is clear that key antitumor properties of resveratrol are linked to its anti-inflammatory effects.

Resveratrol and SIRT-1

The sirtuin family of proteins possesses NAD+-dependent deacetylase activity and/or ADP ribosyltransferase activity. The seven mammalian sirtuins (Sirt1–7) are localized differently within the cell and have a variety of functions **[55]**. Sirt1 is the most extensively studied member of the family and regulates diverse biological processes ranging from cell proliferation, differentiation and apoptosis, to metabolism **[56]**.

In fact, SIRT-1 plays a context-dependent role in metabolic regulation, for instance by mediating effects in metabolic stress situations, such as high-fat-diet-induced obesity **[57].** Furthermore, it confers protection against aging-associated metabolic diseases such as glucose intolerance and cancer. In light of the growing number of patients suffering from metabolic diseases, compounds that activate SIRT-1 directly or indirectly might offer protection against the onset of metabolic damage and promote healthy aging.

Resveratrol was identified as the most potent activator of SIRT-1 **[58]**. Recently, however, it was shown that resveratrol may not activate SIRT-1 directly, but rather exerts its effects on SIRT-1 through activation of

AMPK, although additional direct SIRT-1 activation is not completely excluded **[59]**. Resveratrol treatment in mice fed a high-calorie diet consistently improved various health parameters, including glucose homeostasis, endurance, and survival **[57]** and it has therefore been suggested to act as a calorie restriction mimetic. In Timmers et al. study **[60]** a dietary supplementation of trans-resveratrol was given to obese volunteers over 30 days. Whole-body energy expenditure, mitochondrial function and other parameter have been examined: the results showed that resveratrol supplementation lowers energy expenditure and improves metabolic profile as well as general health parameters.

Anti-diabetes activity

Diabetes mellitus is a complex metabolic disease and is divided into different types: type 1 and type 2 diabetes. Type 1 diabetes results from the autoimmune destruction of β cells and patients with this kind of diabetes are dependent on exogenous insulin. Type 2 diabetes is characterized by defects in insulin secretion and action.

In the last few years, rodent studies and experiments *in vitro* provided evidences that resveratrol may be helpful in preventing and treating some metabolic diseases, including diabetes **[61]**. In general, the management of diabetes involves in three main aspects: reduction of blood glucose, preservation of β cells, and, in the case of type 2 diabetes, improvement in insulin action. Data in literature indicate that the beneficial effects of resveratrol in relation to this pathology comprise all of these aspects.

The maintenance of blood glucose in the physiological range is pivotal in diabetes, since increased glycemia causes numerous diabetic complications. The anti-hyperglycemic effect of resveratrol observed in diabetes animals is thought to result from its stimulatory action on intracellular glucose transport. Increased glucose uptake by different cells isolated from diabetic rats was found in the presence of resveratrol. Interestingly, in experiments on isolated cells, resveratrol was able to stimulate glucose uptake in absence of insulin **[62].** The stimulation of glucose uptake induced by resveratrol seems to be due to increased action of glucose transporter in the plasma membrane (GLUT4) **[63].**

Type 2 diabetes develops slowly and is usually accompanied by insulin resistance. Initially, blood glucose is maintained in the physiological range because of the compensatory increase in insulin secretion. This compensatory mechanism impedes and delays the diagnosis of diabetes. Moreover, chronic overstimulation of β cells causes their exhaustion and degradation, leading over time to insufficient secretion of insulin **[64].** In animals with hyperinsulinemia, resveratrol was established to effectively reduce blood insulin and this inhibition was found to result from metabolic changes in β cells **[65].**

Since chronic overstimulation of β cells is known to induce their degradation, inhibition of insulin secretion by resveratrol may attenuate these unfavorable effects.

The protective action of this polyphenol on the endocrine pancreas may also involve other mechanisms. One of them is the inhibitory influence of resveratrol on cytokine action. Studies *in vitro* and *in vivo* confirmed the important role of the inhibition of cytokine action in the mechanism activated by resveratrol in order to protects pancreatic β cells **[66].**

Moreover, animal studies provided evidence that resveratrol may be useful to improve insulin action in type 2 diabetes. The improvement in insulin action caused by resveratrol seems to result from different effects. One of them is reduced adiposity. Resveratrol-induced reduction in body fat content was demonstrated in mice and rats on a hypercaloric diet **[67].** Moreover, resveratrol ingestion was found to cause effects that are similar to those induced by calorie reduction **[68].** Consistent with animal studies, experiments *in vitro* revealed reduced ATP content and decreased accumulation of triglycerides in isolated rat adipocytes exposed to resveratrol **[69].**

In conclusion, the preventive and therapeutic action of this compound in relation to diabetes is complex and involves in different effects. Elucidation of these beneficial properties of resveratrol is necessary to enable clinical human studies.

Resveratrol and skin

UV radiation, in particular UVB, is known to elicit a variety of adverse effects, such as erythema, sunburn, inflammation, hyperplasia, hyperpigmentation, immunosuppression, premature skin aging, and photocarcinogenesis **[70].** UVB exposure is known to cause excessive generation of ROS, which if remains uncounteracted creates a situation of oxidative stress in the skin**[71].** Topical application of resveratrol to SKH-1 hairless mice was found to inhibit UVB-mediated: skin edema, inflammation, cyclooxygenase (COX), and ornithine decarboxyase (ODC), enzyme activity, ODC protein expression, lipid peroxidation and generation of hydrogen peroxide ($H_2O_2$) **[72]**.

UV radiation is also known to cause significant leukocyte infiltration, which may be responsible for an additional outburst of ROS in the system. Some data demonstrated that resveratrol significantly inhibits UVB-mediated infiltration of leucocytes, suggesting the photochemopreventive potential of this compound and indicating that the antioxidant property may be responsible for the biological effects of resveratrol.

Antiviral activity

Resveratrol has been shown to be a potent antiviral molecule against various types of DNA and RNA viruses. However, even if the antiviral activity of resveratrol is becoming evident, the cellular pathways that lead to its protective activity are still far from being elucidated. Interestingly, some of the molecular pathways regulated by resveratrol such as p53, NF-κB or PML (promyelocytic leukemia protein) are also

main players in the control of virus infection. In addition, the activation of SIRT1 by resveratrol considered to be one of the main mediators of its activity, may also contribute to this regulation **[73].** Further studies are required to clarify the contribution of SIRT1 in the regulation of the mentioned molecular pathways activated by resveratrol and their roles in the control of virus infection.

The activity of resveratrol in protection from viral infection and putative molecular mechanisms that mediate its activity are summarized in Table 1 **[73].**

| Virus | Effect of resveratrol treatment | Possible mechanism of action of resveratrol |
|---|---|---|
| HSV-1, HSV-2 | Inhibition of virus replication *in vitro* and *in vivo* | Suppression of the NF-κB pathway activation |
| VZV | Inhibition of VZV replication *in vitro* | |
| HCMV | Inhibition of HCMV replication *in vitro* | Inhibition of the virus -induced EGFR activation |
| EBV | Inhibition of EBV early antigen induction *in vitro* | |
| HIV-1 | I) Activation of the lytic cycle of HIV- 1 *in vitro*<br>II) inhibition of HIV-1 replication when used synergistically with nucleoside analogues *in vitro*<br>III) attenuation of the Tat-induced HIV-1 LTR transactivation *in vitro* | I) activation of EGR-1<br>II) prolongation of the S-phase of the cell cycle<br>III) SIRT1 activation |
| Influenza A virus | Inhibition of influenza A virus replication *in vitro* and *in vivo* | Inhibition of the protein kinase C activity |
| Polyomavirus | Inhibition Polyoma DNA synthesis *in vitro* | |

**Table 1.** Modulation of virus replication by resveratrol.

### 1.2.2 - METABOLISM AND BIOAVAILABILITY

Resveratrol is rapidly absorbed and metabolized, mainly as sulfo- and glucuro- conjugates which are excreted in urine. This high metabolic rate probably allows the transport, the distribution and the excretion of resveratrol. In rodents, the gut epithelium has been shown to be highly implicated in the metabolic

process, resulting in polar resveratrol metabolism products which then need specific transporters to cross cell membranes **[74]**.

In a study by Walle et al. **[75]**, $^{14}$C-labeled resveratrol has been administered both orally and intravenously. Measurement of total radioactivity demonstrated high absorption after oral intake of resveratrol, without distinguishing resveratrol from its metabolites. The maximum plasma concentration was observed 1 h after oral intake and a second peak was present after 6 h, probably as a result of enteric recirculation of conjugate metabolites following reabsorption after intestinal hydrolysis. There was no second peak in any subject after the intravenous administration, showing no enterohepatic cycle.

## 1.3 - QUERCETIN

Quercetin (Fig. 3) is categorized as a flavonol one of the six subclasses of flavonoid compounds (Table 2). Flavonoids are a family of plant compounds that share a similar flavones backbone ( a three-ringed molecule with hydroxyl [OH] groups attached).a multitude of other substitutions can occur, giving rise to the subclasses of flavonoids and the different compounds found within these subclasses.

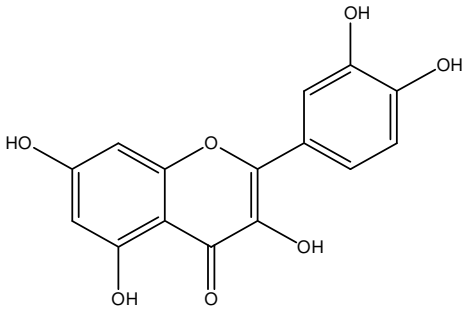



**Figure 3.**

| Subclass | Select Example |
|---|---|
| Flavones | Apigenin, Luteolin |
| Flavonols | Quercetin, Kaempferol |
| Flavanones | Hesperidin, Narigenin |
| Flavanols (also called catechins) | Epicatechin, Gallocatechin |
| Anthocyanidins | Cyanidin, Malvidin |
| Isoflavones | Genistein, Daidzein |

**Table 2.** Flavonoid subclasses and examples.

Flavonoids also occur as either glycosides (with attached sugar) or as aglicones (without attached sugars) **[76]**. Quercetin glucosides are found in a variety of foods including apples, berries, grapes, onion, shallots, tea and tomatoes. the aglycone form of quercetin is found in much lesser amounts in diet. Two of the better food sources are onion and shallots, but depending upon which part of these foods is eaten. For example, quercetin in shallots flesh is about 99.2 % quercetin glucosides and 0.8 % quercetin aglycone. In dry shallot skin the composition is almost the opposite 83,3% quercetin aglycone and 16.7% quercetin glucoside **[77]**. Similar differences exist with onions. The flesh of onions contains mostly quercetin glucosides, with only trace amounts of quercetin aglycone. Like shallots, the skin and outermost layers of an onion have much more quercetin aglycone **[78]**. Growing conditions might significantly influence the amount of quercetin in food, with evidence indicating that organically grown tomatoes have significantly higher quercetin aglycone content than conventionally produced tomatoes.

### 1.3.1 - BIOLOGICAL ACTIVITY

Antioxidant activity

Quercetin has been shown to be an excellent *in vitro* antiossidant. Within the flavonoid family, quercetin is the most potent scavenger of ROS, including $O_2^{\cdot -}$ and RNS like $NO^{\cdot}$ and $ONOO^{-}$**[79].** These antioxidant capacities of this compound are attributed to the presence of two antioxidant pharmacophores, cathecol group and OH group in position 3, within the molecule that have the optimal configuration for free radical scavenging **[80]**. In fact, when quercetin reacts with a free radical, it donates a proton and becomes a radical itself, but the resulting unpaired electron is localized by resonance, making the quercetin radical too low in energy to be reactive **[81]**.

Quercetin can also protect against the more obvious environmental causes of free radicals, such as smoking. Cigarette tar is a source of free radicals, which have been found to damage erythrocyte membranes. Begum and Terao **[82]** found that the quercetin aglycone and its conjugate metabolites (quercetin-3-O-β-glucuronide and quercetin-3-O-β-glucoside) could protect erythrocytes from the membrane damage that is caused by smoking.

New evidence supports that quercetin not only has antioxidant capabilities but more importantly may act as a signaling molecule. In fact, it has been shown that this compound can act as antioxidant leading to the formation of quinones and prooxidants **[83-84]**. Furthermore, quercetin can be metabolized to quinone/quinone methide metabolite such as o-semiquinone and o-quinone. O-semiquinone has the capability to generate $O_2^{-}$ in cell culture whereas o-quinone depletes glutathione (GSH) in the presence of excess ascorbate **[85-86].** The depletion of GSH and generation of $O_2^{-}$ through these quercetin metabolites suggest a prooxidant effect. The formation of quinone/quinone methide metabolites may lead to a low

level production of oxidants, which may in turn activate certain signaling cascades. By acting as a sensor as well as a signaling molecule, $H_2O_2$ is an important regulator of signal transduction. An additional mechanism of action of quercetin then is through regulating $H_2O_2$ levels: $H_2O_2$ regulates endothelial cell function such as proliferation, inflammatory responses, apoptosis, and endothelium-dependent vasorelaxation **[87]**.

Anti-inflammatory activity

Quercetin is known to possess strong anti-inflammatory capacities. Several *in vitro* studies have shown that flavonoids are capable of inhibiting LPS-induced cytokine production. For instance, quercetin inhibits LPS-induced TNFα production in macrophages and LPS-induced IL-8 production in lung cell **[88]**.

A possible explanation for these anti-inflammatory effects of quercetin may be found in the interplay between oxidative stress and inflammation: ROS are not only involved in the occurrence of oxidative stress, but also in the promotion of inflammatory processes via activation of transcription factors such as NF-κB and activator proteins (AP)-1 which induce the production of cytokines like TNFα **[89]**. Consequently, scavenging ROS would not only prevent the occurrence of oxidative stress but also help mitigate inflammation.

In addition quercetin, in according with several other *in vitro* studies, inhibits the production of inflammation-production enzymes (cyclooxygenase and lipoxygenase) **[90]**, NO production and nitric oxide synthase (NOS) expression **[91]**.

Cardiovascular activity

Consumption of flavonoids found in fruits, vegetables, and beverages such as tea and wine are inversely correlated with mortality from coronary heart disease. The several studies demonstrated an inverse relationship between flavonoid intake and occurrence of myocardial infarction **[92]**. The cardiovascular benefits of flavonoid-rich foods have been attributed to their antioxidant effects (inhibiting the oxidation of low density lipoproteins (LDL) **[93]**), NO mediated vasodilatory effects, reducing platelet aggregation **[94]**, and activating Nrf2-induced phase II detoxification through antioxidant response element (ARE)-mediated gene expression **[95]**. Quercetin is the most prevalent flavonoid in the human diet and it has been shown to engage in redox cycling reactions in cellular system thereby endowing both antioxidant and prooxidant (by generating $H_2O_2$) properties **[96-97]**. The effect of quercetin on vessel function are not clear and especially whether the proposed generation of $H_2O_2$ can modulated eNOS activity and vessel relaxation. In fact, vascular endothelial cells have the capacity to release H2O2 and endothelium-derived H2O2 stimulates EDHF. The endothelium produces and releases several vasoactive mediators involved in vessel tone, such as NO, prostacyclins, and EDHF.

Moreover, quercetin, in human, inhibits platelet aggregation and thrombus formation **[98]**. Quercetin appears to be effective in improving blood pressure and might have an effect on cholesterol in human.

Antitumor activity

Apart from scavenging ROS, another important effect of quercetin is to regulate cell cycle by modulating several molecular targets, including p21, cyclin B, p27, cyclin-dependent kinases and topoisomerase II, even if the mechanisms involved have not been elucidated yet. Depending on the cell type and tumor origin, quercetin is able to block the cell cycle at G2/M or at the G1/S transition. In particular, quercetin causes G2/M arrest in human esophageal squamous cell carcinoma cell line through up-regulation of p73 and p21waf1 and subsequent down-regulation of cyclin B1, both at the mRNA and protein levels **[99]**.In human breast carcinoma cell lines low doses of quercetin inhibit proliferation. Cell-cycle arrest occurs at the G1 phase through the induction of p21 and through the concomitant decrease of phosphorylation of the retinoblastoma protein (pRb) **[100]**.In the same cell model, quercetin downregulates the cyclin B1 and cyclin-dependent kinase (CDK) 1, which are essential in the progression to the G2/M phases of the cell cycle **[101]**. Similarly, in the human lung cancer cells, quercetin glucuronides induce cell-cycle arrest at G2/M phase by increasing the expressions of proteins such as cyclin B **[102]**. A similar antiproliferative effect has also been observed both for highly or moderately aggressive prostate cancer cell lines, whereas no effect has been found for poorly aggressive prostate cancer cells **[103]**.

In add, quercetin has shown a pro-apoptotic activity. The capability of quercetin to induce apoptosis in cancer cells (via both the intrinsic and extrinsic pathways) undoubtedly renders this molecule an interesting tool in the oncology field. The pro-apoptotic effects of quercetin may result from multiple pathways. First, in several kind of cell lines, quercetin treatment increases cytosolic $Ca^{2+}$ levels and reduces the mitochondrial membrane potential (MMP), thus promoting activation of caspase-3, -8 and -9 **[104]**. Second, quercetin is a potent enhancer of TNF-related **[105]**. Third, the anti-proliferative and pro-apoptotic effects could be related to the capability of quercetin to directly bind tubulin, provoking the depolymerization of cellular microtubules **[106]**.

Several studies have investigated the role of p53 in the antiproliferative and proapoptotic action of quercetin on tumor cell lines. In fact, quercetin causes cell-cycle arrest and apoptosis by inducing p53 phosphorylation and by stabilizing p53 both at the mRNA and protein level **[107]**. The presence of p53 limits the effect of quercetin, since when p53 is inhibited, cells become more sensitive to quercetin related cytotoxicity and quercetin-related apoptosis. The effect of the presence or absence of p53 on quercetin induced cytotoxicity and apoptosis is consistent with the involvement of this polyphenol in the oxidative cell balance.

The studies of quercetin on cellular models offer an almost exhaustive explanation of the mechanisms that link this compound to the oxidative cell balance and to the control of cell-cycle phases; these results invite major attention to study quercetin in more complex and sophisticated animal models.

Allergy, Asthma, and Atopic Disease

In vitro quercetin inhibits histamine release by mast cells and basophils **[108]** suggesting an antiallergy effect. Animal evidence indicates that quercetin might have therapeutic potential for allergic airway disease. Several studies conducted in guinea pigs have reported that quercetin, provided orally or administered via inhalation, has anti-asthmatic activity **[109]**. In murine models of allergic airway inflammation and asthma, quercetin had pronounced anti-inflammatory effects **[110]**, reduced eosinophil and neutrophil counts and infiltration in in lung tissue, and inhibited asthmatic reactions **[111]**.

In addition, quercetin inhibits anaphylactic contraction of giunea pig ileum smooth muscle *in vitro* **[112]**.

Human epidemiological research reports an inverse association between intakes of quercetin and asthma incidence **[113]**. Human intervention studies investigating quercetin for asthma and atopic disease are currently lacking. However, two studies have investigated the effects of an enzymatically-modified isoquercitrin (a quercetin glycoside) on allergic symptoms. In these studies, this specific quercetin glycoside provided a statistically significant relief of ocular symptoms, but no statistically significant relief of nasal symptoms caused by pollen **[114]**.

In addition, other studies reported that quercetin might reduce erythema and burning sensation. Presumably this occurred in part because quercetin inhibited niacin-induced human mast cell prostaglandin D2 release **[115]**.

Antiviral and antibacterial activity

Quercetin has *in vitro* antiviral activity against reverse transcriptase of HIV and other retroviruses like *Herpes simplex* virus type 1, polio-virus type 1, respiratory syncytial virus (RSV) **[116]**, and hepatitis C **[117]**.

Quercetin has *in vitro* antibacterial activity against five microorganisms (*Actinobacillus actinomycetemcomitans, Actinomyces viscosus, Porphyromonas gingivalis, Fusobacterium nucleatum,* and *Actinomyces naeslundii*) associated with onset and progression of periodontal disease **[118]**. It also has *in vitro* and *in vivo* activity against H. pylori **[119]**.

Quercetin exerts immune and inflammation modulating activity in several murine models of autoimmunity. For example, in experimental allergic encephalomyelitis a T-helper 1 (Th1) cell-mediated inflammatory demyelinating autoimmune disease model of multiple sclerosis, quercetin ameliorated this patology by blocking IL-12 signaling and Th1 differentiation **[120]**.

Skin and quercetin

As mentioned above, quercetin has the highest antiradical activity compared to other flavonoids and its properties include ROS scavenging, inhibition of peroxidation and metal ions chelation. These evidences suggest the possible effectiveness of quercetin topical administration to prevent UVB radiation-induced skin damage **[121]**. Acute UV skin exposure induces an immediate inflammatory response with erythema and leukocyte infiltration. It has been suggested that the modulation of redox-sensitive transcription factors such as nuclear factor (NF)-κB by ROS is a central and early event in the induction of inflammatory reactions **[122]**. The NF-κB is an oxidative stress sensitive factor that activates multiple target genes involved in the expression of several pro-inflammatory mediators. In agreement with the inflammatory character of the UVB irradiation, it induces a dose-dependent increase in myeloperoxidase (MPO) activity **[123]**. The topical application of a formulation containing quercetin significantly decreased the UVB-induced increase of MPO in the skin **[124]**.

The antioxidants are mainly concentrated in the epidermis compared to the dermis; in fact, the UVB radiation exposure induces a greater decrease in the epidermis antioxidant systems compared to the dermis **[125]**. Therefore, the epidermis can be considered the first line of skin defense and the glutathione redox status has been confirmed as an early and sensitive sensor of UVB-induced epidermal oxidative stress **[126]**. In fact, a dose-dependent depletion of GSH was detected in the skin after UVB irradiation. In Casagrande et al. study **[127]** it has been demonstrated that the topical treatment with a formulation containing quercetin significantly prevented the UVB irradiation-induced GSH depletion.

During multiple intermittent UV exposure, repeated processes induce the formation of severely damaged collagen and ultimately skin wrinkling and reduced elasticity. The degradation of the extracellular matrix is a consequence of metalloproteinases activity in epidermal keratinocytes and fibroblasts. This process is followed by formation of imperfectly repaired collagen **[128]**. Furthermore, the UVB irradiation induction of proteinases expression in the epidermis has been recently demonstrated; it also induces gene transcription factors for metalloproteinases. The proteinases may be produced by a variety of cells including macrophages and neutrophils; Casagrande et al. study **[127]** also demonstrated that the formulations containing quercetin inhibited the proteinases secretion/activity in the skin. These evidences further strengthen the possible use of topical formulations containing quercetin to prevent UVB radiation skin damages.

### 1.3.2 - METABOLISM AND BIOAVAILABILITY

Quercetin is found in far greater amounts in the diet as glycosides than as quercetin aglycone. When quercetin glycosides are ingested, glycosyl groups can be released during chewing, digestion, and

absorption. As an example, enzymes in the mouth and intestine can hydrolyze quercetin glycosides to aglycones. **[129]**. Several human studies have been conducted to compare the bioavailability of quercetin

aglycone and glycosides. A study of absorption in ileostomy patients revealed absorption of 24 % of the pure aglycone and 52 % of quercetin glycosides **[130].** Other studies have confirmed that the absorpition of quercetin is considerably enhanced by its conjugation with a sugar group **[131]**.

After absorption, quercetin is metabolized in various organs, such as the small intestine, colon, liver and kidney **[132]**. Metabolites formed in the small intestine and liver are mainly the result of phase II metabolism by biotransformation enzymes and therefore include methylated, sulfated and glucuronidated forms **[133]**. Moreover, bacterial ring fission of the aglycone occurs both in the small intestine and in colon, resulting in breakdown of the backbone structure of quercetin and the subsequent formation of smaller phenolics **[132]**. Normally, human quercetin plasma concentration is in low nanomolar range, but upon quercetin supplementation it may increase to high nanomolar or low micromolar range **[134]**. A recent study regarding the tissue distribution in rats and pigs has shown that, upon quercetin supplementation, the highest accumulation of the flavonoid and its metabolites is found in lungs (rats) and liver and kidney (pigs) **[135]**. It has been shown that the half-lives of quercetin metabolites are rather high (from 11 to 28 h). This indicates that, upon repeated quercetin supplementation, they could attain a considerable plasma level and pharmacological function of dietary quercetin, including antioxidant activity, should be exerted exclusively by its conjugated metabolites **[136].**

## 1.4-FATTY ACIDS

Fatty acids are classified as saturated fatty acid (SFA), monounsaturated fatty acid (MUPA), and polyunsaturated fatty acid (PUFA). Omega-3 (n-3), omega-6 (n-6), and omega-9 (n-9) unsaturated fatty acid structures are based on the position of the first double bond at the third, sixth or ninth position from the methyl (omega) terminal of the aliphatic carbon chain.

Humans and other mammals can synthesize saturated fatty acids and some monounsaturated fatty acids from carbon groups in carbohydrates and proteins; they lack the enzymes necessary to insert a *cis* double bond at the n-6 or the n-3 position of a fatty acid **[137]**. Consequently, omega-6 and omega-3 fatty acids are essential nutrients. The parent fatty acid of the omega-6 series is linoleic acid (18:2n-6), and the parent fatty acid of the omega-3 series is linolenic acid (18:3n-3.). Humans can synthesize long-chain (20 carbons

or more) omega-6 fatty acids, such as dihomo-gamma-linolenic acid (DGLA; 20:3n-6) and arachidonic acid (AA; 20:4n-6) from linoleic acid and long-chain omega-3 fatty acids, such as eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) from linolenic acid through a series of desaturation (addition of a double bond) and elongation (addition of two carbon atoms) reactions.

Unlike the linolenic and linoleic acid, oleic acid (18:1n-9), is consumed in substantial amounts in the typical Western diet and is not an essential fatty acid. There is little eicosatrienoic acid (ETA; 20:3n-9) in cell membranes, however, probably because of the overwhelming competition from dietary linoleic acid for the relevant desaturase and elongase enzymes.

### 1.4.1 - PHARMACOLOGICAL ACTIVITIES

Omega-6 and omega-3 PUFA are important structural components of cell membranes. When incorporated into phospholipids, they affect cell membrane properties such as fluidity, flexibility, permeability and the activity of membrane bound enzymes **[138]**.

DHA is selectively incorporated into retinal cell membranes and postsynaptic neuronal cell membranes, suggesting it plays important roles in vision and nervous system function. DHA is found at very high concentrations in the cell membranes of the retina; the retina conserves and recycles DHA even when omega-3 fatty acid intake is low **[139]**. Animal studies indicate that DHA is required for the normal development and function of the retina. Moreover, these studies suggest that there is a critical period during retinal development when inadequate DHA will result in permanent abnormalities in retinal function.

The phospholipids of the brain's gray matter contain high proportions of DHA and AA, suggesting they are important to central nervous system function **[140]**. Brain DHA content may be particularly important, since animal studies have shown that depletion of DHA in the brain can result in learning deficits. It is not clear how DHA affects brain function, but changes in DHA content of neuronal cell membranes could alter the function of ion channels or membrane-associated receptors, as well as the availability of neurotransmitters **[141]**.

Eicosanoids, derived from 20-carbon PUFA, are potent chemical messengers that play critical roles in immune and inflammatory responses. During an inflammatory response, DGLA, AA, and EPA in cell membranes can be metabolized by enzymes known as cyclooxygenases and lipoxygenases to form prostaglandins and leukotrienes **[142]**.

AA is the progenitor of both $PGE_2$ and $LTB_4$ via the cyclooxygenase and 5-lipoxygenase enzymatic pathways, respectively. $PGE_2$ and $LTB_4$ have pro-inflammatory biological actions. $PGE_2$ can cause pain and vasodilation

and $LTB_4$ is a chemoattractant and activator of neutrophils; together they can cause vascular leakage and extravasation of fluid.

EPA, the n-3 homologue of AA, can inhibit AA metabolism competitively via the cyclooxygenase and 5-lipoxygenase enzymatic pathways and, thus, can suppress production of the n-6 eicosanoid inflammatory mediators. EPA is a potential cyclooxygenase substrate for the synthesis of $PGE_3$, which also has inflammatory activity **[143, 144]**. EPA is also a 5-lipoxygenase substrate and can lead to the formation of $LTB_5$, but $LTB_5$ has little inflammatory activity compared with $LTB_4$ **[145]**.

In those who consume typical Western diets, the amount of AA in cell membranes is much greater than the amount of EPA, resulting in the formation of more eicosanoids derived from AA than EPA. However, increasing omega-3 fatty acid intake enhances the EPA content of cell membranes, resulting in higher proportions of eicosanoids derived from EPA **[146]**.

Many antiinflammatory pharmacotherapies are directed at inhibiting the production of these inflammatory mediators and thus possibilities exist for therapies that incorporate n-3 and n-9 dietary fatty acids. However, there are competitive interactions between dietary PUFAs; thus, in any examination of the effects of dietary n-3 or n-9 PUFAs, it is important to consider also the background dietary n-6 PUFAs.

The results of epidemiological studies and randomized controlled trials suggest that replacing dietary SFAs with omega-6 and omega-3 PUFA lowers LDL cholesterol and decreases cardiovascular disease risk.

A reduction in omega-6 fatty acids leads to skin lesions, anemia, increased platelet aggregation, thrombocytopenia, fatty liver, delayed wound healing, increased susceptibility to infections, diarrhea, growth delay in childhood.

The lack of omega-3 fatty acids is characterized by neurological symptoms, reduced visual acuity, skin lesions, developmental delay, compromised learning ability, abnormal electroretinogram.

Maintaining an optimal omega-3/omega-6 ratio is essential in the prevention of some pathologic conditions as well as in the therapy of diseases due to immuno-allergic causes and to lipid metabolism errors. In particular, a correct omega-3/omega-6 ratio is important: to prevent cardiovascular diseases and to control their risk factors **[147, 148]**; to prevent and treat skin and immuno-allergic affections **[149]**; in congenital metabolic pathologies, such as adreno-leukodystrophy and phenylketonuria.

In addition, fatty acids are essential components of natural lipids, which determine the physiological structure and function of the human skin **[150]**. They are present in the epidermis, especially in the stratum corneum, the outermost layer, and cell membranes **[151]**. Many effects of fatty acids can be linked to changes in membrane lipid composition affecting cell signaling mechanisms originating from membranes **[152]**. Skin aging may influence epidermal lipids and free fatty acid composition, and their physiological functions may be involved in aging process. In fact, the levels of SFAs such as palmitic acid and stearic acid,

PUFAs such as linoleic acid and 11,14,17-eicosatrienoic acid , one of the omega-3 polyunsaturated acids, were decreased

in aged skin by 15%, 31%, 7%, and 56%, compared with those in young skin, respectively **[153]**.Moreovere, also photoaging process may affect the fatty acids composition in human skin **[153]**.

# 2 - AIM

This PhD project aims to overcome the problems related to poor bioavailability of some natural polyphenols and sterols, through the synthesis of the corresponding esters with saturated and unsaturated fatty acids.

The limitation of pharmaceutical, cosmetic and dietary application of polyphenols and sterols is due to their poor solubility and stability in lipophilic media: these features are also undermining their oral and dermal bioavailability. The synthesis of more lipophilic derivatives, especially esters, could lead to an increase in lipophilicity, and then in the bioavailability and permeability, in particular in the skin.

The selected natural compounds have all proven biological activities and are widely described in literature. The choice of saturated and unsaturated fatty acids as acid components for the esterification is due to their beneficial activities: thus, the synthesis of such esters would introduce in a single molecule (prodrug) two moieties both pharmacologically active.

# 3 - MATERIALS AND METHODS

## 3.1 - MATERIALS

β-sitosterol (purity grade 60%), quercetin, oleic acid (purity grade 90%), linolenic acid (purity grade 70%), linoleic acid (purity grade 95%), palmitoyl chloride, stearoyl chloride were purchased from Sigma Aldrich (Milan, Italy); resveratrol was purchased from Polichimica (Bologna, Italy). All solvents, deuterated solvents, reagents and enzymes used were purchased from Sigma Aldrich.

## 3.2 - METHODS

### 3.2.1 - INFRARED SPECTROSCOPY ANALYSIS (FT-IR)

Fourier Transform Infrared Spectroscopy (FT-IR) is an analytical technique used to identify functional groups in the examined molecules.

Measurements have been performed by using a SPECTRUM ONE FT-IR spectrometer (Perkin Elmer). Spectra have been acquired by DATA MANAGER 2 software (Perkin Elmer).

*Sample preparation:*

Samples in solid form and samples in liquid/semi-solid form have been treated differently:

- Solid form sample:

  The sample is mixed at a ratio of 1:100 w/w with potassium bromide and homogenized in an agate mortar; the powder is then pressed, without applying vacuum, under a 9 tons pressure for 4 minutes, to form a thin tablet that is gently put on a suitable support for the analysis. The acquisition of spectra have been performed using the following parameters:

  | | |
  |---|---|
  | *Wavelenght:* | 4000 - 450 $cm^{-1}$ |
  | *Resolution:* | 4 $cm^{-1}$ |
  | *Scan repetitions:* | 8 |
  | *Background:* | KBr tablet |

- Liquid/semi-solid form sample:

  The substance is dropped between two sodium chloride windows. The acquisition of spectra have been performed using the following parameters:

  *Wavelenght:* 4000 - 600 $cm^{-1}$

  *Resolution:* 4 $cm^{-1}$

  *Scan repetitions:* 4

  *Background:* NaCl window

### 3.2.2 - NUCLEAR MAGNETIC RESONANCE ANALYSIS (NMR)

NMR is a technique used to determine the structure of the investigated compounds.

NMR spectra have been registered, depending on different needs, by using two different instruments:

- Varian Mercury Plus 200, operating at 200 MHz, coupled with Sun software
- Bruker Advance 500, operating at 500 MHz, coupled with Bruker X-Win NMR 3.0 software.

*Sample preparation:*

8-10 mg ($^{1}$H-NMR) or 50 mg ($^{13}$C-NMR) of substance have been dissolved in chloroform ($CDCl_3$) or dimethyl sulfoxide (DMSO) depending on their solubility.

### 3.2.3 - ULTRAVIOLET-VISIBLE SPECTROSCOPY ANALYSIS (UV-Vis)

UV-vis allow the determination of the maximum absorption wavelength of the analyzed molecule. This value helps the identification of the analytes. Measurement have been performed by using a Jasco V-530 UV-vis spectrophotometer in different wavelength ranges, depending on the solvent:

- Methanol: 210-600 nm
- Chloroform: 235-600nm

*Sample preparation:*

Weighted quantities of the samples have been dissolved in the appropriate solvent (methanol or chloroform) and spectra have been registered using quartz cuvettes. Solvent backgrounds have been subtracted.

### 3.2.4 - HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

HPLC is a chromatographic technique which allows to separate two or more components in an analyte, based on differential affinity of the compounds for a stationary phase, placed inside the chromatographic column, and a mobile phase, flowing through it. The stationary phase and the mobile phase are chosen

according to the sample to be analyzed. Depending on the stationary phase used, HPLC analysis can be carried out in normal phase or reversed phase:

- Normal phase: in this case the column inner packing is polar, commonly made of silica
- Reversed phase: the column packing is apolar, commonly made of derivatized silica

The choice of the mobile phase is closely related to the stationary phase employed: usually in normal phase HPLC apolar solvents are used, such as hexane and *ter*-butanol, whereas in reversed phase HPLC polar solvents such as methanol, acetonitrile and water are used. In addition, HPLC can be performed using one or more mobile phases: if the composition of the mobile phase is constant, the elution is performed in isocratic conditions, while if the composition of the mobile phase changes during the analysis the chromatographic separation is described as a gradient elution.

The samples to be analyzed are dissolved in a suitable solvent, usually similar or identical to the mobile phase.

After leaving the column, the mobile phase enters a detector, needed to provide qualitative and quantitative information on the analyte components. The most common detectors are based on UV-vis light absorption. DAD (Diode Array Detector) allows to record the absorbance spectrum of the analyte in a specified range of wavelenghts.

β-sitosterol, resveratrol and their respective derivatives analyses were performed by using an HPLC system equipped with a quaternary pump Waters Delta Prep 600E, an injector Rheodyne 7125 with a 20 µl loop, a thermostat Column Block Heater Mod. 7940 Hichrom Ltd., a detector DAD 2996. The acquired data were processed by software Empawer 2.

Quercetin and its derivatives were performed by using an HPLC system equipped with a quaternary pump Merck Hitachi L-7100, an injector Rheodyne 7125 with a 20 µl loop, a thermostat Column Block Heater Mod. 7940 Hichrom Ltd., a detector DAD Hewlett Packard HP 1050. The acquired data were processed by software HP CHEM.

### 3.2.5 - MASS SPECTROMETRY (MS)

All synthesized derivatives were analyzed by using a LCQ Advantage mass spectrometer, equipped with ion trap and ESI (Electrospray Ionization) source.

### 3.2.6 - ANTIOXIDANT ACTIVITY

In collaboration with Dr. Angela Maria Rizzo from Dept. “Scienze Molecolari Applicate ai Biosistemi”, Università degli Studi, Milan, the antioxidant activity of resveratrol and its tri-esters was tested using ABTS (2,2’-azinobis-[3-ethylbenzthiazoline-6-sulphonic acid]) and DPPH (1,1-diphenyl-2-picrylhydrazyl) assays.

TEAC (Trolox Equivalent Antioxidant Capacity) and $IC_{50}$ values of the compounds were calculated and compared.

ABTS assay

The ABTS assay involves the oxidation of ABTS (2,2'-azinobis[3-ethylbenzothiazoline-6-sulphonate]) to a radical cation, $ABTS^{\bullet+}$. $ABTS^{\bullet+}$ is enzymatically pre-generated and the antioxidant or sample to be analyzed is added to the reaction mixture. This results in the disappearance of $ABTS^{\bullet+}$, which is measured by the decrease in absorbance; the ABTS radical cation has an absorption maximum at 735 nm.

This method is based on the capacity of different components to scavenge ABTS radical cation compared to a standard antioxidant (Trolox, vitamin E analogue) in a dose-response curve.

**Stock solutions:**

- Solution 1: potassium persulfate ($K_2S_2O_8$) 2.5 mM in water
- Solution 2: ABTS 7.4 mM in water
- Solution 3: obtained by mixing Solution 1 and Solution 2 in equal quantities and allowing them to react for 12 h at room temperature in the dark.
- Solution 4: Trolox 1 mM in methanol, used to prepare standard solutions
- Solution 5: obtained by dilution of 10 ml of Solution 3 to 100 ml of methanol ($Abs_{735}$ = approximately 0.700)
- Solution 6: sample solution

**Standard solutions preparation** (Table 3)

| Standard solution | Solution 4 vol. (µl) | Solution 5 vol. (µl) | Solvent vol. (µl) | Trolox conc. (µM) |
|---|---|---|---|---|
| 1 | 0 | 950 | 50 | 0 |
| 2 | 5 | 950 | 45 | 5 |
| 3 | 10 | 950 | 40 | 10 |
| 4 | 15 | 950 | 35 | 15 |
| 5 | 20 | 950 | 30 | 20 |
| 6 | 25 | 950 | 25 | 25 |
| 7 | 30 | 950 | 20 | 30 |

**Table 3.**

**Results acquisition:**

- for each acquisition Solution 5, solvent and Solution 4 (in the prescribed order) were added in a cuvette, then the mixture was stirred and after 3 minutes its absorbance at 735 nm was acquired using a UV-vis spectrophotometer;
- the calibration curve was plotted inserting the data in a graph (absorbance vs. Trolox concentration);
- the samples were analyzed and the results were interpolated with the calibration curve and the TEAC (Trolox Equivalent Antioxidant Capacity) values were obtained.

DPPH assay

The DPPH assay makes use of a stable free radical DPPH• (1,1-diphenyl-2-picrylhydrazyl). The reaction involves a colour change from violet to yellow that can be easily monitored using a spectrophotomer at 520 nm.

**DPPH solution preparation**: prepared by dissolving 27 mg of DPPH with 25 ml of ethanol 70%, stirred until complete dissolution and then stored in the dark.

**Samples preparation**: an increasing volume of analyte (50 µl by 50 µl) was added to different test tubes; ethanol 70% was added to a final volume of 1 ml and then 500 µl di DPPH was added. The mixture was stirred and stored for 30 minutes in the dark.

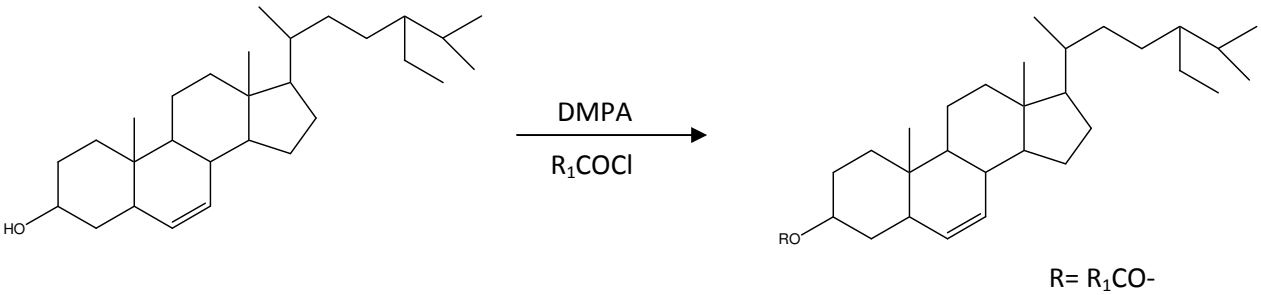
**Results acquisition:** the samples were analyzed using an UV-vis spectrophotometer (520 nm), obtaining absorbance values relevant to the residual amounts of DPPH•: then $IC_{50}$ value was obtained by interpolation, plotting a graph with the calculated residual amounts of DPPH• vs. sample concentration. $IC_{50}$ value (µg/ml) represents the concentration of sample leading to a 50% decrease of DPPH•: the higher the $IC_{50}$ value, the lower the antioxidant activity.

# 4 - EXPERIMENTAL PART

## 4.1 - β-SITOSTEROL

### 4.1.1 - CHEMICAL SYNTHESIS OF β-SITOSTEROL FATTY ESTERS

β-sitosterol esters were obtained both with saturated (palmitic acid) and unsaturated (oleic and linolenic acid) fatty acids.




**Scheme 1.**

Under nitrogen flow, β-sitosterol was dissolved in 20 ml anhydrous toluene and the solution was stirred at room temperature. Then dimethyl-aminopydirine (DMPA) was added and, using an ice bath, the temperature was decreased to 0°C; afterwards, the chloride was added dropwise. The obtained reaction mixture was carried to room temperature and magnetically stirred for 12 h, repairing from daylight.

The mixture was washed with saturated $NaHCO_3$ water solution and then with water (3 x 20 ml). The organic layer was dried over anhydrous $Na_2SO_4$ and evaporated under vacuum (77 mbar, 40 °C).

When the acyl chloride was not commercially available, it was prepared as follows:

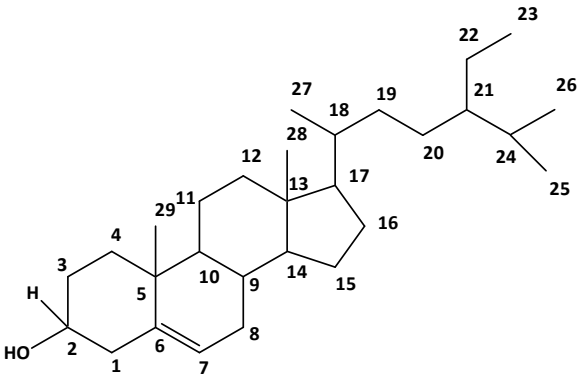
$$R_1COOH + (COCl)_2 \xrightarrow[3h]{\text{anhydrous toluene}} R_1COCl$$

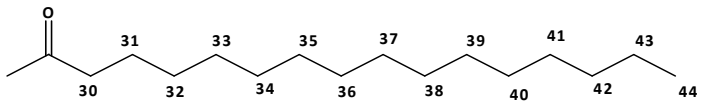
**Scheme 2.**

The unsaturated fatty acid was dissolved in 20 ml anhydrous toluene and the solution was stirred at room temperature under anhydrous conditions. Then, using an ice bath, the temperature was decreased to 0 °C and oxalyl chloride was added dropwise. The reaction mixture was carried to room temperature and magnetically stirred for 3 h. The reaction was monitored using IR spectroscopy. At the end of the reaction the solution was evaporated under vacuum (77 mbar, 40 °C) in inert atmosphere: the obtained acyl chloride was employed without further purification for the subsequent reaction.

All synthesized compounds were analyzed by FT-IR, $^{1}$H-NMR, $^{13}$C-NMR, UV and mass spectrometry. The instrumental results interpretation for all compounds is reported below; as an example, spectra are reported only for lynolenoyl-β-sitosterol.

## PALMITOYL β-SITOSTEROL




R= 


### Reaction parameters:

**Molar ratio palmitoyl chloride:β-sitosterol 1:1**

| | |
|---|---|
| β-sitosterol | $1.21x10^{-3}$ mol, 0.50 g, |
| Palmitoyl chloride | $1.21x10^{-3}$ mol, 0.33 g, 0.36 ml |
| DMPA | $1.73x10^{-3}$ mol, 0.21 g, |
| Reaction temperature | Room temperature |
| Reaction time | 12 h |
| Yield | 70% |

$C_{45}H_{80}O_2$ MW = 653.2

**TLC:** Hexane/Ethyl acetate/Ethyl Ether 5/2/1and spot visualization with iodine vapor

**UV/Vis** in MeOH λ (nm) =261; 280

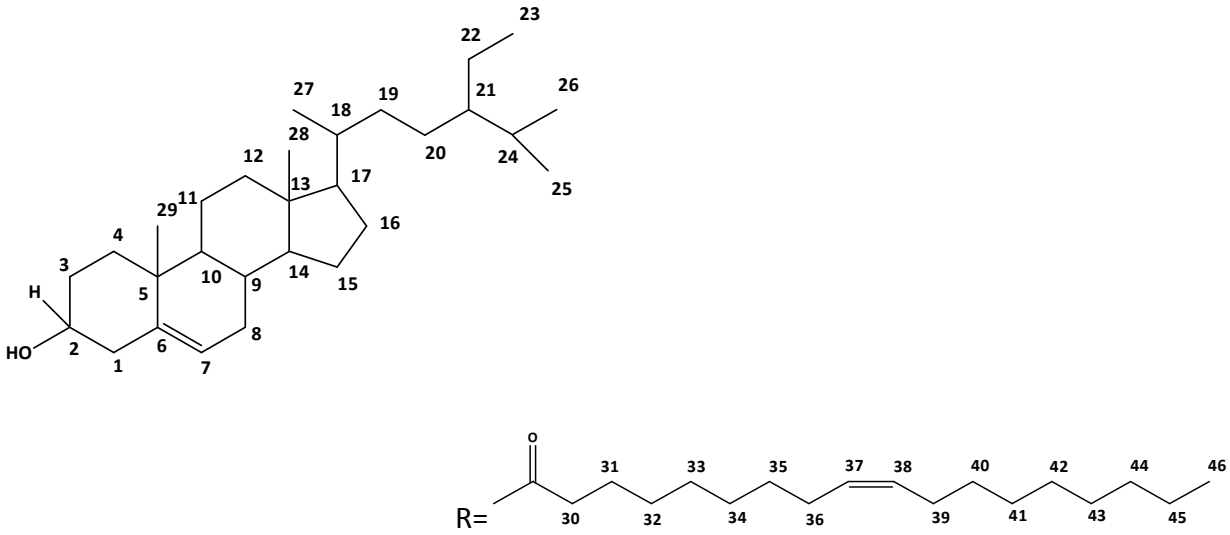
**$^1$H-NMR ($CDCl_3$):** δ = 5.38-5.36 (m, 1 H, $H_7$); 4.69-4.55 (m, 1 H, $H_2$); 2.35-2.22 (m, 4 H, $H_1$, $H_{30}$); 2.04-0.80 (m, 74 H, $CH_2$ and $CH_3$).

**$^{13}$C-NMR ($CDCl_3$):** 173.245 CO; 140.012 $C_6$; 122.623 $C_7$; 73.897 $C_2$, 57.022 $C_{14}$; 56.989 $C_{17}$; 50.274 $C_{10}$; 46.040 $C_{21}$; 42.225 $C_{13}$; 39.873 $C_{12}$; 38.373 $C_1$; 37.215 $C_4$; 36.797 $C_5$; 35.154 $C_{18}$; 34.828 $C_{19}$; 34.528 $C_{30}$;; 32.089 $C_9$; 32.024 $C_{42}$; 29.987 $C_{32}$; 29.865 $C_{35}$; 29.647 $C_{39}$, $C_{41}$; 29.520 $C_{33}$, $C_{34}$; 29.504 $C_{36}$, $C_{37}$; 29.433 $C_{24}$; 29.440 $C_{38}$, $C_{40}$; 28.572 $C_{16}$; 27.406 $C_3$; 26.129 $C_8$; 25.123 $C_{31}$; 24.516 $C_{20}$; 23.417 $C_{22}$; 21.199 $C_{11}$; 20.031 $C_{25}$, $C_{26}$; 19.602 $C_{23}$; 19.243 $C_{29}$; 19.015 $C_{27}$; 14.226 $CH_3$; 12.125, 12.077 $C_{28}$.

**FT-IR (KBr)**

| **1/λ ($cm^{-1}$)** | **Attribution** |
|---|---|
| 2952.16, 2853.91 | ν C $sp^3$ - H |
| 1735.11 | ν C=O |
| 1465.63 | δ $CH_2$ |
| 1168.12, 1135.45 | ν C-O |

## OLEOYL β-SITOSTEROL




### Reaction parameters:

**Molar ratio oleic acid:β-sitosterol 1:1**

| | |
|---|---|
| Oleic acid | $1.21x10^{-3}$ mol, 0.34 g, 0.38 ml |
| Oxalyl chloride | $7.64x10^{-3}$ mol, 0.96 g, 0.64 ml |
| B-sitosterol | $1.21x10^{-3}$ mol, 0.50 g |
| DMPA | $1.73x10^{-3}$ mol, 0.21 g |
| Reaction temperature | Room temperature |
| Reaction time | 12 h |
| Purification | Gravimetric column (Ethyl acetate/hexane 9.5/0.5) |
| Yield | 35% |

$C_{47}H_{81}O_2$ MW = 678.21

**TLC:** Hexane/Ethyl acetate/Ethyl Ether 5/2/1and spot visualization with iodine vapor

**UV/Vis** in MeOH λ (nm) = 234; 268

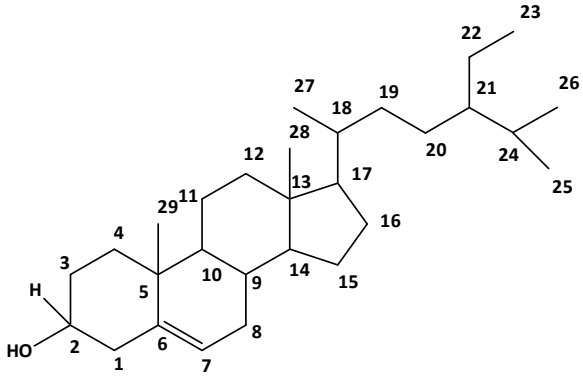
**$^1$H-NMR ($CDCl_3$):** δ = 5.37-5.31 (m, 3 H, $H_7$, $H_{37}$, $H_{38}$); 4.63-4.59 (m, 1 H, $H_2$); 2.33-2.23 (m, 4 H, $H_1$, $H_{30}$); 2.02-0.80 (m, 73 H, $CH_2$ and $CH_3$).

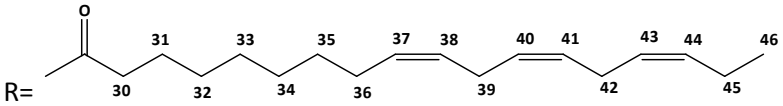
**$^{13}$C-NMR ($CDCl_3$):** 173.375 CO; 139.876 $C_6$; 130.264, 129.987 $C_{37}$, $C_{38}$, 122.870 $C_7$; 74.011 $C_2$, 56.927 $C_{14}$; 56.375 $C_{17}$; 49.998 $C_{10}$; 45.897 $C_{21}$; 42.225 $C_{13}$; 39.886 $C_{12}$; 38.501 $C_1$; 37.115 $C_4$; 36.842 $C_5$; 35.246 $C_{18}$; 34.767 $C_{19}$; 34.205 $C_{30}$; 32.107 $C_9$; 31.989 $C_{44}$; 29.964 $C_{35}$; 29.880 $C_{40}$; 29.780 $C_{42}$; 29.579 $C_{41}$;29.502 C24; 29.436 C $_{43}$; *2*9.402 $C_{32}$; 29.305 $C_{33}$, $C_{34}$; 28.614 $C_{16}$; 27.541, 27.479$C_{36}$, $C_{39}$; 27.429 $C_3$; 26.219 $C_8$; 25.098 $C_{31}$; 23.987 $C_{20}$; 23.275 $C_{22}$;22.745 $C_{45}$; 21.207 $C_{11}$; 20.009 $C_{25}$, $C_{26}$; 19.372 $C_{23}$; 19.125 $C_{29}$; 18.872 $C_{27}$; 14.377 $CH_3$; 12.132, 12.096 $C_{28}$.

**FT-IR (NaCl)**

| **1/λ ($cm^{-1}$)** | **Attribution** |
|---|---|
| 2928.21, 2854.15 | ν C $sp^3$ - H |
| 1736.71 | ν C=O |
| 1464.76 | δ $CH_2$ |
| 1173.20 | ν C-O |
| 799.98 | δ HC=CH *trans* |

## LINOLENOYL β-SITOSTEROL




### Reaction parameters:

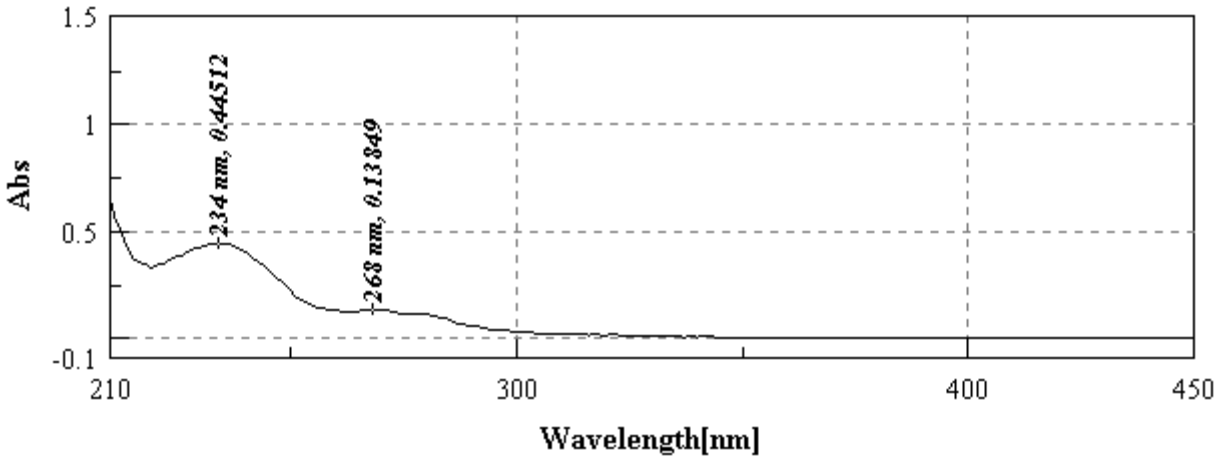
**Molar ratio linolenic acid:β-sitosterol 1:1**

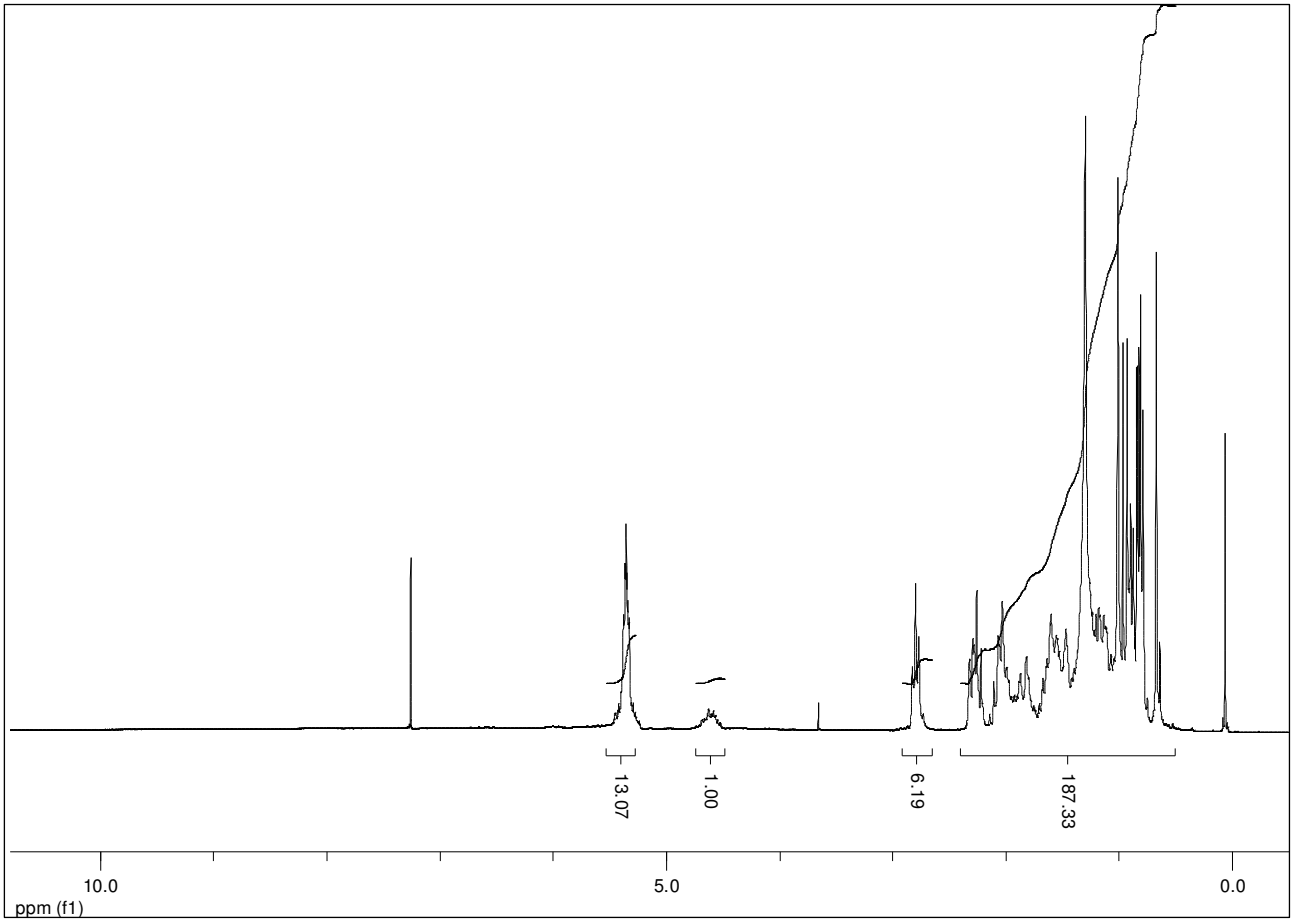
| | |
|---|---|
| Linolenic acid | $1.21x10^{-3}$ mol, 0.34 g, 0.37 ml |
| Oxalyl chloride | $7.64x10^{-3}$ mol, 0.96 g, 0.64 ml |
| β-sitosterol | $1.21x10^{-3}$ mol, 0.50 g |
| DMPA | $1.73x10^{-3}$ mol, 0.21 g |
| Reaction temperature | Room temperature |
| Reaction time | 12 h |
| Purification | Gravimetric column (Ethyl acetate/hexane 9.5/0.5) |
| Yield | 42% |

$C_{47}H_{77}O_2$ MW = 674.21

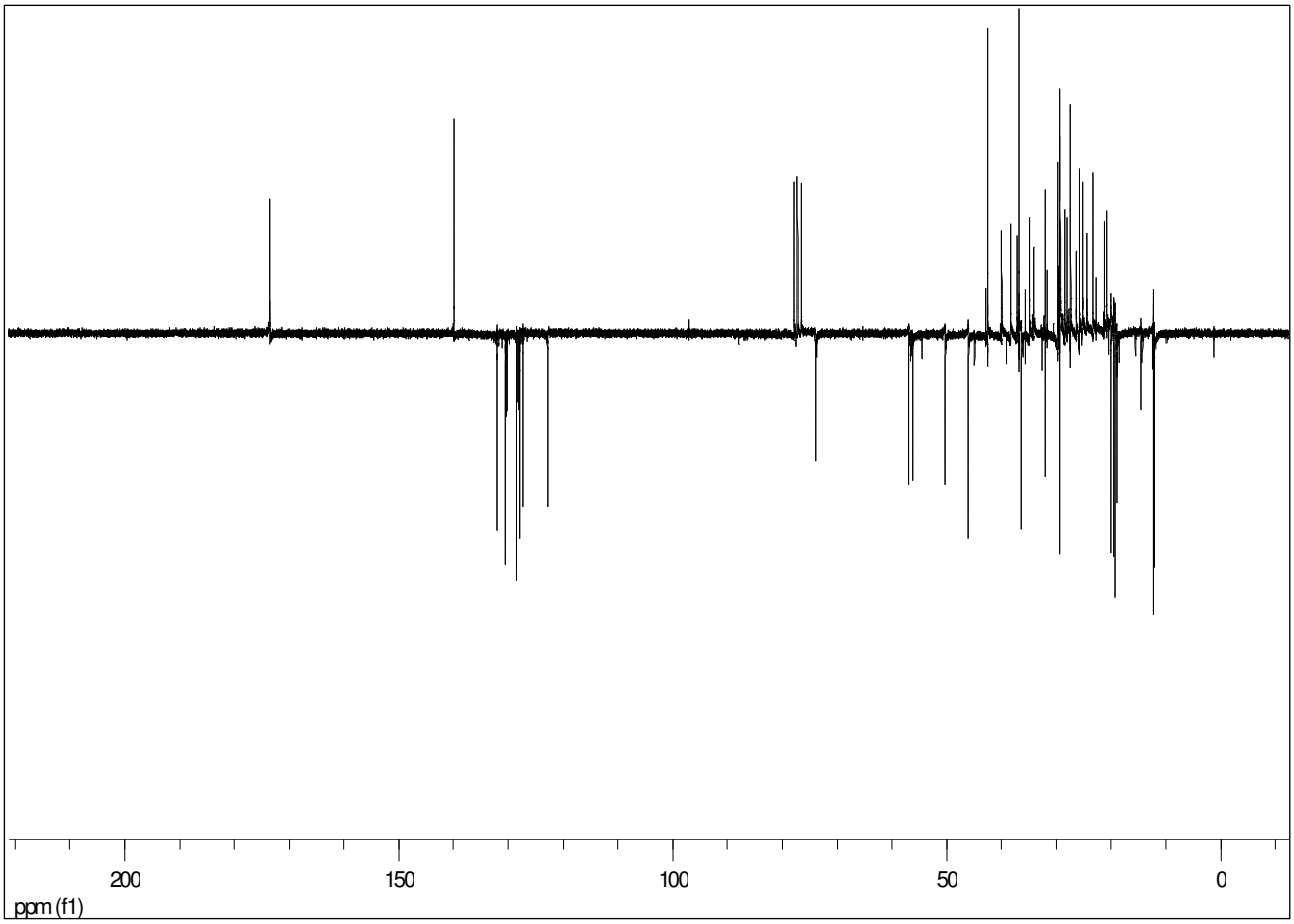
**TLC:** Hexane/Ethyl acetate/Ethyl Ether 5/2/1and spot visualization with iodine vapor

**UV/Vis** in MeOH λ (nm) = 234; 268



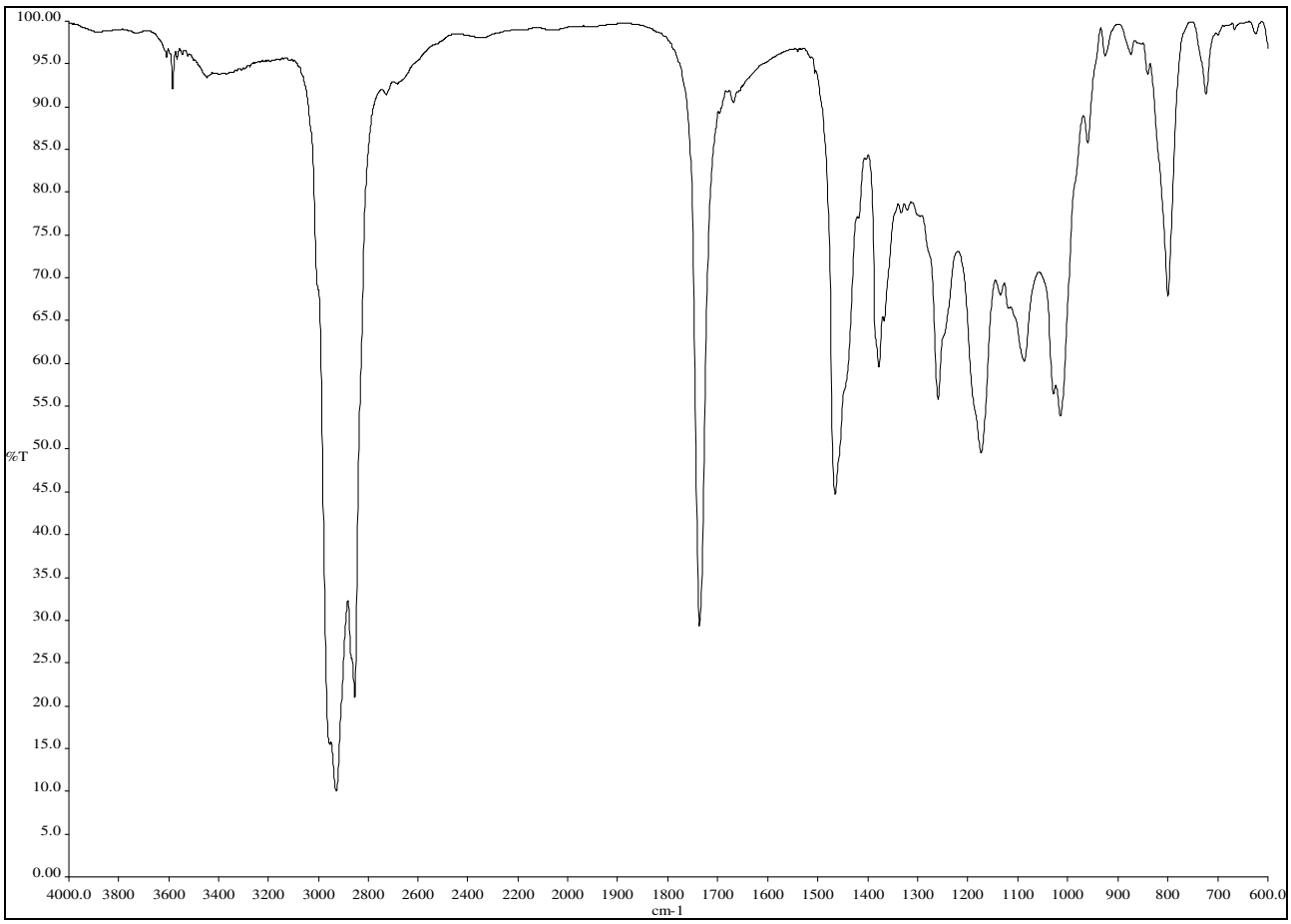



**$^{1}$H-NMR (500 MHz, $CDCl_3$):** δ = 5.43-5.27 (m, 7 H, $H_7$, $H_{37}$, $H_{38}$, $H_{40}$, $H_{41}$, $H_{43}$, $H_{44}$); 4.63-4.57 (m, 1 H, $H_2$,); 2.80 (t, J=5.5, 4 H, $H_{39}$, $H_{42}$), 2.32-2.26 (m, 4 H, $H_1$, $H_{30}$); 2.10-0.83 (m, 61 H, $CH_2$ and $CH_3$).




**$^{13}$C-NMR ($CDCl_3$):** δ = 173.477 CO; 139.935 $C_6$; 132.153-127.342 $C_{37}$, $C_{38}$, $C_{40}$, $C_{41}$, $C_{43}$, $C_{44}$; 122.796 $C_7$; 73.910 $C_2$, 56.922 $C_{14}$; 56.278 $C_{17}$; 50.270 $C_{10}$; 46.072 $C_{21}$; 42.542 $C_{13}$; 39.958 $C_{12}$; 38.389; 37.230 $C_1$; 36.821 $C_5$; 36.374 $C_{18}$; 34.919 $C_{19}$; 34.177 $C_{30}$; 32.101 $C_9$; 29.790-29.305 $C_{32}$-$C_{35}$; 29.403 $C_{24}$; 28.464 $C_{16}$; 28.039 $C_{36}$; 27.418 $C_3$; 26.342 $C_8$; 25.842 $C_{39}$; 25.741 $C_{42}$; 25.259 $C_{31}$; 24.516 $C_{20}$; 23.304 $C_{22}$; 21.258 $C_{11}$; 20.766 $C_{45}$; 20.031 $C_{25}$, $C_{26}$; 19.531 $C_{23}$; 19.265 $C_{29}$; 19.000 $C_{27}$; 14.484 $CH_3$; 12.204, 12.075 $C_{28}$.

**FT-IR(NaCl)**




| **1/λ ($cm^{-1}$)** | **Attribution** |
|---|---|
| 2932.11, 2854.15 | ν C $sp^3$ - H |
| 1734.41 | ν C=O |
| 1464.01 | δ $CH_2$ |
| 1175.57 | ν C-O |
| 803.55 | δ HC=CH *trans* |

### 4.1.2 - HPLC METHOD DEVELOPMENT

First, the solubility of β-sitosterol, oleic and linolenic acids and their esters in methanol and in acetonitrile was assessed. β-sitosterol, as well as oleic and linolenic acids, are very soluble in both methanol and acetonitrile, whereas their esters dissolve less easily in both solvents, but sufficiently to be analyzed in reverse phase HPLC. Then, several trials were performed in order to select the best analysis conditions, changing different HPLC parameters: stationary phase, mobile phase, flow rate. The following trials were performed:

| Stationary phase | Mobile phase | Flow rate (ml/min) | λ (nm) | T °C |
|---|---|---|---|---|
| ODS Hypersil 250x4.6mm | MeOH/$H_2O$ 80:20 | 0.8 ml/min | 210 | Room temp. |
| ODS Hypersil 250x4.6mm | MeOH/$H_2O$ 75:25 | 0.8 ml/min | 210 | Room temp. |
| Symmetry Shield 250x4.6mm | MeOH/$H_2O$ 75:25 | 0.4 ml/min | 210 | Room temp. |
| X Bridge 250x4.6 mm | MeOH/$H_2O$ 75:25 | 0.4 ml/min | 210 | Room temp. |
| Symmetry Shield 250x4.6mm | ACN/$H_2O$ 80:20 | 0.4 ml/min | 210 | Room temp. |
| Symmetry Shield 250x4.6mm | ACN/$H_2O$ 80:20 | 0.8 ml/min | 210 | Room temp. |
| Symmetry Shield 250x4.6mm | ACN/$H_2O$ 90:10 | 0.8 ml/min | 210 | Room temp. |
| Alltima C-8 250x4.4 mm | ACN/$H_2O$ 90:10 | 0.8 ml/min | 210 | Room temp. |
| Alltima C-8 250x4.4 mm | ACN/$H_2O$ 95:5 | 0.8 ml/min | 210 | Room temp. |
| Alltima C-8 250x4.4 mm | ACN/ $H_2O$ 95:5 | 1 ml/min | 210 | Room temp. |
| Alltima C-8 250x4.4 mm | ACN 100% | 0.8 ml/min | 210 | Room temp. |
| Alltima C-8 250x4.4 mm | ACN 100% | 1 ml/min | 210 | Room temp. |

**Table 4.** Conditions used in method development.

All the trials reported in Table 4 were performed injecting β-sitosterol, oleic acid, linolenic acid, and their esterification products, separately.

Acetonitrile (100%) resulted the best solvent to be used as mobile phase for our analysis, since the trials performed using a mixture of methanol and water showed a low peak resolution. Long retention time of the analytes made us to select a flow rate of 1.0 ml/min and a C-8 column, which resulted also in a good peak separation.

A wavelength of 210 nm was chosen in order to detect both β-sitosterol and fatty acids.

The selected analysis condition are:

- Mobile phase: Acetonitrile 100%
- Stationary phase: Alltima C-8
- Flow rate: 1 ml/min
- λ: 210 nm
- Room temperature

The chromatograms of β-sitosterol (Fig. 4), linolenic acid (Fig. 5), oleoyl β-sitosterol (Fig. 6) and linolenoyl β-sitosterol (Fig. 7) are reported below.

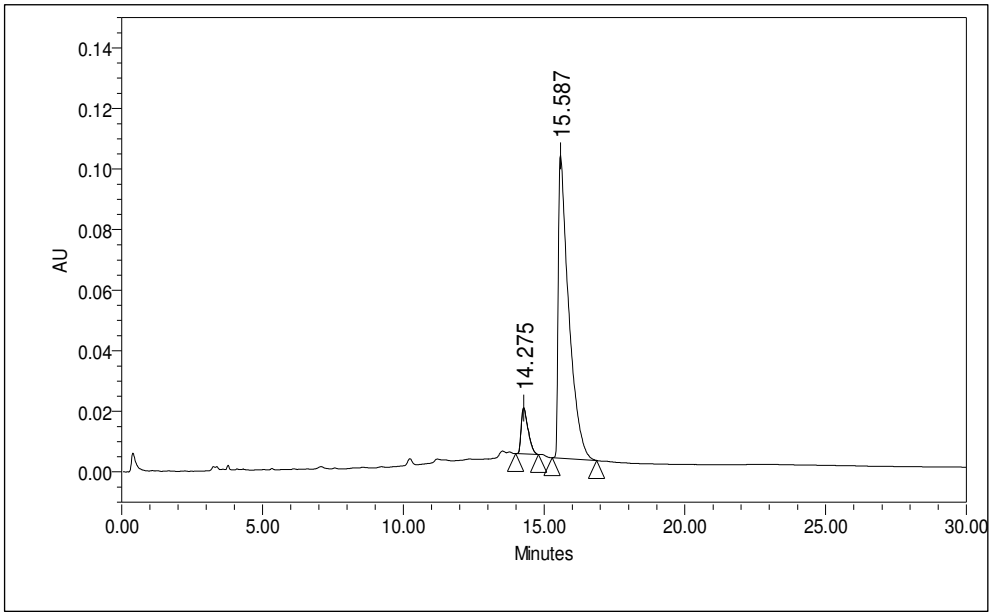



**Figure 4.** Chromatogram of β-sitosterol.

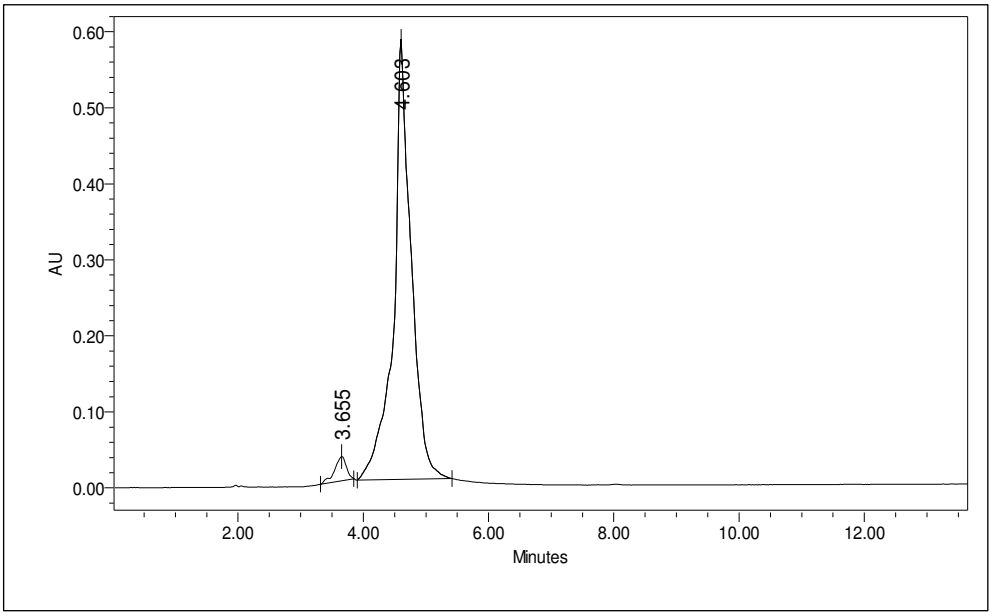



**Figure 5.** Chromatogram of linolenic acid.

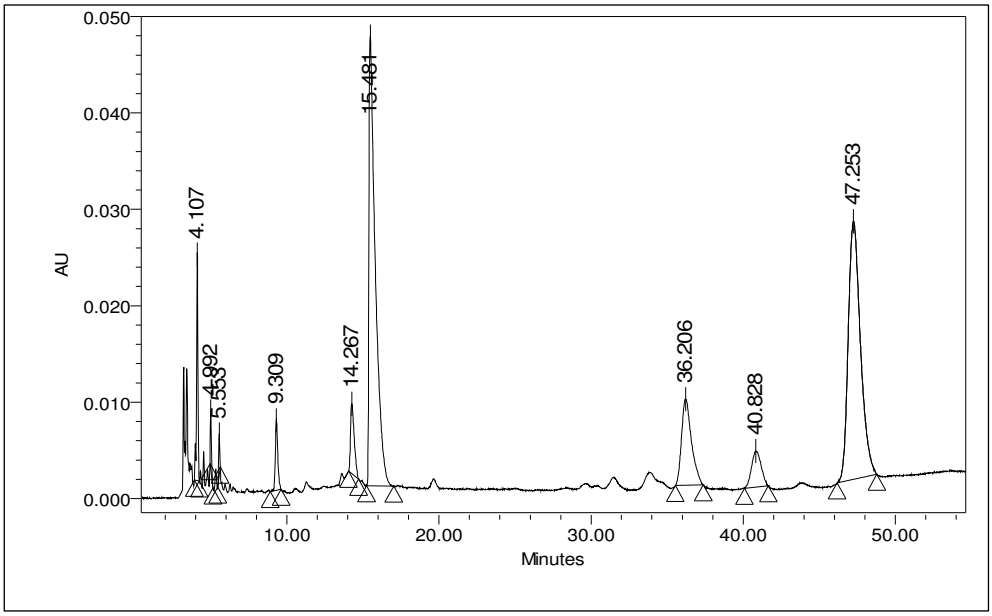



**Figure 6.** Chromatogram of oleoyl β-sitosterol.

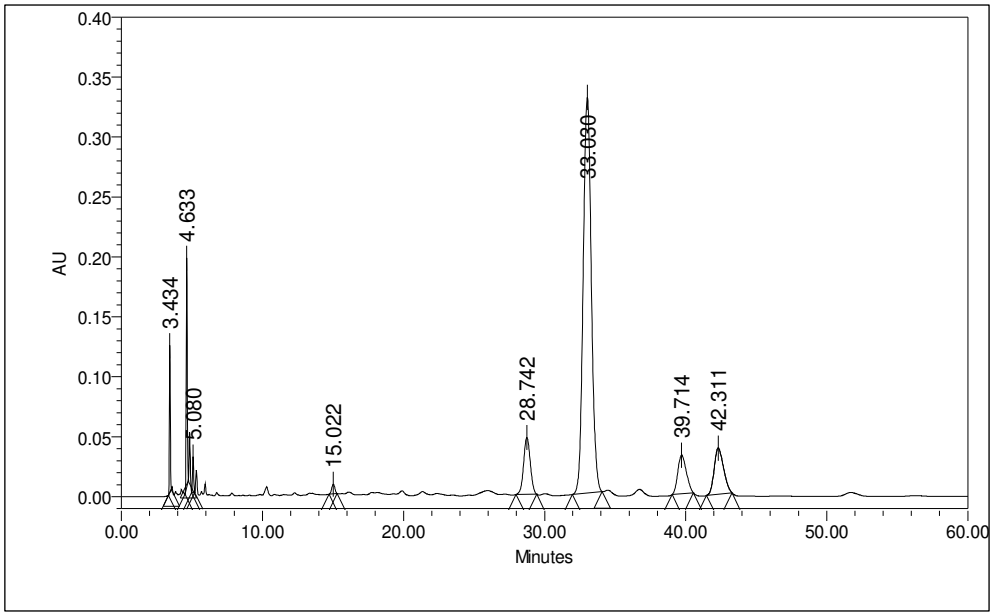



**Figure 7.** Chromatogram of linolenoyl β-sitosterol.

As seen in Fig. 4, β-sitosterol displayed two peaks having a similar retention time (14.275 and 15.587), due to its purity grade: 60%, the remaining 40% were other sterols. In addition, the purity grades of oleic and linolenic acids (Fig. 5) were 90% and 70%, respectively: as a result, the esters obtained using β-sitosterol and fatty acids showed multiple peaks having similar retention times.

### 4.1.3 - BIOCATALYTIC SYNTHESIS OF β-SITOSTEROL FATTY ESTERS

In the first phase of the study, different enzymes were screened in order to select the most suitable for the proposed aim, namely the esterification of β-sitosterol with oleic acid and linolenic acid.

In this phase different trials were performed in screw-cap test tubes, with a high amount of enzyme (140 U/mg), a low concentration of substrates (1 mg/ml), used with a 1:1 molar ratio, and using heptane as reaction solvent (2 ml), since the bio-catalysts used prefer hydrophobic solvents: such conditions were shown to be the best ones in order to promote the full development of the reaction.

The enzymes used in this phase were:

- *Amano* lipase
- Lipase from *Candida cylindracea*
- Lipase from *Rhizopus oryzae*
- Lipase from *Candida rugosa*
- Lipase from *Aspergillum oryzae*

TLC analyses were performed at different times of the reactions, showing that lipase from *Candida cylindracea* promotes the reaction with oleic acid, whereas lipase from *Candida rugosa* promotes the esterification of β-sitosterol with both oleic and linolenic acids.

The second phase of this study was the optimization of the reaction conditions. Different parameters were screened: solvent, reaction temperature, lipase units, reaction time. Several trials in 2 ml of solvent were performed, changing one parameter at a time, and keeping the other variables constant. TLC technique was used to assess the results of the trials. The following parameters were changed:

- Solvent: heptane, toluene, acetone, acetonitrile, hexane, isopropyl ether
- Reaction temperature: 25 °C, 30 °C, 40 °C
- Enzyme units : 140 U/mg, 70 U/mg , 35 U/mg, 28 U/mg, 14 U/mg

The conditions found in this phase of the study are shown in Tables 5, 6, 7.

**Oleoyl β-sitosterol**

| | |
|---|---|
| **Lipase from *Candida rugosa*** | 35 U/mg |
| β-sitosterol | 1 mg/ml |
| Oleic acid | 0.68 mg/ml * |
| Reaction solvent | Toluene |
| Reaction temperature | 40 °C |

**Table 5.**

| | |
|---|---|
| **Lipase from *Candida cylindracea*** | 14 U/mg |
| β-sitosterol | 1 mg/ml |
| Oleic acid | 0.68 mg/ml * |
| Reaction solvent | Heptane |
| Reaction temperature | 40 °C |

**Table 6.**

**Linolenoyl β-sitosterol**

| | |
|---|---|
| **Lipase from *Candida rugosa*** | 35 U/mg |
| β-sitosterol | 1 mg/ml |
| Linolenic acid | 0.67 mg/ml * |
| Reaction solvent | Toluene |
| Reaction temperature | 40 °C |

**Table 7.**

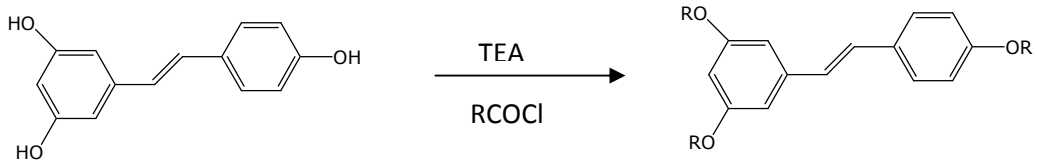
***** = Fatty acid (oleic or linolenic) and β-sitosterol were used with a molar ratio of 1:1.

Using the conditions found in the previous phase of the study, reactions on a semi-preparative scale (25 ml) were performed in order to evaluate the reaction's kinetics and time. Samples (0.5 ml) were taken at fixed times (4 h, 24 h, 42 h, 48 h, 52 h, 72 h), centrifuged to eliminate the enzyme, dried under $N_2$ flow, dissolved in the mobile phase and subsequently analyzed by HPLC using the conditions previously reported.

## 4.2 - RESVERATROL

### 4.2.1 - CHEMICAL SYNTHESIS OF RESVERATROL FATTY ESTERS

Resveratrol esters were obtained both with saturated (palmitic and stearic acid) and unsaturated (oleic, linoleic, and linolenic acid) fatty acids.



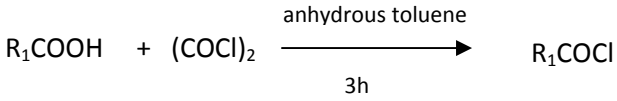

R= $R_1CO$-

**Scheme 3.**

Under nitrogen flow, resveratrol was dissolved in 20 ml anhydrous toluene and the solution was stirred at room temperature. Then TEA was added and, using an ice bath, the temperature was decreased until 0°C; afterwards the chloride was added dropwise. The reaction mixture thus obtained was carried to room temperature and magnetically stirred, repairing from daylight.

The mixture was washed with saturated $NaHCO_3$ water solution and then with water (3 x 20 ml). The organic layer was dried over anhydrous $Na_2SO_4$ and evaporate under vacuum (77 mbar, 40 °C).

When the acyl chloride was not commercially available, it was prepared as follows:

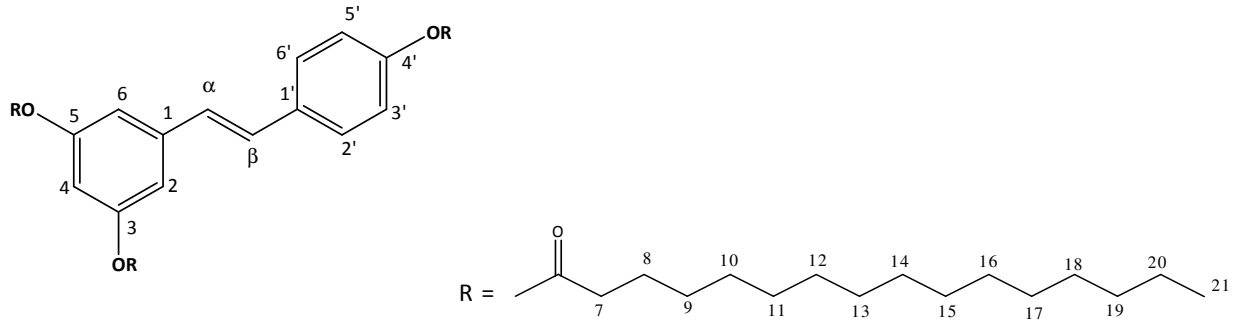



**Scheme 4.**

The unsaturated fatty acid was dissolved in 20 ml anhydrous toluene and the solution was stirred at room temperature under anhydrous conditions. Then, using an ice bath, the temperature was decreased to 0 °C and oxalyl chloride was added dropwise. The reaction mixture was carried to room temperature and magnetically stirred for 3 h. The reaction was monitored by IR. At the end of the reaction the solution was evaporated under vacuum (77 mbar, 40 °C) in inert atmosphere: the obtained acyl chloride was employed without further purification the for subsequent reaction.

All synthesized compounds were analyzed by FT-IR, $^{1}$H-NMR, $^{13}$C-NMR, UV and mass spectrometry. The instrumental results interpretation for all compounds is reported below; as example, 3,5,4'-tri-oleoyl resveratrol spectra are reported.

## 3,5,4'-TRIPALMITOYL RESVERATROL




**Reaction parameters:**

**Molar ratio palmitoyl chloride:resveratrol 1:3**

| | |
|---|---|
| Resveratrol | $2.2x10^{-3}$ mol, 0.50 g |
| Palmitoyl chloride | $6.6x10^{-3}$ mol, 1.81 g, 2 ml |
| TEA | $6.6x10^{-3}$ mol, 0.67 g, 0.92 ml |
| Reaction temperature | Room temperature |
| Reaction time | 3 h |
| Yield | 70% |

**$C_{62}H_{96}O_6$** MW = 943.65

**TLC:** Hexane/Acetone 1/1

**m/z:** 965 $[M+H]^+$

**UV/Vis** in MeOH λ (nm) = 232; 291

**$^1$H-NMR (, $CDCl_3$):** δ = 7.48 (d, $J_{2'-3'}$ = 8.8, 2 H, $H_{2'}$, $H_{6'}$); 7.02-7.12 (m, 5 H, $H_2$,$H_6$, $H_{3'}$, $H_{5'}$, $H_\alpha$); 7.01 (d, $J_{\beta,\alpha}$ = 15.8, 1 H, $H_{\beta'}$); 6.80 (t, $J_{4-6}$ = 1.8, 1 H, $H_4$); 2.55 (t, J = 7.3, 6 H, $H_7$); 1.79-1.68 (m, 6 H, $H_8$); 1.35-1.26 (m, 72 H, $H_9$-$H_{20}$,); 0.91 (t, J=5.9, 9 H, $CH_3$,).

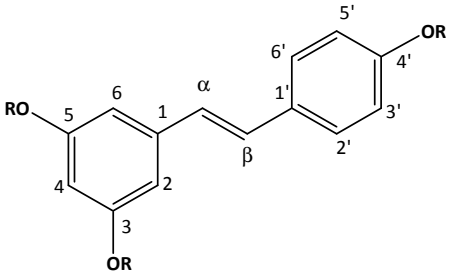
**$^{13}$C-NMR ($CDCl_3$):** δ =172.242 CO($C_{4'}$); 171.902 CO($C_3$), CO($C_5$); 151.724 C4'; 150.853 $C_3$, $C_5$; 139.707 $C_1$; 134.631 $C_{1'}$, 129.865 $C_\alpha$; 127.774 $C_{2'}$, $C_{6'}$; 127.516 $C_\beta$; 122.053 $C_{3'}$, $C_{5'}$, 116.946 $C_2$, $C_6$; 114.560 $C_4$; 34.624 $C_7$; 32.108 $C_{19}$; 29.865 $C_{12}$; 29.835 $C_{17}$; 29.797 $C_9$; 29.638 $C_{16}$, $C_{18}$; 29.524 $C_{13}$, $C_{14}$; 29.441 $C_{17}$; 29.320 $C_{10}$,$C_{11}$; 25.153 $C_8$* 25.100 $C_8$*; 22.849 $C_{20}$; 14.227 $CH_3$.

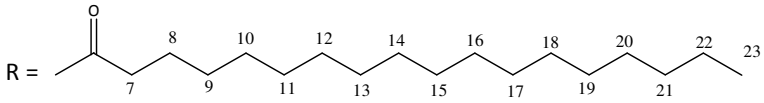
*= the two chains give two different signals since they are not equivalent

**FT-IR (KBr)**

| **1/λ ($cm^{-1}$)** | **Attribution** |
|---|---|
| 3064.65 | ν C $sp^2$ - H |
| 2954.16 ,2919.52, 2850.56 | ν C $sp^3$ - H |
| 1753.12 | ν C=O |
| 1612.42, 1599.20, 1581.78 | ν C=C |
| 1467.90 | δ $CH_2$ |
| 1198.66, 1139.73 | ν C-O |
| 971.67 | δ HC=CH *trans* |
| 720.95 | δ HC= aromatic |

## 3,5,4'-TRISTEAROYL RESVERATROL




**Reaction parameters:**

**Molar ratio stearoyl chloride:resveratrol 1:3**

| | |
|---|---|
| Stearoyl chloride | $6.6x10^{-3}$ mol, 1.81 g, 2 ml |
| Resveratrol | $2.2x10^{-3}$ mol, 0.50 g |
| TEA | $6.6x10^{-3}$ mol, 0.67 g, 0.92 ml |
| Reaction temperature | Room temperature |
| Reaction time | 3 h |
| Yield | 63% |

**$C_{68}H_{114}O_6$** MW = 1026.52

**TLC:** Hexane/Acetone 1/1

**m/z:** 1049 $[M+H]^+$

**UV/Vis** in MeOH λ (nm) = 228; 300; 310

**$^1$H-NMR ($CDCl_3$):** δ = 7.48 (d, $J_{2'-3'}$ = 8.8, 2 H, $H_{2'}$, $H_{6'}$);7.02-7.12 (m, 5 H, $H_2$,$H_6$, $H_{3'}$, $H_{5'}$, $H_\alpha$); 7.01 (d, $J_{\alpha-\beta}$= 15.8, 1 H, $H_\beta$); 6.79 (t, $J_{4-6}$ = 2.2, 1 H, $H_4$); 2.55 (t, J = 7.3, 6 H, $H_7$); 1.79-1.68 (m, 6 H, $H_8$); 1.33-1.26 (m, 84 H, $H_9$-$H_{22}$); 0.91 (t, J=5.9, 9 H, $CH_3$).

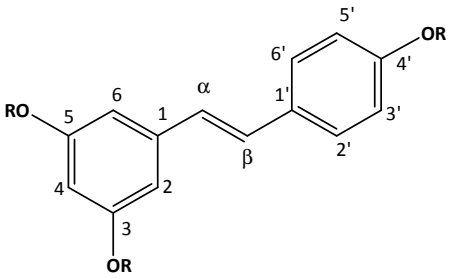
**$^{13}$C-NMR ($CDCl_3$):** δ =172.242 CO($C_{4'}$); 171.894 CO($C_3$), CO($C_5$); 151.724 C4'; 150.845 $C_3$, $C_5$; 139.707 $C_1$; 134.631 $C_{1'}$; 129.857 $C_\alpha$; 127.766 $C_{2'}$, $C_{6'}$; 127.509 $C_\beta$; 122.053 $C_{3'}$, $C_{5'}$; 116.946 $C_2$, $C_6$; 114.552 $C_4$; 34.616 $C_7$; 32.101 $C_{21}$; 29.873 $C_{12}$, $C_{13}$; 29.838 $C_{17}$; 29.631 $C_{19}$ $C_{16}$; 29.524 $C_{14}$ ,$C_{13}$; 29.434 $C_{18}$, $C_9$, $C_{20}$; 29.312 $C_{10}$,$C_{11}$; 25.145 $C_8$* 25.100 $C_8$*; 22.842 $C_{22}$; 14.219 $CH_3$.

*= the two chains give two different signals since they are not equivalent

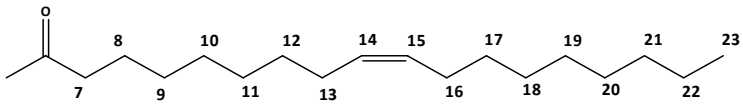
**FT-IR (KBr)**

| **1/ λ ($cm^{-1}$)** | **Attribution** |
|---|---|
| 3064.65 | ν C $sp^2$ - H |
| 2954.16 ;2918.28; 2850.06 | ν C $sp^3$ - H |
| 1759.80 | ν C=O |
| 1612.40; 1600.05; 1587.78 | ν C=C |
| 1467.86 | δ $CH_2$ |
| 1198.68; 1140.27 | ν C-O |
| 971.02 | δ HC=CH *trans* |
| 720.97 | δ HC= aromatic |

## 3,5,4'-TRIOLEOYL RESVERATROL




R =




**Reaction parameters:**

**Molar ratio oleic acid:resveratrol** **1:3**

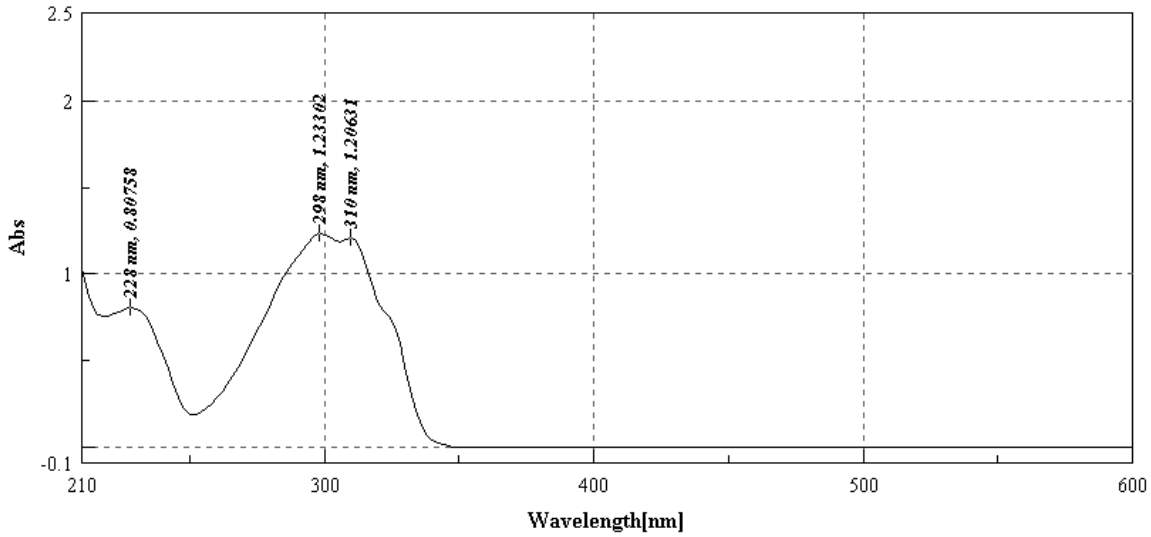
| | |
|---|---|
| Oleic acid | $6.6x10^{-3}$ mol, 1.85 g, 2.08 ml |
| Oxalyl chloride | $4.38x10^{-2}$ mol, 5.52 g, 3.51 ml |
| Resveratrol | $2.2x10^{-3}$ mol, 0.5 g |
| TEA | $6.6x10^{-3}$ mol, 0.67 g, 0.92 ml |
| Reaction temperature | Room temperature |
| Reaction time | 20 h |
| Yield | 69% |

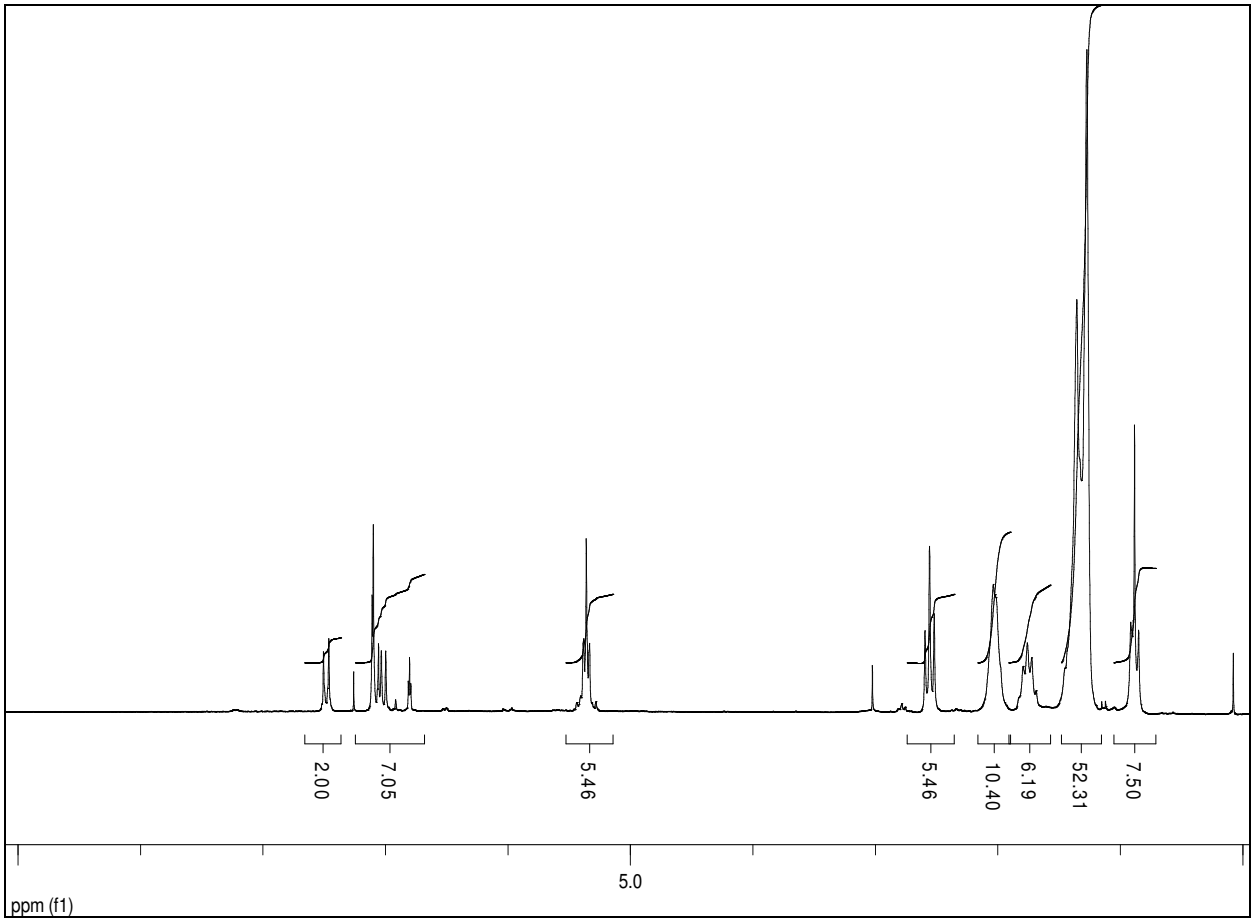
**$C_{68}H_{108}O_6$** MW = 1021.58

**TLC:** Hexane/Acetone 1/1

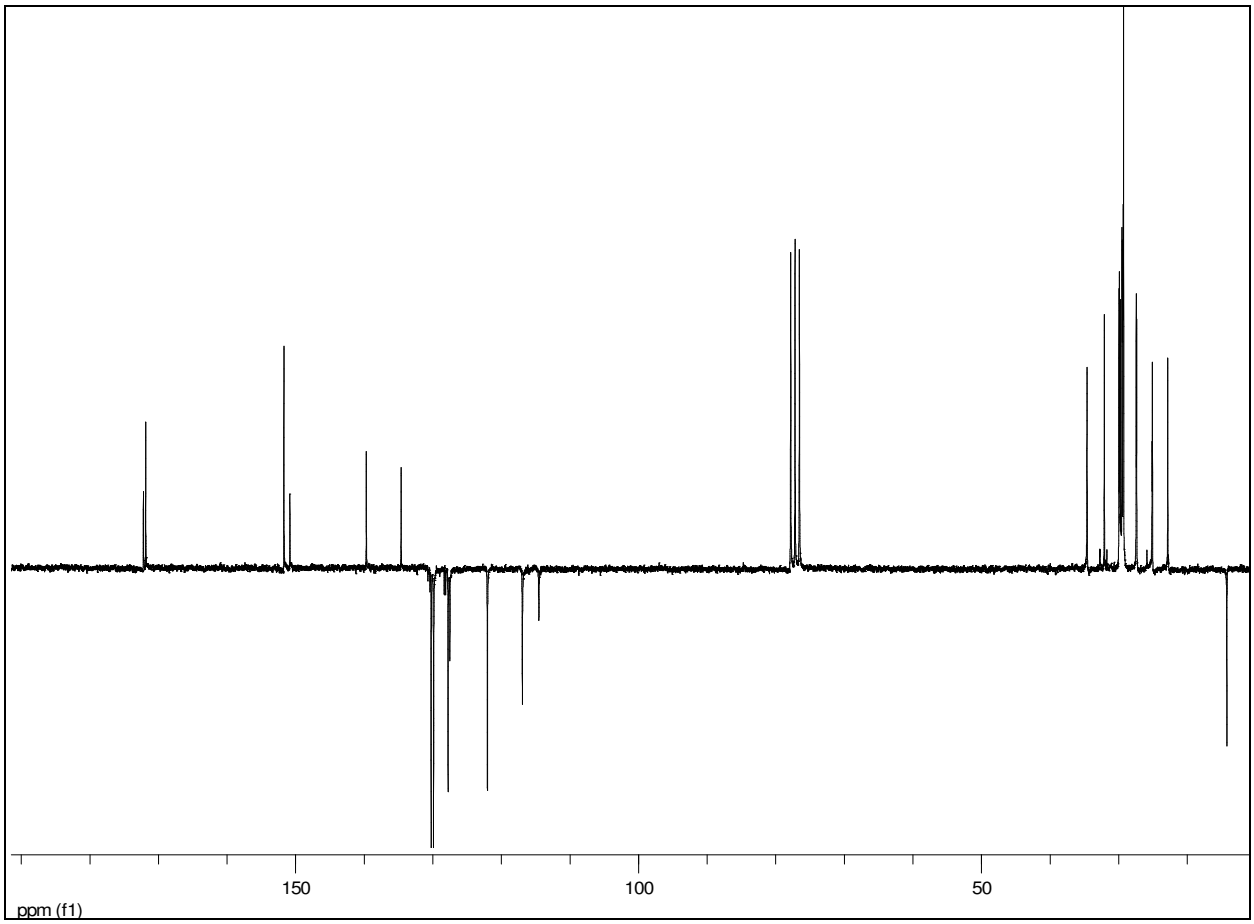
**m/z:** 1022 $[M+H]^+$

**UV/Vis** in MeOH λ (nm) = 228; 298; 310





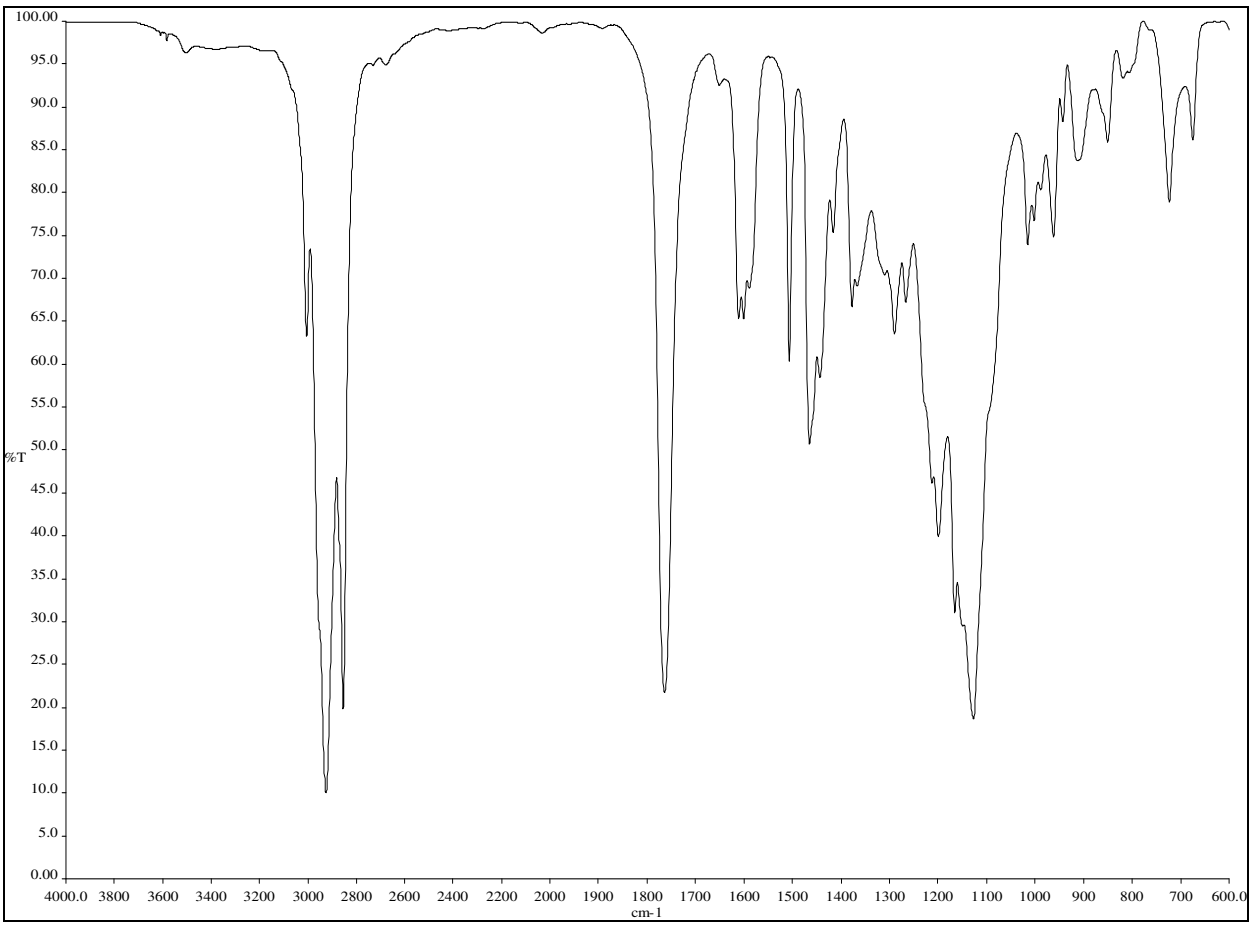

**$^{1}$H-NMR (500 MHz, $CDCl_3$):** δ = 7.48 (d, $J_{2'-3'}$ = 8.4, 2 H, $H_{2'}$, $H_{6'}$);7.10-7.02 (m, 5 H, $H_2$,$H_6$, $H_{3'}$, $H_{5'}$, $H_\alpha$); 7.00 (d, $J_{\alpha-\beta}$= 15.8, 1 H, $H_\beta$); 6.80 (t, $J_{4-6}$ = 1.8, 1 H, $H_4$); 5.35 (m, 6H, $H_{14}$, $H_{16}$); 2.55 (t, J = 7.3, 6 H, $H_7$); 2.35-2.03 (m, 12 H, $H_{13}$, $H_{16}$); 1.79-1.68 (m, 6 H, $H_8$); 1.35-1.20 (m, 60 H, $H_9$-$H_{12}$ and $H_{17}$-$H_{22}$); 0.91 (t, J=5.9, 9 H, $CH_3$).




**$^{13}$C-NMR (MHz, $CDCl_3$):** δ =172.220 $CO(C_{4'})$ ; 171.871 $CO(C_3)$, $CO(C_5)$; 151.717 C4'; 150.845 $C_3$, $C_5$; 139.707 $C_1$; 134.631 $C_{1'}$; 130.251 $C_{14}$; 129.903 $C_{15}$, $C_\alpha$; 127.781 $C_{2'}$, $C_{6'}$; 127.524 $C_\beta$; 122.053 $C_{3'}$, $C_{5'}$; 116.946 $C_2$, $C_6$; 114.552 $C_4$; 34.906 $C_7$; 32.085 $C_{21}$; 29.964 $C_{12}$, 29.880 $C_{17}$; 29.669 $C_{19}$; 29.509 $C_{18}$ ; 29.479 $C_{20}$; 29.335 $C_9$; 29.282 $C_{10}$, $C_{11}$; 27.441 $C_{13}$-$C_{16}$; 27.373 $C_{16}$-$C_{13}$ ; 25.137, 25.077 $C_{8*}$; 22.840 $C_{22}$; 14.277 $CH_3$.

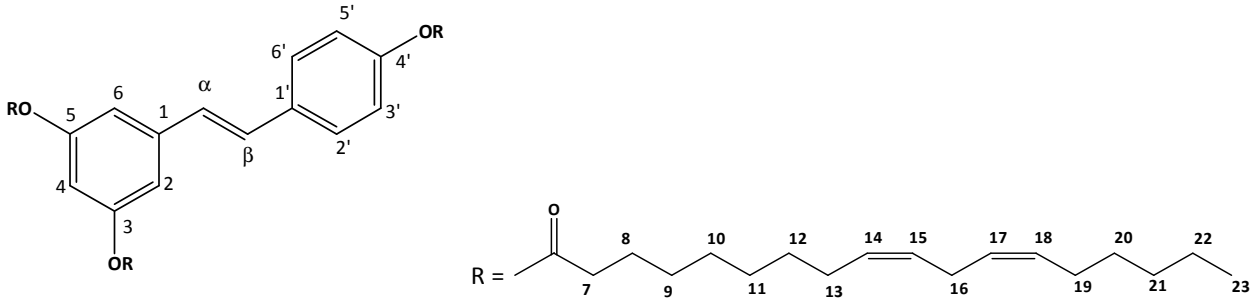
*= the two chains give two different signals since they are not equivalent

**FT-IR (NaCl)**




| **1/ λ ($cm^{-1}$)** | **Attribution** |
|---|---|
| 3064.65 | ν C $sp^2$ - H |
| 3005.07; 2925.38; 2854.23 | ν C $sp^3$ - H |
| 1764.11 | ν C=O |
| 1610.66; 1600.36; 1589.14 | ν C=C |
| 1464.75 | δ $CH_2$ |
| 1198.51; 1165.62; 1127.11 | ν C-O |
| 961.91 | δ HC=CH *trans* |
| 724.19 | δ HC= aromatic |

## 3,5,4'-TRILINOLEOYL RESVERATROL




**Reaction parameters:**

**Molar ratio linoleic acid:resveratrol 1:3**

| | |
|---|---|
| Linoleic acid | $6.6x10^{-3}$ mol, 1.84 g, 2.04 ml |
| Oxalyl chloride | $4.36x10^{-2}$ mol, 5.50 g, 3.45 ml |
| Resveratrol | $2.2x10^{-3}$ mol, 0.5 g |
| TEA | $6.6x10^{-3}$ mol, 0.67 g, 0.92 ml |
| Reaction temperature | Room temperature |
| Reaction time | 20 h |
| Yield | 58% |

**$C_{68}H_{102}O_6$** MW = 1015.43

**TLC:** Hexane/Acetone 1/1

**m/z:** 1016$[M+H]^+$

**UV/Vis in MeOH** λ (nm) = 228; 298; 310

**$^1$H-NMR ($CDCl_3$):** δ = 7.48 (d, $J_{2'-3'}$ = 8.8, 2 H, $H_{2'}$, $H_{6'}$);7.11-7.03 (m, 5 H, $H_2$,$H_6$, $H_{3'}$, $H_{5'}$, $H_\alpha$); 6.95 (d, $J_{\alpha-\beta}$= 16.0, 1 H, $H_\beta$); 6.80 (t, $J_{4-6}$ = 2.2, 1 H, $H_4$); 5.35 (m, 12H, $H_{14}$, $H_{16}$,$H_{17}$, $H_{18}$,); 2.78 (t, J=5.5, 6 H, $H_{16}$,); 2.55 (t, J = 7.3, 6 H, $H_7$,); 2.35-2.03 (m, 12 H, $H_{13}$, $H_{19}$,); 1.79-1.66 (m, 6 H, $H_8$,); 1.37-1.23 (m, 42 H, $H_9$-$H_{12}$ and $H_{20}$-$H_{22}$,); 0.89 (t, J=6.9, 9 H, $CH_3$,).

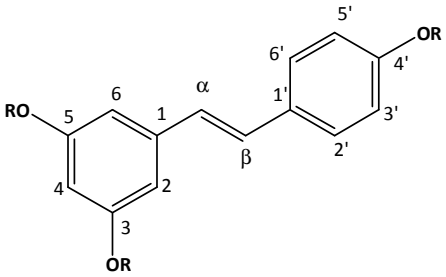
**$^{13}$C-NMR ($CDCl_3$):** δ =172.331 CO($C_{4'}$) ; 171.995 CO($C_3$), CO($C_5$); 151.671 C4'; 150.792 $C_3$, $C_5$; 139.707 $C_1$; 134.623 $C_{1'}$; 130.441 $C_{14}$; 130.221 $C_{17}$; 129.850 $C_\alpha$; 128.342 $C_{18}$; 128.137 $C_{15}$; 127.812 $C_{2'}$, $C_{6'}$; 127.478 $C_\beta$; 122.084 $C_{3'}$, $C_{5'}$; 116.984 $C_2$, $C_6$; 114.598 $C_4$; 34.609 $C_7$; 31.745 $C_{21}$; 29.812 $C_{12}$; 29.555 $C_{19}$; 29.365 $C_9$ $C_{20}$; 29.305 $C_{10}$, $C_{11}$; 27.418 $C_{13}$; 25.872 $C_{16}$ ; 25.137, 25.084 $C_{8*}$; 22.773 $C_{22}$; 14.250 $CH_3$.

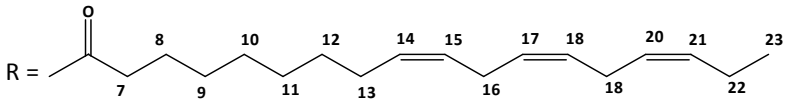
*= the two chains give two different signals since they are not equivalent

**FT-IR (NaCl)**

| **1/ λ ($cm^{-1}$)** | **Attribution** |
|---|---|
| 3064.65 | ν C $sp^2$ - H |
| 3008.07; 2927.64; 2855.64 | ν C $sp^3$ - H |
| 1764.11 | ν C=O |
| 1611.15; 1600.59, 1588.92 | ν C=C |
| 1464.72 | δ $CH_2$ |
| 1198.85; 1165.83, 1127.41 | ν C-O |
| 961.92 | δ HC=CH *trans* |
| 728.86 | δ HC= aromatic |

## 3,5,4'-TRILINOLENOYL RESVERATROL




**Reaction parameters:**

**Molar ratio linolenic acid:resveratrol 1:3**

| | |
|---|---|
| Linolenic acid | $6.6x10^{-3}$ mol, 1.83 g, 2.00 ml |
| Oxalyl chloride | $4.00x10^{-2}$ mol, 5.00 g, 2.38 ml |
| Resveratrol | $2.2x10^{-3}$ mol, 0.5 g |
| TEA | $6.6x10^{-3}$ mol, 0.67 g, 0.92 ml |
| Reaction temperature | Room temperature |
| Reaction time | 20 h |
| Yield | 43% |

**$C_{68}H_{96}O_6$** MW = 1009.53

**TLC:** Hexane/Acetone 1/1

**m/z:** 1022 $[M+H]^+$

**UV/Vis in MeOH** λ (nm) = 228; 294; 310

**$^1$H-NMR ($CDCl_3$):** δ = 7.48 (d, $J_{2'-3'}$ = 8.8, 2 H, $H_{2'}$, $H_{6'}$);7.10-7.05 (m, 5 H, $H_2$,$H_6$, $H_{3'}$, $H_{5'}$, $H_\alpha$); 7.01 (d, $J_{\alpha-\beta}$= 15.8, 1 H, $H_\beta$); 6.80 (t, $J_{4-6}$ = 2.1, 1 H, $H_4$); 5.35 (m, 18 H, $H_{14}$, $H_{16}$,$H_{17}$, $H_{18}$, $H_{20}$, $H_{21}$); 2.81 (t, J=5.5, 12 H, $H_{16}$, $H_{19}$); 2.55 (t, J = 7.3, 6 H, $H_7$); 2.35-2.03 (m, 12 H, $H_{13}$, $H_{22}$); 1.79-1.71 (m, 6 H, $H_8$); 1.35-1.25 (m, 24 H, $H_9$-$H_{12}$ $H_{17}$); 0.91 (t, J=5.9, 9 H, $CH_3$).

**$^{13}$C-NMR (MHz, $CDCl_3$):** δ =172.227 CO($C_{4'}$) ; 171.879 CO($C_3$), CO($C_5$); 151.717 C4'; 150.838 $C_3$, $C_5$; 139.715 $C_1$; 134.631 $C_{1'}$; 132.161 $C_{21}$; 130.426 $C_{14}$; 129.873 $C_\alpha$; 128.531 $C_{17}$-$C_{18}$;128.471 $C_{17}$-$C_{18}$; 128.031 $C_{15}$; 127.789 $C_{2'}$, $C_{6'}$; 127.516 $C_\beta$; 127.365 $C_{20}$; 122.061 $C_{3'}$, $C_{5'}$; 116.954 $C_2$, $C_6$; 114.552 $C_4$; 34.601 $C_7$; 29.775 $C_{12}$; 29.335 $C_9$; 29.282 $C_{10}$,$C_{11}$; 27.411 $C_{13}$; 25.835 $C_{16}$; 25.759 $C_{19}$; 25.130-25.069 $C_{8*}$; 20.743 $C_{22}$; 14.401 $CH_3$.

*= the two chains give two different signals since they are not equivalent

**FT-IR (NaCl)**

| **1/ λ ($cm^{-1}$)** | **Attribution** |
|---|---|
| 3064.65 | ν C $sp^2$ - H |
| 3005.07; 2925.38; 2854.23 | ν C $sp^3$ - H |
| 1764.11 | ν –C=O |
| 1610.66; 1600.36; 1589.14 | ν –C=C aromatic |
| 1464.75 | δ $CH_2$ |
| 1198.51; 1165.62; 1127.11 | ν C-O |
| 961.91 | δ HC=CH *trans* |
| 724.19 | δ HC= aromatic |
| 3064.65 | ν C $sp^2$ - H |

### 4.2.2 - HPLC METHOD DEVELOPMENT

The solubility of resveratrol and its esters was screened: resveratrol resulted very soluble in methanol, whereas its derivatives showed a lower solubility. Thanks to these solubility tests we also found out that the addition of a little amount of water leads to the re-precipitation of resveratrol esters: hence, 100% methanol was chosen as mobile phase.

Two different stationary phases were screened: C-18 Puro-sphestar 250x4.6 mm and C-8 Alltima 250x4.6 mm. C-8 Alltima was selected, because of the higher resolution achieved.

Different flow rates were tested as well: a 0.8 ml/min flow resulted in a good peak resolution, so it was selected for the analysis.

The final analysis conditions are:

- Mobile phase: 100% methanol
- Stationary phase: C-8 Alltima 250x4.6 mm
- Flow rate: 0.8 ml/min
- λ: 300 nm
- Room temperature

The chromatograms of resveratrol (Fig. 8), 3,5,4'-tripalmitoyl resveratrol (Fig. 9), 3,5,4'-trioleoyl resveratrol (Fig. 10), 3,5,4'-trilinolenoyl resveratrol (Fig. 11) are reported below.

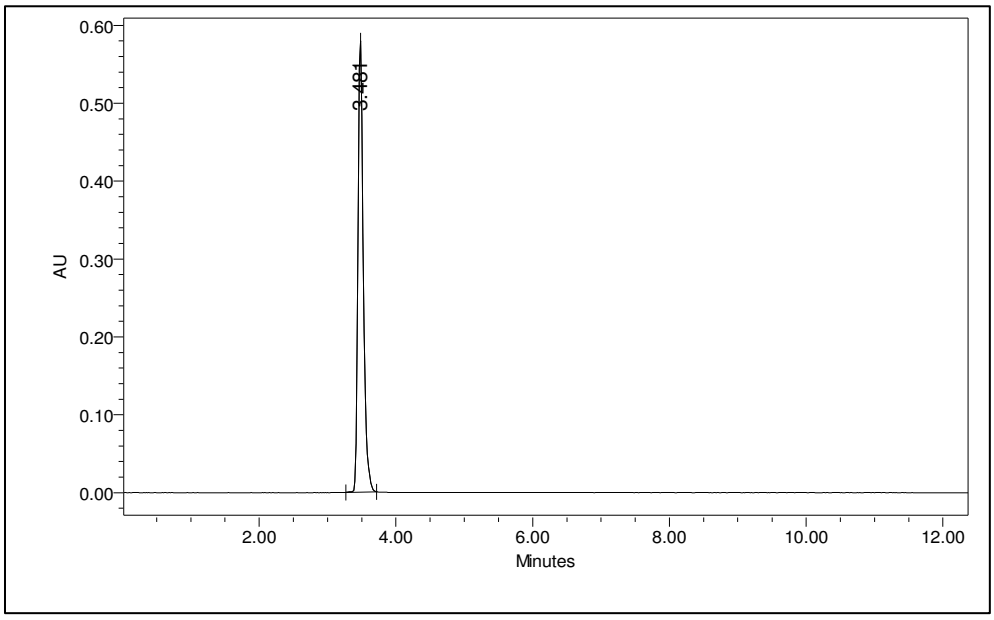



**Figure 8.** Chromatogram of resveratrol.

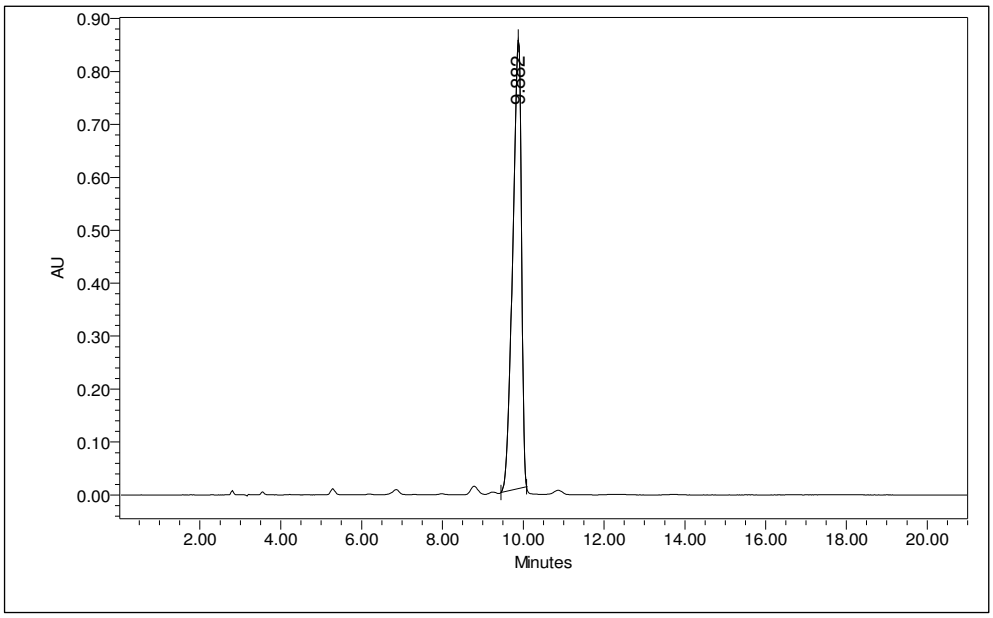



**Figure 9.** Chromatogram of 3,5,4'-tripalmitoyl resveratrol.

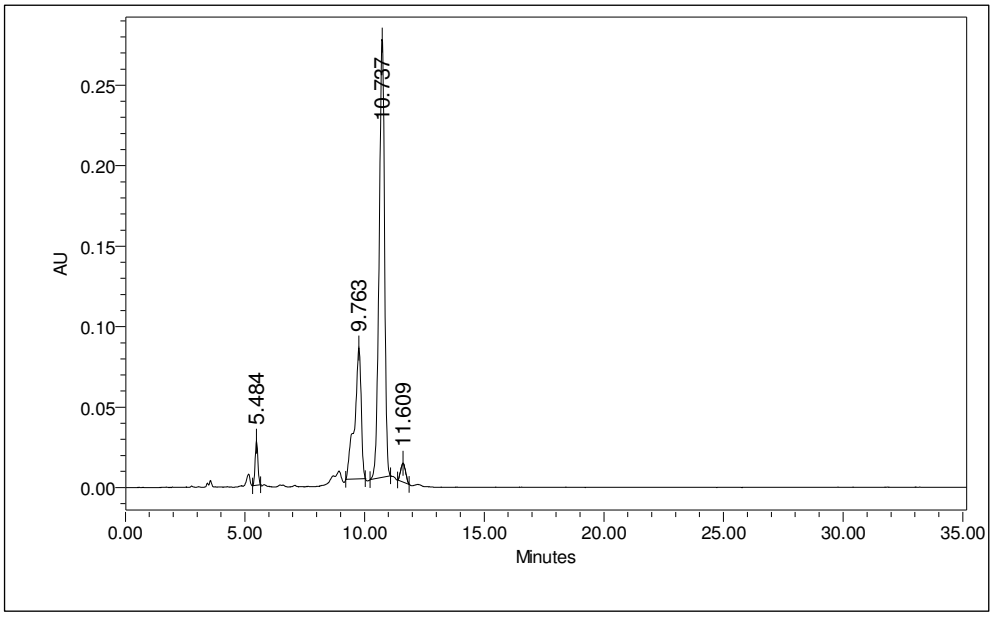



**Figure 10.** Chromatogram of 3,5,4'-trioleoyl resveratrol.

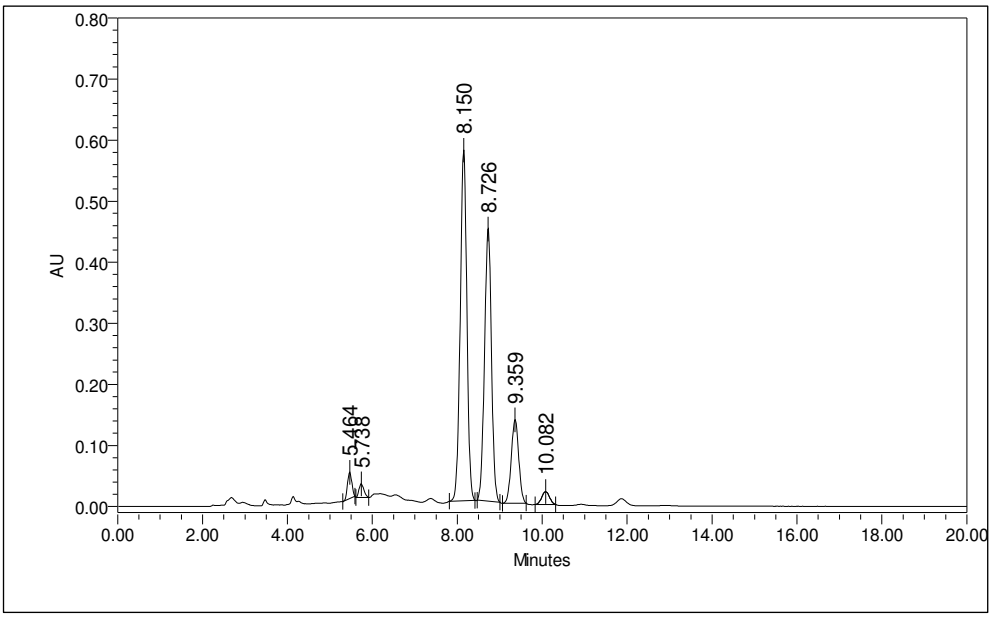



**Figure 11.** Chromatogram of 3,5,4'-trilinolenoyl resveratrol.

As seen in Figures 10 and 11, the triesters obtained from oleic and linolenic acids showed multiple peaks having similar retention times, due to the purity grade of the starting fatty acids, which was 90% for oleic acid and 70% for linolenic acid. The tri-esters obtained starting from palmitoyl chloride (Fig. 9), stearoyl chloride and linoleic acid had a higher purity, hence their chromatograms showed a main peak.

### 4.2.3 - BIOCATALYTIC SYNTHESIS OF RESVERATROL FATTY ESTERS

Since chemical synthesis of resveratrol mono and diesters is not possible and given the presence in literature **[154]** of synthesis of these compounds by biocatalytic hydrolysis of triester, in the first phase of the study a screening on enzymes and reaction conditions was performed. The aim of this screening was to select the most suitable enzymes and reaction conditions for the synthesis of mono and diesters of resveratrol starting from triester.

Different trials were performed in screw-cap test tubes, kept at 30 °C under magnetic stirring, using 2 ml of solvent, 2 mg/ml of substrate (trioleyol resveratrol) and 20 mg/ml of enzyme. These trials are reported in Table 8.

| Solvent | Enzyme |
|---|---|
| Toluene (95%) / Water (5%) | Protease |
| Toluene (95%) / Water (5%) | not immobilized CAL-B |
| Toluene (95%) / Water (5%) | *PS-Amano* lipase |
| Toluene (95%) / Water (5%) | *Lipase G* |
| Acetate buffer pH=7 / Acetone (10%) | Protease |
| Acetate buffer pH=7 / Acetone (10%) | not immobilized CAL-B |
| Acetate buffer pH=7 / Acetone (10%) | *PS-Amano* lipase |
| Acetate buffer pH=7 / Acetone (10%) | *Lipase G* |
| Tris buffer / HCl pH=8 / DMSO (2%) | Lipase from *Candida rugosa* |
| Tris buffer / HCl pH=8 / DMSO (2%) | Lipase from *Rhizopus arrhizus* |
| Tris buffer / HCl pH=8 / DMSO (2%) | *PS-Amano* lipase |
| Tris buffer / HCl pH=8 / DMSO (2%) | Acylase from *Aspergillus melleus* |
| Tris buffer / HCl pH=8 / DMSO (2%) | PPL (lipase from hog pancreas) |

**Table 8.**

All the trials listed in Table 8 were performed using trioleoyl resveratrol as substrate and analyzing the hydrolysis products by TLC (1/1 hexane/acetone). For each trial a blank reaction (set with only solvent and substrate) was carried out, in order to exclude any false result due to substrate degradation. TLC examination showed that only Acylase I from *Aspergillus melleus* and PPL in TRIS buffer / HCl solution (pH=8) were able to catalyze the hydrolysis of triester.

Once the enzymes and the reaction medium have been selected, a new set of trials was prepared, increasing the substrate concentration (4 mg/ml): this increment resulted in a higher hydrolysis with both

enzymes. The subsequent step was to perform tests on a larger scale (25 ml), in order to assess the time course of the reaction. At fixed times (4 h, 24 h, 48 h, 72 h, 96 h, 120 h), samples (0.5 ml) were taken, extracted with chloroform and then with ethyl acetate, treated with anhydrous $Na_2SO_4$, dried under $N_2$ flow, dissolved in the mobile phase and subsequently analyzed by HPLC using the conditions previously reported. These analyses showed that both enzymes promote the maximum production of mono and diesters at 96 h. A further increase of reaction time resulted in the formation of resveratrol, and thus in the complete hydrolysis of triester.

In the case of biotransformation with Acylase I from *Aspergillus melleus* the reaction was set up on a semi-preparative scale (100 ml) in order to isolate the final products in a sufficient amount for the structural characterization. To 400 mg of trioleoyl resveratrol dispersed in 4 ml of DMSO, 2 g of Acylase I from *Aspergillus melleus* and 100 ml of TRIS buffer/HCl pH=8 (prepared dissolving 1.2114 g of TRIS in 100 ml deionized water and adding HCl dropwise until pH 8 is reached) were added.

After 96 hours the reaction mix was extracted with chloroform (2 x 40ml) and with ethyl acetate (1 x 40 ml). The organic layers were pooled and washed with saturated $NaHCO_3$ water solution and then with water (3 x 20 ml). The new organic layer was dried over anhydrous $Na_2SO_4$ and evaporated under vacuum; then, the evaporation residue was analyzed by HPLC.

In order to characterize each analyte, the evaporation residue was separated using a chromatographic column with 7/3 hexane/acetone as mobile phase and a 1/60 product/silica ratio. Four different fractions were obtained in this separation, and subsequently analyzed.

## 4.2.4 - ORAL BIOAVALABILITY STUDIES OF RESVERATROL AND 3,5,4'-TRIOLEOYL RESVARATROL IN THE RAT

### 4.2.4.1 - STUDY PROTOCOL

In order to check the oral bioavailability of 3,5,4'-trioleoyl resveratrol, preliminary tests were performed on rats, in collaboration with Dr. Giuseppe Rossoni from Dept. of Pharmacology, Chemotherapy and Medical Toxicology, Università degli Studi, Milan.

For these studies 32 male Wistar rats were purchased from Charles River Italy (Calco, LC, Italy). Their initial weight was 250 ± 10 g and they were kept under automatically controlled conditions of temperature (21 ± 1 °C), humidity (55 ± 5%) and light (12 h light + 12 h dark). Both water (Milan water supply network) and food (Mucedola Srl, Settimo Milanese, MI, Italia) were freely accessible to animals.
All of the tests were performed in accordance with the Italian Government guidelines for laboratory animals and conformed with European Community Directives drawn up in November 1986 (86/609/EEC).
Seven days after their arrival, the rats were randomly divided in two different experimental groups of 16 animals (2 control rats + 14 treated rats), then starved for 24 hours before each treatment. Resveratrol was administered to the first group (20 mg/ml), whereas 3,5,4'-trioleoyl resveratrol was administered to the second group (90 mg/ml, corresponding to 20 mg/ml of resveratrol). Both resveratrol and 3,5,4'-trioleoyl resveratrol were suspended in carboxymethyl cellulose (0.5 w/v) and administered to the animals orally, by using a gastric probe. The rats were anesthetized (sodium thiopental, 50 mg/kg i.p.) at fixed times (0.5, 1, 2, 3, 4, 6, 24 hours) in order to take blood samples, that were subsequently used to assess the bioavailability of resveratrol and its ester. Blood was collected from vena cava by using heparinized syringes, then placed in plastic tubes and centrifuged at 2000 x g for 15 minutes at 4 °C, in order to allow the separation of plasma. The plasma thus obtained was stored at -80 °C until it was analyzed (less than one week). Subsequently, plasma was extracted by using a suitable solvent and analyzed by HPLC/DAD.

### 4.4.2.2- EXTRACTION METHOD DEVELOPMENT

Before analyzing plasma samples by HPLC, the extraction method was developed.
Two samples of plasma from control rats were added with solutions of resveratrol and 3,5,4'-trioleoyl resveratrol (at the same concentration), respectively. These samples were extracted using different procedures:
Method 1: 70 µl of each sample were added with 10 µl of caffeine solution in methanol, then the resulting solutions were stirred for 30 seconds. Subsequently, the samples were added with 100 µl of acetonitrile,

stirred for 1 minute, centrifuged for 10 minutes at 800 rpm and the supernatant dried under $N_2$; then the residues were re-dissolved in 50 µl of HPLC mobile phase (100% methanol).

Method 2: 70 µl of each sample were added with 1 ml of acetone, stirred for 30 seconds, centrifuged for 1 minute at 14000 rpm and the supernatant dried under $N_2$; then the residues were re-dissolved in 50 µl of HPLC mobile phase (100% methanol).

Method 3: 70 µl of each sample were added with 10 µl of caffeine solution in methanol, then the resulting solutions were stirred for 30 seconds. After, the samples were added with 100 µl of acetone, stirred for 1 minute, centrifuged for 10 minutes at 800 rpm and the supernatant dried under $N_2$; then the residues were re-dissolved in 50 µl of HPLC mobile phase (100% methanol).

The extracts obtained using each method were analyzed by HPLC (analysis condition previously described) and % recovery was calculated (sample area/standard area) in order to select the best extraction procedure. Resveratrol and 3,5,4'-trioleoyl resveratrol standard solutions were prepared at the same concentration of the solutions added in the samples.

Standard areas and sample areas along with their respective % recovery values are reported in Table 9.

| | Reveratrol area | Trioleoyl resveratrol area | Resveratrol recovery (%) | Trioleoyl resveratrol recovery (%) |
|---|---|---|---|---|
| Standard | 3230428 | 5102418 | - | - |
| Method 1 | 194453 | 11832 | 6.02 | 0.23 |
| Method 2 | 1545366 | 2910922 | 47.83 | 57.04 |
| Method 3 | 1080340 | 2530835 | 33.44 | 30.16 |

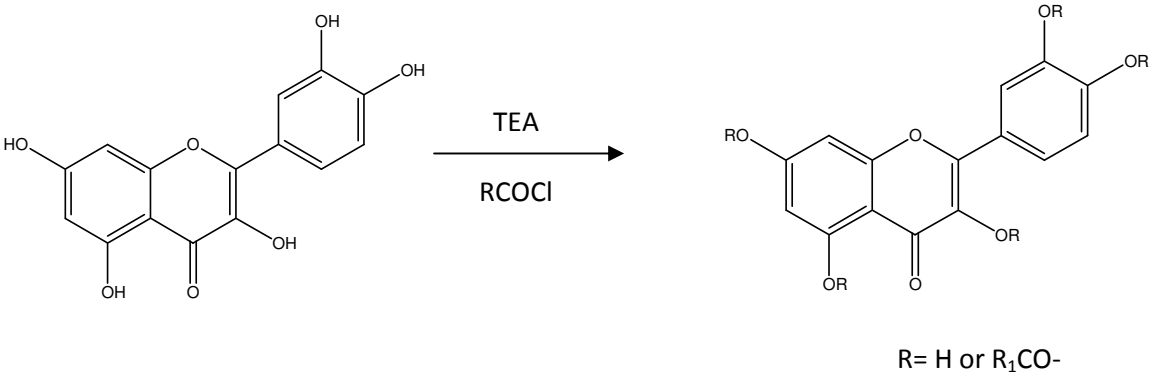
**Table 9.**

Method 2 resulted the most efficient. Hence, the rat plasma samples were extracted using this method and subsequently analyzed by HPLC in triplicate.

## 4.3 – QUERCETIN

### 4.3.1 - CHEMICAL SYNTHESIS OF QUERCETIN FATTY ESTERS

Quercetin esters were obtained both with saturated (palmitic and stearic acid) and unsaturated (oleic, linoleic, and linolenic acid) fatty acids.




**Scheme 5.**

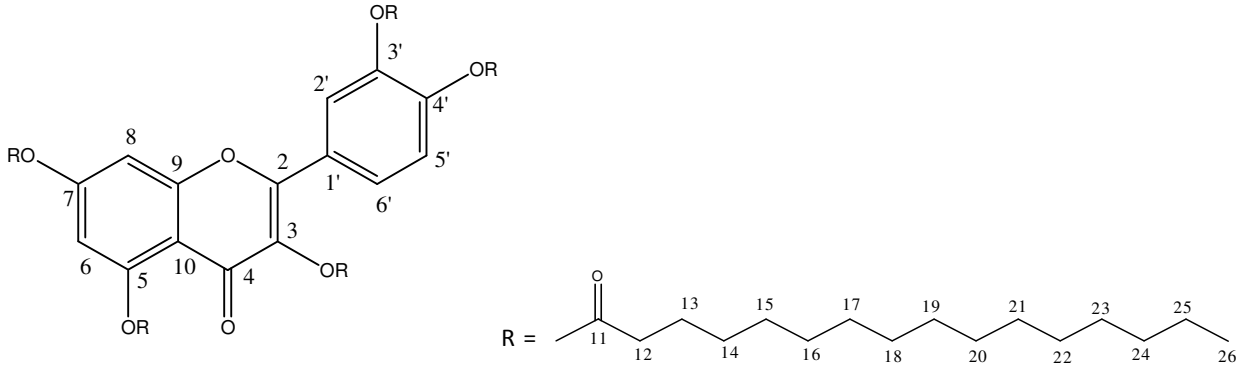
Under nitrogen flow, quercetin was dissolved in 10 ml of anhydrous dioxane and the solution was stirred at room temperature. Then TEA was added and, using an ice bath, the temperature was decreased until 0°C; afterwards the chloride was added dropwise. The reaction mixture thus obtained was stirred, repairing from daylight.

The mixture was evaporated under vacuum (107mbar, 40°C); the residue was dissolved in 20 ml of chloroform and washed with saturated $NaHCO_3$ water solution and then with water (3 x 20 ml). The organic layer was dried over anhydrous $Na_2SO_4$ and evaporate under vacuum.

When the acyl chloride was not commercially available, it was prepared following the procedure previously described at page 44.

All synthesized compounds were analyzed by FT-IR, $^{1}$H-NMR, $^{13}$C-NMR, UV and mass spectrometry. The instrumental results interpretation for all compounds is reported below; as an example, 3,5,7,3',4'-pentaoleoyl quercetin, 3,7,3',4'-tetraoleoyl quercetin and 3,7,(3'/4')-tri-oleoyl quercetin spectra are reported.

## 3,5,7,3',4'-PENTAPALMYTOIL QUERCETIN




**Reaction parameters:**

**Molar ratio palmytoil chloride:quercetin 1:7.5**

| | |
|---|---|
| Quercetin | $3.3x10^{-4}$ mol, 0.10 g, |
| Palmytoil chloride | $2.47x10^{-3}$ mol, 0.68 g, 0.75 ml |
| TEA | $2.47x10^{-3}$ mol, 0.25 g, 0.35 ml |
| Reaction temperature | Room temperature |
| Reaction time | 3 h |
| Purification | Crystallization with ethanol |
| Yield | 40% |

**$C_{95}H_{160}O_{12}$** MW = 1495,18

**TLC:** Hexane/Acetone 9/1. In order to evidence the presence of residual quercetin, ethyl acetate 100% was used.

**m/z:** 1496 $[M+H]^+$

**UV/Vis** in $CHCl_3$ λ (nm) = 256; 300

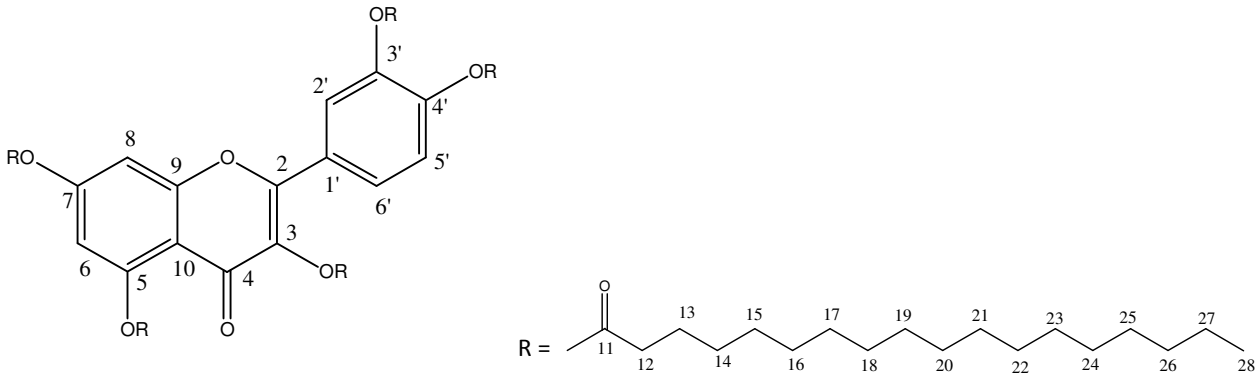
**$^1$H-NMR ($CDCl_3$):** δ = 7.70 (dd, $J_{6'-5'}$ = 8.1, $J_{6'-2'}$ = 2.0, 1 H, $H_{6'}$); 7.65 (d, $J_{2'-6'}$ = 2.0, 1 H, $H_{2'}$); 7.32 (d, $J_{5'-6'}$ = 8.1, 1 H, $H_{5'}$); 7.30 (d, $J_{6-8}$ = 2.5, 1 H, $H_8$); 6.84 (d, $J_{8-6}$ = 2.5, 1 H, $H_6$); 2.72 (t, J = 7.6, 2 H, $H_{12}$ in $C_5$); 2.63-2.52 (m, 8 H, $H_{12}$); 1.78-1.67 (m, 10 H, $H_{13}$), 1.52-1.26 (m, 120 H, $H_{14}$-$H_{25}$); 0.87 (t, J = 5.9, 15 H, $CH_3$).

**$^{13}$C-NMR ($CDCl_3$):** δ = 172.09 CO($C_5$) ; 170.92, 170.76, 170.73, 170.66 CO; 170.11 $C_4$; 157.11 $C_9$; 154.56 $C_7$; 153,79 $C_2$; 150.88 $C_5$, 144.74 $C_{4'}$; 142.56 $C_{3'}$; 134.39 $C_3$; 128.02 $C_{1'}$; 126.49 $C_{6'}$; 123.99 $C_{5'}$, $C_{2'}$; 115.08 $C_{10}$; 114,04 $C_6$, 108.95 $C_8$, 34.62, 34.33, 34.27, 34.01 $C_{12}$; 31.12 $C_{24}$; 29.88 -29.26 $C_{14}$-$C_{23}$, 25.12, 24.95, 24.87, 24.67 $C_{13}$; 22.86 $C_{25}$; 13.23 $CH_3$.

**FT-IR (KBr)**

| **1/λ ($cm^{-1}$)** | **Attribution** |
|---|---|
| 2956.78; 2917.82; 2850.03 | ν C $sp^3$- H |
| 1769.36 | ν C=O |
| 1641.86 | ν C=O α,β unsaturated |
| 1468.42; 1439.79 | δ C=C |
| 1268.31; 1242.04; 1208.39; 1189.18; 1114.42 | ν C-O |

## 3,5,7,3',4'-PENTASTEAROYL QUERCETIN




**Reaction parameters:**

**Molar ratio stearoyl chloride:quercetin 1:7.5**

| | |
|---|---|
| Quercetin | $3.3x10^{-4}$ mol, 0.10 g |
| Stearoyl chloride | $2.47x10^{-3}$ mol, 0.75 g, 0.84 ml |
| TEA | $2.47x10^{-3}$ mol, 0.25 g, 0.35 ml |
| Reaction temperature | Room temperature |
| Reaction time | 3 h |
| Purification | Crystallization with ethanol |
| Yield | 57% |

**$C_{105}H_{180}O_{12}$** MW = 1635,7

**TLC:** Hexane/Acetone 9/1. In order to evidence the presence of residual quercetin, ethyl acetate 100% was used.

**m/z:** 1636 $[M+H]^+$

**UV/Vis** in $CHCl_3$ λ (nm) = 257; 298

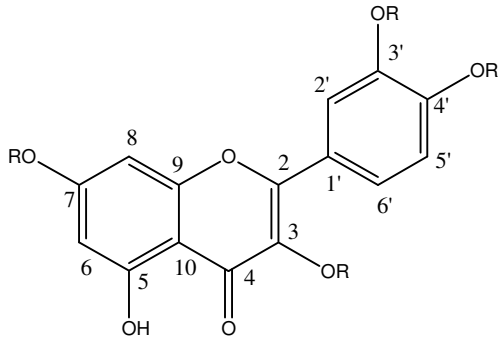
**$^1$H-NMR ($CDCl_3$):** δ = 7.70 (dd, $J_{6'-5'}$ = 8.3, $J_{6'-2'}$ = 1.8, 1 H, $H_{6'}$); 7.66 (d, $J_{2'-6'}$ = 1.8, 1 H, $H_{2'}$); 7.32 (d, $J_{5'-6'}$ = 8.3, 1 H, $H_{5'}$); 7.31 (d, $J_{6-8}$ = 2.2, 1 H, $H_8$); 6.83 (d, $J_{8-6}$ = 2.2, 1 H, $H_6$); 2.73 (t, J = 7.6, 2 H, $H_{12}(C_5)$); 2.62-2.52 (m, 8 H, $H_{12}$); 1.78-1.71 (m, 10 H, $H_{13}$), 1.55-1.26 (m, 100 H, $H_{14}$-$H_{27}$); 0.88 (t, J = 5.6, 15 H, $CH_3$).

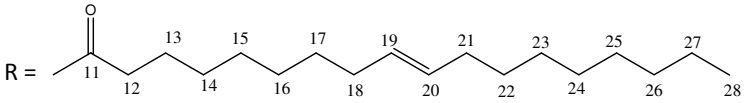
**$^{13}$C-NMR ($CDCl_3$):** δ = : 172.25 (CO)$C_5$ ; 171.04, 170.87 , 170.79 CO; 170.17 $C_4$; 157.09 $C_9$; 154.53 $C_7$; 153,80 $C_2$; 150.81 $C_5$, 144.71 $C_{4'}$; 142.52 $C_{3'}$; 134.43 $C_3$; 128.00 $C_{1'}$; 126.57 $C_{6'}$; 124.05 $C_{5'}$, $C_{2'}$; 115.04 $C_{10}$; 114,10 $C_6$, 109.07 $C_8$, 34.63, 34.35, 33.40, 34.27, 34.02 $C_{12}$; 32.16 $C_{26}$; 29.95-29.28 $C_{14}$- $C_{25}$; 25.13, 24.97, 24.88, 24.69 $C_{13}$; 22.91 $C_{27}$; 13.34 $CH_3$.

**FT-IR (KBr)**

| **1/λ ($cm^{-1}$)** | **Attribution** |
|---|---|
| 2956.62; 2918.33; 2850.34 | ν C $sp^3$- H |
| 1764.58 | ν C=O |
| 1654.61 | ν C=O α,β unsaturated |
| 1468.24; 1437.97 | δ C=C |
| 1266.03; 1233.10; 1208.25; 1178.50; 1112.17 | ν C-O |

## 3,7,3',4'-TETRASTEAROYL QUERCETIN




**Reaction parameters:**

**Molar ratio stearoyl chloride:quercetin 1:4**

| | |
|---|---|
| Quercetin | $3.3x10^{-4}$ mol, 0.10 g |
| Stearoyl chloride | $1.32x10^{-3}$ mol, 0.40 g, 0.45 ml |
| TEA | $2.47x10^{-3}$ mol, 0.13 g, 0.18 ml |
| Reaction temperature | 10 °C |
| Reaction time | 3 h |
| Yield | 53% |

**$C_{87}H_{146}O_{10}$** MW = 1369,18

**TLC:** Hexane/Acetone 9/1. In order to evidence the presence of residual quercetin, ethyl acetate 100% was used.

**m/z:** 1370 $[M+H]^+$

**UV/Vis** in $CHCl_3$ λ (nm) = 260; 300

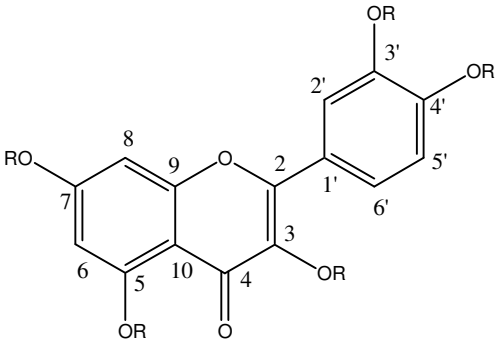
**$^{1}$H-NMR ($CDCl_3$):** δ =12.12 (s, 1 H, OH exch. $D_2O$), 7.72 (dd, $J_{2'-5'}$ =2.1, $J_{6'-5'}$ =8.4, 2 H, $H_{2'}$, $H_{6'}$), 7.35 (d, $J_{5'-6'}$=8.4, 1 H, $H_{5'}$), 6.84 (d, $J_{8-6}$ = 2.1, 1 H, $H_8$), 6.58 (d, $J_{6-8}$ = 2.1, 1 H, $H_6$), 2.67-2.52 (m, 8 H, $H_{12}$); 1.75-1.71 (m, 8 H, $H_{13}$); 1.26-1.05 (m, 112 H, $H_{14}$-$H_{27}$); 0.88 (t, J=6.1, 12 H, $CH_3$)

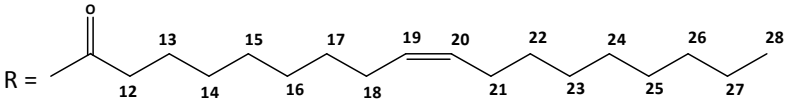
**$^{13}$C-NMR ($CDCl_3$):** δ = 176.52 $C_4$; 171.30, 170.86 , 170.76, 170.17 CO ; 161.96 $C_5$; 156.71 $C_7$; 156.18 $C_9$; 155.75 $C_2$; 145.02 $C_{4'}$; 142.60 $C_{3'}$; 132.48 $C_3$; 127.72 $C_{1'}$; 126.64 $C_{6'}$; 124.22 $C_{2'}$, $C_5$; 108.98 $C_{10}$; 105.69 $C_6$, 101.34 $C_8$; 34.63, 34.33, 34.27, 33.97 $C_{12}$; 32.14 $C_{26}$; 29.93-29.27 $C_{14}$-$C_{25}$; 26.54, 25.12, 24.89, 24.68 $C_{13}$; 22.90 $C_{27}$; 13.31 $CH_3$

**FT-IR (NaCl)**

| **1/λ ($cm^{-1}$)** | **Attribution** |
|---|---|
| 3436.32 | ν OH |
| 2956.72; 2918.51; 2850.40 | ν C $sp^3$- H |
| 1766.06 | ν C=O |
| 1654.93 | ν C=O α,β unsaturated |
| 1468.28; 1437.94 | δ C=C |
| 1266.39; 1233.69; 1195.61; 1178.02; 1144.98 | ν C-O |

## 3,5,7,3',4'-PENTAOLEOYL QUERCETIN




**Reaction parameters:**

**Molar ratio oleic acid:quercetin 1:7.5**

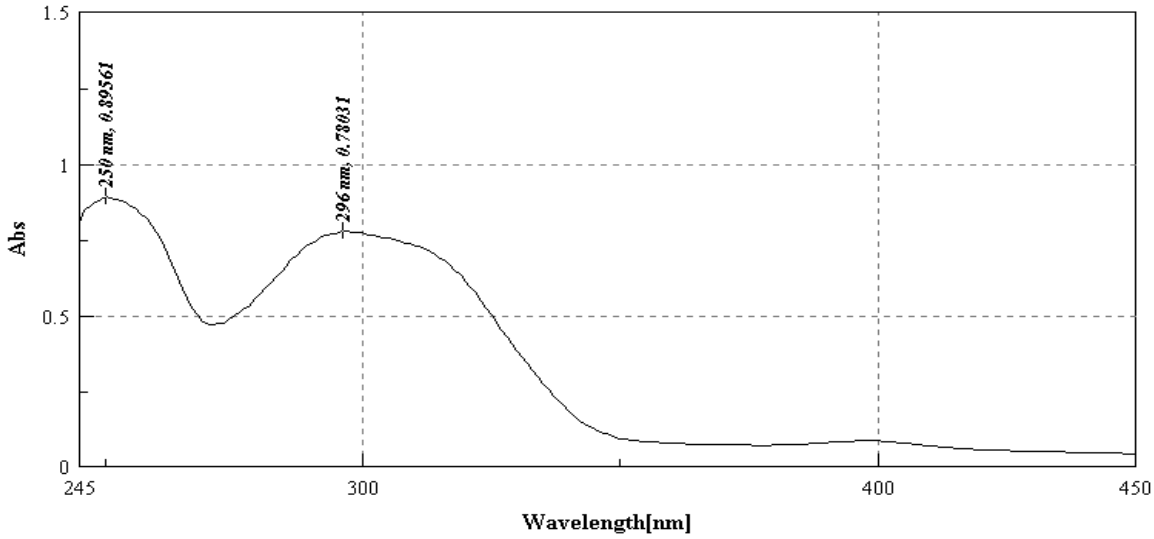
| | |
|---|---|
| Oleic acid | $2.47x10^{-3}$ mol, 0.70 g, 0.77 ml |
| Oxalyl chloride | 0.016 mol, 2.07 g, 1.31 ml |
| Quercetin | $3.3x10^{-4}$ mol, 0.10 g |
| TEA | $2.47x10^{-3}$ mol, 0.25 g, 0.35 ml |
| Reaction temperature | Room temperature |
| Reaction time | 20 h |
| Yield | 60% |

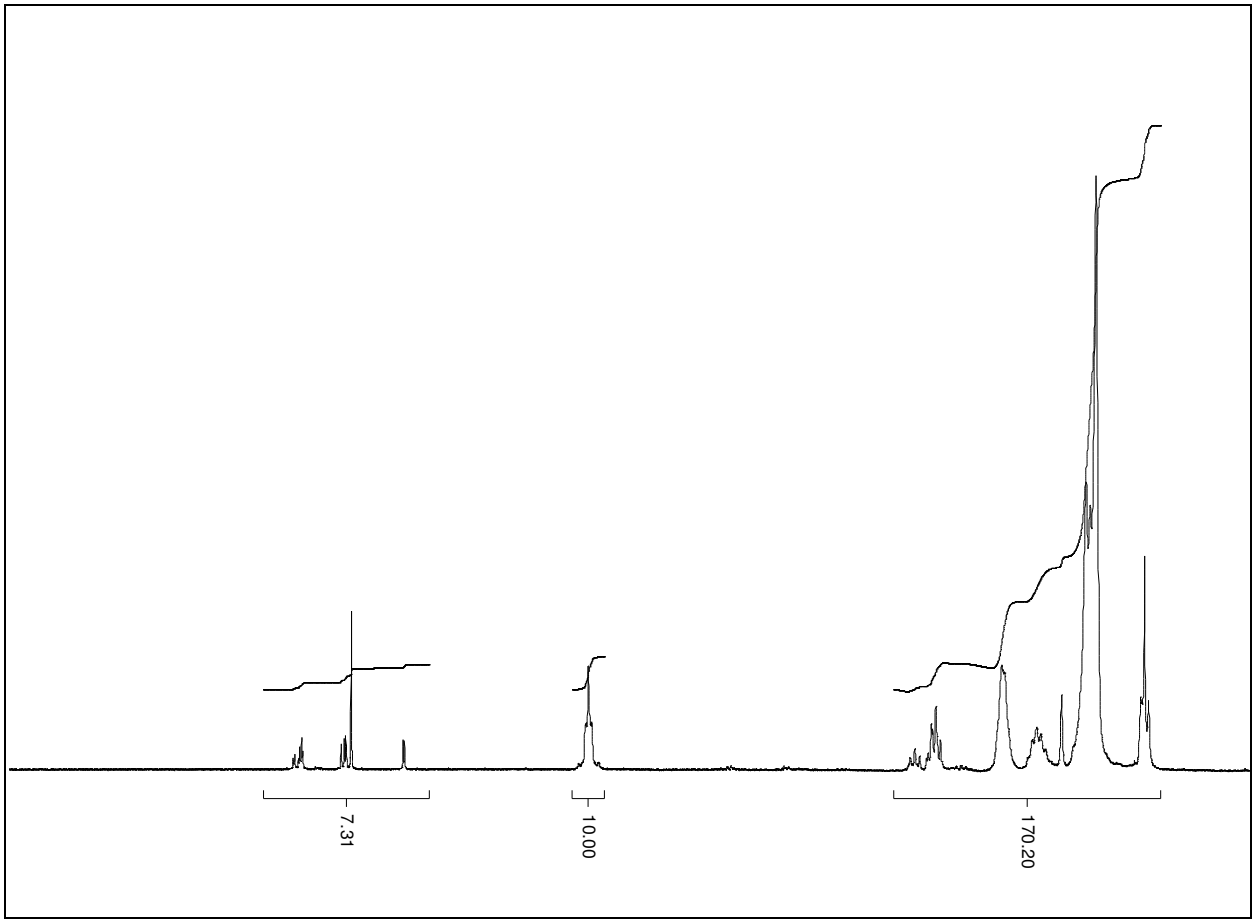
**$C_{105}H_{170}O_{12}$** MW = 1624,47

**TLC:** Hexane/Acetone 9/1. In order to evidence the presence of residual quercetin, ethyl acetate 100% was used.

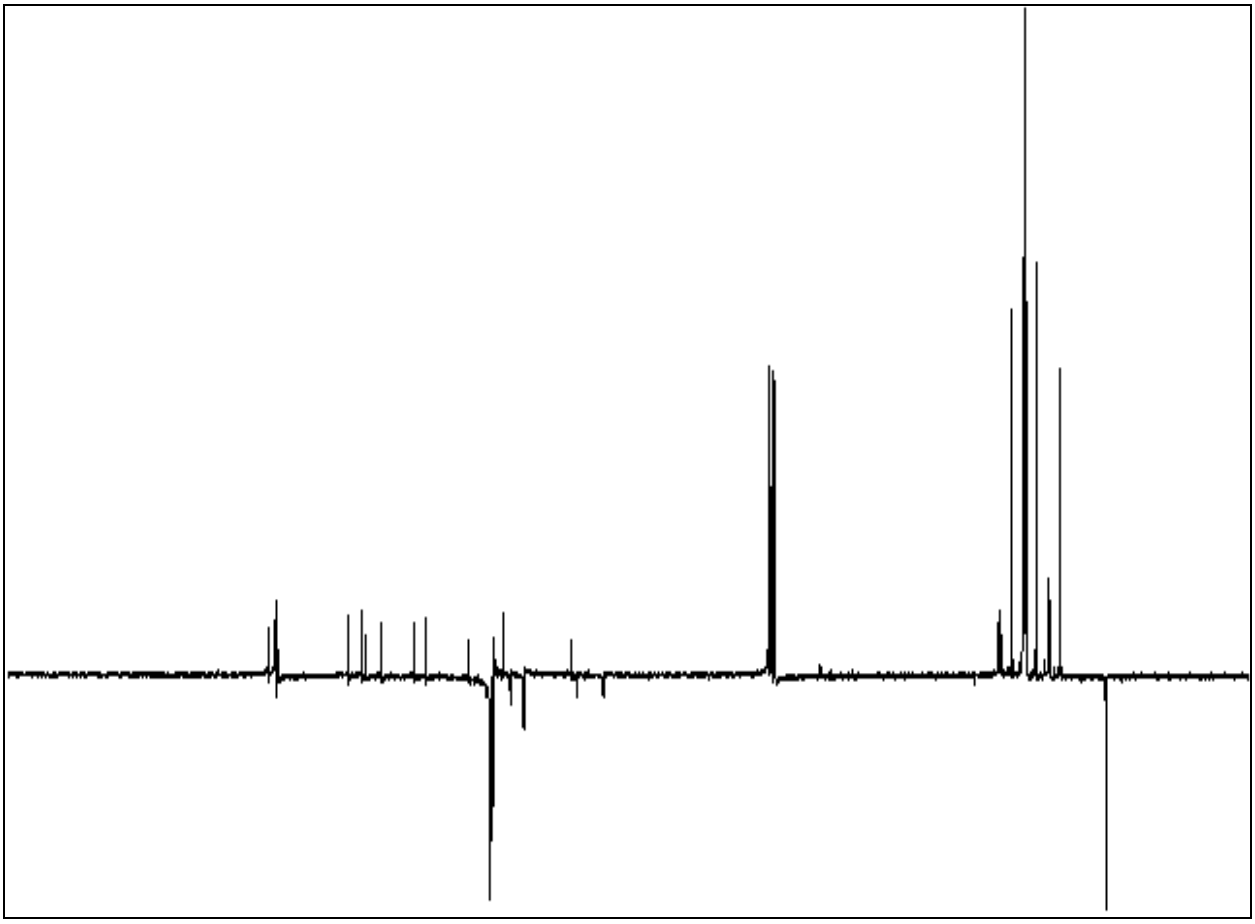
**m/z:** 1625 $[M+H]^+$

**UV/Vis** in $CHCl_3$ λ (nm) = 250; 296



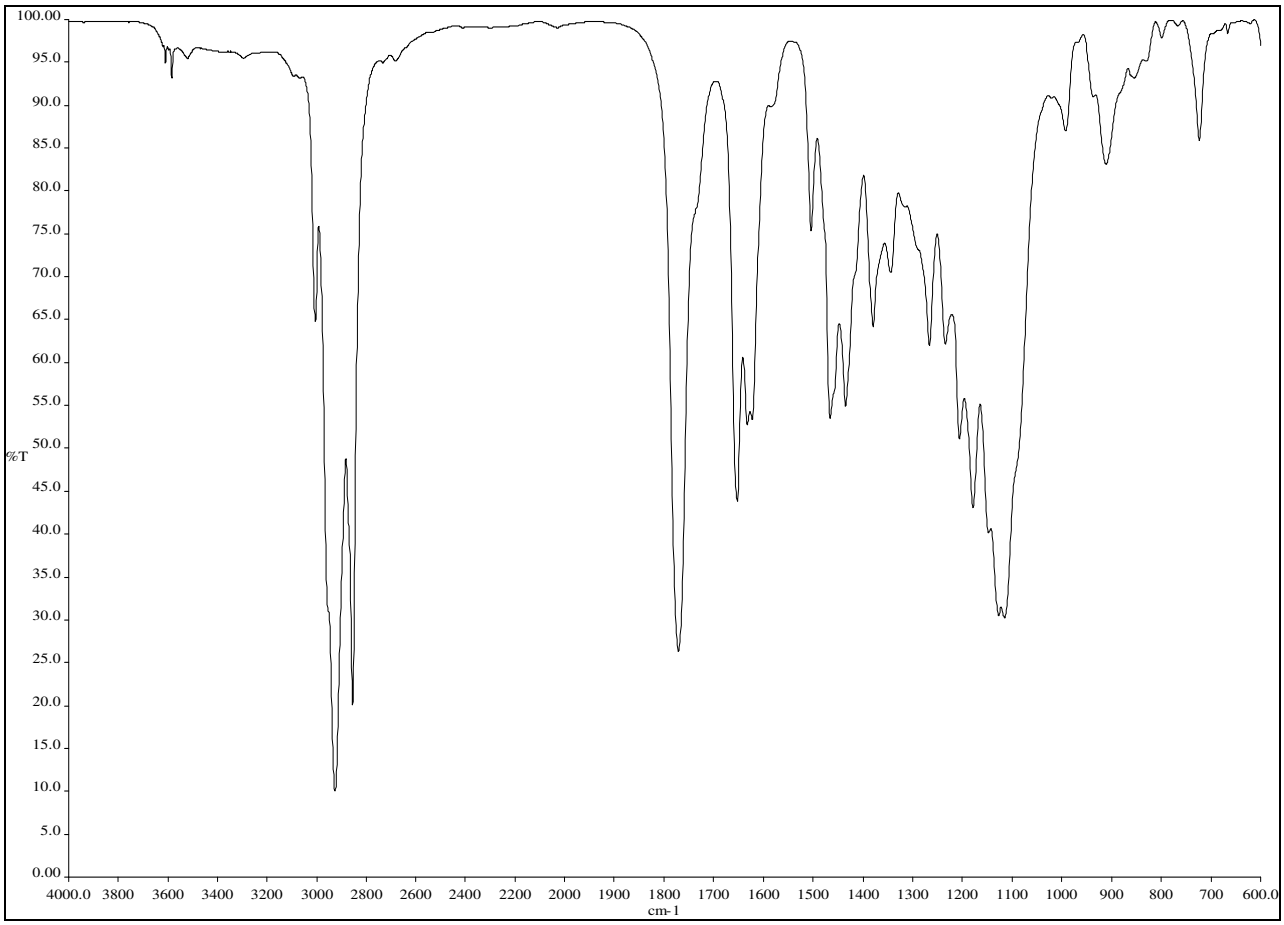



**$^1$H-NMR (500 MHz, $CDCl_3$):** δ = 7.73 (dd, $J_{6'-5'}$ = 8.0, $J_{6'-2'}$ = 2.0, 1 H, $H_{6'}$); 7.69 (d, $J_{2'-6'}$ = 2.0, 1 H, $H_{2'}$); 7.32 (d, $J_{5'-6'}$ = 8.0, 1 H, $H_{5'}$); 7.31 (d, $J_{6-8}$ = 2.2, 1 H, $H_8$); 6.86 (d, $J_{8-6}$ = 2.2, 1 H, $H_6$); 5.6-5.3 (m, 10 H, $H_{19}$-$H_{20}$); 2.75 (t, J = 7.6, 2 H, $H_{12}(C_5)$); 2.65-2.55 (m, 8 H, $H_{12}$); 2.05 (m, 20 H, $H_{18}$, $H_{21}$); 1.82-1.70 (m, 10 H, $H_{13}$), 1.48-1.20 (m, 100 H, $H_{14}$-$H_{17}$ and $H_{22}$-$H_{27}$); 0.90 (t, J = 6.6, 15 H, $CH_3$).



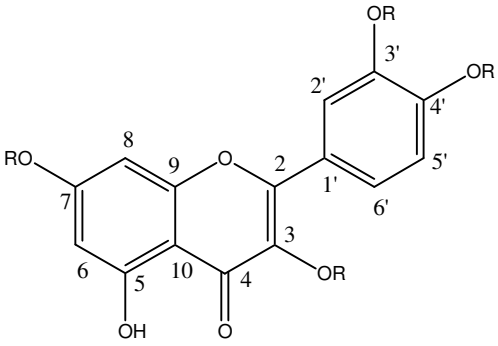
**$^{13}$C-NMR ($CDCl_3$):** δ = 171.27 CO($C_5$); 170.07, 169.91, 169.87, 169.83 CO; 169.25 $C_4$; 156.17 $C_9$; 153.61 $C_7$; 152,9 $C_2$; 149.9 $C_5$, 143.79 $C_{4'}$; 141.61 $C_{3'}$; 133.42 $C_3$; 129.38-128.92 $C_{19}$, $C_{20}$; 127.09 $C_{1'}$; 125.65 $C_{6'}$; 123.13 $C_{5'}$; 123.09 $C_{2'}$; 114.13 $C_{10}$; 113,17 $C_6$, 108.13 $C_8$, 33.69, 33.43, 33.40, 33.34, 33.07 $C_{12}$; 31.26 $C_{26}$; 29.08-28.32 $C_{14}$-$C_{17}$ and $C_{22}$-$C_{25}$; 26.54, 26.52, 26.48, 26.47 $C_{18}$, $C_{21}$; 24.20, 24.17, 24.01, 23.92, 23.37 $C_{13}$; 22.00, 21.98, 21.96 $C_{27}$; 13.40 $CH_3$.

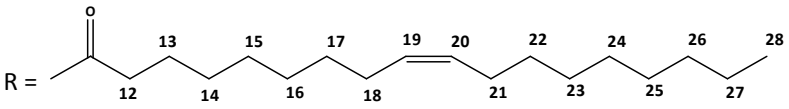
**FT-IR (NaCl)**




| **1/λ ($cm^{-1}$)** | **Attribution** |
|---|---|
| 3005.42; 2924.95; 2854.29 | ν C $sp^3$ - H |
| 1770.90 | ν C=O |
| 1652.88 | ν C=O α,β unsaturated |
| 1465.64; 1434.78 | δ C=C |
| 1266.04; 1234.19; 1205.04; 1178.27; 1114.74 | ν C-O |

## 3,7,3',4'-TETRAOLEOYL QUERCETIN




**Reaction parameters:**

**Molar ratio oleic acid:quercetin 1:5**

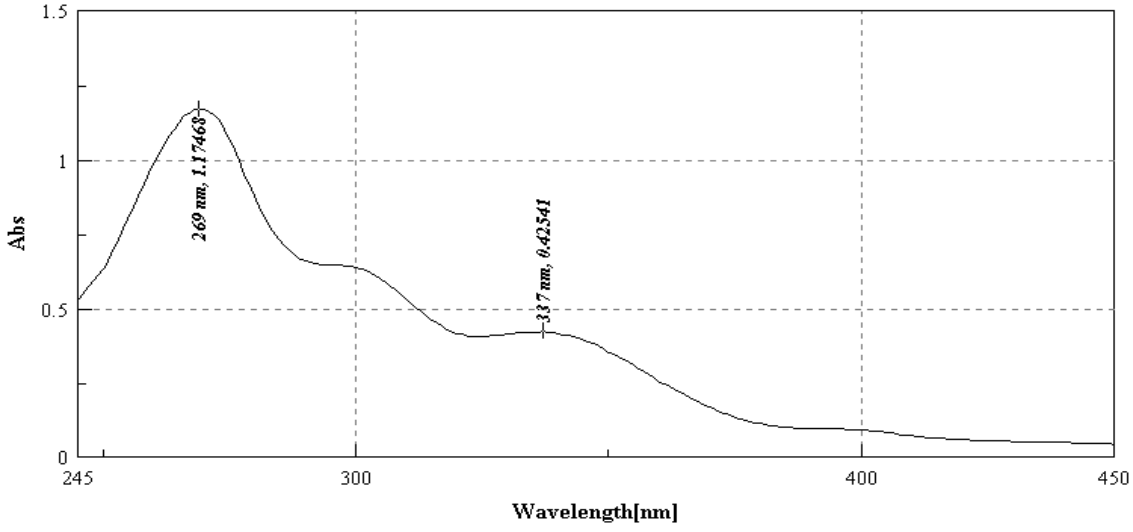
| | |
|---|---|
| Oleic acid | $1.65x10^{-3}$ mol, 0.47 g, 0.52 ml |
| Oxalyl chloride | 0.011 mol, 1.38 g, 0.88 ml |
| Quercetin | $3.3x10^{-4}$ mol, 0.10 g |
| TEA | $1.65x10^{-3}$ mol, 0.17 g, 0.23 ml |
| Reaction temperature | Room temperature |
| Reaction time | 20 h |
| Yield | 65% |

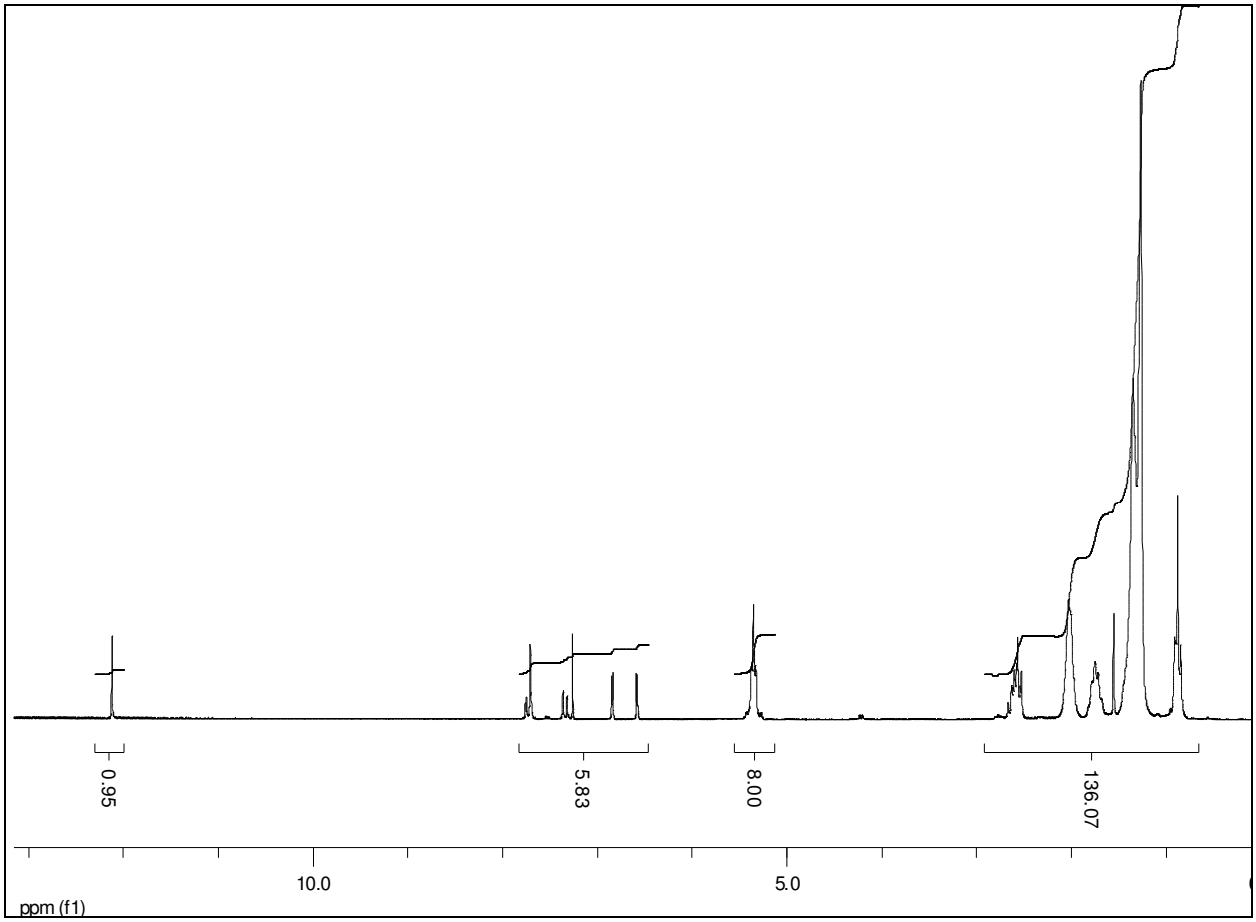
**$C_{87}H_{138}O_{11}$** MW = 1359,47

**TLC:** Hexane/Acetone 9/1. In order to evidence the presence of residual quercetin, ethyl acetate 100% was used.

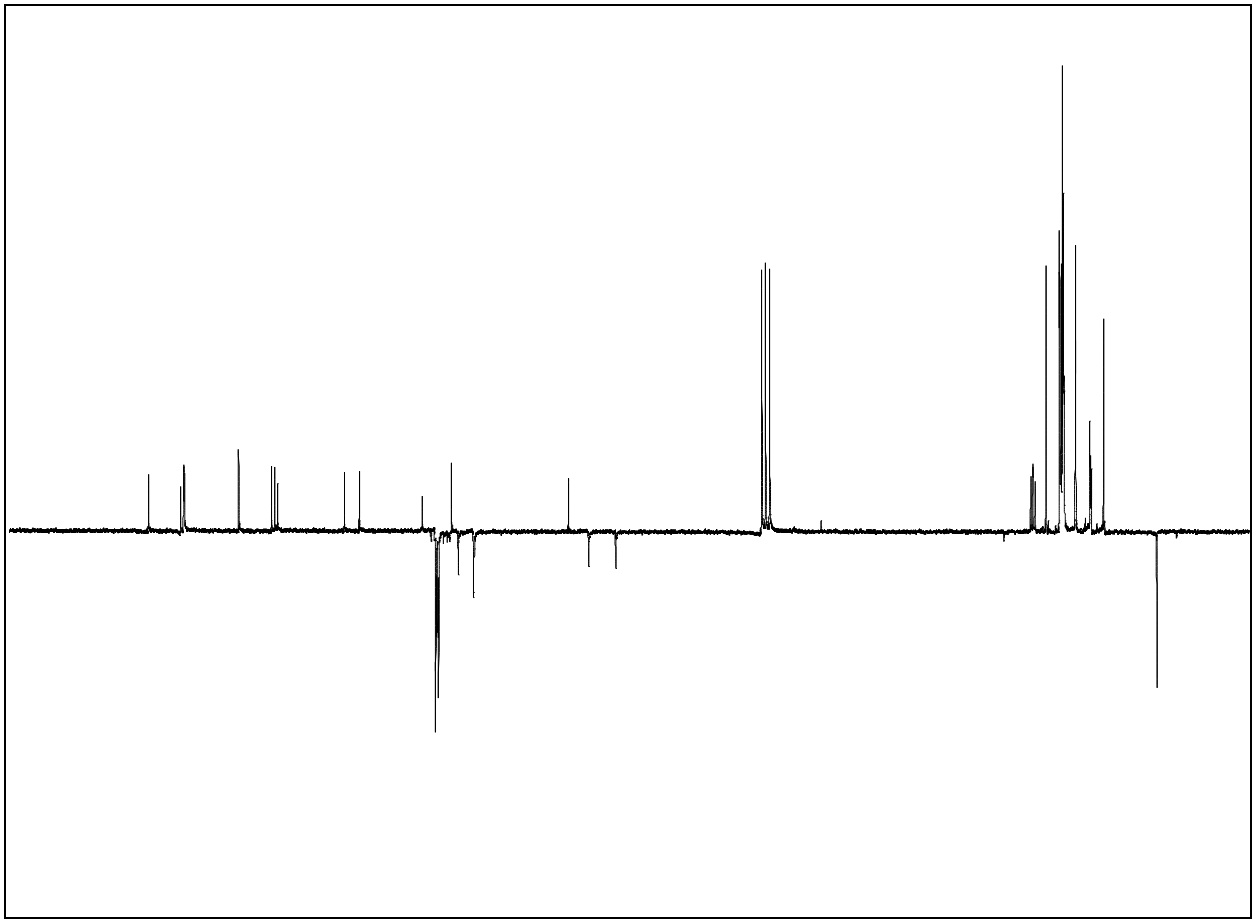
**m/z:** 1360 $[M+H]^+$

**UV/Vis** in $CHCl_3$ λ (nm) = 269; 337



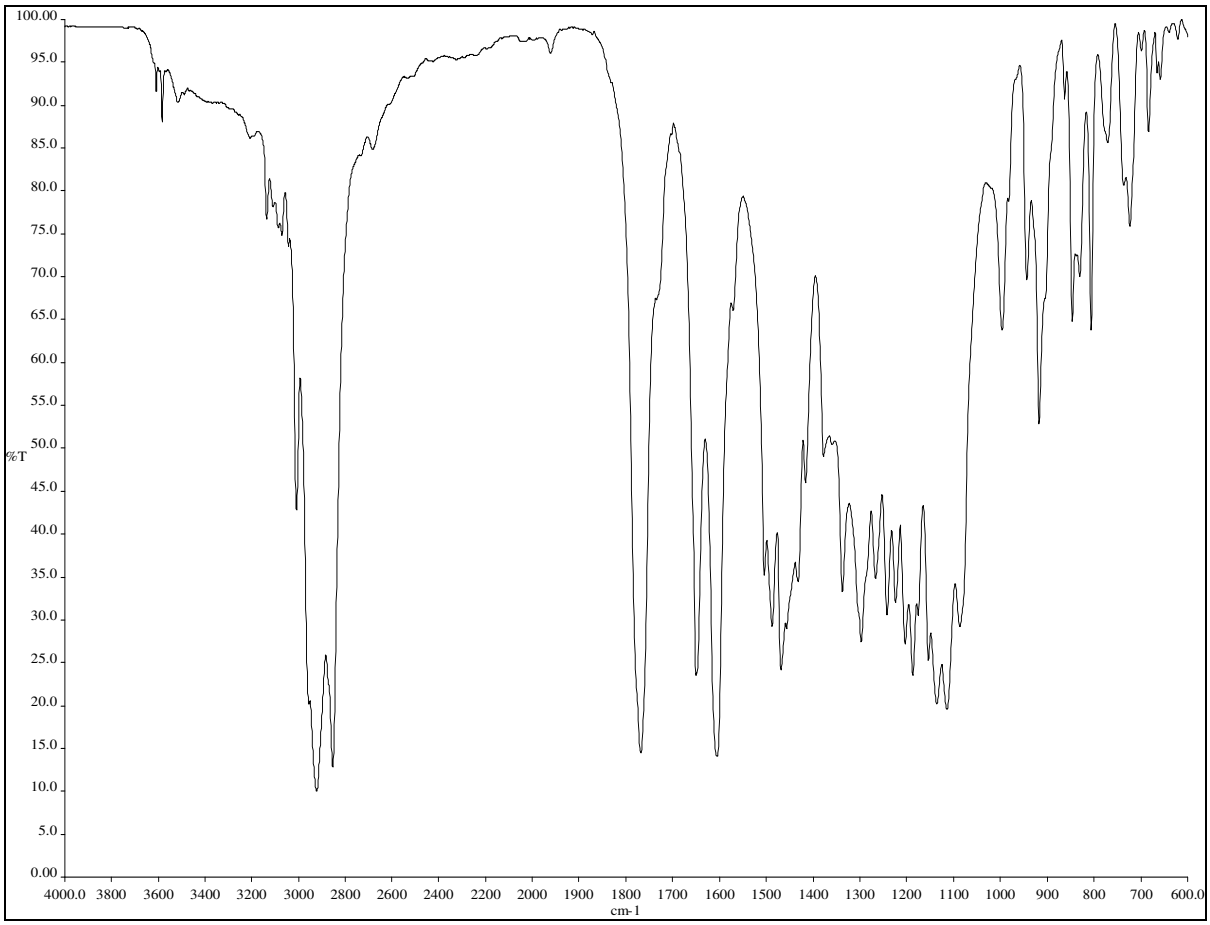



**$^1$H-NMR (500 MHz, $CDCl_3$):** δ =12.14 (s, 1 H, OH exch. $D_2O$), 7.75 (dd, $J_{2'-5'}$ =2.0, $J_{6'-5'}$ =8.5, 1 H, $H_{6'}$),7.73 (d, J= 2.0, 1 H, $H_{2'}$),7.37 (d, $J_{5'-6'}$=8.5, 1 H, $H_{5'}$), 6.87 (d, $J_{8-6}$ = 2.0, 1 H, $H_8$), 6.61 (d, $J_{6-8}$ = 2.0, 1 H, $H_6$), 5.41-5.35 (m, 8 H, $H_{19}$-$H_{20}$); 2.67-2.57 (m, 8 H, $H_{12}$); 2.05 (m, 16 H, $H_{18}$, $H_{21}$); 1.81-1.74 (m, 8 H, $H_{13}$); 1.44-1.29 (m, 80H, $H_{14}$-$H_{17}$ and $H_{22}$-$H_{27}$); 0.90 (t, J=6.6, 12 H, $CH_3$).



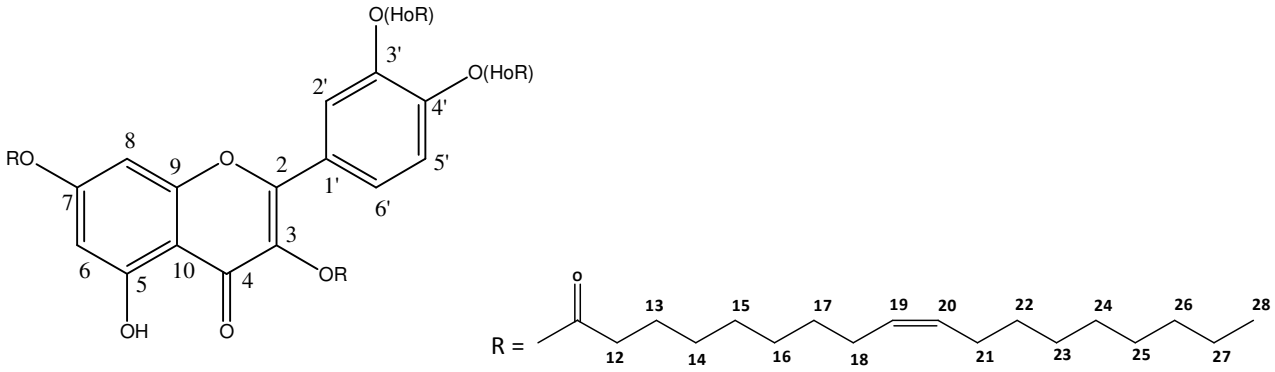
**$^{13}$C-NMR ($CDCl_3$):** δ =174.60 $C_4$; 170.34, 169.93, 169.89, 169,81 CO; 161.04 $C_5$; 155.78 $C_7$; 155.26 $C_9$; 154.83 $C_2$; 144.08 $C_{4'}$; 141.67 $C_{3'}$; 131.57 $C_3$; 129.40-128.90 $C_{19}$, $C_{20}$; 126.78 $C_{1'}$; 125.76 $C_{6'}$; 123.24 $C_{2'}$, $C_5$; 108.06 $C_{10}$; 104.77 $C_6$, 100.57 $C_8$; 33.71, 33.39, 33.34, 33.04 $C_{12}$; 31.26 $C_{26}$; 29.07-28.31 $C_{14}$-$C_{17}$ and $C_{22}$-$C_{25}$; 26.54, 26.52, 26.47 $C_{18}$, $C_{21}$; 24.19, 24.17, 24.07, 23.92, $C_{13}$; 21.98 $C_{27}$; 13.40 $CH_3$.

**FT-IR (NaCl)**




| **1/λ ($cm^{-1}$)** | **Attribution** |
|---|---|
| 3517.78 | ν OH |
| 3135.90; 3070.52; 3008.25 | ν C $sp^2$ - H |
| 2922.13; 2853.12 | ν C $sp^3$ - H |
| 1767.38 | ν C=O |
| 1650.65 | ν C=O α,β unsaturated |
| 1605.48; 1488.18; 1467.84 | δ C=C |
| 1296.70; 1223.94; 1202.91; 1186.96; 1113.52 | ν C-O |

## 3,7,(3'/4')-TRIOLEOYL QUERCETIN




**Reaction parameters:**

**Molar ratio oleic acid:quercetin 1:4**

| | |
|---|---|
| Oleic acid | $1.32x10^{-3}$ mol, 0.37 g, 0.41 ml |
| Oxalyl chloride | 8.87x10-3 mol, 1.12 g, 0.7 ml |
| Quercetin | $3.3x10^{-4}$ mol, 0.10 g |
| TEA | $1,32x10^{-3}$ mol, 0.13 g, 0.18 ml |
| Reaction temperature | Room temperature |
| Reaction time | 20 h |
| Yield | 14% |

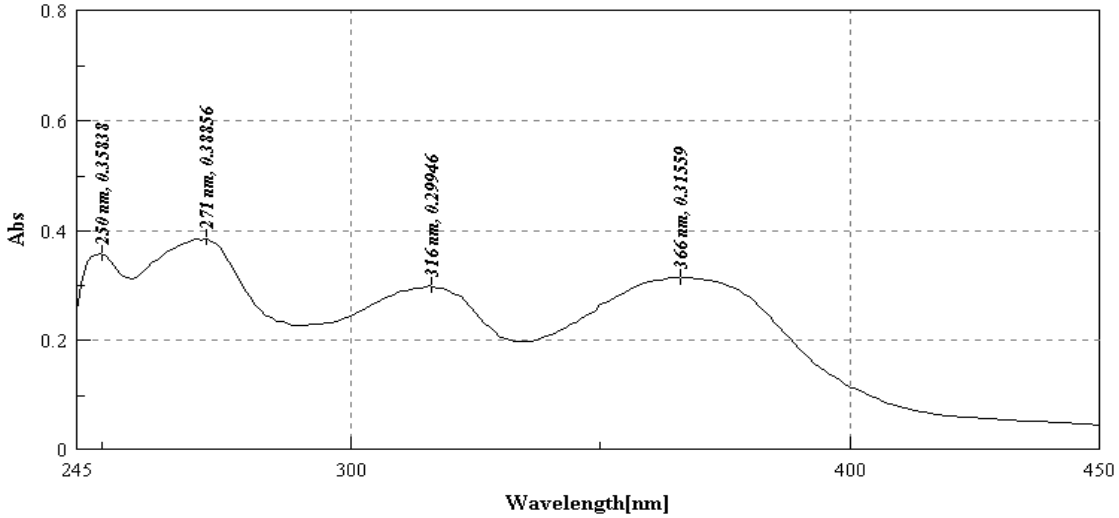
Using this molar ratio (1:4) both tri and tetraesters of quercetin were obtained. The two esters were then separated by chromatography, using a silica gel gravimetric columm (eluent hexane:acetone 9:1, ratio raw material/silica 1:60).

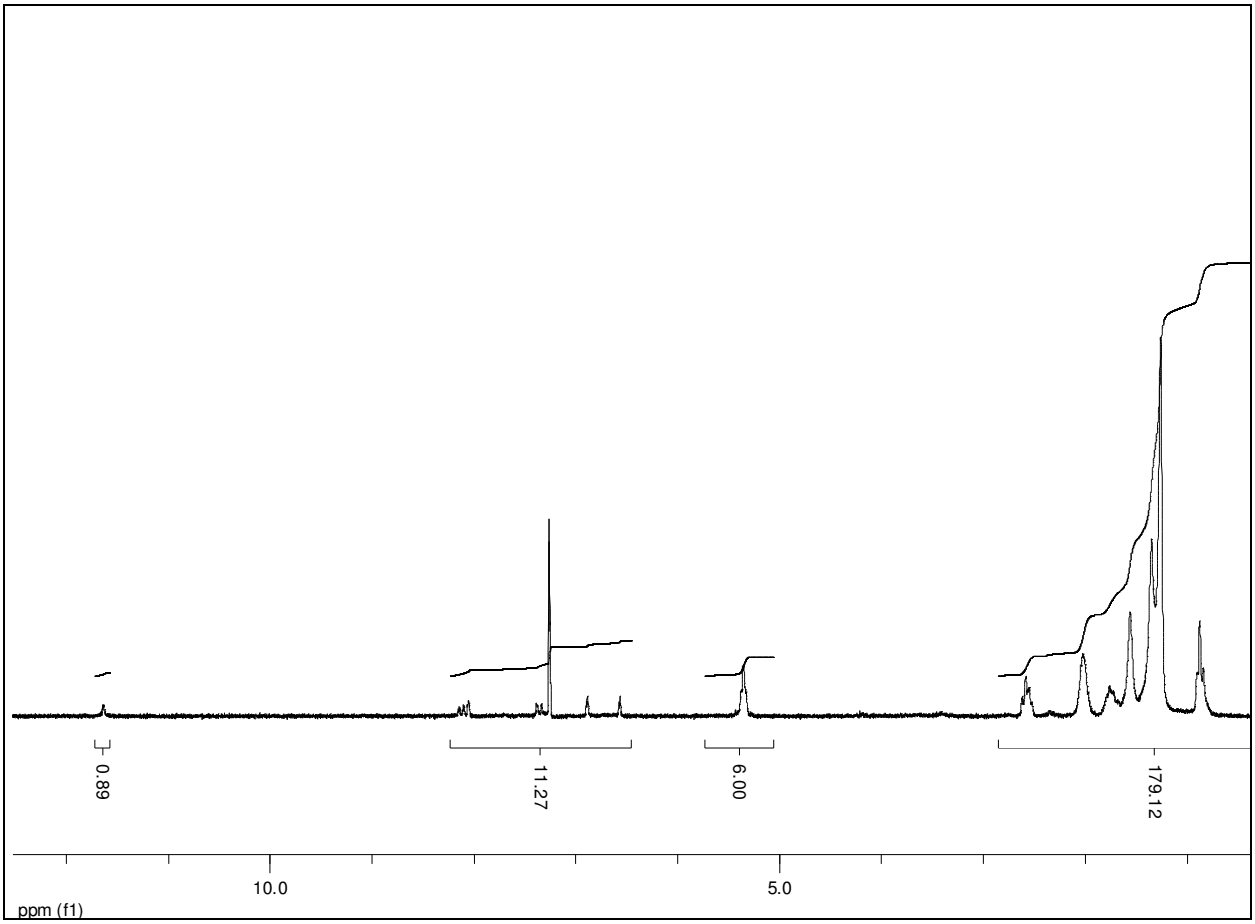
**$C_{69}H_{106}O_{10}$** MW = 1095,47

**TLC:** Hexane/Acetone 9/1. In order to evidence the presence of residual quercetin, ethyl acetate 100% was used.

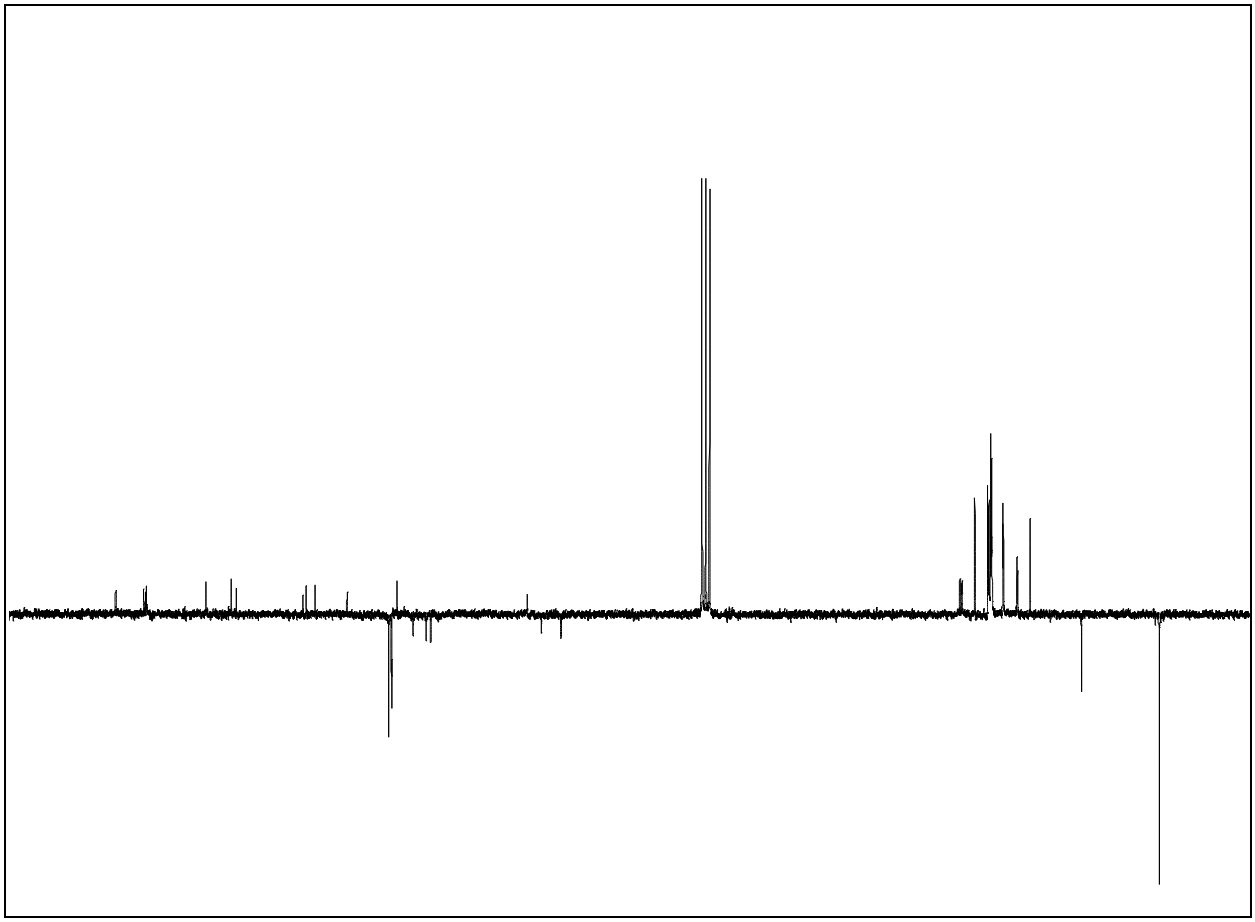
**m/z:** 1096 $[M+H]^+$

**UV/Vis** in $CHCl_3$ λ (nm) = 250; 271; 316; 366



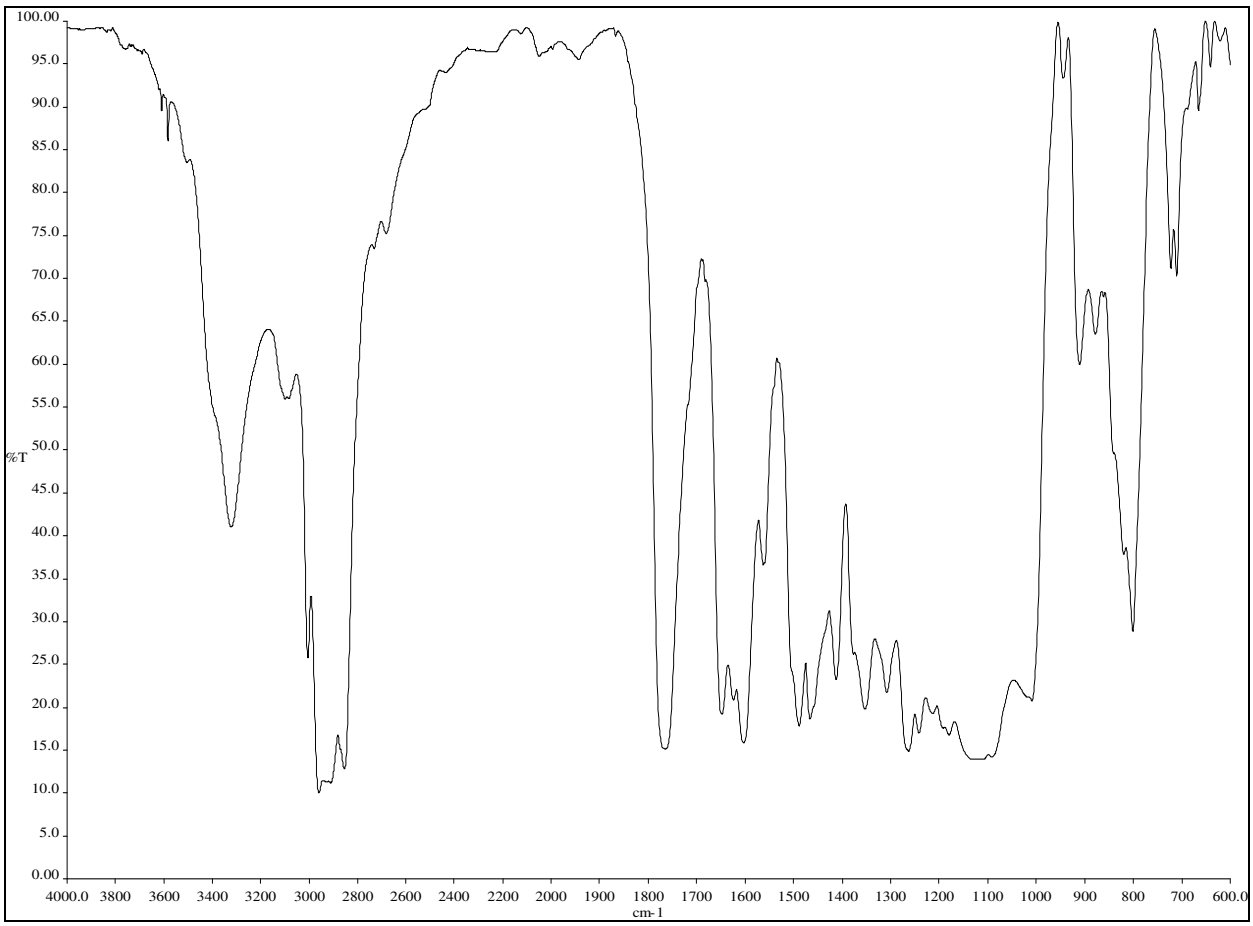



**$^{1}$H-NMR (500 MHz, $CDCl_3$):** δ = 11.61 (s, 1 H, OH exch. $D_2O$), 8.14 (dd, $J_{6'-5'}$=8.5, $J_{6'-2'}$ = 2.1, 1 H, $H_{6'}$), 8.08 (d, $J_{2'-6'}$ = 2.0, 1 H, $H_{2'}$), 7.38 (d, $J_{5'-6'}$ = 8.5, 1 H, $H_{5'}$), 6.90 (d, $J_{8-6}$ = 1.9, 1 H, H8), 6.8-6.7 (s, 1 H, OH exch. $D_2O$), 6.59 (d, $J_{6-8}$ = 2.0, 1H, $H_6$), 5.40-5.31 (m, 8 H, $H_{19}$-$H_{20}$); 2.06-2.57 (m, 6 H, $H_{12}$); 2.06-2.02 (m, 12 H, $H_{18}$, $H_{21}$); 1.82-1.78 (m, 6 H, $H_{13}$), 1.48-1.28 (m; 60 H, $H_{14}$-$H_{17}$ and $H_{22}$-$H_{27}$), 0.92 (t, J=5.9, 9 H, $CH_3$).



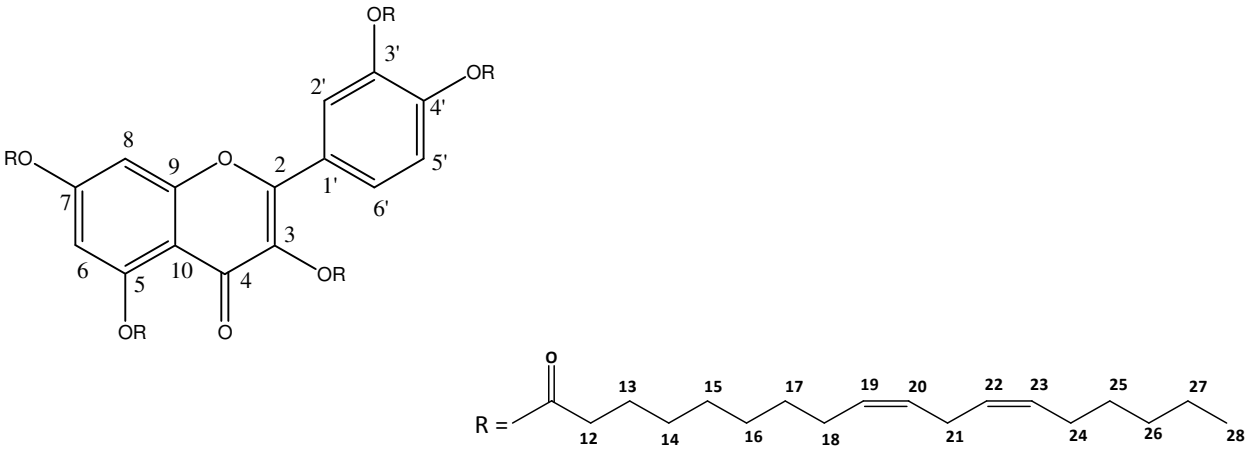
**$^{13}$C-NMR ($CDCl_3$):** δ = 175.15 $C_4$; 170.46, 170.22, 169.99 CO; 159.98 $C_5$; 155.82 $C_7$; 154.98 $C_9$; 143.79 $C_2$; 143.22 $C_{4'}$; 141.69 $C_{3'}$; 136.39 $C_3$;129.40-128.94 $C_{19}$, $C_{20}$; 128.09 $C_{1'}$; 125.32 $C_{6'}$; 123.11 $C_{5'}$; 122.39 $C_{2'}$; 106.27 $C_{10}$; 103.87 $C_6$; 100.57 $C_8$; 33.73, 33.43, 33.37 $C_{12}$; 31.21 $C_{26}$; 29.07-28.34 $C_{14}$-$C_{17}$ and $C_{22}$-$C_{25}$; 26.54, 26.48 $C_{18}$, $C_{21}$; 24.20, 24.09 $C_{13}$; 21.98 $C_{27}$; 13.40 $CH_3$.

**FT-IR (NaCl)**




| **1/λ ($cm^{-1}$)** | **Attribution** |
|---|---|
| 3321.56 | ν OH |
| 3099.91; 3005.01 | ν C $sp^2$ - H |
| 2959.22; 2854.40 | ν C $sp^3$ - H |
| 1765.07 | ν C=O |
| 1647.08 | ν C=O α,β unsaturated |
| 1602.99; 1562.62; 1488.98; 1465.92 | δ C=C |
| 1308.35; 1262.86; 1179.44; 1111.40 | ν C-O |

## 3,5,7,3',4'-PENTALINOLEOYL QUERCETIN




**Reaction parameters:**

**Molar ratio linoleic acid:quercetin 1:7.5**

| | |
|---|---|
| Linoleic acid | $2.47x10^{-3}$ mol, 0.69 g, 0.76 ml |
| Oxalyl chloride | 0.016 mol, 2.07 g, 1.31 ml |
| Quercetin | $3.3x10^{-4}$ mol, 0.10 g |
| TEA | $2.47x10^{-3}$ mol, 0.25 g, 0.35 ml |
| Reaction temperature | Room temperature |
| Reaction time | 20 h |
| Yield | 85% |

**$C_{105}H_{160}O_{12}$** MW = 1613,47

**TLC:** Hexane/Acetone 9/1. In order to evidence the presence of residual quercetin, ethyl acetate 100% was used.

**m/z:** 1614 $[M+H]^+$

**UV/**Vis in $CHCl_3$ λ (nm) = 281

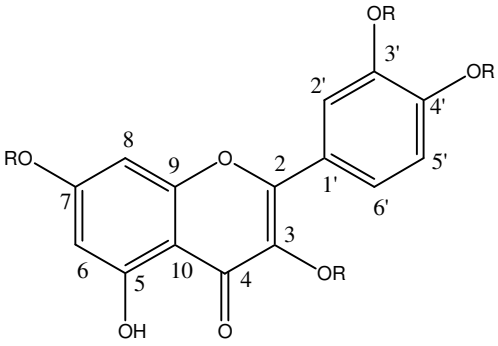
**$^{1}$H-NMR ($CDCl_3$):** δ = 7.70 (dd, $J_{6'-5'}$ = 8.2, $J_{6'-2'}$ = 1.8, 1 H, $H_{6'}$); 7.66 (d, $J_{2'-6'}$ = 1.8, 1 H, $H_{2'}$); 7.33 (d, $J_{5'-6'}$ = 8.2, 1 H, $H_{5'}$); 7.32 (d, $J_{6-8}$ = 2.4, 1 H, $H_8$); 6.84 (d, $J_{8-6}$ = 2.1, 1 H, $H_6$); 5.46-5.26 (m, 20 H, $H_{19}$,$H_{20}$,$H_{22}$, $H_{23}$); 2.77 (t, J = 5.5, 10 H, $H_{21}$ ); 2.69-2.41 (m, 10 H, $H_{12}$); 2.10 (m, 20 H, $H_{18}$, $H_{24}$); 1.78-1.71 (m, 10 H, $H_{13}$), 1.36-1.07 (m, 70 H, $H_{14}$-$H_{17}$ and $H_{25}$-$H_{27}$); 0.89 (t, J = 6.1, 15 H, $CH_3$).

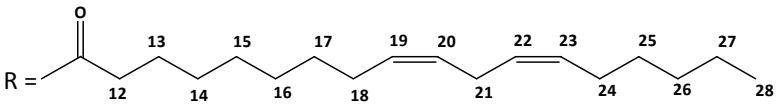
**$^{13}$C-NMR ($CDCl_3$):** δ = 172.19 CO($C_5$); 170.98, 170.79, 170.74, 170.18 CO; 169.79 $C_4$; 157.10 $C_9$; 154.53 $C_7$; 153,84 $C_2$; 150.81 $C_5$, 144.71 $C_{4'}$; 142.53 $C_{3'}$; 134.33 $C_3$; 130.47-128.08 $C_{19}$, $C_{20}$, $C_{22}$, $C_{23}$; 128.01 $C_{1'}$; 126.58 $C_{6'}$; 124.05 $C_{2'}$, $C_{5'}$; 115.05 $C_{10}$; 114.08 $C_6$, 109.05 $C_8$, 34.60, 34.31, 34.25, 34.08, 33.98 $C_{12}$; 31.74 $C_{26}$; 29.87-29.06 $C_{14}$-$C_{17}$ and $C_{25}$; 27.43 $C_{18}$, $C_{24}$; 25.87$C_{21}$; 25.11, 24.93, 24.83; 24.67, 24.43$C_{13}$; 22.80 $C_{27}$; 13.29 $CH_3$.

**FT-IR (NaCl)**

| **1/λ ($cm^{-1}$)** | **Attribution** |
|---|---|
| 3009.81; 2926.55; 2855.30 | ν C $sp^3$ - H |
| 1771.48 | ν C=O |
| 1653.51 | ν C=O α,β unsaturated |
| 1464.98; 1433.96 | δ C=C |
| 1265.09; 1233.19; 1203.86; 1177.03; 1115.73 | ν C-O |

## 3,7,3',4'-TETRALINOLEOYL QUERCETIN




**Reaction parameters:**

**Molar ratio linoleil acid:quercetin 1:5**

| | |
|---|---|
| Linoleic acid | $1.65x10^{-3}$ mol, 0.46 g, 0.51 ml |
| Oxalyl chloride | 0.011 mol, 1.38 g, 0.88 ml |
| Quercetin | $3.3x10^{-4}$ mol, 0.10 g |
| TEA | $1.65x10^{-3}$ mol, 0.17 g, 0.23 ml |
| Reaction temperature | Room temperature |
| Reaction time | 20 h |
| Yield | 56% |

**$C_{87}H_{140}O_{11}$** MW = 1351,47

**TLC:** Hexane/Acetone 9/1. In order to evidence the presence of residual quercetin, ethyl acetate 100% was used.

**m/z:** 1352 $[M+H]^+$

**UV/Vis** in $CHCl_3$ λ (nm) = 273; 339

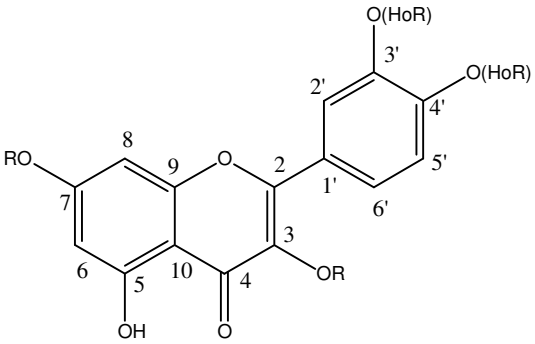
**$^{1}$H-NMR ($CDCl_3$):** δ = 12.12 (s, 1 H, OH exch. $D_2O$), 7.72 (dd, $J_{2'-5'}$ =2.1, $J_{6'-5'}$ =8.2, 2 H, $H_{2'}$ , $H_{6'}$), 7.34 (d, $J_{5'-6'}$=8.2, 1 H, $H_{5'}$), 6.84 (d, $J_{8-6}$ = 1.8, 1 H, $H_8$), 6.58 (d, $J_{6-8}$ = 1.8, 1 H, $H_6$), 5.45-5.29 (m, 16 H, $H_{19}$-$H_{20}$,$H_{22}$-$H_{23}$); 2.77 (t, J=5.5, 8 H, $H_{21}$,); 2.66-2.53 (m, 8 H, $H_{12}$); 2.00 (m, 16 H, $H_{18}$, $H_{24}$); 1.75-1.56 (m, 8 H, $H_{13}$); 1.29-1.25 (m, 56H, $H_{14}$-$H_{17}$ and $H_{25}$-$H_{27}$); 0.88 (t, J=6.8, 12 H, $CH_3$).

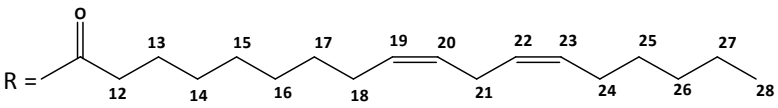
**$^{13}$C-NMR ($CDCl_3$):** δ = 176.52 $C_4$; 171.29 ,170.85, 170.81, 170.74 CO; 161.96 $C_5$; 156.70 $C_7$; 156.19 $C_9$; 155.74 $C_2$; 145.00 $C_{4'}$; 142.60 $C_{3'}$; 133.48 $C_3$; 130.48-128.08 $C_{19}$, $C_{20}$, $C_{22}$, $C_{23}$; 127.74 $C_{1'}$; 126.69 $C_{6'}$; 124.21 $C_{2'}$, $C_5$; 108.98 $C_{10}$; 105.70 $C_6$, 101.35 $C_8$; 34.63, 34.31, 34.26, 33.96 $C_{12}$; 31.75 $C_{26}$; 29.85-29.25 $C_{14}$-$C_{17}$ and $C_{25}$; 27.43 $C_{18}$, $C_{24}$;25.87 $C_{21}$; 25.11, 24.99, 24.89 $C_{13}$; 22.80 $C_{27}$; 14.30 $CH_3$.

**FT-IR (NaCl)**

| **1/λ ($cm^{-1}$)** | **Attribution** |
|---|---|
| 3009.59; 2927.35; 2855.56 | ν C $sp^3$ - H |
| 1772.46 | ν C=O |
| 1654.85 | ν C=O α,β unsaturated |
| 1465.42; 1433.99 | δ C=C |
| 1266.25; 1235.50; 1196.53; 1179.28; 1127.48 | ν C-O |

## 3,7,(3'/4')-TRILINOLEOYL QUERCETIN




**Reaction parameters:**

**Molar ratio linoleic acid:quercetin 1:4**

| | |
|---|---|
| Linoleic acid | $1.32x10^{-3}$ mol, 0.37 g, 0.41 ml |
| Oxalyl chloride | $8.87x10^{-3}$ mol, 1.12 g, 0.7 ml |
| Quercetin | $3.3x10^{-4}$ mol, 0.10 g |
| TEA | $1,32x10^{-3}$ mol, 0.13 g, 0.18 ml |
| Reaction temperature | Room temperature |
| Reaction time | 20 h |
| Yield | 16% |

Using this molar ratio (1:4) both tri and tetraesters of quercetin were obtained. The two esters were then separated by chromatography, using a silica gel gravimetric columm (eluent hexane:acetone 9:1, ratio raw material/silica 1:60).

**$C_{69}H_{100}O_{10}$** MW = 1089.32

**TLC:** Hexane/Acetone 9/1. In order to evidence the presence of residual quercetin, ethyl acetate 100% was used.

**m/z:** 1090 $[M+H]^+$

**UV/Vis** in $CHCl_3$ λ (nm) = 269; 311; 359

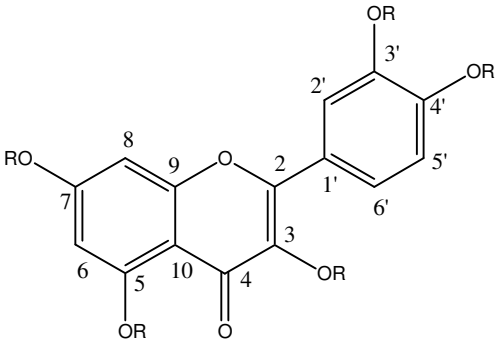
**$^{1}$H-NMR ($CDCl_3$):** δ = 11.64 (s, 1 H, OH exch. $D_2O$), 8.12 (dd, $J_{6'-5'}$=8.5, $J_{6'-2'}$ = 2.1, 1 H, $H_{6'}$), 8.05 (d, $J_{2'-6'}$ = 1.8, 1 H, $H_{2'}$), 7.36 (d, $J_{5'-6'}$ = 8.5, 1 H, $H_{5'}$), 6.88 (d, $J_{8-6}$ = 2.1, 1 H, $H_8$), 6.85-6.70 (s, 1 H, OH exch. $D_2O$), 6.57 (d, $J_{6-8}$ = 2.1, 1H, $H_6$), 5.45-5.26 (m, 12 H, $H_{19}$-$H_{20}$,$H_{22}$-$H_{23}$); 2.77 (t, J=5.5, 6 H, $H_{21}$); 2.66-2.53 (m, 6 H, $H_{12}$); 2.07-2.00 (m, 12 H, $H_{18}$, $H_{24}$); 1.75-1.59 (m, 6 H, $H_{13}$), 1.30-1.21 (m, 42H, $H_{14}$-$H_{17}$ and $H_{25}$-$H_{27}$), 0.88 (t, J=6.7, 9 H, $CH_3$).

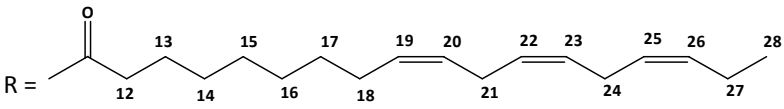
**$^{13}$C-NMR ($CDCl_3$):** δ = 176.52 $C_4$; 171.39, 171.15, 170.91 CO; 160.90 $C_5$; 156.71 $C_7$; 155.88 $C_9$; 144.71 $C_2$; 144.11 $C_{4'}$; 142.59 $C_{3'}$; 137.37 $C_3$; 130.47-128.10 $C_{19}$, $C_{20}$, $C_{22}$, $C_{23}$; 129.03 $C_{1'}$; 126.27 $C_{6'}$; 124.02 $C_5$; 123.29 $C_{2'}$; 107.20 $C_{10}$; 104.78 $C_6$, 101.47 $C_8$; 34.64, 34.34, 34.28 $C_{12}$; 31.75 $C_{26}$; 29.85-29.26 $C_{14}$-$C_{17}$ and $C_{25}$; 27.43 $C_{18}$,;25.87 $C_{21}$, $C_{24}$; 25.16, 25.00 $C_{13}$; 22.80 $C_{27}$; 14.30 $CH_3$.

**FT-IR (NaCl)**

| **1/λ ($cm^{-1}$)** | **Attribution** |
|---|---|
| 3351.56 | ν OH |
| 3009.50; 3005.01 | ν C $sp^2$ - H |
| 2927.46; 2855.61 | ν C $sp^3$ - H |
| 1771.37 | ν C=O |
| 1651.79 | ν C=O α,β unsaturated |
| 1605.85; 1562.62; 1492.00; 1465.84 | δ C=C |
| 1307.00; 1268.23; 1194.05; 1133.49 | ν C-O |

## 3,5,7,3',4'-PENTALINOLENOYL QUERCETIN




**Reaction parameters:**

**Molar ratio linolenil acid:quercetin 1:7.5**

| | |
|---|---|
| Linolenil acid | $2.47x10^{-3}$ mol, 0.69 g, 0.75 ml |
| Oxalyl chloride | 0.016 mol, 2.07 g, 1.31 ml |
| Quercetin | $3.3x10^{-4}$ mol, 0.10 g |
| TEA | $2.47x10^{-3}$ mol, 0.25 g, 0.35 ml |
| Reaction temperature | Room temperature |
| Reaction time | 20 h |
| Yield | 74% |

**$C_{105}H_{150}O_{12}$** MW = 1587.18

**TLC:** Hexane/Acetone 9/1. In order to evidence the presence of residual quercetin, ethyl acetate 100% was used.

**m/z:** 1588 $[M+H]^+$

**UV/Vis** in $CHCl_3$ λ (nm) = 259; 284

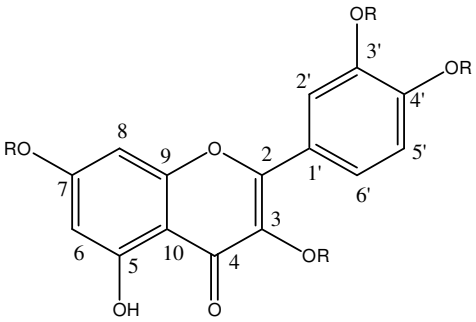
**$^{1}$H-NMR ($CDCl_3$):** δ = 7.70 (dd, $J_{6'-5'}$ = 8.5, $J_{6'-2'}$ = 2.1, 1 H, $H_{6'}$); 7.65 (d, $J_{2'-6'}$ = 1.8, 1 H, $H_{2'}$); 7.32 (d, $J_{5'-6'}$ = 8.5, 1 H, $H_{5'}$); 7.31 (d, $J_{6-8}$ = 2.1, 1 H, $H_8$); 6.84 (d, $J_{8-6}$ = 2.1, 1 H, $H_6$); 5.58-5.24(m, 30 H, $H_{19}$-$H_{20}$,$H_{22}$-$H_{23}$, $H_{25}$-$H_{26}$); 2.84 (t, J=5.8, 20 H, $H_{21}$, $H_{24}$); 2.65-2.55 (m, 10 H, $H_{12}$); 2.26-2.00 (m, 20 H, $H_{18}$, $H_{27}$,); 1.77-1.59 (m, 10 H, $H_{13}$,), 1.36-1.21 (m, 40 H, $H_{14}$-$H_{17}$,); 0.94 (t, J = 7.6, 15 H, $CH_3$).

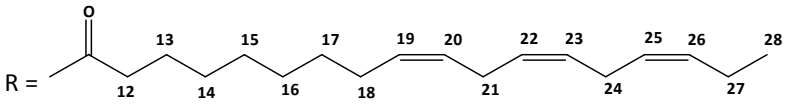
**$^{13}$C-NMR ($CDCl_3$):** δ = 172.20 CO($C_5$); 170.98, 170.83, 170.74 CO; 169.79 $C_4$; 157.09 $C_9$; 154.53 $C_7$; 153.85 $C_2$; 150.80 $C_5$; 144.70 $C_{4'}$; 142.53 $C_{3'}$; 134.33 $C_3$; 133.18-127.93 $C_{19}$, $C_{20}$, $C_{22}$, $C_{23}$, $C_{25}$, $C_{26}$; 127.33 $C_{1'}$; 126.58 $C_{6'}$; 124.05 $C_{2'}$, $C_{5'}$; 115.05 $C_{10}$; 114.10 $C_6$, 109.07 $C_8$; 35.49, 34.60, 34.30, 34.25, 33.99 $C_{12}$; 29.84-29.05 $C_{14}$-$C_{17}$; 27.42 $C_{18}$; 25.75 $C_{24}$; 25.85 $C_{21}$; 25.10, 24.91, 24.82, 24.67, 24.43 $C_{13}$; 22.79, 20.77 $C_{27}$; 14.48, 14.28 $CH_3$.

**FT-IR (NaCl)**

| **1/λ ($cm^{-1}$)** | **Attribution** |
|---|---|
| 3011.02; 2928.57; 2855.35 | ν C $sp^3$ - H |
| 1772.17 | ν C=O |
| 1654.88 | ν C=O α,β unsaturated |
| 1463.34; 1433.80 | δ C=C |
| 1265.47; 1232.57; 1203.31; 1177.68; 1123.19 | ν C-O |

## 3,7,3',4'-TETRALINOLENOYL QUERCETIN




**Reaction parameters:**

**Molar ratio linolenil acid:quercetin 1:5**

| | |
|---|---|
| Linolenil acid | $1.65x10^{-3}$ mol, 0.46 g, 0.50 ml |
| Oxalyl chloride | 0.011 mol, 1.38 g, 0.88 ml |
| Quercetin | $3.3x10^{-4}$ mol, 0.10 g |
| TEA | $1.65x10^{-3}$ mol, 0.17 g, 0.23 ml |
| Reaction temperature | Room temperature |
| Reaction time | 20 h |
| Yield | 48% |

**$C_{87}H_{122}O_{11}$** MW = 1344.75

**TLC:** Hexane/Acetone 9/1. In order to evidence the presence of residual quercetin, ethyl acetate 100% was used.

**m/z:** 1588 $[M+H]^+$

**UV/Vis** in $CHCl_3$ λ (nm) = 269; 301; 340

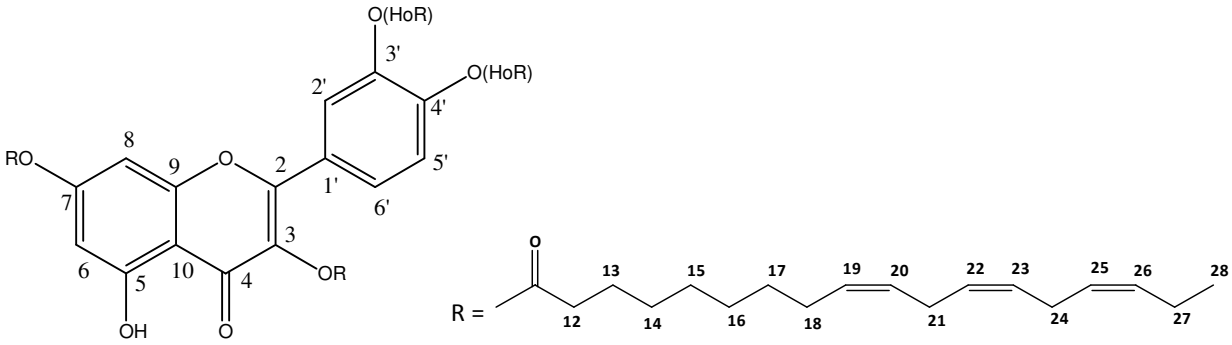
**$^{1}$H-NMR ($CDCl_3$):** δ =12.12 (s, 1 H, OH exch. $D_2O$), 7.73 (dd, $J_{2'-5'}$ =2.1, $J_{6'-5'}$ =8.2, 2 H, $H_{2'}$, $H_{6'}$), 7.34 (d, $J_{5'-6'}$=8.2, 1 H, $H_{5'}$), 6.84 (d, $J_{8-6}$ = 2.1, 1 H, $H_8$), 6.58 (d, $J_{6-8}$ = 2.1, 1 H, $H_6$), 5.46-5.24 (m, 24 H, $H_{19}$-$H_{20}$,$H_{22}$-$H_{23}$,$H_{25}$-$H_{26}$); 2.81 (t, J=5.5, 16H, $H_{21}$, $H_{24}$); 2.66-2.52 (m, 8 H, $H_{12}$); 2.15-2.00 (m, 16 H, $H_{18}$, $H_{27}$); 1.78-1.56 (m, 8 H, $H_{13}$); 1.27-1.21 (m, 32 H, $H_{14}$-$H_{17}$); 0.94 (t, J=7.6, 12 H, $CH_3$).

**$^{13}$C-NMR ($CDCl_3$):** δ = :176.52 $C_4$; 171.29, 170.85, 170.81, 170.73 CO; 161.96 $C_5$; 156.70 $C_7$; 156.19 $C_9$; 155.74 $C_2$; 145.00 $C_{4'}$; 142.60 $C_{3'}$; 132.48 $C_3$; 132.19-127.75 $C_{19}$, $C_{20}$, $C_{22}$, $C_{23}$, $C_{25}$, $C_{26}$; 127.74 $C_{1'}$; 126.69 $C_{6'}$; 124.21 $C_{2'}$, $C_{5'}$; 108.98 $C_{10}$; 105.70 $C_6$, 101.36 $C_8$; 34.62, 34.30, 34.26, 33.95 $C_{12}$; 29.83-29.24 $C_{14}$-$C_{17}$; 27.43 $C_{18}$; 25.76 $C_{24}$; 25.86 $C_{21}$; 25.08, 24.98, 24.89 $C_{13}$; 22.80, 20.78 $C_{27}$; 14.50, 14.30 $CH_3$.

**FT-IR (NaCl)**

| **1/λ ($cm^{-1}$)** | **Attribution** |
|---|---|
| 3011.23; 2928.63; 2855.47 | ν C $sp^3$ - H |
| 1771.61 | ν C=O |
| 1655.42 | ν C=O α,β unsaturated |
| 1487.78; 1455.55 | δ C=C |
| 1266.37; 1235.64; 1196.68; 1179.25; 1126.98 | ν C-O |

## 3,7,(3'/4')-TRILINOLENOYL QUERCETIN




**Reaction parameters:**

**Molar ratio linolenic acid:quercetin 1:4**

| | |
|---|---|
| Linolenic acid | $1.32x10^{-3}$ mol, 0.37 g, 0.41 ml |
| Oxalyl chloride | $8.87x10^{-}3$ mol, 1.12 g, 0.7 ml |
| Quercetin | $3.3x10^{-4}$ mol, 0.10 g |
| TEA | $1,32x10^{-3}$ mol, 0.13 g, 0.18 ml |
| Reaction temperature | Room temperature |
| Reaction time | 20 h |
| Yield | 15% |

Using this molar ratio (1:4) both tri and tetraesters of quercetin were obtained. The two esters were then separated by chromatography, using a silica gel gravimetric columm (eluent hexane:acetone 9:1, ratio raw material/silica 1:60).

**$C_{69}H_{94}O_{10}$** MW = 1084.32

**TLC:** Hexane/Acetone 9/1. In order to evidence the presence of residual quercetin, ethyl acetate 100% was used.

**m/z:** 1085 $[M+H]^+$

**UV/Vis** in $CHCl_3$ λ (nm) = 270; 313; 362

**$^{1}$H-NMR ($CDCl_3$):** δ = 11.64 (s, 1 H, OH exch. $D_2O$ ), 8.12 (dd, $J_{6'-5'}$=8.5, $J_{6'-2'}$ = 1.8, 1 H, $H_{6'}$), 8.05 (d, $J_{2'-6'}$ = 2.1, 1 H, $H_{2'}$), 7.36 (d, $J_{5'-6'}$ = 8.5, 1 H, $H_{5'}$), 6.89 (d, $J_{8-6}$ = 1.8, 1 H, $H_8$), 6.84-6.69 (s, 1 H, OH exch. $D_2O$), 6.57 (d, $J_{6-8}$ = 1.8, 1H, $H_6$), 5.46-5.31 (m, 18 H, $H_{19}$-$H_{20}$,$H_{22}$-$H_{23}$,$H_{25}$-$H_{26}$); 2.81 (t, J=5.5, 12 H, $H_{21}$,$H_{24}$); 2.62-2.53 (m, 6 H, $H_{12}$); 2.15-2.01 (m, 12 H, $H_{18}$, $H_{27}$); 1.76-1.56 (m, 6 H, $H_{13}$), 1.26-1.17 (m, 24H, $H_{14}$-$H_{17}$), 0.98 (t, J=7.6, 9 H, $CH_3$).

**$^{13}$C-NMR ($CDCl_3$):** δ = 176.08 $C_4$; 171.37, 171.14, 170.90 CO; 160.90 $C_5$; 156.73 $C_7$; 155.89 $C_9$; 144.71 $C_2$; 144.12 $C_{4'}$; 142.59 $C_{3'}$; 137.34 $C_3$; 132.20-127.33 $C_{19}$, $C_{20}$, $C_{22}$, $C_{23}$, $C_{25}$, $C_{26}$; 129.02 $C_{1'}$; 126.24 $C_{6'}$; 124.03 $C_{5'}$; 123.31 $C_{2'}$; 107.20 $C_{10}$; 104.79 $C_6$, 101.48 $C_8$; 34.65, 34.34, 34.28 $C_{12}$;29.83-29.36 $C_{14}$-$C_{17}$; 27.43 $C_{18}$;25.86 $C_{21}$; 25.76 $C_{24}$; 25.16, 25.00 $C_{13}$; 20.78 $C_{27}$; 14.50, 14.30 $CH_3$.

**FT-IR (NaCl)**

| **1/λ ($cm^{-1}$)** | **Attribution** |
|---|---|
| 3368.98 | ν OH |
| 3010.86 | ν C $sp^2$ - H |
| 2927.77; 2854.68 | ν C $sp^3$ - H |
| 1771.16 | ν C=O |
| 1652.15 | ν C=O α,β unsaturated |
| 1606.40; 1491.44; 1463.99 | δ C=C |
| 1306.08; 1266.97; 1194.39; 1127.76 | ν C-O |

### 4.3.2 - HPLC METHOD DEVELOPMENT

At first, the solubility of quercetin and its esters was screened: quercetin resulted very soluble in methanol, whereas its derivatives were practically insoluble in methanol.

The solubility in organic solvent was screened and quercetin esters resulted very soluble in hexane, while quercetin was shown to be insoluble in that solvent. This difference in solubility led to choose two different HPLC methods: one for quercetin and the other for its esters.

The HPLC method employed for resveratrol was suitable also for quercetin:

- Mobile phase: 100% methanol
- Stationary phase: C-8 Alltima 250x4.6 mm
- Flow rate: 0.8 ml/min
- λ: 300 nm
- Room temperature

Initially quercetin esters were analyzed as follows:

- Mobile phase: 99.9% Hexane and 0.1% isopropanol
- Stationary phase: silica Ascentis 15x4.6 mm (3 μm)
- Flow rate: 1.5 ml/min
- λ: 254 nm
- Room temperature

This normal phase method overcame the solubility troubles, but it resulted in a low resolution, so the isopropanol percentage was slightly increased: hexane (99.8%) / isopropanol (0.2%). This composition allowed to achieve a higher resolution. Different stationary phases were tried as well, and the silica Ascentic 15x4.6 mm (3 μm) was chosen.

The final analysis conditions were:

- Mobile phase: 99.9% Hexane and 0.2% isopropanol
- Stationary phase: silica Ascentis 15x4.6 mm (3 μm)
- Flow rate: 1.5 ml/min
- λ: 254 nm
- Room temperature

The chromatograms of 3,5,7,3’,4’-pentaoleoyl quercetin (Fig. 12), 3,7,3’,4’-tetraoleoyl quercetin (Fig. 13), 3,7,3’-tri-oleoyl quercetin (Fig. 14) are reported below.

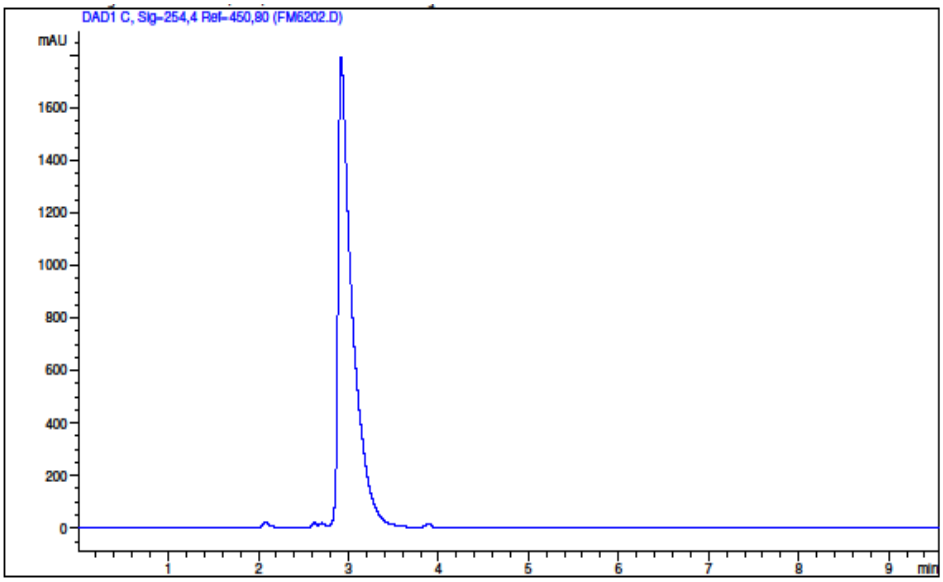



**Figure 12.** Chromatogram of 3,5,7,3’,4’-pentaoleoyl quercetin (RT= 2.933 min).

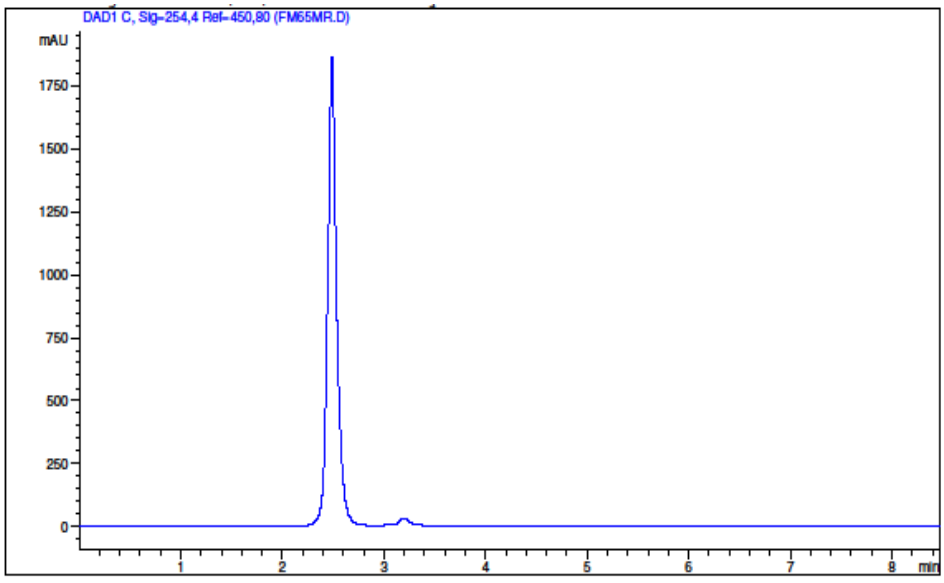



**Figure 13.** Chromatogram of 3,7, 3’,4’-tetraoleoyl quercetin (RT=2.490 min).

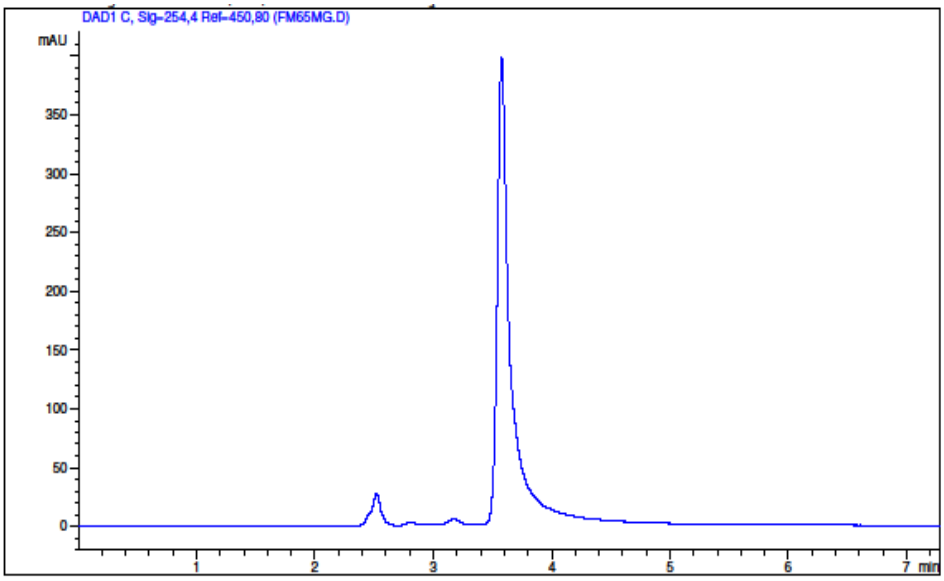



**Figure 14.** Chromatogram of 3,7,(3'/4')-trioleyol quercetin (RT= 3.582 min).

### 4.3.3 - LIPOSOMES

The topical application of quercetin inhibits oxidative skin damage and the inflammatory processes induced by UV radiation. Low skin permeability and poor solubility of quercetin in aqueous media reduce its topical use: these features make the development of pharmaceutical formulations difficult. In addition to synthesis of prodrugs (quercetin esters), other strategies can be used in order to improve quercetin topical delivery, such as liposome formulation.

In collaboration with Dipartimento Farmaco Chimico Tecnologico, University of Cagliari, quercetin and its derivatives were incorporated into phospholipid vesicles. The incorporation of quercetin esters into liposomes would allow the delivery not only of quercetin, but also of fatty acids, a class of molecules known to be active in the skin.

In order to better incorporate quercetin derivatives into phospholipid vesicles, two different hydrophilic penetration enhancers (PEs), propylene glycol (PG) and polyethylene glycol 400 (PEG), were added to the formulations. Two different quercetin derivatives-loaded Penetration Enhancer containing Vesicles (PEVs) formulations (Formulation A e B) were produced, as reported below:

- Formulation A:
  - Soybean lecithin (0.15 g)
  - PEG 400 (1.0 g)
  - Quercetin / quercetin derivative (0.005 g)
  - Water (1.5 ml)

- Formulation B:
  - Soybean lecithin (0.15 g)
  - Propylene glycol (1.0 g)
  - Quercetin / Quercetin derivative (0.005 g)
  - Water (1.5 ml)

The esters of quercetin used in these experimental formulations were: 3,5,7,3',4'-pentastearoyl quercetin, 3,5,7,3',4'-pentaoleoyl quercetin, 3,7,3',4'-tetraoleoyl quercetin, 3,5,7,3',4'-pentalinoleoyl quercetin, 3,7,3',4'-tetralinoleoyl quercetin, 3,5,7,3',4'-pentalinolenoyl quercetin and 3,7,3',4'-tetralinolenoyl quercetin.

The use of a mixture of PE/water (40% v/v) as hydrophilic phase improved the quercetin and derivatives solubility in water, due to their high solubilizing power, and enabled the incorporation of ~2 mg/ml of drug in PEVs without any sign of drug precipitation during or after fabrication. Previous findings demonstrated

that PEVs are powerful enhancers for dermal delivery, due to the synergistic effect of phospholipid vesicles and PE.

#### 4.3.3.1 - VESICLE PREPARATION

The quercetin ester was dissolved in a PE/water solution (40% v/v) and added to a flask containing soybean lecithin. Lipids were left swelling in the solution overnight. Vesicles were obtained by sonicating for 3 minutes (5 seconds on and 5 seconds off) the dispersions with a Soniprep 150 ultrasonic disintegrator (MSE Crowley, UK).

#### 4.3.3.2 - VESICLE CHARACTERIZATION

Each vesicle suspension was purified from the non-incorporated drug by exhaustive dialysis against distilled water at 4 °C for 1 hour, using dialysis tubing (Spectra/Por® membranes: 12–14 kDa MW cut-off, 3 nm pore size; Spectrum Laboratories Inc., USA). Incorporation efficiency (E%), expressed as the percentage of the amount of drug initially used, was determined by high performance liquid chromatography (HPLC) after disruption of vesicles with 0.025% non-ionic Triton X-100. Quercetin content was quantified at 255 and 367 nm using a chromatograph Alliance 2690 (Waters, Italy). The column was a SunFire C18 (3.5 µm, 4.6 × 150 mm). The mobile phase was a mixture of acetonitrile, water and acetic acid (94.8:5:0.2, v/v), delivered at a flow rate of 1.0 mL/min.

The average diameter and polydispersity index (P.I.) of the samples (6 replicates) were determined by Photon Correlation Spectroscopy (PCS) using a Zetasizer nano-ZS (Malvern Instrument, UK). Samples were backscattered by a helium–neon laser (633 nm) at an angle of 173° and a constant temperature of 25 °C. The P.I. was used as a measure of the width of the size distribution: P.I. less than 0.4 indicates a homogenous and monodisperse population. Zeta potential was estimated using the Zetasizer nano-ZS by means of the M3-PALS (Phase Analysis Light Scattering) technique, which measures the particle electrophoretic mobility in a thermostated cell. All the samples were analyzed 24 h after their preparation.

# 5 - RESULTS

## 5.1 - β-SITOSTEROL

### 5.1.1 - BIOCATALYTIC SYNTHESIS OF β-SITOSTEROL FATTY ESTERS

The esters of β-sitosterol and fatty acid were already obtained by chemical synthesis; nevertheless, we synthesized these compounds using the enzymatic approach, which represents a natural way to obtain them.

In the first phase of this study, five enzymes were screened: only lipases from *Candida rugosa* and *Candida cylindracea* resulted active. The subsequent step was to evaluate the suitability between these enzymes with different organic solvents, such as heptane, toluene, acetone, acetonitrile, hexane and isopropyl ether: among them, heptane and toluene resulted to be the most convenient. Then, different trials were set up at 25, 30 and 40 °C, evidencing that the esterification rate was higher at 40 °C. Finally, different amounts of enzyme were screened: lipase from *Candida rugosa* resulted more efficient at 35 U/mg, whereas lipase from *Candida cylindracea* at 14 U/mg.

After setting up these reaction conditions, we assessed that lipase from *Candida rugosa* was able to esterify β-sitosterol with both oleic and linolenic acids, while *Candida cylindracea* esterified β-sitosterol with only oleic acid. In order to evaluate the reaction kinetics and time, three reactions in semi-preparative scale were performed and at fixed times (4 h, 24 h, 42 h, 48 h, 52 h, 72 h) samples were taken and analyzed using HPLC.

In this step, the synthetic esters were used as reference standards for HPLC analysis. The courses of the biotrasformations are reported in the figures that show the amount of ester formed, calculated as area under the peaks in relation to time. The resulting reaction kinetics are reported below (Figures 15, 16 and 17).

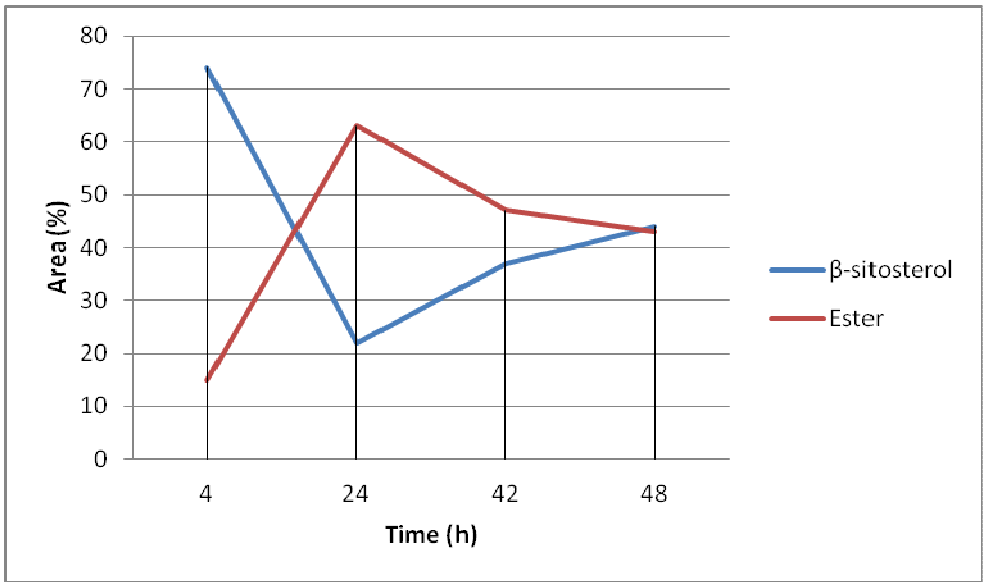



**Figure15.** Progress of the esterification of oleic acid with β-sitosterol by *Candida rugosa* lipase in relation to time

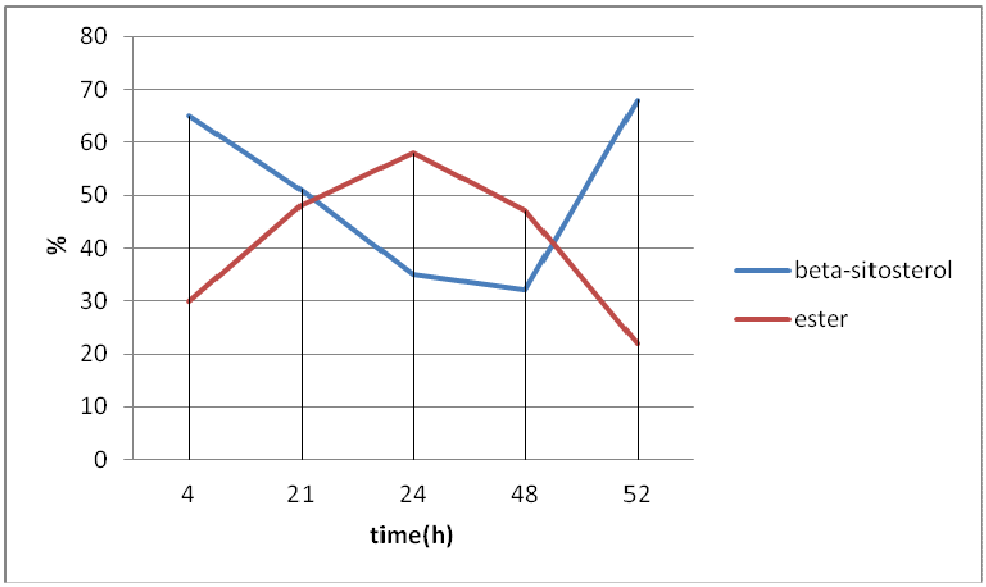



**Figure 16.** Progress of the esterification of oleic acid with β-sitosterol by *Candida cylindracea* lipase in relation to time.

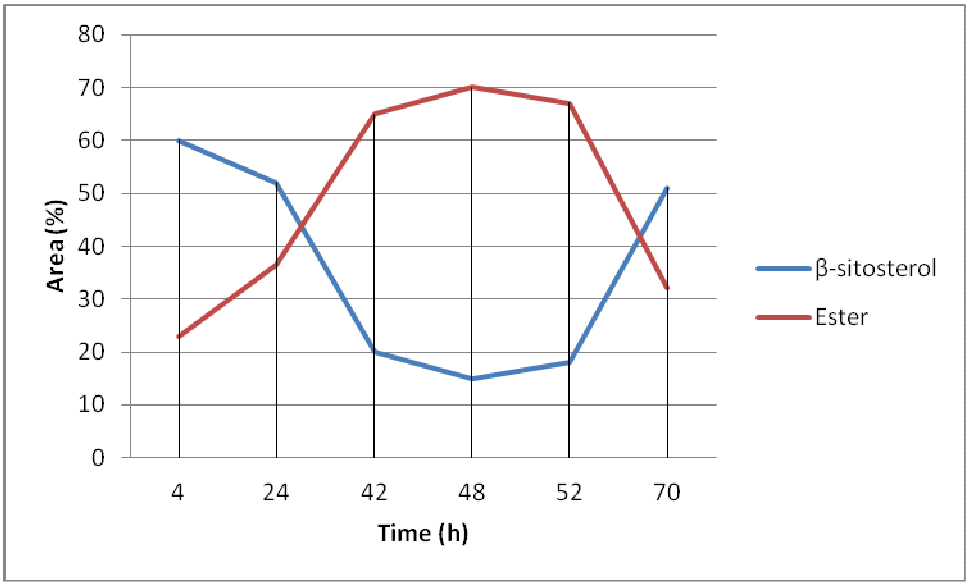



**Figure 17.** Progress of the esterification of linolenic acid with β-sitosterol by *Candida rugosa* lipase in relation to time.

As shown in the figures, the esterification of β-sitosterol with oleic acid by *Candida rugosa* lipase (Fig. 15) and by *Candida cylindracea* lipase (Fig 16) reaches its maximum at 24 h with a 60% yield, whereas the esterification of β-sitosterol using linolenic acid by *Candida rugosa* lipase (Fig 17) is maximum at 48 h, leading to a yield of 70%. Beyond these times the equilibrium of the reaction shifts towards the hydrolysis, due to the abundance of water in the reaction environment.

$$\mathbf{RCOOH + R'OH \rightleftharpoons RCOOR' + H_2O}$$

These data are confirmed by the amount of β-sitosterol during the reaction, indicated by blue lines (Fig. 15, 16, 17).

These results showed that lipase from *Candida cylindracea* is chemoselective for oleic acid.

## 5.2 - RESVERATROL

### 5.2.1 - BIOCATALYTIC SYNTHESIS OF RESVERATROL FATTY ESTERS

Since chemical synthesis of resveratrol mono and diesters is not possible and given the presence in literature of synthesis of these compounds by biocatalytic hydrolysis of triester, in the first phase of the study a screening on enzymes and reaction conditions was performed. This preliminary screening showed that a small amount of water (5%) in the reaction medium was not sufficient for promoting the triester hydrolysis; on the contrary, TRIS buffer/HCl (pH 8) allowed this hydrolysis. Only PPL and Acylase I from *Aspergillus melleus* were suitable to the proposed synthesis.

Preliminary TLC analyses showed that the reaction with Acylase I from *Aspergillus melleus* led to the formation of different hydrolysis products, and in the selected reaction conditions the final amount of triester was low. The reaction promoted by PPL yielded to the formation of only one hydrolysis product, and using this enzyme there was a higher amount of triester at the end of the reaction. These results made us to focus on the first enzyme: hence, a semi-preparative scale reaction using Acylase I from *Aspergillus melleus* was set up in order to obtain a sufficient amount of substance for its analytical characterization.

The reaction mixture was extracted and analyzed by HPLC (Figure 18).

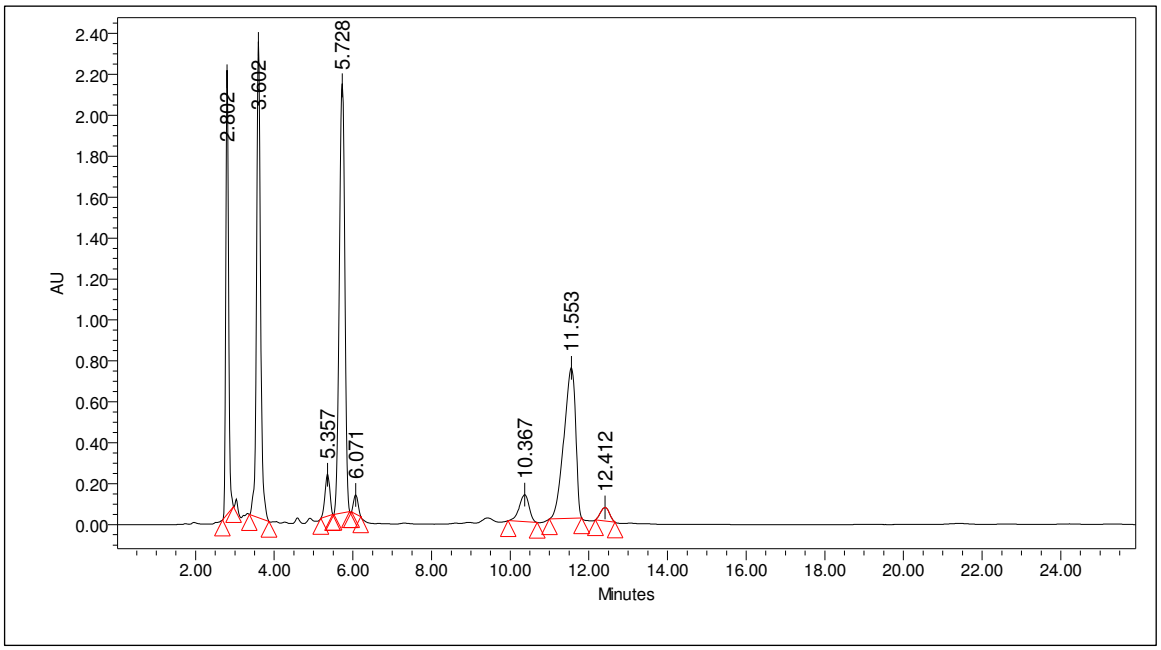



**Figure 18.**

The HPLC/DAD analysis (Fig. 18) showed that the starting triester was still present (RT=11.55 min). Peaks at 2.80 min, 3.60 min, 5.73 min correspond to other products in the reaction mixture.

In order to characterize each analyte, the raw residue (200 mg) was separated using a chromatographic column with 7/3 hexane/acetone as mobile phase and a 1/60 product/silica ratio. Four different fractions were obtained in this separation, and subsequently analyzed by MS and $^{1}$H-NMR.

The first fraction (15 mg) corresponded to the starting substrate (trioleoyl resveratrol), according both to $^{1}$H-NMR and mass spectrometry results. The result of MS analysis of the second fraction (30 mg) was 758 $[M+H]^{+}$, in compliance with the molecular weight of a diester. The $^{1}$H-NMR analysis (Fig. 19) confirmed this evidence and indicated that the free -OH group was in position 4'. In fact, signals corresponding to H atoms in positions 2',6' and 3',5' underwent higher shifts (7.40 ppm and 6.85 ppm, respectively). Furthermore, no asymmetry in the signals of disubstituted ring was evidenced: if the unesterified hydroxyl group was in positions 3 or 5, the meta-disubstituted ring would lose its symmetry.

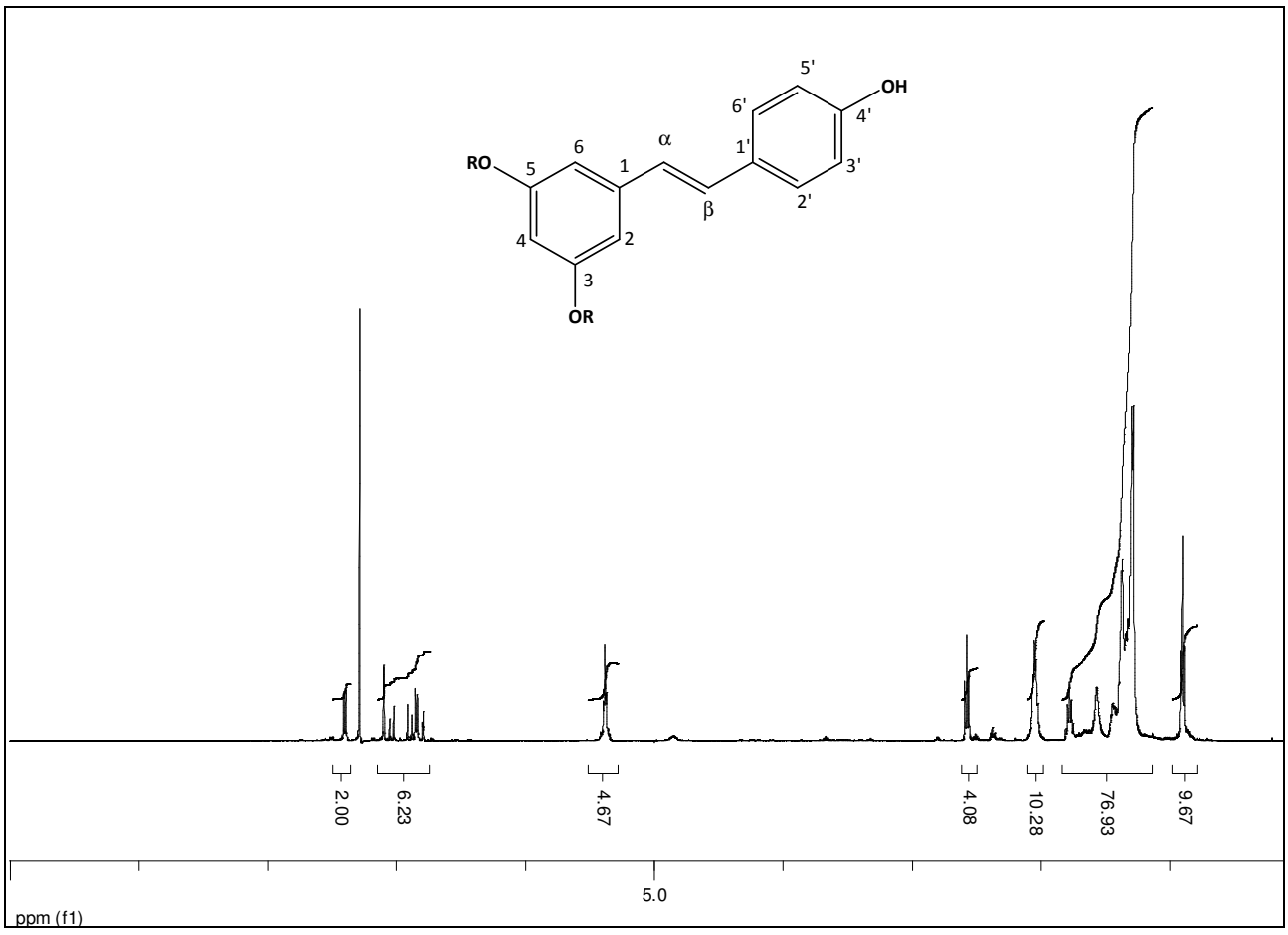



**Figure 19.** $^{1}$H-NMR spectrum of the 3,5-dioleoyl-resveratrol.

The MS analysis result of the third fraction (20 mg) was 493 $[M+H]^{+}$, which corresponds to the molecular weight of a monoester, whereas $^{1}$H-NMR analysis showed an ambiguous result, due to its low solubility.

The $^{1}$H-NMR analysis of the fourth fraction (10 mg) showed that it was oleic acid; the mass spectrometry yielded no results, since fatty acids are not detectable using ESI analysis.

### 5.2.2 - ORAL BIOAVALABILITY STUDIES OF RESVERATROL AND 3,5,4'-TRIOLEOYL RESVARATROL IN THE RAT

Once the extraction procedure had been defined, resveratrol and its tri-oleoyl ester were extracted from rat plasma samples and analyzed by HPLC: the bioavailability results are reported in Figure 20.

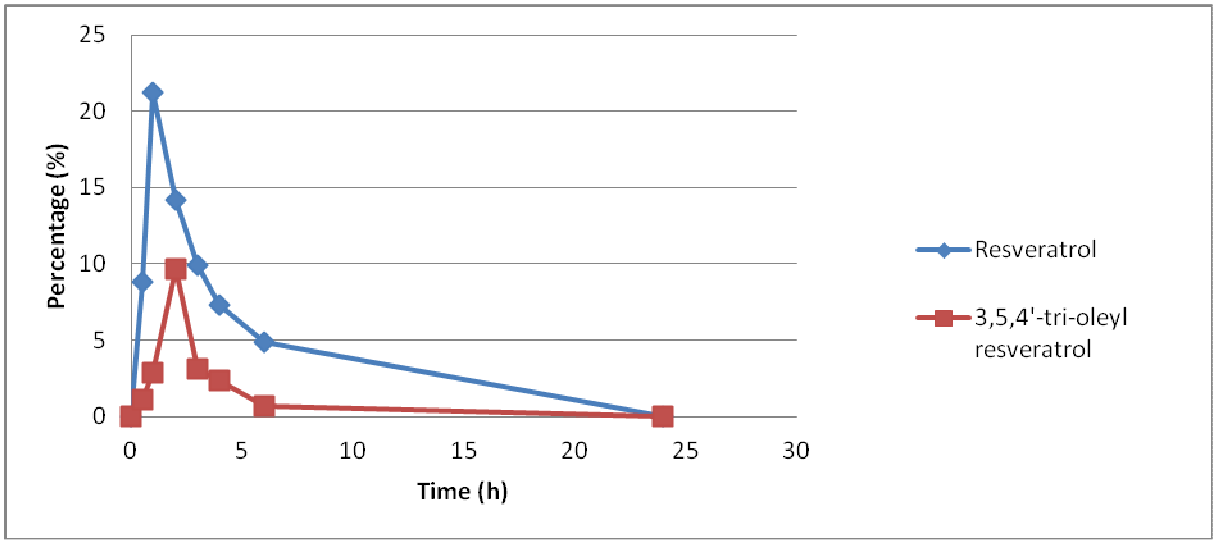



**Figure 20.** Bioavailability of resveratrol and 3,5,4'-tri-oleoyl resveratrol after oral administration.

When administered orally, 3,5,4'-tri-oleoyl resveratrol was expected to release, at least in part, free resveratrol: in the plasma, however, only the presence of tri-ester was detected. In addition, as seen in figure 20, the oral administration of tri-oleoyl ester led to a lower bioavailability compared with resveratrol: hence, these results demonstrate that the oral administration of 3,5,4'-tri-oleoyl resveratrol did not improve the bioavailability of resveratrol.

## 5.3 - QUERCETIN

### 5.3.1 - CHARACTERIZZATION OF QUERCETIN POLYESTERS

In order to identify the unesterified -OH groups in the molecular structure, tetra and trioleoyl quercetin esters were analyzed by bi-dimensional NMR techniques, such as HMBC (Heteronuclear Multiple-Bond Correlation Spectroscopy), HSQC (Heteronuclear Single-Quantum Correlation Spectroscopy). Also pentaester was analyzed by these techniques, in order to provide a reference for tetra and triester. The $^{1}$H-NMR analysis of tetra-oleoyl ester evidenced for $H_6$ (6.61 ppm) a downfield shift compared with that of the corresponding pentaester ($H_6$ 6.86 ppm,); this data suggested that the 5 position could be unesterified.

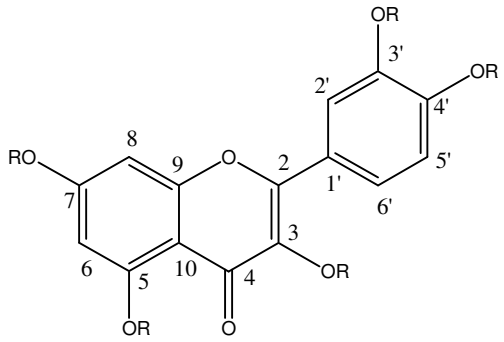



**Figure 21.** General structure of quercetin pent-esters.

Furthermore, the $^{13}$C-NMR analysis of tetra-ester showed that $C_4$ (174.60 ppm) had a higher frequency in comparison with that in the pentaester (169.25 ppm) and confirming that the hydrolized –OCO- group is in position 5. This hypothesis was confirmed by HMCQ and HSQC analyses.

The identification of free hydroxyl groups in trioleoyl quercetin led to ambiguous results. The $H_6$ and $C_4$ shifts allowed us to understand that position 5 was unesterified as in tetra-ester, while the comparison between the $H_{6'}$, $H_{2'}$,.and $H_{5'}$ shifts in penta and tetraester (7.73, 7.69 and 7.32 ppm) with that in triester (8.14, 8.08 and 7.38 ppm) allow to hypnotize that the second free -OH group could be either in 3' or in 4' position. Not even $^{13}$C-NMR and 2D-NMR techniques were able to distinguish which position was unesterified.

Thus, computational studies, in collaboration with Dr. Contini, Dipartimento di Scienze Molecolari Applicate ai Biosistemi, University of Milan, were performed. To make the calculation easier, the studies were performed on quercetin acetylated esters: 3,5,7,3',4'-pentaacetyl quercetin, 3,7,3',4'-tetraacetyl quercetin, 3,7,3'-triacetyl quercetin and 3,7,4'-triacetyl quercetin. Penta and tetraesters were included in the study to

rule out any systematical error. Initially, a conformational research ( molecular mechanic, lowmode method, ff MMFF94x, gas phase) was performed and eight of the most stable conformation were chosen for both quercetin triacetyl esters, while for penta and tetraester only the most stable conformation was selected. Then, the geometry optimization of molecules was performed following a quantum mechanical method (DFT mPW1B95/6-31+G(d,p)) and either $^{1}$H- and $^{13}$C-NMR analyses were simulated for all the selected conformations (GIAO method, mPW1B95/TZVP). The results of the computational study were fitted with the experimental values on a regression curve, and regression coefficients ($R^2$) were calculated for both $^{1}$H- and $^{13}$C-NMR. Figure 22 shows the regression curve obtained comparing the $^{1}$H-NMR calculated values for 3,7,4'-triacetyl quercetin vs experimental values, while the comparison between the calculated data for3,7,3'-triacetyl quercetin and the experimental results are displayed in Figure 23.

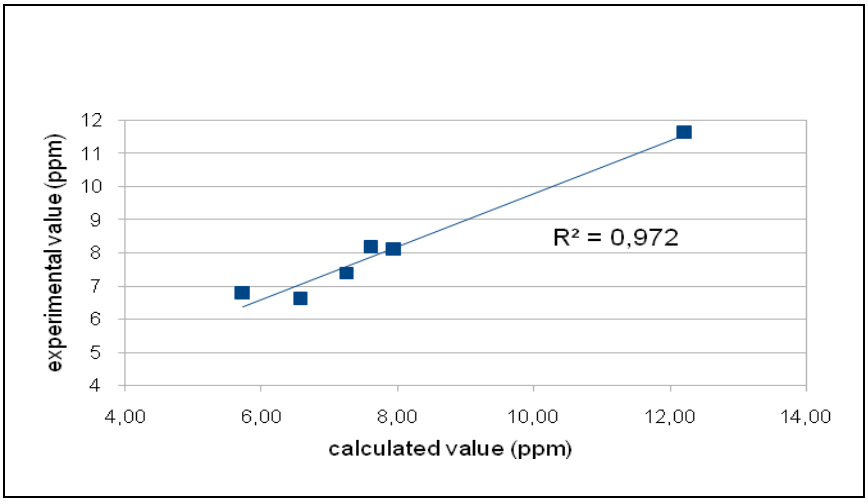



**Figure 22.** Regression curve of 3,7,4'-triacetyl quercetin calculated values vs. experimental values.

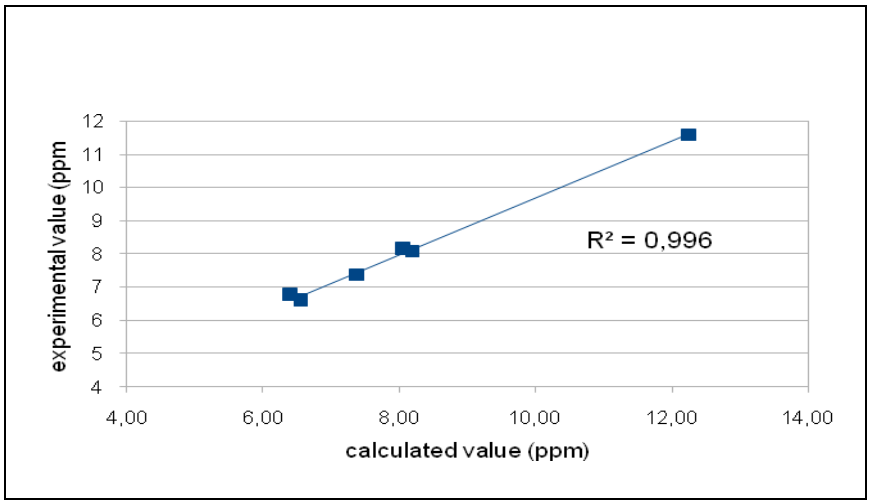



**Figure 23 .** Regression curve of 3,7,3'-triacetyl quercetin calculated values vs. experimental values.

As seen in Figure 22, the regression curve of 3,7,4'-triacetyl quercetin reported a $R^2$ value of 0.972, while for 3,7,3'-triacetyl quercetin the $R^2$ value is 0.996 (Figure 23): this data indicate that the unesterified hydroxyl group of the synthesized quercetin triester could be more probably in position 4'. The data from computational study on $^{13}$C-NMR, however, were not indicative to assess whether the free -OH group was in position 4' or 3'; these ambiguous data could be due to the molecular conformation, hence, other computational studies are still in progress.

### 5.3.2 - LIPOSOMES

As an example, the results of pentaoleoyl and tetraoleoyl esters incorporation in liposomes are displayed in Table 10.

Mean size of PEVs, measured by PCS, was around 200 nm for all the loaded formulations, which were always larger than the empty ones. The difference in size between empty and corresponding loaded vesicles was related to the composition of the samples.

PEVs were quite homogeneously dispersed and values were always repeatable. Zeta potential values were always highly negative (around –50 mV), indicative of a good storage stability against vesicle aggregation and fusion. Drug incorporation into the vesicles at a percentage ranging from 44 to 57 was achieved by all the prepared formulations, showing their good loading capacity.

| | | MD(nm) | P.I. | ZP (mV) | E (%) |
|---|---|---|---|---|---|
| Lecithin/PG | Empty | 57 ± 10 | 0.30 | –51 ± 7 | |
| | Pentaoleolyl quercetin | 183 ± 10 | 0.35 | –64 ± 4 | 57 ± 8 |
| | Tetra-oleolyl quercetin | 195 ± 5 | 0.30 | –52 ± 2 | 44 ± 9 |
| Lecithin/PEG | Empty | 153 ± 10 | 0.28 | –51 ± 4 | |
| | Penta-oleolyl quercetin | 186 ± 5 | 0.29 | –32 ± 3 | 55 ± 6 |
| | Tetra-oleolyl quercetin | 205 ± 6 | 0.19 | –51 ± 6 | 45 ± 4 |

**Table 10.**

## 5.4. - ANTIOXIDANT ACTIVITY

The antioxidant activity of resveratrol, quercetin and their respective esters was assessed by ABTS and DPPH assays. For ABTS assay the results were expressed as TEAC, whereas for DPPH assay as $IC_{50}$. These tests were selected since they are the most used to assess the antioxidant activity of polyphenols.

The results relevant to the antioxidant activity of resveratrol tri-esters are shown in Figures 24 and 25.

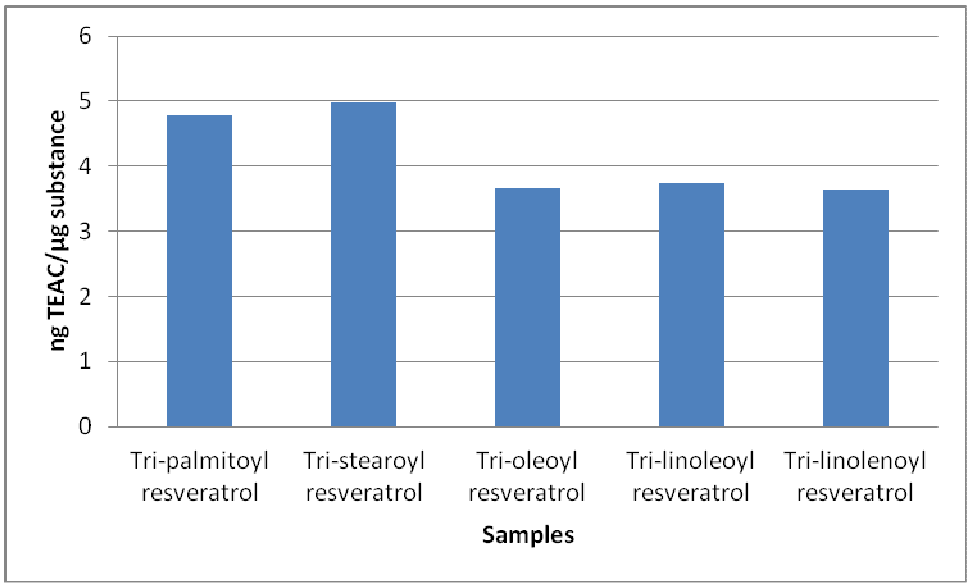



**Figure 24.** ABTS assay: antioxidant activity of resveratrol tri-esters expressed as TEAC.

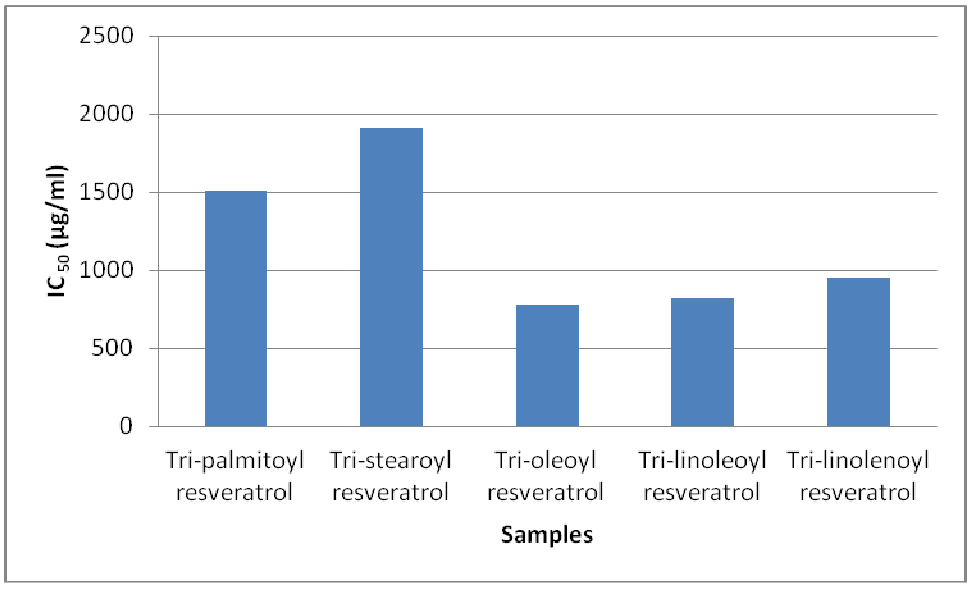


**Figure 25.** DPPH assay: antioxidant activity of resveratrol tri-esters expressed in $IC_{50}$.

As seen in Figures 24 and 25, ABTS assay show that the triesters with saturated fatty acids (tripalmitoyl and tristearoyl resveratrol) have a higher activity than esters with unsaturated fatty acids, while DPPH assay shows opposite results. However, differences in antioxidant activity resulting from ABTS assay among resveratrol esters could not be considered significant, since their results were of few ng TEAC/µg substance: data expressed in such small units are more probably subject to errors. In addition, the dissolution medium used in ABTS assay contained a considerable amount of water: resveratrol esters are scarcely soluble in such solvent, hence it could jeopardize the results of this test. On the contrary, DPPH assay could be considered more reliable, as the medium was mainly ethanol, which allows a better dissolution of resveratrol tri-esters. In addition, the DPPH results showed larger differences among resveratrol esters antioxidant activity. In conclusion, we can state that resveratrol esters with unsaturated fatty acids show a more potent antioxidant activity compared with saturated fatty acids esters.

Both of the assays performed displayed a significantly lower activity of the tri-ester compared with resveratrol: this result shows that the -OH groups of resveratrol have a key role for its antioxidant activity.

In addition, preliminary tests on 3,5-dioleoyl resveratrol showed a higher antioxidant activity in comparison with 3,5,4'-trioleoyl resveratrol.

The results relevant to the antioxidant activity of quercetin esters are shown in Figures 26 and 27.

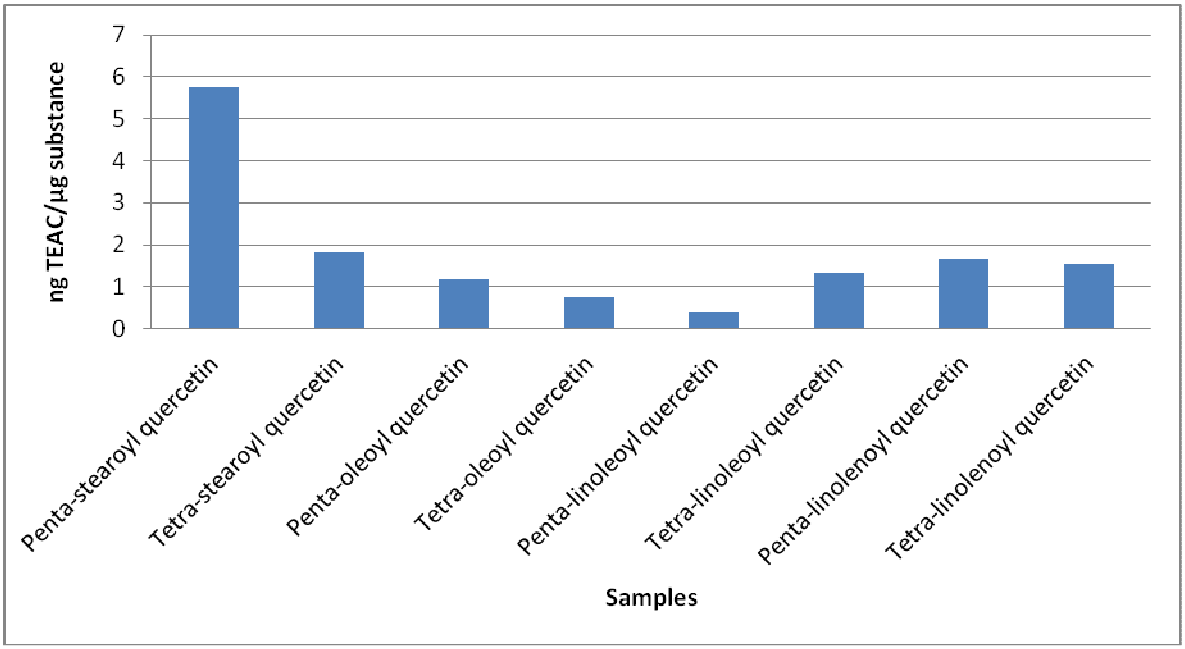



**Figure 26.** ABTS assay: antioxidant activity of resveratrol tri-esters expressed as TEAC.

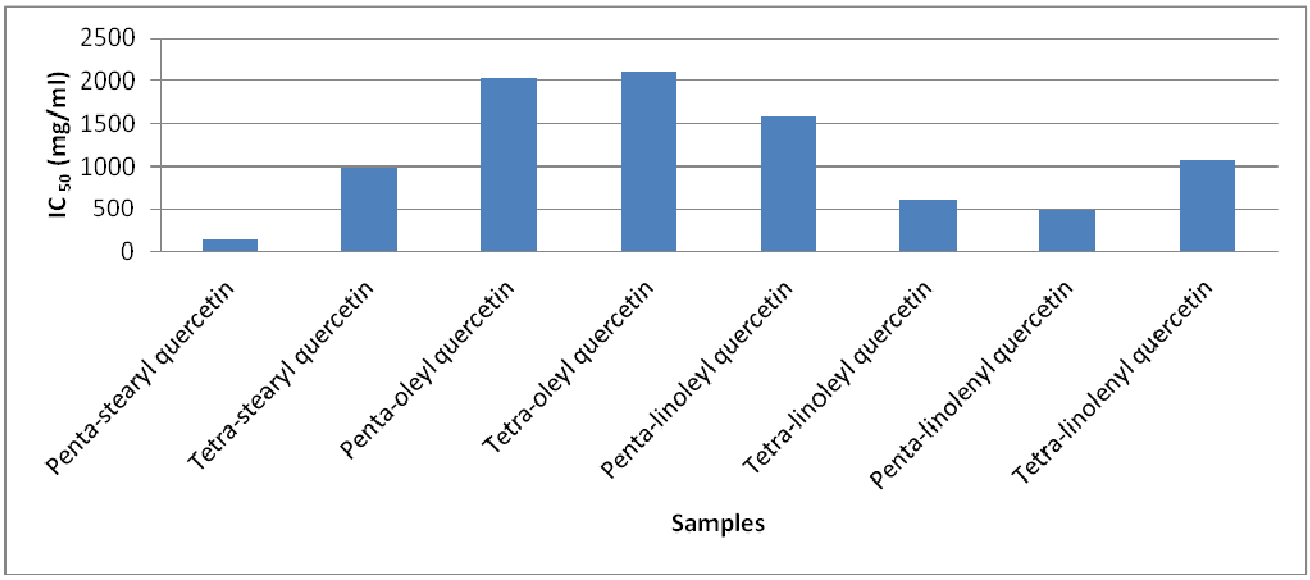



**Figure 27.** DPPH assay: antioxidant activity of resveratrol tri-esters expressed in $IC_{50}$.

As seen in the Figures reported above, both assay showed that the difference in antioxidant activity between penta and tetraesters with the same fatty acid was not considerable. This evidence indicate that -OH group at position 5, which is free in all analyzed tetra-esters, is not essential for the antioxidant activity of quercetin. The higher activity of pentastearoyl quercetin resulting from both assays could be explained by its hydrolysis. As for resveratrol, also quercetin in both assays displayed a dramatically higher antioxidant activity compared with its esters.

These data show that the synthesis of esters is intended mainly to enhance the absorption and permeability properties of polyphenols; once absorbed, the esters are supposed to hydrolyze and release free polyphenols.

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